

Principles & Practice of
**Clinical
Virology**

Sixth Edition

Editors

Arie J. Zuckerman
Jangu E. Banatvala
Barry D. Schoub
Paul D. Griffiths
Philip Mortimer



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Preface

The current (Sixth Edition) *Principles and Practice of Clinical Virology* consists of 39 chapters and 968 pages, contrasting with the 16 chapters and 590 pages in the First Edition, published in 1987. This is a manifestation of not only the wealth of new knowledge acquired on those virus infections listed in the First Edition, but also the discovery of newly-recognized emerging infections, the improvement or development of new vaccines, for example human papilloma virus vaccines, and an increasing repertoire of antiviral agents for treatment. Molecular techniques are now increasingly used for the diagnosis and monitoring of viral infections but their use and assessment is dependent on having a clear understanding of some of the basic virology covered in each of the chapters.

All chapters have been thoroughly revised and there are a number of new contributors, joining the cadre of internationally-recognized experts. Of particular note is a new chapter on vaccinology, covering the principles

relating to the development and use of vaccines generally, which will be a useful complement to the specific vaccines described, where appropriate, in the various chapters. The two chapters on nosocomial infections have been rewritten and enlarged and will be particularly useful for those having to advise on the management of hospital-acquired infections. There are chapters on virus infections common in developing countries, including zoonoses which, because of climate change and speed of travel by air, may need to be identified and managed in resource-rich countries.

This edition emphasizes the rapid accumulation of new information in such fields as retroviruses, particularly HIV, SARS, hepatitis C and influenza, including avian influenza.

As in previous editions, attempts have been made to limit references to key publications of historical importance and to recent review articles.

October 2008

Past, Present and Future of Clinical Virology: an Overview

Medical virology is an eclectic science, born a century ago of the germ theory of disease and the realization that pathogenic microbes smaller than bacteria must exist. The circumstances surrounding its birth are exemplified by the early study of influenza. Epidemic influenza had first been clearly documented in the years 1889–1891, and this led to the description of *Haemophilus influenzae* as its causative agent. That organism proved to be a secondary invader, however, and by the time the next influenza pandemic appeared in 1918 few believed that it was responsible for influenza. By then, too, there was a growing conviction that influenza and several other common diseases such as measles and herpes simplex were due to a different life form, one that passed through bacteria-retaining filters and could not be propagated in the laboratory on inert growth media.

Although the word ‘virus’ had been loosely used for many years previously, the first clues about the life form that eventually took that name came with the independent description around 1915, by Twort and d’Herelle, of bacteriophages. These were viruses observed to cause patches of lysis on lawns of bacteria grown on agar, a phenomenon that could then be reproduced with high dilutions of filtrates derived from the same patches. About the same time comparable observations were being made on plants with filtrates derived from lesions on their leaves. In respect to human viruses, though, the first quarter of the twentieth century can be regarded as a Dark Age in which there was accurate observation of virus diseases but little understanding of their pathogenesis and none at all of the biology of the viruses that caused them.

Fortunately, veterinary studies were more instructive; both investigations of viral infections of animals that might be analogous to human ones, and others in which animal susceptibilities to human viruses were beginning to be used for clinical diagnosis and vaccine development. Into the former class fell the studies by Loeffler and Frosch on Foot and Mouth Disease (around 1900). These showed that very highly dilute filtrates derived from acute

lesions of cattle readily transmitted the infection. Into the latter class fell the successful transmissions of measles (1908), polio (1909) and yellow fever (1926) infections to rhesus monkeys, of vaccinia and herpes simplex to rabbits (around 1915), and of influenza to ferrets (1933). As has continued to be the case, such transmissions were very instructive; but they were inconvenient as a routine and have since become increasingly controversial, both in their use of animals and, in the case of primates, in their impact on endangered species. Moreover, animal studies have not been forward-looking, harking back as they do to the nineteenth century propagation of vaccinia on the flanks of calves and of attenuated rabies virus in the spinal cords of rabbits. Though this was the bedrock on which clinical virology was built, inoculation of animals with human viruses has proved to have distinct limitations.

The next recognizable phase in the development of clinical virology saw the first applications of tissue culture to the propagation of viruses and the adoption of fertile eggs as a convenient substrate for virus growth. Before antibiotics the former was hopelessly prone to contamination, but the latter much less so. A pioneer (around 1930) in the use of fertile eggs was the American biologist Ernest Goodpasture, and his technique was soon applied by Macfarlane Burnet to the study of influenza viruses and by Max Theiler to the production of yellow fever vaccine.

Meanwhile, physicians were using convalescent and immune sera in the prevention and treatment of infections such as measles, and applying increasingly sophisticated serological techniques to diagnose virus infections in the laboratory, based on the host immune response. As well as having a therapeutic role, sera had become both routine diagnostic specimens and standard diagnostic reagents.

In Germany in the late 1930s electron microscopy was first used to examine structured virus particles. Staining and light microscopy had previously been used to visualize ‘elementary bodies’ including pox virions, but with the electron microscope viruses smaller than these could be visualized if suspended in electron-dense salt solutions.

This 'negative' staining did not find diagnostic applications until the 1960s, but it meant that for the first time viruses were being seen with certainty, and 'seeing was believing'. Demonstrably, viruses were particulate intracellular micro-organisms, capable of limited survival outside the living cell but of growth only within it. It might seem obvious now, but it had taken over half a century to arrive at that conclusion.

World War II was a stimulus to the study of virology, particularly in respect of those diseases, such as infectious hepatitis, that in several theatres of war put troops out of action for weeks at a stretch. For reasons that only came to be understood later, hepatitis afflicted both those newly exposed to battlefield or other insanitary conditions and, after a longer interval, those transfused with plasma to treat blood loss. The next period in the development of virology saw, from 1950, the introduction of *in vitro* monolayer cell culture as the substrate of choice for the isolation and propagation of viruses. This was greatly enabled by the availability of antibiotics to suppress contamination, and its eventual impact on virology was as far-reaching as, in the 1880s, had been Koch's use of semi-solid media to isolate pathogens on the development of bacteriology. A swathe of human and animal viruses was grown on monolayers, and cell culture transformed both virus diagnostics and vaccine manufacture. Outstanding was the development and distribution of cell-grown killed and attenuated polio vaccines, followed, in the 1960s, by measles, mumps and rubella vaccines.

As the pace of virus discoveries slackened at the end of the 1960s it became apparent that there were diseases for which isolation of a causative virus in cell culture was difficult, if not impossible. Prominent among these refractory viruses were the agents of hepatitis and gastroenteritis, and these were the centre of attention in a further phase of virological development in the 1970s and 1980s. It was also a time of rapid expansion of diagnostic services, benefitting from the discovery of Australia antigen (the surface antigen of hepatitis B virus), of Epstein-Barr virus and its association with human lymphatic and pharyngeal tumours, and of the two very prevalent human gastroenteritis viruses, rotavirus and norovirus. None of these agents could be grown in conventional cell monolayers, yet they were responsible for much acute and chronic disease. Their detection was added to the diagnostic repertoire, first in the developed, then in the developing world, where they were particularly important pathogens.

The same era saw the eradication of smallpox, based on exhaustive case finding, contact observation, vaccination and, where necessary, the use of diagnostic tools such as electron microscopy. The magnitude of that global health achievement is now more fully appreciated as

international bodies struggle with the complexities of a second generation of virus-eradication schemes, against polio and measles.

It is difficult to set recent events in a proper historical context, but the most significant developments since the mid 1980s have probably been the emergence of novel infections, the impact of molecular biology and the synthesis and use of effective, relatively nontoxic, antiviral substances. HIV is a virus with a profound effect on the immune system; hepatitis C is an insidious infection whose varied pathogenesis remains poorly understood; and the infectious zoonotic form of dementia, vCJD (which is almost certainly not due to a virus), points to the possibility of finding other unusual pathogens that will fall within the ambit of clinical virology. Some of these may be more common than vCJD has so far proved to be. All the most important recent technical innovations in virology have been based on molecular biology, including the expression of synthetic virus proteins, for example the antigens of hepatitis B, hepatitis C and papilloma viruses, and the ability through the polymerase chain reaction (PCR) to amplify virus oligonucleotides for diagnostic purposes. Other applications of PCR have allowed virus 'fingerprinting', entire genome sequencing and even the synthesis of whole viruses. Antivirals have facilitated successful transplantation and transformed the prognosis for HIV and hepatitis patients. They may have a role in mitigating the impact of epidemic influenza.

The history of a subject gives context to the state it is currently in, but it does not necessarily show how it will develop in the future. It is for instance predictable that as national vaccination programmes against hepatitis B and high-virulence papilloma viruses are rolled out, the incidence of two important viral cancers will slowly decline; but the foregoing also offers examples of developments in virology that were wholly unpredictable. It cannot be said when pandemic influenza will next occur, or whether a comprehensive vaccine will ever be devised against HIV, let alone what new viruses might soon emerge. Quite likely other forms of intracellular parasite than the presently-recognized viruses will appear, and they may possibly be shown to be responsible for 'orphan' diseases, not least important chronic ones such as multiple sclerosis. Virologists, aware of the rapid and somewhat haphazard development of their subject to date, should avoid the hubristic notion that almost all there is to know has already been found out. Instead they should look forward to the surprises the future holds with an open mind, curiosity and even relish.

Further reading: there is as yet no comprehensive history of virology, but original reports of discoveries since 1900 are easily found in the series of various learned journals. Textbooks, from *Filterable Viruses* (1928), edited by

Thomas Rivers, through to more modern ones such as the continuing series first published in 1953 and also early on edited by Rivers, demonstrate in their successive editions how the subject has advanced. A scholarly attempt at an historical approach was A.P. Waterson and L. Wilkinson's *Short History of Virology* (1977), but it has not been

brought up to date since. More popular and discursive is *The Virus Hunters* by Greer Williams (1978). Williams was a professional journalist who interviewed some of the leading virologists of the mid twentieth century and reported what he learned from them in accessible prose. That book, too, deserves to be brought up to date.

Diagnostic Approaches

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INTRODUCTION

Human virus infections may affect all ages and assume any degree of severity. They may be acute or chronic, be recurrent or elicit lifelong immunity. They are acquired through various routes via contact with humans, animals or the environment. They present as various syndromes involving fever, rash, arthralgia/myalgia, respiratory or gastrointestinal disorders and occasionally serious organ malfunction with deaths from pneumonia, cardiac, liver or kidney failure or encephalitis. They have to be rapidly distinguished from bacteriological and other infectious and non-infectious diagnoses if the appropriate clinical management is to be given.

Host factors are crucial to the outcome of virus infections. For any virus infection, age may be critical to determining outcome, those at extremes of age being more vulnerable as a consequence of lack of immunocompetence, inexperience of vaccination and waning of immunity. For some infections, gender and race may confer advantages or disadvantages, but malnutrition, pre-existing organ damage and social neglect are always potentially disadvantageous. Thus, in assessing prognosis and deciding on the investigation, management and treatment of virological infections, the individual patient must be carefully considered in their medical and social context. Any natural tendency towards spontaneous immune-mediated clearance of a virus is likely to be compromised if factors such as these are unfavourable.

A substantial part of clinical virology is taken up with the investigation and treatment of patients either constitutionally or iatrogenically immunosuppressed, or suffering from an existing immunocompromising infection, such as

human immunodeficiency virus (HIV). These patients often need pre-emptive and continuing investigation if intensive treatment for other conditions is not to be nullified by overwhelming virological and other opportunistic infections. The management of these patients must be planned and rigorous, and will differ from that of other virological patients.

Because virus infections are contagious, diagnosis cannot be confined to a consideration of the individual patient. Two questions may be crucial: where did the infection originate? And who may contract it next? Each question clearly gives rise to the potential for wider investigation and possible action to protect contacts through behaviour modification, isolation or prophylaxis with drugs or vaccines. If the infection is sufficiently contagious or is life-threatening, more extensive public health measures may be required and the clinical diagnosticians must not lose sight of the possible implications of their conclusions for the wider community.

Clinical virology in the 1980s was characterized by the widespread use of enzyme-linked immunosorbent assay (ELISA) technology, and in the 1990s by the entry into routine diagnostic use of molecular methods for virus detection. During the early years of the twenty-first century real-time polymerase chain reaction (PCR) and virus quantification have come of age, alongside increasing automation of molecular diagnostics. Concurrently, the emphasis and priorities of diagnostic virology laboratories have shifted. This is in response to the availability of rapid diagnostic methods, the identification of new viruses many of which are non- or poorly cultivable, the increasing availability of effective antiviral agents, the emergence of antiviral resistance, the increasing number

of immunocompromised patients in whom opportunistic viral infections are life-threatening, and the cost pressures on pathology services.

This chapter will provide, firstly, an overview of diagnostic techniques set against this background and presented in order of historical development. Secondly, it will highlight the ways in which these techniques may be applied to arrive at accurate diagnosis thereby facilitating effective management of virus infections, including prevention of their onward spread.

ELECTRON MICROSCOPY

Electron microscopy (EM) is the only technique available for directly visualizing viruses, and therefore has many applications beyond purely diagnostic ones. With the advent of alternative diagnostic methods, EM retains a limited role in the clinical setting for the diagnosis of viral gastroenteritis and examination of skin lesions for herpes and pox viruses.

Preparation of specimens for EM and the technique of negative staining are straightforward and quick, and the method is a 'catch-all' approach to detecting viruses. However, it has a limit of sensitivity of approximately 10^6 viral particles per millilitre of fluid, making negative results unreliable. Vast numbers of virions are present during acute skin and gastrointestinal disease and a diagnosis is easily made, but later in the course of infection viral shedding is reduced below the level of detection. Although sensitivity can be enhanced by antibody-induced clumping of virus (immune EM) or ultracentrifugation, it is unrealistic to undertake these methods routinely. The advantages and disadvantages of EM are summarized in Table 1.1.

The survival of EM within the routine clinical virology laboratory hinges on the emergence of alternative, more sensitive methods of diagnosis. Many centres now use latex agglutination for rotavirus diagnosis, and PCR is more sensitive than EM for detection of herpesviruses in vesicular fluid (Beards *et al.*, 1998) and for the detection of noroviruses (previously called Norwalk-like

viruses) (O'Neill *et al.*, 2001). Thus, the future of EM in clinical virology is in some doubt. However, one of the first indications for EM was for the rapid diagnosis of smallpox and, in the era of bioterrorism, EM may continue to play a role in specialist centres in the event of a bioterrorist attack.

HISTOLOGY/CYTOLOGY

Direct microscopic examination of stained histology or cytology specimens can on occasion provide the first indication that a virus may be responsible for a pathological process, for example the intranuclear (early) or basophilic (late) inclusions seen in interstitial nephritis in renal transplant biopsies due to BK virus, changes in cervical cytology seen in association with human papilloma virus (HPV) and the nuclear inclusions seen in erythroid precursor cells in Parvovirus B19 infection. Moreover, the particular viral aetiology can be confirmed by specific antigen/genome staining using labelled antibody or *in situ* hybridization techniques (see below).

VIRUS ISOLATION

Many of the advances in clinical virology have come about because of the ability to grow viruses in the laboratory. Historically, viruses were propagated in laboratory animals and embryonated eggs, but most virus-isolation techniques now rely on cultured cells. With appropriate specimens and optimal cell lines, this technique can be highly sensitive and specific, with a presumptive diagnosis made on the basis of a characteristic cytopathic effect (CPE). The particular diagnosis can then be confirmed by haemadsorption (certain viruses, influenza and measles for example, cause adherence of erythrocytes to infected cells in a monolayer because the viral antigens expressed include a haemagglutinin) or by immunofluorescence (IF) using a virus-specific antibody labelled with a fluorescent dye. The judicious selection of two or three cell lines, such as a monkey kidney line, a human continuous cell line and a human fibroblast line will allow the detection of the majority of cultivable viruses of clinical importance, such as herpes simplex virus (HSV), Varicella zoster virus (VZV), cytomegalovirus (CMV), enteroviruses, respiratory syncytial virus (RSV), adenovirus, parainfluenza viruses and influenza viruses. In addition, the ability to grow virus from a clinical specimen demonstrates the presence of viable virus (albeit viable within the chosen cell line)—this is not necessarily the case with detection of a viral antigen or genome. For example, following initiation of antiviral therapy for genital herpes, HSV antigen can be detected from serial genital swabs for longer than by virus

Table 1.1 Electron microscopy

Advantages	Disadvantages
'Catch-all'	Requires skilled staff
Detects unculturable viruses	Large capital outlay
Economical running costs	Poor sensitivity
Adaptable, for example immunoelectron microscopy confirms cytopathic effect	
Rapid (same day)	

propagation in cell culture. This implies that antigen is persisting in the absence of viral replication and underlines the importance of correct interpretation of laboratory results. However, failure to isolate a virus does not guarantee that the virus is not present. Virus isolation has also been shown to be diagnostically less sensitive than molecular amplification methods such as PCR for HSV and several other viruses (see below), for example for the diagnosis of herpes simplex encephalitis.

The benefits of virus isolation (Table 1.2) include: the ability to undertake further characterization of the isolate, such as drug susceptibility (see later) or phenotyping; and the identification of previously unrecognized viruses, for example human metapneumovirus (van den Hoogen *et al.*, 2001), severe acute respiratory syndrome (SARS)-associated coronaviruses (Drosten *et al.*, 2003) and human enteroviruses 93 and 94, associated with acute flaccid paralysis (Junttila *et al.*, 2007). On the other hand, routine cell culture techniques available in most laboratories will not detect a number of clinically important viruses such as gastroenteritis viruses, hepatitis viruses, Epstein–Barr virus (EBV), human herpesvirus 6, 7 and 8 (HHV-6, -7, -8) and HIV. Other than HSV and some enteroviruses, most isolates of which will grow in human fibroblast cells within three days, the time taken for CPE (or, for example, haemadsorption) to develop for most clinical viral isolates is between 5 and 21 days, which is often too long to influence clinical management. For this reason, a number of modifications to conventional cell culture have been developed to yield more rapid results. These include centrifugation of specimens on to cell monolayers, often on cover slips, and immunostaining with viral protein-specific antibodies at 48–72 hours post inoculation (shell vial assay) (e.g. Stirk and Griffiths, 1988). Such techniques can also be undertaken in microtitre plates (O’Neill *et al.*, 1996). Certain changes, for example in haemadsorption or pH, may precede the CPE and therefore can be used to expedite detection of virus. Similarly, PCR techniques (see later) can be used to detect virus in cell culture supernatants before the appearance of CPE.

The role of conventional cell culture for routine diagnosis of viral infections has been a subject of active debate within the virology community (Carman, 2001; Ogilvie, 2001). Many laboratories are discontinuing or downgrading virus isolation methods in favour of antigen or genome detection for the rapid diagnosis of key viral infections, for example respiratory and herpes viruses. Nevertheless, it is important for certain reference and specialist laboratories to maintain the ability to employ this methodology to obtain live virus isolates and allow unexpected and emergent viruses to be grown and recognized.

SEROLOGY

This term is often used to refer to diagnostic tests for the detection of specific antibodies. More properly, the term encompasses any testing of blood serum samples for the presence of a specific antigen or antibody. However, as both antigen and antibody assays are often applied to whole blood or plasma, or indeed to body fluids other than blood (e.g. cerebrospinal fluid (CSF), oral crevicular fluid), it is helpful to use the term to span all such testing. As will be seen, most of the techniques used for viral antigen detection can also be used for detection of specific antibody, and vice versa.

Antigen Detection

Immunofluorescence

IF is one of the most effective rapid diagnostic tests. Direct IF involves the use of indicator-labelled virus-specific antibody to visualize cell-associated viral antigens in clinical specimens. The indirect method utilizes a combination of virus-specific antibody (of a nonhuman species) and labelled anti-species antibody. Usually, the label used is fluorescein. The indirect method is more sensitive, since more label can be bound to an infected cell. Results can be available within 1–2 hours of specimen receipt. The success of the technique depends on adequate collection of

Table 1.2 Virus isolation

Advantages	Disadvantages
‘Catch-all’ (as long as viable within the chosen cell line(s))	Only detects ‘viable’ virus
Sensitive	Slow (conventional cell culture)
Generates isolate for further study, for example phenotyping	Multiple cell lines required
Can be adapted for a more rapid result	Labour intensive and requires skilled staff
	Safety concerns, laboratory security

Table 1.3 Antigen detection by immunofluorescence

Advantages	Disadvantages
Rapid (same day)	Labour intensive and requires skilled staff
Sensitive for some viruses (e.g. RSV)	Dependent on high-quality specimen Interpretation is subjective

cells in the particular sample to be examined, for example epithelial cells for respiratory virus antigens or peripheral blood mononuclear cells (PBMCs) for CMV. An advantage of IF is that microscopic examination of the fixed cells can determine the presence of adequate cell numbers for analysis (Table 1.3). Whenever it is employed, however, a trained microscopist is required to interpret results, which remain subjective.

The most common use of this technique is for the diagnosis of respiratory viral infections. A panel of reagents can be used to detect RSV, parainfluenza viruses, influenza A and B, metapneumovirus and adenovirus in multiple wells of a microscope slide. Compared to cell culture this technique is rapid and sensitive, especially for detection of RSV. The ideal specimen is a nasopharyngeal aspirate or a well-taken throat/nasal swab, most usually obtained from infants with suspected bronchiolitis, in whom a rapid result is invaluable for correct clinical management and implementation of infection control measures. There is increasing evidence that community- or nosocomial-acquired respiratory viruses lead to severe disease in immunocompromised patients (reviewed in Ison and Hayden, 2002), and it is important that bronchoalveolar lavage specimens from such patients with respiratory disease are also tested for these viruses, in addition to bacterial and fungal pathogens.

IF has been used widely for the direct detection of HSV and VZV in vesicle fluid, and has advantages over EM in both sensitivity and specificity. Detection and semi-quantification of CMV antigen-containing cells in blood can also be undertaken by direct IF (CMV/pp65 antigenaemia assay). This technique involves separating PBMCs and fixing them on a slide, followed by staining with a labelled monoclonal antibody directed against the matrix protein pp65. The frequency of positive cells can predict CMV disease in the immunocompromised patient (van der Bij *et al.*, 1989) and has been used quite extensively, though it is labour intensive. It needs large numbers of PBMCs, making it unsuitable for some patients. In addition, it requires a rapid processing of blood specimens if a reduction in sensitivity of detection is to be avoided (Boeckh *et al.*, 1994). PCR has therefore

become the method of choice for qualitative and quantitative detection of CMV as well as several other viruses.

Enzyme-linked Immunoassay (EIA), Chemiluminescent and Fluorescence-based Immunoassay

Solid-phase systems for antigen detection are still used widely. EIA is based on the capture of antigen in a clinical specimen to a solid phase (such as the base and walls of a well in a microtitre plate, or multiple magnetic microparticles/beads) via a capture antibody, and subsequent detection uses an enzyme-linked specific antibody that produces a colour change in the presence of a suitable substrate. EIA is also readily used for the detection of specific antibody as well as antigen (see later).

Elaboration of capture and detector antibody species has increased the sensitivity of EIA antigen-detection assays, which are widely used for hepatitis B virus (HBV) surface antigen (HBsAg) and 'e' antigen (HBeAg) detection and for HIV p24 antigen detection. Neutralization of the antigen reactivity by the appropriate immune serum can be used to confirm the specificity of the antigen reactivity. In primary HIV infection, HIV p24 antigen is present in the blood prior to the development of antibodies. Therefore, assays which detect this antigen in addition to anti-HIV antibodies reduce the diagnostic 'window period', that is the time from acquisition of infection to its first becoming detectable (Hashida *et al.*, 1996). Similar assays that detect hepatitis C core antigen in addition to anti-HCV (hepatitis C virus) antibodies have been proposed for testing donated blood.

Molecules with chemiluminescent properties, for example acridinium ester, which produces chemiluminescence in the presence of hydrogen peroxide, can be conjugated to antibodies/antigens and used instead of enzymes for immunoassay detection. Fluorescent labels are another alternative. The fluorescence emissions of chelates of certain rare earth metals—lanthanides, for example Europium—are relatively long-lived. Thus, the presence of an antigen or antibody labelled with a lanthanide chelate can be detected by measuring fluorescence intensity at a delayed time point after excitation, background fluorescence having completely died away. This is the principle of the time-resolved fluorescence assay (TRFA). Both chemiluminescent and TRF methods are very sensitive and highly amenable to automation in commercial systems.

Particle Agglutination Assays

Small latex particles coated with specific antibody will agglutinate in the presence of antigen, and their clumping together can then be observed by the naked eye. This

rapid assay can be used for rotavirus diagnosis, with an equivalent sensitivity to EM.

Immunochromatography

Several immunochromatography or ‘dipstick’ tests have been developed commercially as point of care tests (POCTs) that can be used either in the laboratory, in the clinic or at the patient’s bedside for specific antigen detection. Examples include tests for influenza A and for rotavirus. Such dipstick tests require no special expertise and are quick to perform.

A detector reagent (virus-specific antibody conjugated to a coloured indicator) is impregnated at one end of a base membrane in a disposable ‘dipstick’. A capture antibody is coated on the membrane at the test region. When the clinical specimen is added to the sample pad, any viral antigen present in the specimen binds to the detector reagent and is carried along the membrane by capillary action. As the specimen passes over the test region coated with capture antibody, the viral antigen-bound detector reagent is immobilized. A coloured band proportional to the amount of virus present in the sample develops. The excess unbound detector reagent moves further up the membrane and is immobilized at the control band by an anti-detector antibody, and a second coloured line appears. Thus, two coloured lines on the test stick indicate the presence of virus. In the absence of virus in the patient’s sample only the control band appears.

Antibody Detection

Viral infections generate a host immune response, and this can be used for diagnostic purposes. The classical response pattern following an acute infection is illustrated in Figure 1.1. The functional nature of this response is extremely variable. In some instances the antibodies are neutralizing and can be assessed for this activity (e.g. polioviruses). Other infections are controlled more effectively by T-cell responses, though the detection of antibody may still be used diagnostically.

Traditional methods of antibody detection did not distinguish between IgG and IgM responses, and diagnosis was simply based on seroconversion or a significant rise in antibody titre between acute and convalescent samples (10–14 days apart). The complement fixation test (CFT) was widely used in this way, though assay insensitivity and the cross-reactivity of many antigens used within the assay limited its clinical usefulness and, importantly, the diagnosis could only be made a week or more after the acute illness. The principle of complement fixation is that a specific reaction between an antigen and an antibody takes up complement. If a measured amount of complement is added to a reaction

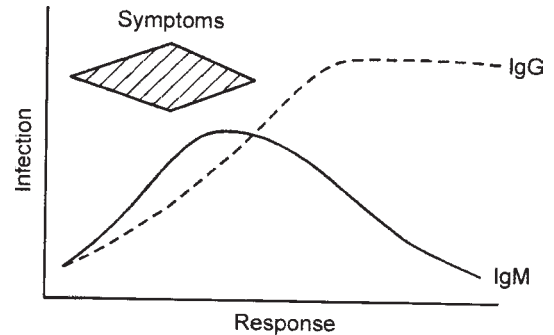


Figure 1.1 Typical evolution of antibody responses following an acute viral infection.

in which both antigen and specific antibody are present, the uptake of complement can be detected by a second ‘detector’ reaction in which sensitized red cells are added to the system. Failure to lyse the red cells signals that complement fixing antibody has been detected, giving a positive test result. Currently, complement-fixation assays are mostly used for the retrospective diagnosis of ‘atypical’ pneumonia (*Chlamydia psittaci/pneumoniae*, coxiella, influenza or mycoplasma).

Serum or plasma is the specimen of choice for antibody detection, but oral crevicular fluid (obtained by rubbing an absorbent sponge around the gums) can be used as a non-invasive alternative. This may be useful for outreach surveillance studies (Hope *et al.*, 2001; MMWR, 2007) or in children (Holm-Hansen *et al.*, 2007). In patients with viral central nervous system (CNS) infections, the CSF may be tested for virus antibodies, and the antibody concentration compared with serum to confirm intrathecal antibody synthesis.

Enzyme-linked Immunoassay (EIA) for Antibody Detection

Solid-phase enzyme immunoassays (EIAs), in which one of the reagents is immobilized on a plastic or other surface (e.g. magnetic beads), are used extensively in diagnostic laboratories. The use of synthetic peptides or recombinant antigens instead of whole viral lysates, and improvements in signal detection, have led to more sensitive, specific and rapid methods for measuring virus-specific antibody levels. The immunoassay format is versatile, and new assays can be designed quickly to cope with clinical demands, for example the investigation of new viruses such as SARS-associated coronaviruses. As indicated above, several non-isotopic, non-enzymatic reagent labelling and detection methods have been developed, such as chemiluminescence- and fluorescence-based methods. These are very sensitive, very rapid and highly amenable to incorporation into commercial automated systems.

Solid-phase immunoassays for the detection of antibody are essentially of three types (Figure 1.2):

- *Indirect assays.* Viral antigen is immobilized on to a solid phase. Specific antibody in the patient serum sample binds to this antigen and, after a washing step, is detected by an enzyme-labelled anti-human immunoglobulin. In this way, either specific IgG or IgM can be detected, depending on the indicator immunoglobulin (Figure 1.2a,b). Detection of IgM species is dependent on the prevailing level of IgG, and a high level of specific IgG may reduce the sensitivity of an IgM assay for the same virus. If rheumatoid factor is present in the clinical sample it may lead to false-positive IgM reactions (Figure 1.2c).
- *Capture assays.* IgG or IgM species are captured on to the solid phase by anti-human immunoglobulin, after which antigen and then labelled antibody is added. This is the preferred method for IgM assays, as it reduces the potential for interference by rheumatoid factor (Figure 1.2d).
- *Competitive assays.* In this case, a labelled antibody in the EIA system competes for binding to immobilized antigen with antibody in the clinical sample. This assay may improve the specificity of the assay diagnosis (Figure 1.2e).

Other Antibody Detection Methods

Immunoblot (western blot) methods can be useful for confirmation of certain infections, such as human T lymphotropic virus (HTLV) and HCV infection. These are based on the detection by antibodies within a serum sample of multiple antigenic epitopes previously separated and blotted onto a membrane. Nonspecific reactions within EIAs can be clarified in these systems, since the nonspecific antibodies will react with the nonviral antigenic epitopes, and the specific ones with the viral epitopes. Immunoblot assays are expensive and technically demanding.

Other antibody-detection techniques include haemagglutination inhibition, latex agglutination (in which antibody is captured by antigen-coated particles) and IF (most widely used for EBV diagnosis), and these techniques still have a significant role in clinical laboratories.

The diagnosis of acute infection by detection of specific antibody in body fluids is particularly suited to situations in which detection of the virus itself is difficult and time-consuming, or where virus excretion is likely to have ceased by the time of investigation, such as hepatitis A, rubella and parvovirus B19. There are situations, however, where IgM is produced over a prolonged period, or in response to re-infection, as is the

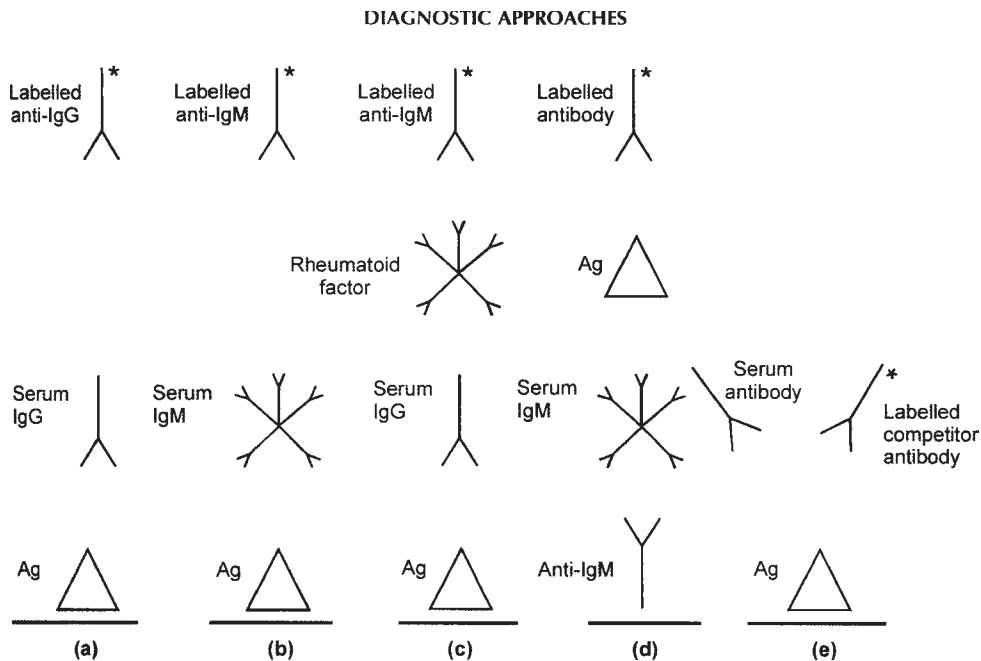


Figure 1.2 EIA formats (a) indirect IgG assay; (b) indirect IgM assay; (c) rheumatoid factor interference in IgM assay (indirect); (d) IgM capture assay; (e) competitive assay. Note that the solid horizontal lines represent the solid phase.

case for CMV. In these cases, past infection can better be distinguished from recent infection by antibody avidity tests. These are based on the principle that antibody responses mature over time, with high-avidity antibodies predominating at the later stage. By using a chaotropic agent (e.g. urea) during the EIA washing stage, low-affinity antibodies (representing recent infection) will be preferentially dissociated from antigen compared to higher-affinity antibodies (Blackburn *et al.*, 1991).

Antibody detection is also essential for diagnosis of, and screening for, persistent infections where antibodies are detectable in the presence of virus replication, such as HIV and HCV. The availability of sensitive and specific assays allows widespread screening for immunity against, for example, HBV, rubella, VZV and hepatitis A.

Despite recent advances in antibody-detection techniques, there remain inherent limitations to this form of virological diagnosis (Table 1.4). It is highly dependent on the ability of the individual to mount appropriate immune responses to infection. Thus, these methods have a limited role for diagnosing viral infections in severely immunocompromised patients (Paya *et al.*, 1989). Every effort must then be made to detect the virus itself. Transfusion or other receipt of blood products may lead to spurious serological results; for instance, leading to a false interpretation that a seroconversion, indicating acute infection, has occurred. The major role of antibody-detection tests in transplant patients is in identifying immune status at the baseline in order to forecast the risk of primary infection, re-infection or reactivation during subsequent immunosuppression (see later).

Interpretation of Serological Assays

Viral serology should take account of the patient's history and symptoms, and sometimes additional information may need to be sought. For example, the detection of very low levels of HBsAg in the absence of any other markers of hepatitis B infection might be explained by very recent immunization (because the vaccine itself is recombinant HBsAg) rather than hyperacute infection with this virus. The diagnosis of a primary virus infection can be made by demonstrating seroconversion from a negative to a positive specific IgG antibody response, or by detecting virus-specific IgM. A fourfold rise in IgG antibody titre between acute and convalescent samples can also be indicative of a primary infection (e.g. by CFT). Detection of virus-specific IgG without IgM in a single sample, or no change in virus antibody titre between acute and convalescent phase sera, indicates exposure to the virus at some time in the past. Results of antibody-detection assays can be complicated by a number of factors: the age of the patient (the production of serum IgG or IgM antibodies can be absent or impaired in the immunocompromised, neonates and the elderly), receipt of blood products with passive antibody transfer, maternal transfer of IgG antibodies and nonspecific elevation of certain virus antibodies due to recent infection with other viruses. The last phenomenon is particularly common with herpes virus infections, which have group-specific cross-reacting epitopes. IgM antibodies may persist for extended periods of time following primary infection, and may also be produced as a result of reactivation of latent infection, although not reliably so (e.g. CMV, EBV). The production of

Table 1.4 Serology

Advantages	Disadvantages
Specific IgG assays good indicator of prior infection	Retrospective (e.g. rising CFT titres). CFTs insensitive, especially to assess previous infection
Capture IgM assays good indicator of recent infection	Cross-reactivity and interference
May allow retrospective diagnosis if no acute specimen obtained	Poor sensitivity for diagnosing some congenital infections, for example CMV
Readily automated	Not appropriate for the immunocompromised
Rapid (same day)	Spurious results possible following receipt of blood products
Diagnosis of unculturable or poorly culturable viruses, for example hepatitis B	
Can use non-invasive samples such as saliva or urine	

CFT = complement fixation test.

virus-specific intrathecal antibody (requiring demonstration of an intact blood–brain barrier) can confirm the diagnosis of viral CNS infection, for example subacute sclerosing panencephalitis. Antibody-detection assays may be complemented and confirmed by molecular assays, for example PCR for HCV RNA in the presence of hepatitis C antibody or PCR for HIV provirus, used for the investigation of infants born to HIV-infected mothers.

MOLECULAR AMPLIFICATION TECHNIQUES

This is the most rapidly developing area in diagnostic virology, providing both qualitative and quantitative results. PCR and other molecular amplification techniques have now been applied to the diagnosis of virtually all human viruses and, in general, the sensitivity of these assays far exceeds that of other virus detection systems. However, the interpretation of results in a clinical setting may be difficult. A number of commercial kits and automated systems are now available, with the advantages of improved quality control and reduced inter-laboratory variability. The advantages and disadvantages of molecular techniques are summarized in Table 1.5. These issues will be discussed following a brief review of the techniques available.

The Principle of the Polymerase Chain Reaction (PCR)

This technique uses a thermostable DNA polymerase to extend oligonucleotide primers complementary to the viral DNA genome target (Saiki *et al.*, 1988). Consecutive cycles of denaturation, annealing and extension result in an exponential accumulation of target DNA. This is

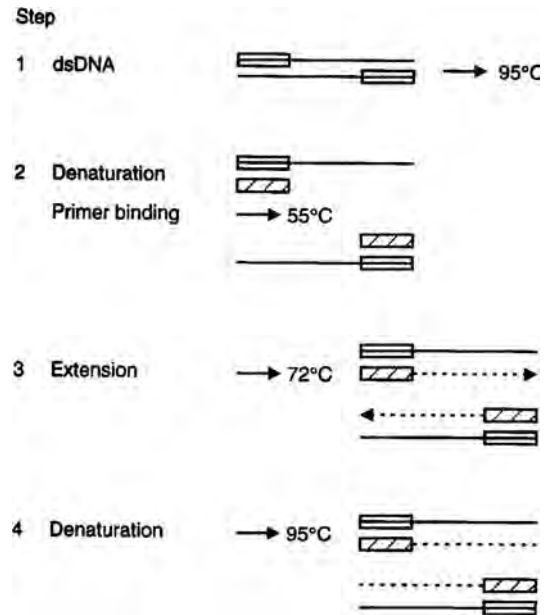


Figure 1.3 Polymerase chain reaction.

limited only by substrate (nucleotide) availability and possible competition between target genome and nontarget amplicons for reaction components (Figure 1.3). RNA genomes require transcription to complementary DNA (reverse transcription) prior to the PCR reaction. Undertaking a second round of PCR on the first-round amplicon can increase the overall sensitivity of detection (nested PCR). The second round uses a different set of PCR primers internal to the first set, and can therefore act as confirmation that the correct amplicon was produced by the first-round reaction.

Table 1.5 Molecular assays

Advantages	Disadvantages
Speed—results available in a few hours	Commercial assays expensive (but becoming relatively less so)
High sensitivity—gold standard for many viruses	High set-up costs (equipment) for ‘in-house’ assays
Wide range of applications/versatility	Susceptible to contamination
Increasing availability of automation	Rigorous quality-control systems required for ‘in-house’ molecular diagnostics
Increasing availability of commercial assay kits with built-in quality control	Some assays lack clinical validation
Increasing availability of external quality-assurance programmes	Lack of availability and expertise outside specialist centres
Amplicon can be used for sequencing/genotyping	No isolate available for phenotyping
Can be highly specific to viral subtype	Target sequence must be known and highly conserved
Quantification readily possible	
Detection of uncultivable viruses (e.g. acute HCV infection)	
Can use non-invasive samples, for example urine, saliva	

Primers

The correct choice of primers is an important determinant of the success of any PCR. The nucleic acid sequence of at least a part of the viral genome needs to be known, and primers must target a very well-conserved region. This can be done using multiple alignment programs; however, the final success of the PCR depends on the availability of sequence data from a range of different viral isolates. Otherwise, unusual viral variants may not be detected. This issue is as important for commercial assays as it is for 'in-house' assays, as was demonstrated by suboptimal HIV subtype detection by a commercial quantitative PCR (qPCR) assay (Arnold *et al.*, 1995). Other important aspects of primer design include the avoidance of secondary structure or complementarity between primers (leading to so-called primer-dimer amplification artefacts). Computer programs used to design primer sequences address these problems.

Preparation of Clinical Specimens for PCR

Viral gene detection methods do not rely on persistence of viral infectivity within the clinical specimen and in one respect this is a major advantage over traditional methods of virus detection. Specimens should be transported and stored in the refrigerator or freezer prior to analysis, but less meticulousness is required than to achieve virus isolation. However, viral RNAs are susceptible to nucleases, present in all biological material, and certain specimen types (e.g. intraocular fluids (Wiedbrauk *et al.*, 1995) and urine (Chernesky *et al.*, 1997)) contain inhibitors of PCR. PCR is therefore susceptible to false-negative results, and specimens for qualitative and especially qPCR require careful preparation. Each assay needs to be evaluated for individual specimen type and patient group. For blood samples, the anticoagulant heparin is contraindicated because it inhibits the PCR reaction. It is generally recommended that for viral quantification ethylenediamine tetraacetic acid (EDTA) anticoagulated blood is separated as soon as possible, after which the plasma can be stored frozen until analysis. If multiple tests are to be undertaken on one sample, it should be aliquoted on receipt to avoid multiple freeze-thawing. A number of different nucleic acid extraction methods are available. The choice depends on the nature of the clinical specimen and whether the target is RNA or DNA.

Detection of Conventional PCR Product

The PCR product of any specific reaction has a known size, and can therefore be detected on an agarose gel, usually by staining with ethidium bromide (or other, potentially safer, fluorescent stains such as SYBR Green) in comparison with a molecular weight ladder. However,

more than one specific band may be seen, or the band may not be of the expected size. For this reason, detection of the product by hybridization with a specific nucleic acid probe is desirable. A microtitre plate format with a colorimetric end point read by a standard spectrophotometer may be used, for example (Gor *et al.*, 1996). Many commercial PCR assays employ this system. The addition of such a step enhances the specificity of the assay, and may improve sensitivity.

Multiplex PCR

Since more than one viral target is frequently sought in each specimen, multiplex assays have been devised in which multiple sets of primers (against different targets) are combined within one PCR reaction (for example Dingle *et al.* 2004). Each set of primers requires specific conditions for optimal amplification of the relevant target, and the development of a multiplex system requires a detailed evaluation of these conditions to ensure that the efficiency of amplification for any one target is not compromised. Identification of the specific product in this system may be based on the different size of amplicons, or the use of different probes. Figure 1.4 illustrates a multiplex PCR for RSV A (RA), RSV B (RB) and human metapneumovirus with agarose gel-based detection. Increasingly, conventional multiplex PCR has been replaced by multiplex real-time PCR (see below).

Quantification

Conventional PCR is inherently a qualitative assay. Initial attempts to quantify with PCR involved the simultaneous analysis of samples with a known target genome copy number and comparing the intensity of bands on an agarose gel with that of the test specimen. However, the efficiency of amplification within any one PCR reaction can be exquisitely sensitive to changes in reaction conditions and inhibitory factors present in the clinical specimen. It is therefore important that internal standards (within the same PCR reaction) are used for quantitative competitive (qcPCR) assays. These control sequences should mimic the target genome as closely as possible, yet be detectable as a distinct entity on final analysis. This can involve the incorporation of restriction enzyme sites at which the control amplicon, but not the target sequence, can subsequently be cleaved (Fox *et al.*, 1992), or involve use of a control sequence of different size (Piatak *et al.*, 1993). Commercial assays often use a jumbled sequence as a control, with subsequent use of probes against both control and target sequences. In all cases, since the number of input control genomes is known, simple proportions can be applied to the signals to generate a quantitative value for the clinical specimen (Figure 1.5a). Real-time

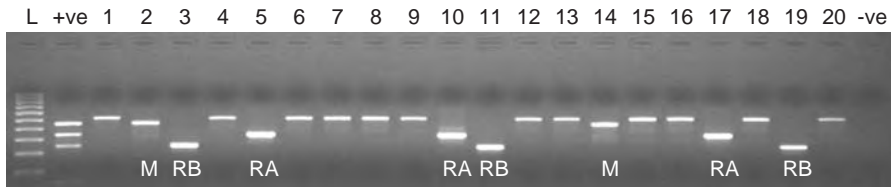


Figure 1.4 A 2% agarose gel of ethidium bromide-stained products from an internally-controlled nested multiplexed reverse transcriptase PCR for RSV A (RA), RSV B (RB) and human metapneumovirus (M). Lanes 1–20 represent 20 clinical samples; L size markers; +ve positive control; –ve negative control. Each clinical sample has been spiked with an internal control from the hepatitis delta genome. The internal control reaction is out-competed by the amplification of target genome in this assay. The asterisk indicates the position of the internal control when target viruses were not detected. (Source: Based on a figure in Dingle *et al.*, 2004.)

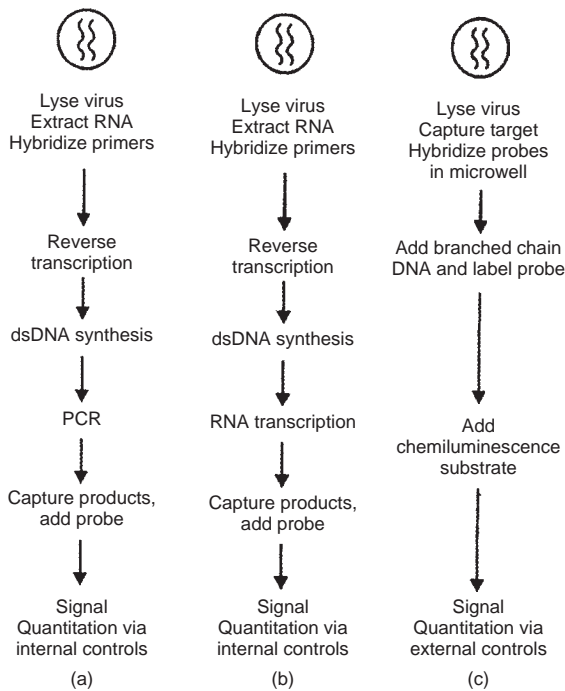


Figure 1.5 Quantitative molecular methods for HIV plasma RNA. (a) PCR; (b) NASBA; (c) bDNA.

PCR methods, as described in the next section, have now largely replaced these qcPCR assays.

Real-time PCR

The conventional PCR method described above aims to maximize the amplification reaction and depends on end-point detection of product. More sensitive detection methods allow the kinetics of the amplification to be measured, and may require fewer cycles of amplification for the product to be detected.

Real-time PCR systems allow the reactions to be undertaken within a closed system, and fluorescence generated

by the assay can be measured without further manipulation. Some of these systems produce very rapid temperature cycling times and, by also abandoning post-PCR detection procedures, this means that PCR tests can be completed within minutes. Many of the signalling technologies (reviewed in Mackay, 2004) rely on energy transfer between a donor fluorophore and a proximal acceptor molecule (fluorescence resonance energy transfer, FRET). The simplest of these involves the use of molecules, such as SYBR green, which spontaneously intercalate into dsDNA and then fluoresce when exposed to a suitable wavelength of light. Specificity of the PCR reaction for the correct product (rather than artefacts) is provided by analyzing a decrease in fluorescence at the melting (denaturation) temperature specific for that product.

5' nuclease or TaqMan oligoprobes (see Figure 1.6a) utilize the intrinsic 5' → 3' endonuclease activity of *Taq* DNA polymerase. A short target-specific probe, in which the fluorescence of the fluorophore at the 5' end is quenched by the fluorophore at the 3' end, binds to the relevant amplicon, and subsequent hydrolysis of this probe increases fluorescence (Morris *et al.*, 1996).

The method of choice for amplicon detection in the LightCycler system employs linear oligoprobes (or 'kissing' probes: see Figure 1.6c), one bearing a donor fluorophore and the other an acceptor fluorophore. Adjacent hybridization of the two probes on the denatured amplicon DNA results in a FRET signal due to interaction between the donor and acceptor.

Somewhat similarly to 5' nuclease probes, hairpin oligoprobes (see Figure 1.6c) carry a fluorophore and quencher at opposite ends. The labels are held in close proximity by homologous base-pairing of the distal ends of the oligonucleotide into a hairpin structure. Hybridization of the probe to the target separates fluorophore and quencher, resulting in increased fluorescence. The self-fluorescing amplicon concept is similar to that of the hairpin oligoprobe except that the fluorophore and quencher are attached to opposite ends of a primer (rather than a probe), distal complementary sequences of which

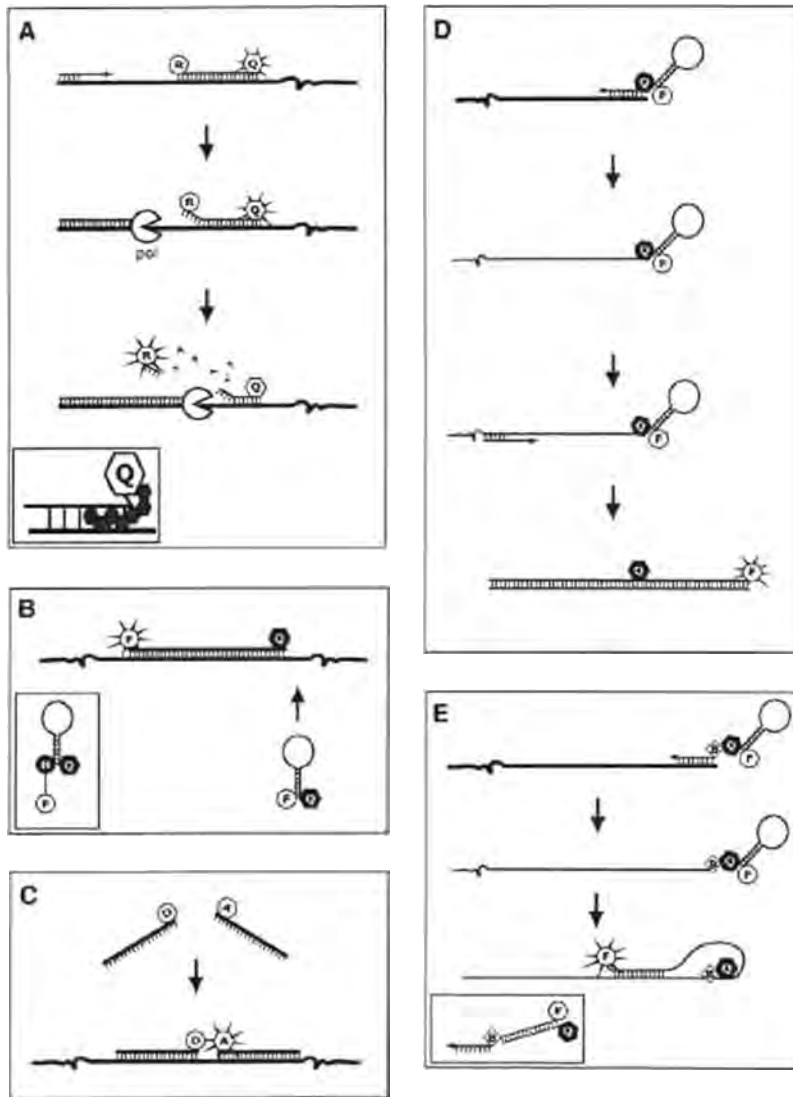


Figure 1.6 Oligoprobe chemistries. (a) 5' nuclease oligoprobes. As the DNA polymerase (pol) progresses along the relevant strand, it displaces and then hydrolyzes the oligoprobe via its 5' → 3' endonuclease activity. Once the reporter (R) is removed from the extinguishing influence of the quencher (Q, open), it is able to release excitation energy at a wavelength that is monitored by the instrument and different from the emissions of the quencher. Inset shows the non-fluorescent quencher (NFQ) and minor groove binder (MGB) molecule that make up the improved MGB nuclease-oligoprobes. (b) Hairpin oligoprobes. Hybridization of the oligoprobe to the target separates the fluorophore (F) and nonfluorescent quencher (Q, closed) sufficiently to allow emission from the excited fluorophore, which is monitored. Inset shows a wavelength-shifting hairpin oligoprobe incorporating a harvester molecule. (c) Adjacent oligoprobes. Adjacent hybridization results in a FRET signal due to interaction between the donor (D) and acceptor (A) fluorophores. This bimolecular system acquires its data from the acceptor's emissions in an opposite manner to the function of nuclease oligoprobe chemistry. (d) Sunrise primers. The opposite strand is duplicated so that the primer's hairpin structure can be disrupted. This separates the labels, eliminating the quenching in a similar manner to the hairpin oligoprobe. (e) Scorpion primers. The primer does not require extension of the complementary strand; in fact it blocks extension to ensure that the hairpin in the probe is only disrupted by specific hybridization with a complementary sequence designed to occur downstream of its own, nascent strand. Inset shows a duplex scorpion that exchanges the stem-loop structure for a primer element terminally labelled with the fluorophore and a separate complementary oligonucleotide labelled with a quencher at the 5' terminus. (Source: Reproduced from Mackay *et al.*, 2002. *Nucleic Acids Research* 30(6) pp. 1292–1305 Figure 3 (A-E), with permission from Oxford University Press.

keep the fluorophore and quencher in close proximity (see Figure 1.6d). On hybridization of the primer to its target, the fluorophore and quencher are separated from one another, and irreversibly incorporated into the PCR product.

Due to the limited number of fluorophoric labels available and the significant overlap in their emission spectra, quantification of multiplex reaction products is difficult and often not possible for more than two or three targets. Moreover, one channel is required for the detection of an internal control in order to confirm satisfactory extraction and amplification (see 'Quality Control', later). Nevertheless, a recent study reported a 'pentaplex' assay using 5' nuclease probes for four viral targets (influenza A, influenza B, adenovirus and enterovirus) together with an internal control sequence (Molenkamp *et al.*, 2007). Development of novel chemistries and improvements in real-time instrumentation and software should allow more fluorophores to be multiplexed and enhance real-time PCR assays.

The major advantage of real-time PCR is that it is inherently semi-quantitative: the quantity of target sequence present in the initial reaction mixture determines the number of temperature cycles required for a threshold fluorescence signal to be reached. An external standard curve is used to determine the relationship between cycle threshold (Ct) and input target copy number. An example of real-time detection of a calibration series for the detection of hepatitis C is shown in Figure 1.7. The dynamic range of real-time PCR of at least eight log₁₀ copies of template surmounts the problem encountered by many qPCR reactions of inability to quantify high virus loads if sensitivity at the lower end of the assay is also to be maintained

(Garson *et al.*, 2005). In addition, intra- and inter-assay variability is reduced in comparison with qPCR (Abe *et al.*, 1999; Locatelli *et al.*, 2000).

The disadvantages of real-time PCR compared with conventional PCR include an inability to monitor the size of the amplicon or to perform a nested PCR reaction without opening the system; incompatibility of some systems with certain fluorescent chemistries; and (as discussed above) relatively limited capability for multiplexed reactions because of the few non-overlapping fluorophores available. In addition, the start-up costs of real-time PCR may be prohibitive. Despite these difficulties, real-time PCR is now used routinely in many diagnostic virology laboratories, for both qualitative and quantitative applications. As with conventional PCR, real-time PCR has proven cost-effective in high-throughput laboratories when compared with traditional culture-based methods of viral diagnosis.

PCR Contamination and Control Reactions

PCR is highly susceptible to contamination from amplified products generated in a previous reaction, from target sequences cloned in plasmid vectors and from other infected clinical specimens. By contrast, a false-negative result can arise from inadequate nucleic acid extraction from a sample, or from inhibitory factors in the PCR reaction; or the sensitivity of the assay, though reduced, may not be completely inhibited. Relevant controls within each PCR run are essential for a correct interpretation of a positive or negative result, and these are highlighted in Table 1.6. The limit of sensitivity for each assay must be assessed. This can be undertaken by serial dilutions

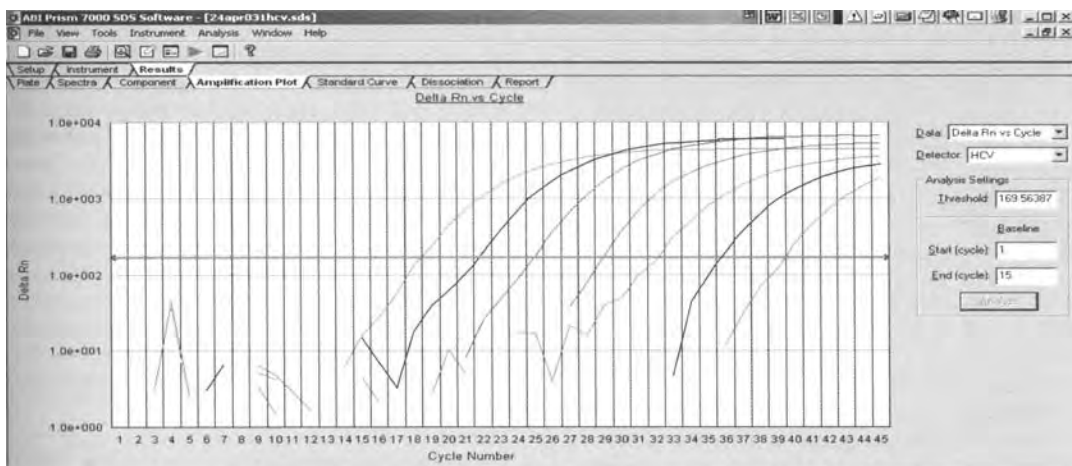


Figure 1.7 Real-time PCR detection of a hepatitis C calibration series from 10 million IU/ml down to 10 IU/ml in 1 log steps. x axis = cycle number; y axis = log fluorescence. The sample with the highest virus load (10^7 IU/ml) requires 39 cycles of PCR before reaching a detectable level. Results obtained by Dr Jeremy Garson.

Table 1.6 PCR—recommended controls

Negative controls	
Extraction control	to control for contamination during extraction (use negative clinical material)
Reagent control	to control for contamination of reagents (use solvent in which extracted nucleic acid is suspended)
Positive controls	
Extraction control	Use positive clinical material
Control genome/run control	To control for PCR efficiency, specifically to assess sensitivity
Alternate target/internal control	to control for inhibition of reaction

of a tissue culture supernatant of known median tissue culture infective dose (TCID₅₀) or virion concentration (as measured by EM), or of a preparation of purified viral genome provided at a standardized concentration. Alternatively, plasmid containing the target genome may be used, but many laboratories are reluctant to introduce plasmids into the molecular biology area because of the risk of widespread contamination with plasmid amplicons.

There are two specific procedures designed to reduce PCR contamination. Firstly, extraneous DNA contaminating PCR reagents can be inactivated by subjecting 'clean' PCR reagents to ultraviolet irradiation. This introduces thymidine dimers into the DNA chain, rendering it unamplifiable. More effective is the substitution of dUTP for dTTP in the PCR reaction (Longo *et al.*, 1990); this does not affect specific product detection. The use of uracil DNA glycosylase in any subsequent PCR reaction prevents DNA polymerization of any uracil-containing DNA, but has no effect on thymidine-containing DNA template. Thus any contaminating DNA from a previous reaction is not amplified.

Physical Organization of the Laboratory for PCR

The physical requirements for undertaking 'in-house' PCR reactions are demanding (Victor *et al.*, 1993). A 'clean room' is required for preparation and aliquoting of reagents. This must be protected from any possible contamination with viral nucleic acid. A separate area is also required for nucleic acid extraction, although this can be undertaken in a diagnostic area. A dedicated PCR room is required for setting up reactions and siting thermal cyclers. Finally, another room is required for any post-PCR analyses, such as running gels. Dedicated laboratory coats and equipment are required for each of these areas, and strict adherence to protocol by all staff is essential.

The provision of such a dedicated set of rooms for molecular biology is a challenge for busy, crowded, diagnostic virology laboratories. Nevertheless, it is paramount that diagnostic PCR reactions are undertaken with the

risk of contamination minimized, and every effort must be made to provide the relevant space if such assays are to enter the routine diagnostic armamentarium. Some of the newer automated commercial assays incorporate several of the above steps within a self-contained machine. However, it is unwise to use such assays outside of a laboratory environment in which staff are well trained in this type of work.

The Range of Other Amplification Systems

Other, mostly commercial, amplification systems include the ligase chain reaction (LCR), which, as with PCR, requires a thermal cycler. Nucleic acid sequence-based amplification (NASBA), transcription mediated amplification (TMA), strand displacement amplification (SDA) and branched chain DNA (bDNA) do not require any specialized thermal cycler. A number of newer technologies particularly suited to the simultaneous detection of multiple viral (and nonviral) targets in individual samples are also coming into use.

Ligase Chain Reaction

LCR involves hybridization of two oligonucleotide probes at adjacent positions on a strand of target DNA, which are joined subsequently by a thermostable ligase. The reaction also takes place on the complementary strand so multiple rounds of denaturation, annealing and ligation lead to an exponential amplification of the viral DNA target (Hsuih *et al.*, 1996). RNA targets require prior reverse transcription.

Nucleic Acid Sequence-based Amplification

This technique uses RNA as a target, utilizing three enzyme activities simultaneously: reverse transcriptase (RT), RNase H and a DNA-dependent RNA polymerase (Guatelli *et al.*, 1990). A DNA primer incorporating the T7 promoter hybridizes to the target RNA and is extended by RT. RNase degrades the RNA strand, and the RT then

utilizes a second primer to produce double-stranded DNA. Subsequently, T7 polymerase forms multiple copies of RNA from this DNA template. This method is suited to the detection of RNA viruses, or mRNA transcripts of DNA viruses. In addition, it can be turned into a quantitative assay using internal controls (Figure 1.5b). Different detection formats for the amplified RNA product have been developed, including electrochemiluminescence and molecular beacon detection technologies, and adapted for rapid detection of various viruses, for example West Nile and St Louis encephalitis (Lanciotti and Kerst, 2001). TMA techniques are very similar. Commercial TMA systems have been used to detect and quantify HIV and HCV nucleic acid sequences.

Strand Displacement Amplification

In this technology, an oligonucleotide primer containing a restriction enzyme site binds to its complementary (target) nucleic acid. An exonuclease-deficient DNA polymerase is used in the presence of dGTP, dATP, dUTP and a dCTP containing an α -thiol group (dCTP α S) to produce double-stranded DNA containing a restriction enzyme site. Upon binding, the restriction enzyme nicks the strand without cutting the complementary thiolated strand. The exo-DNA polymerase recognizes the nick and extends the strand from the site, displacing the previously-created strand. The recognition site is repeatedly nicked and restored by the restriction enzyme and exo-DNA polymerase, with continuous displacement of DNA strands containing the target segment. The process becomes exponential with the addition of an antisense primer containing the appropriate recognition site. SDA technology has been established in a fully-automated system known as BDProbeTec.

Hybridization Methods

These methods are based on the hybridization of a labelled oligonucleotide probe to a unique complementary piece of viral genome, and can be undertaken either on a solid phase or *in situ*. The short probes are 20–30 bases in length and can be RNA (riboprobe) or DNA. The bDNA assay is a modification of the probe assay principle and, unlike the other molecular methods described so far, it uses a *signal* amplification system rather than amplifying a *target* genome. A single-stranded genome (RNA or DNA) is hybridized to an assortment of hybrid probes, which in turn are captured on to a solid phase by further complementary sequences. Branched DNA amplifier molecules then mediate signal amplification via enzyme-labelled probes with a chemiluminescent output. This method can also provide quantitative results (De-war *et al.*, 1994; van Gemen *et al.*, 1993) (Figure 1.5c).

The hybrid-capture assay is another hybridization-based signal-amplification system in which riboprobes hybridize with DNA targets. These RNA-DNA hybrids are captured and detected by means of a labelled monoclonal antibody, which has been developed commercially and used extensively for the detection of HPV genome in cervical brushings/washings.

New Molecular Techniques

Loop-mediated Isothermal Amplification Assay

The loop-mediated isothermal amplification assay (LAMP) is another method for rapid amplification of DNA under isothermal conditions. It is based on the principle of autocycling strand-displacement DNA synthesis (Notomi *et al.*, 2000). The enzyme required is a DNA polymerase with high strand-displacement activity. A high degree of target specificity is achieved by the use of two outer primers and two inner primers, with each of the inner primers recognizing independent target sequences. The LAMP reaction results in the production of a mixture of stem-loop DNAs of different stem lengths, and cauliflower-like structures comprising multiple loops. These products can be detected by gel electrophoresis and appropriate staining, or (because pyrophosphate ion is a by-product of DNA synthesis) by monitoring the accumulation of precipitated magnesium pyrophosphate in a simple turbidometer (Mori *et al.*, 2004). Further assay refinements include the employment of an initial RT step, so as to be able to apply the technique to an RNA target, and the use of an additional pair of 'loop primers' to accelerate the LAMP reaction (Nagamine *et al.*, 2002). Very sensitive, specific and fast LAMP assays have been reported for detection of West Nile virus (Parida *et al.*, (2004)) and noroviruses (Yoda *et al.*, 2007). As the technique does not require sophisticated equipment, it is potentially valuable for use in resource-poor countries.

Microarrays

Microarrays consist of arrays of single-stranded DNA oligonucleotide probes spotted on to specific locations on a small glass slide, membrane or coated quartz microchip surface. Tens of thousands of spots can be contained on one microarray. DNA and RNA is extracted from experimental or clinical samples, and then target sequences are amplified and labelled with fluorescent markers. When these labelled nucleic acids are hybridized to complementary sequences in the array, the amount of label can be monitored at each spot, thereby indicating whether or not the complementary nucleic acid sequence was present in the original sample. Microarrays, for example the Pneu-moVir from GENOMICA SA are now entering routine

laboratory use for the diagnosis of multiple viral respiratory tract pathogens at reasonable economic cost compared with multiple multiplexed conventional PCR-based assays. In the future it is likely that they will be developed for the simultaneous detection of multiple infectious agents—bacterial, viral and fungal—in individual patient samples. By detecting specific target sequences, not only should they be able to detect the presence of a specific pathogen but they should also determine whether that pathogen demonstrates genotypic drug resistance.

Micro-bead Suspension Array Multiplex PCR

This technology combines multiplex PCR for simultaneous amplification of multiple target sequences with a coloured micro-bead detection system. For each pathogen, target-specific capture probes are covalently linked to a specific set of colour-coded beads. Labelled PCR products are captured by the bead-bound capture probes in a hybridization suspension. A dual-laser detection device identifies the colour of each bead (corresponding to a particular pathogen) and determines whether labelled PCR product is present on the bead or not, so indicating the presence or absence of the particular pathogen in the original sample. The technology offers the potential for the rapid simultaneous detection and quantification of up to 100 different analytes within a single sample. Recent studies have applied it successfully to respiratory virus diagnosis (Mahony *et al.*, 2007) and to determination of HPV type (Schmitt *et al.*, 2006).

Quality Control for Molecular Methods

Compared to more traditional virological methods, molecular biological ones are expensive. There have recently been initiatives to make all molecular assays in Europe compliant with the *in vitro* diagnostic directive (IVDD). However, many laboratories in the United Kingdom and elsewhere continue to use well-validated molecular assays developed in-house. This makes it difficult for each laboratory to evaluate each molecular assay, and there is likely to be considerable inter-assay and inter-laboratory variation (Valentine-Thon, 2002). If a noncommercial assay is employed, the critical reagents should be batch tested, and it is vital that a known low-level positive ‘run control’ is used to monitor within- and between-analytical run variability. To supplement these internal quality control measures, it is very important to participate in external quality assessment (EQA), since major clinical and therapeutic decisions are made on the basis of molecular assay results. An EQA service sends participating clinical laboratories samples on a regular basis, which they test as if they had come from patients. Results are returned to the EQA centre, which provides a report that compares

a participant’s performance with that of all laboratories and/or groups of laboratories using similar test methods. These may be commercial assays or developed ‘in-house’. Programmes such as Quality Control for Molecular Diagnostics (www.qcmd.com) provide EQA schemes for blood-borne viruses and other pathogens such as CMV, enterovirus and respiratory viruses. Participation in such schemes represents a significant but essential expenditure for diagnostic laboratories.

Automation of Molecular Techniques

With the use of highly-sophisticated robotics, the component processes of molecular assays—extraction of nucleic acid, real-time PCR reaction set-up, amplification and detection—can also be automated. Thus, it is possible for a single machine to perform a specific diagnostic nucleic acid test on a patient sample and deliver a very rapid result without any technical expertise being required at all, for example the GeneExpert System. Such self-contained, fully-integrated systems are currently very expensive however, prohibiting widespread use.

Clinical Value of Molecular Techniques

The application of qualitative and quantitative molecular analysis to human viral infections has provided new insights into the natural history of infections such as HIV, HBV, HCV and the herpesviruses. This includes the nature of viral persistence and latency, viral replication and turnover rate, and an understanding of the response to antiviral therapies. Molecular diagnostic assays have not merely increased sensitivity over alternative methods; they have resulted in the identification of a number of new viruses associated with respiratory disease: coronaviruses NL63 (van der *et al.*, 2004) and HKU1 (Woo *et al.*, 2005), and human bocavirus (Allander *et al.*, 2005).

Diagnosis of Virus Infection and Disease

Infection is revealed by the detection of virus in a clinical specimen. The infection may be asymptomatic or symptomatic (disease). However, the key determinant for correct diagnosis is the sensitivity of the assay, with a goal of detecting viral genome if it is present. A sensitive qualitative assay is relevant, for instance, in the diagnosis of HIV in infants (proviral DNA in PBMCs) (Lyll *et al.*, 2001) or acute HCV (plasma/serum RNA) infection (Aarons *et al.*, 2004). Before introducing such an assay into routine use, the sensitivity and specificity of the new test must be established, according to the formulae in Table 1.7. Note that in this instance, these parameters are compared to an existing gold standard assay (true positives or negatives) and therefore relate purely

Table 1.7 Evaluation of a new diagnostic assay

Parameter	Description	Formula
Sensitivity	Proportion of true positives correctly identified by test	$\frac{\text{true positive results}}{(\text{true positives} + \text{false negatives})}$
Specificity	Proportion of true negatives correctly identified by test	$\frac{\text{true negative results}}{(\text{true negatives and false positives})}$
Positive predictive value	Proportion of patients with positive test results who are correctly diagnosed	$\frac{\text{sensitivity} \times \text{prevalence}}{\text{sensitivity} \times \text{prevalence} + (1 - \text{specificity}) \times (1 - \text{prevalence})}$
Negative predictive value	Proportion of patients with negative test results who are correctly diagnosed	$\frac{\text{sensitivity} \times (1 - \text{prevalence})}{(1 - \text{sensitivity}) \times \text{prevalence} + \text{specificity} \times (1 - \text{prevalence})}$
Likelihood ratio	Indicates how much a given diagnostic test result will raise or lower the pretest probability of the target disorder	$\frac{\text{sensitivity}}{(1 - \text{specificity})}$

to a comparison between assays. Since molecular assays are usually more sensitive than existing assays, it is often necessary to confirm by other means that the samples positive solely by the molecular assay are indeed true positives. This can be done by confirming the identity of the PCR product, or by correlation with another marker of infection (for instance, seropositivity, where appropriate) or with the clinical background. Thus, an expanded gold standard, including positives by both existing and new assay, is used for sensitivity and specificity calculations. A useful concept in evaluating a diagnostic test is the likelihood ratio (Table 1.7), which indicates how much a given diagnostic test result will raise or lower the pre-test probability of the target disorder (Altman and Bland, 1994).

As discussed above, the nature of viral disease has had to be redefined in the light of qualitative and quantitative molecular data. Increasingly, it is possible to detect the presence of virus at low genome copy number in the absence of symptoms. This may make the interpretation of positive results problematic, and requires close clinical–virological liaison. Two approaches are possible:

1. *Qualitative detection of viral genome at a site that is normally virus-free.* A good example is the diagnosis of viral encephalitis, in which detection of HSV, CMV, VZV or enterovirus genome is diagnostic (Jeffery *et al.*, 1997). Qualitative PCR offers significant advantages in terms of speed over traditional methods of viral diagnosis and, indeed, it has been very difficult traditionally to propagate herpesviruses in cell culture from CSF samples. It is unclear whether this is a reflection of a low level

of virus, or whether there is a preponderance of disrupted, non-infectious virus produced from brain tissue into the CSF. Early diagnosis and treatment of CNS infection can improve prognosis in herpes simplex encephalitis (Raschilas *et al.*, 2002), or can reduce unnecessary treatment and hospitalization as in the case of enteroviral meningitis (Nigrovic and Chiang, 2000).

2. *Qualitative detection of virus without an exquisite level of sensitivity.* This is useful where low-level viral shedding may occur in the absence of disease. An example is the use of PCR for HSV1 and HSV2 to determine the cause of oral/genital ulceration (do Nascimento *et al.*, 1998). Another example is the diagnosis of viral gastroenteritis by detection of rotavirus, norovirus or faecal adenovirus genome in stool samples (O'Neill *et al.*, 2002).

Staging of Infection and Prediction of Disease

For many persistent virus infections with transient or continual low-level viraemia, the onset of symptomatic disease is associated with a higher viral replication rate. This provides the rationale to identify the levels of viraemia that are predictive of disease. Quantitative molecular data on CMV disease in allogeneic bone-marrow transplant patients (Boeckh *et al.*, 1996) and AIDS patients (Spector *et al.*, 1998), and similar molecular data on BK polyomavirus-associated nephropathy in renal transplant recipients (Limaye *et al.*, 2001) demonstrate the usefulness of this approach. The

capacity of a positive laboratory test to predict disease must be established by detailed prospective surveillance protocols, in order to generate positive and negative predictive values (Table 1.7). Since the natural history of viral infections (relationship between replication and disease) may be influenced by factors such as the length and nature of immunosuppression, these parameters should be determined separately for different patient groups, such as human stem cell transplant (HSCT) recipients, solid organ recipients and patients with AIDS. Large prospective studies are therefore required in each case. Standardization within commercial assay systems and/or against international unitage standards will help in this respect.

qPCR for CMV has emerged as the preferred screening method for detection of CMV viremia in patients following allogeneic stem cell and solid organ transplant. Although there are currently no universally-accepted qPCR treatment thresholds at which to start pre-emptive therapy, evidence suggests that one of $\geq 10\,000$ copies/ml whole blood is a safe and effective strategy in clinically stable patients (Verkruijse *et al.*, 2006).

Data from the Multicentre AIDS Cohort Study (MACS) has shown that a high virus load predicts a faster rate of decline of CD4+ cells (Mellors and Rinaldo, 1996). This became a guideline for initiating antiviral therapy. However, more recently the British HIV Association (BHIVA) has moved away from recommending initiation of therapy based primarily on plasma HIV RNA load. It recommends that therapy for asymptomatic established infection should be deferred until the CD4+ cell count is between 200 and 350 cells/ μ l (Gazzard *et al.*, 2006). For hepatitis B infection, there is evidence that a high viral load predicts progression to cirrhosis (Iloeje *et al.*, 2006) and the development of hepatocellular carcinoma (Ohkubo *et al.*, 2002).

Genetic Subtyping (Genotyping)

HCV is a genetically heterogeneous virus with six major genotypes (Simmonds *et al.*, 1993). Some genotypes (namely types 2 and 3) have a more favourable response to interferon-based treatment than others (Chemello *et al.*, 1994; Hadziyannis *et al.*, 2004) and genotyping therefore affects the management of HCV infection. Sequencing is the reference method of HCV genotyping. Alternative methods include a line probe assay (in which biotinylated PCR product from the 5' untranslated region (UTR) is hybridized with subtype-specific oligonucleotide probes attached to a nitrocellulose strip and detected with a streptavidin-alkaline phosphatase conjugate), subtype-specific PCR and restriction fragment length polymorphism (RFLP).

Hepatitis B is a similarly heterogeneous virus. Several studies have shown that genotype B (prevalent in the Far

East) is associated with both a better overall prognosis (Kao *et al.*, 2002; Sakugawa *et al.*, 2002) and a higher rate of interferon-induced HBeAg clearance than hepatitis B genotype C (Wai *et al.*, 2002). HBV genotyping is likely to be used clinically in future, for example in guiding appropriate antiviral treatment. In HBeAg negative, antiHBe positive patients with discordantly high viral loads (2000 IU ml⁻¹), sequencing of HBV for the presence of pre-core and core promoter mutations is becoming a common request.

Over 70 genotypes of HPV are recognized, but not all of these types have the potential to cause lesions that may progress to malignancy. A hybrid capture technique (see above) is widely used to detect the DNA of 'high-risk' (HR) HPV genotypes in cervical brushings/washings, and there is evidence that women with minor cytological disorders can be excluded from colposcopy following a negative HR HPV result (Guyot *et al.*, 2003).

Monitoring Antiviral Therapy

In recent years, viral genome quantification to monitor the effect of specific viral therapy has become part of the clinical management of patients infected with HIV, HBV, HCV and those at risk of developing CMV disease (Berger and Preiser, 2002).

For HIV, regardless of the baseline viral load, a level of 1000 copies/ml has been found to be achievable in the majority of people by four weeks from start of highly active antiretroviral therapy (HAART). Failure to achieve this is strongly associated with failure to depress viral load below 50 copies/ml within 24 weeks (Gazzard *et al.*, 2006). Clinical trial data suggest that reduction of viral load to below 50 copies/ml predicts durability of antiviral response (Montaner *et al.*, 1998; Powderly *et al.*, 1999). Thereafter the purpose of regular monitoring of plasma HIV RNA levels is to monitor the success of therapy, and current protocols recommend subsequent tests at three to four month intervals (see Chapter 39).

Three- to six-monthly monitoring of HBV DNA level is an important tool in assessing response to antiviral treatment as most guidelines propose that suppression of HBV replication is a major therapeutic goal. It may be appropriate to use shorter monitoring intervals (every three months) for lamivudine monotherapy than for other nucleoside/nucleotide analogues because of the propensity for lamivudine resistance to arise (Valsamakis, 2007).

Evidence suggests that a lower baseline hepatitis C viral load predicts a more favourable response to combination therapy (pegylated interferon and ribavirin) for chronic infection (Yuki *et al.*, 1995), and that the required duration of treatment may be shorter (Shiffman *et al.*, 2007). Moreover, HCV RNA quantification has become vital for monitoring the response to therapy. In genotype

1 and 4 infections, if the HCV RNA load has not fallen 100-fold after 12 weeks of treatment, the likelihood of viral RNA remaining undetectable six months after completion of therapy, that is of achieving a sustained viral response (SVR), is very low (negative predictive value: 97–98%) and treatment should be discontinued (NICE, 2004). This leads to cost savings and a reduction in the inconvenience and side effects of treatment (Davis, 2002). Patients who become HCV RNA negative after only four weeks of treatment have the best chance of achieving SVR (as reviewed in Poordad *et al.*, 2008).

Virological monitoring of patients receiving anti-CMV therapy is important. Not only does a high viral load predict CMV disease in a number of risk groups such as solid organ transplant recipients (Fox *et al.*, 1995), HIV infected patients (Spector *et al.*, 1998) and congenitally infected newborn infants (Revello *et al.*, 1999), but persistent viraemia following onset of therapy or virological relapse on therapy is associated with continuing disease. Conversely, in stem cell transplant recipients treated with ganciclovir pre-emptively, clearance of viraemia can be an indicator to stop therapy (Einsele *et al.*, 1995). In all cases of antiviral drug monitoring using qualitative or quantitative molecular assays, a rebound in viral load or failure to suppress viral replication may reflect reduced drug susceptibility. In these cases, it may be appropriate to undertake drug susceptibility assays (see below).

Prediction of Transmission

It is reasonable to assume that a high viral load will predict a propensity to transmit infection. Studies on vertical HIV transmission suggest that the mother's viral load is a better indicator of the risk of vertical transmission than CD4 cell count (O'Shea *et al.*, 1998). The plasma HIV load has been shown to be the main predictor of heterosexual transmission in a study of HIV discordant couples in Uganda (Quinn *et al.*, 2000), and a high HIV load in genital secretions is also associated with efficient heterosexual HIV transmission (Chakraborty *et al.*, 2001). Mother-to-infant hepatitis C transmission is associated with a high HCV load (Dal Molin *et al.*, 2002) and similarly, in a study of 155 HIV and HCV co-infected women, the maternal plasma HCV RNA was significantly higher in those who transmitted HCV to their offspring than in those who did not (Thomas *et al.*, 1998). HBeAg has long been used as a surrogate marker of a high HBV virus load and therefore of high risk of mother-to-infant transmission in pregnant women; but increasingly studies are detecting significant levels of HBV DNA in HBeAg-negative individuals (Berger and Preiser, 2002). Following a number of incidents of transmission

of hepatitis B from HBeAg-negative health care workers (HCWs) to patients, guidance (HSC 2000/020) extended the role of HBV DNA monitoring in the United Kingdom to exclude HCWs with a DNA load of $>10^3$ genome equivalents/ml from practising exposure-prone procedures, whatever their HBeAg status. More recent guidelines allow HBeAg-negative individuals with a baseline DNA load of between 10^3 and 10^5 genome equivalents/ml to practice while on antiviral therapy if their DNA load is reduced to $<10^3$ genome equivalents/ml and is monitored under careful supervision every three months (Department of Health UK, 2007).

Viral Genetic Analysis of Transmission Events

Viral genome sequencing is now a standard method for studying transmission events. Relatedness between viruses is examined against a background of genetic variation ('viral quasispecies'). In such investigations, the choice of gene targets to amplify and sequence is important, and results must be subject to the correct statistical and phylogenetic analysis for reliable evidence of a transmission event. This approach has been particularly important in the investigation of HCWs infected with blood-borne viruses such as HBV (Nguu and Teo, 1997; Zuckerman *et al.*, 1995) and HIV (Blanchard *et al.*, 1998).

Sequence-relatedness between different virus isolates is also essential for virus classification. The data used to generate phylogenetic trees are usually derived from conserved genes, such as those coding for viral enzymes or structural proteins. This type of analysis has been used recently to develop a new classification of the *Retroviridae* (www.ncbi.nlm.nih.gov/ICTVdb/Ictv/fs_retro.htm).

Detection of Antiviral Resistance

Resistance has been documented for virtually all compounds with antiviral activity, and so the emergence of antiviral resistance in clinical practice should come as no surprise. Drug susceptibility depends on the concentration of drug required to inhibit viral replication and so drug resistance is not usually 'all or none', but relative. The genetic basis of resistance is becoming better understood, and specific viral genetic mutations have been associated with resistance.

As the use of antiviral drugs increases, there will be more pressure on diagnostic laboratories to provide assays to determine the causes of treatment failure, of which drug resistance is one. Laboratory assays for drug resistance fall into two major categories: phenotypic and genotypic. Their relative advantages and disadvantages are summarized in Table 1.8.

Table 1.8 Advantages and disadvantages of phenotypic and genotypic antiviral drug resistance assays

	Advantages	Disadvantages
<i>Phenotype</i>	Represents sum of all mutations Quantitative assessment of resistance (IC ₅₀ , IC ₉₅)	Expensive Labour-intensive Slow
	Can assess cross-resistance	Selection of culture-adapted strains (not with recombinant virus assay)
<i>Genotype</i>		
(a) Selective (e.g. point mutation assay, line probe)	Quick Relatively inexpensive (PMA) Semiquantitative (PMA)	Difficult to interpret single mutation in absence of other information
(b) Sequencing	Rapid Comprehensive information Background polymorphisms detected	Expensive Labour-intensive Expertise in genomic analysis required Simultaneous mutations not necessarily on same genome

Phenotypic Assays

Though phenotypic assays have been largely replaced by genotypic methods, the plaque reduction assay remains the gold standard for detecting HSV, CMV and VZV drug resistance. A specific titre (plaque-forming unit) of virus is inoculated on to a permissive cell monolayer, usually within a multiwell plate. These monolayers are overlaid with increasing concentrations of drug in a semisolid medium, thus preventing extracellular virus spread. The plaque reduction associated with drug inhibition can then be calculated, with results expressed as IC 50 or IC 90 (concentrations of drug required to inhibit virus production by 50 or 90%). Alternative methods for HSV include the dye uptake method, which quantifies viable cells within a viral-infected monolayer. The time-consuming nature of VZV and CMV culture techniques has led to the development of rapid culture methods, using viral antigen detection or genome detection to assess drug efficacy (Pepin *et al.*, 1992). All these assays produce different IC 50 values on the same isolates, and standardization is therefore required.

For HIV-resistance testing, recombinant virus assays have been developed. In these, a PCR product amplified directly from plasma virus is recombined with an HIV clone lacking the relevant gene (Hertogs *et al.*, 1998). The fragments can include the RT gene, protease gene and gag cleavage sites, and the resulting recombinant can then be screened for susceptibility to a range of drugs. Since the background clone of virus used grows rapidly in culture, this method is faster than conventional phenotypic assays.

With the development of CCR5 entry inhibitors such as Maraviroc to treat HIV infection, it has become important to be able to determine whether a potential recipient already harbours CXCR4-using (X4) viral quasispecies. Such viruses will be resistant to a CCR5 inhibitor drug and their increasing predominance in the viral 'swarm' is associated with more rapidly advancing HIV disease. The Trofile™ co-receptor tropism assay measures the ability of a patient's virus envelope gene to mediate entry into cells expressing CXCR4 or CCR5.

Phenotypic assays are clearly important since they reflect global determinants of drug resistance, but they require propagation of a virus stock before the assay is undertaken. This process is itself selective, and may lead to the final susceptibility assay being carried out on an unrepresentative species. On the other hand, phenotypic assays based on PCR suffer from the same genetic selection limitations as genotypic methods.

Genotypic Assays

An understanding of the genetic basis of drug resistance and the availability of automated and nonradioactive methods of nucleic acid sequencing have enabled widespread assessment of viral isolates with reduced drug susceptibility. With the increasing use of antiviral medication for a number of infections, most notably HIV and hepatitis B, these assays have become part of a routine diagnostic repertoire. Genotypic assays for drug resistance in CMV have also been developed (Bowen *et al.*, 1997) and mutations associated with multidrug resistance are now recognized (Scott *et al.*, 2007).

Sequence-based methods have largely replaced selective PCR (or point mutation assays, PMAs), line probe assays and RFLP assays for specific mutations associated with resistance. Recent advances in automated nucleic acid sequencing, such as the use of capillary sequencers, allow rapid high-throughput sequencing within a clinical laboratory setting. This has been most widely utilized for HIV and hepatitis B drug resistance assays, but it has other applications, for example the study of nosocomial transmission events. The biggest challenge of this technique is the manipulation and analysis of the data generated. Sequence editing and interpretation is required. With regard to HIV drug resistance, the identification of key resistance mutations depends on interpretation of variable drug susceptibility patterns and on the software systems utilized. When based on a product PCR amplified from the plasma, these techniques provide information only on the majority population within the quasispecies. They cannot exclude different mutations existing on separate genomes.

'Virtual phenotyping', a technique developed by Virco (www.vircolab.com), is a method of interpreting genotypic HIV resistance information with the aid of a large database of samples with paired genotypic and phenotypic data. By searching the database, viruses with genotypes similar to the patient's virus are identified and the average IC 50 of these matching viruses is calculated. This information is then used to estimate the likely phenotype of the patient's virus.

RECOMMENDED DIAGNOSTIC INVESTIGATIONS

Making an accurate virological diagnosis is critically dependent on receiving adequate specimens with information relating to the onset of symptoms and the clinical presentation. Swabs and tissue samples should be collected by trained staff and placed into virus transport media. If sample transport is delayed, samples should

be stored at 4°C or on wet ice (for a maximum of 24 hours). Assays for quantifying virus in blood require rapid specimen transport and appropriate processing and storage prior to analysis. It is the role of the clinical virologist to decide on the most appropriate investigations for any given clinical scenario. Laboratory request forms are important in this respect, and should encourage full documentation of clinical details. The practice of sending a serum sample to the virology laboratory with a request for a general 'screen' should be strongly discouraged. Instead diagnosis should be built upon the concept of syndromic presentation, its initial and continuing investigation, and appropriate management developing out of clinical progress, diagnostic findings and response to treatment. If the clinical presentation proves to be due to a virus infection, the virologist may have a leading role, but liaison with haematological, radiological, pharmacological and other clinical colleagues must be appropriate and continued.

Test Selection

Much of the work presenting to a virology laboratory is straightforward, taking the form of a particular screen (Table 1.9). But in other cases clinicians are presented with a patient whom they suspect may have a viral infection, yet they are unsure which tests to request. In some cases, they may request inappropriate tests. One of the most important roles of the laboratory staff, both technical and medical, is to assist the clinician in obtaining the correct diagnosis by choosing appropriate tests. In some cases sufficient clinical details on request forms will allow test selection in the laboratory (not necessarily the tests requested by the clinician!). In other cases, further information and possibly additional and different sample types will be needed. Many virology laboratories also provide serological testing for nonvirological infections, for example syphilis, toxoplasma and chlamydia (serological and nucleic acid-based testing).

Table 1.9 Examples of suggested screening assays for specified patient groups

Pre-stem cell transplant screen (donor and recipient)	HIV (i.e. combined testing for anti-HIV 1 and 2 and for HIV 1 p24 antigen), anti-HCV, HBsAg, anti-HBcore, anti-HTLV 1, CMV IgG, EBV EBNA IgG, VZV IgG, anti-HSV, syphilis, toxoplasma-Ab
Renal dialysis patients	HIV (i.e. combined testing for anti-HIV 1 and 2 and for HIV 1 p24 antigen), anti-HCV, HBsAg pre-dialysis Anti-HCV, HBsAg three-monthly. HIV repeat testing based on risk assessment
Antenatal screening	HIV (i.e. combined testing for anti-HIV 1 and 2 and for HIV 1 p24 antigen), HBsAg, Rubella IgG, syphilis

Table 1.10 Suggested testing strategy for the investigation of hepatitis and abnormal LFTs, with or without jaundice

First line	HAV-IgM, HBsAg and HBcore IgM, anti-HCV
Second line	EBV-IgM, CMV-IgM, HCV-RNA, hepatitis E IgM (any could be first-line, depending on history)
Third line	Dengue, yellow fever, leptospirosis, enterovirus, adenovirus and others depending on age, clinical details, travel history and so on

In the UK, the Health Protection Agency has proposed a number of national standard operating procedures (www.hpa-standardmethods.org.uk) that can help in suggesting a strategy for testing (e.g. VSOP 6: Hepatitis, jaundice and abnormal LFTs); but as illustrated in Table 1.10, the individual patient needs to be taken into account in order to select a cost-effective strategy for testing. There are also many situations where tests will be requested for which there is no evidence

base or recommendation to support testing, and many laboratories have developed brief clinical/educational comments explaining why such testing is thought not to be appropriate. Examples are given in Table 1.11. Clinical liaison leads to samples being used more appropriately, for example proposing hepatitis C antibody testing for a fatigued ex-drug user. Screening requests such as ‘TORCH’ should be discouraged in favour of test selection in response to specific clinical details. Testing for congenital and perinatal infection is complex, and for a neonate/infant is likely to require access to earlier stored samples such as the maternal antenatal booking sample or the infant’s dried blood spot taken at birth (the Guthrie card). Exposure to rash illness in pregnancy is very common (e.g. chickenpox, B19 virus) and national guidelines exist on rash illness and exposure to rash illness in pregnancy (www.hpa.org.uk/infections/topics_az/pregnancy/rashes/default.htm).

Table 1.12 illustrates some suggested first-line serological tests for common clinical requests in the United Kingdom. Selecting an appropriate repertoire of serological tests for a routine diagnostic laboratory and achieving an appropriate balance between testing samples in-house and sending samples to other laboratories

Table 1.11 Examples of comments for samples where serological testing may not be clinically indicated

Request	Comment
Chronic fatigue screen	Serological tests are of low value for investigating chronic fatigue. We are happy to discuss individual cases. Sample stored
Meningitis/encephalitis screen	Viral serology in the first week of meningitis or encephalitis is usually unhelpful. The sample has been stored. CSF is the best sample
?HSV infection	Serological testing is unlikely to be helpful. Please send appropriate sample (CSF, or swab of skin/mucosal lesion in viral transport medium) for viral culture ± PCR
Abdominal pain/diarrhoea. Viral screen please	There are no useful serological tests for viruses that cause primarily vomiting, diarrhoea or abdominal pain
Intrauterine death (IUD)/miscarriage with ‘TORCH’ screen requested	Serological tests are not routinely performed in cases of IUD/miscarriage unless there are clinical features suggesting a viral aetiology. If this is the case please contact the laboratory
Measles/mumps/rubella (MMR) screen	The assays that measure IgG to measles and mumps have not been validated for the determination of protection against these diseases: they were designed to assist in the diagnosis of acute infection. Therefore, it is not appropriate to use these assays for the purpose of excluding the need to give MMR
Atypical pneumonia/influenza	Testing acute samples for atypical and influenza serology does not contribute to acute patient management. Sample stored. If legionella infection is suspected, please send a urine sample for legionella urinary antigen

Table 1.12 Suggested first-line serological tests for common clinical requests in the United Kingdom

Clinical details	First-line tests
Arthralgia/joint pains	B19 IgM, HBsAg, Rubella IgM. Consider toxoplasmosis IgM if myalgia
Atypical pneumonia/influenza	Acute sample store. See above suggested comment. Testing of convalescent samples provides retrospective diagnosis and epidemiological data
Chronic fatigue	Samples not tested until discussed with clinician
Encephalitis	As indicated by clinical details. See HPA protocol QSOP 48
Endocarditis (culture negative)	Q fever, bartonella, chlamydia species, others as indicated
Glandular fever/lymphadenopathy	EBV-IgM, CMV-IgM, consider toxoplasma IgM and HIV test, bartonella if cat scratch a possibility
Hepatitis/abnormal LFTs	See Table 1.10 (also HPA VSOP 6)
Pregnancy and congenital infection—illness in the mother, abnormal fetal ultrasound findings or possible neonatal infection	Strategy depends on nature of illness, clinical findings and local protocols. ‘TORCH’ screening should be discouraged
Rash illness—maculopapular	Depends on local epidemiology. Consider B19, rubella, measles
Rash illness—vesicular	Depends on local epidemiology. HSV and VZV infections usually best diagnosed with a vesicle swab for PCR-based direct virus detection
Rash illness—exposure in pregnancy	See HPA protocols VSOP 33 and www.hpa.org.uk/infections/topics_az/pregnancy/rashes/default.htm

will depend on the size of laboratory, the population served, ease of testing and the throughput of tests. Many assays are best performed in batches, and in order to maintain an acceptable turnaround time it is not practical to provide a comprehensive repertoire in every laboratory. However, the increasing availability of multiple analyser systems that provide a wide range of cross-discipline immunoassays, random access facilities and automation allows even the smaller laboratory to offer a wide range of serological assays. This has to be balanced against the savings that can be made with economies of scale. In addition, obtaining a battery of immunoassays from a single commercial company may mean that compromises have to be made with certain tests not having the optimum sensitivity and specificity for the particular local population.

FUTURE TRENDS

In the light of rapid assay development and the continuing identification of new viruses, clinical protocols require constant updating. The era of retrospective viral diagnosis is over, replaced by rapid techniques which impact directly on clinical management. Competition for health care resources has meant that new techniques have replaced more traditional methods with limited clinical

value. Clinical virologists have to work closely with their clinical colleagues to establish new diagnostic criteria, develop protocols for use of antiviral drugs and monitor patients with persistent infections. The diversity of diagnostic methods now available makes communication between physicians and clinical virologists more important than ever before. The present multiplicity of traditional and new diagnostic techniques, as described above, suggests that a rationalization is now overdue. This will involve the abandonment of some older technologies, more discriminatory use of newer, especially molecular, ones, and technical collaboration with other pathology disciplines to improve efficiency and shorten turnaround times. Laboratories will need to embrace the electronic patient record (EPR) and remote requesting (‘order communications’), which, if well managed, will improve the quality of service provided by the laboratory, improving patient care. Laboratory information management systems will have to be fully integrated with the EPR in order to maximize the benefits from new information technologies.

A special emphasis must be placed on the needs of the immunocompromised patient population. They may experience life-threatening viral infections, which can present atypically. Ongoing antiviral prophylactic therapy may distort the nature and timing of presentation. Routine monitoring of transplant recipients is important

so that pre-emptive or early therapy can be initiated or immunosuppressive therapy modified as appropriate. Precise monitoring protocols will depend on the patient group concerned, availability of laboratory facilities and budgetary constraints. Nevertheless, in the context of high-risk patients such as those receiving long-term chemotherapy or transplants, the relative cost of virological investigations will be small. This population is constantly changing and expanding with new procedures and immunosuppressive agents, and the range of organisms to be monitored is likely to increase.

Alongside the increase in laboratory automation there is an increase in the integration of clinical virology with clinical microbiology laboratories. A step further is more extensive automation that integrates diagnostic immunoassay facilities across pathological specialities. This leads to economies of scale, though it may reduce the skill base and number of individuals trained in traditional virological techniques such as tissue culture and EM. Already, the distinctions between bacteriology and virology diagnostic techniques are becoming blurred, both providing opportunities for those skilled in molecular diagnostics and strengthening the practice of molecular diagnostics. With increasing automation in molecular techniques, the majority of general microbiology laboratories in the United Kingdom now offer at least a limited repertoire of molecular assays. For example, nucleic acid-based testing for *Chlamydia trachomatis* and other commercially-available assay systems are also being used for presurgery methicillin resistant *Staphylococcus aureus* (MRSA) screening and the detection of bacteria in sterile site samples by a PCR for 16S ribosomal RNA. Microbiology laboratories of the future may have to participate in bioterrorism surveillance for multiple infectious pathogens and collaborate in the rapid development of tests for new diseases of high social impact, as recently shown for the SARS caused by SARS-CoV (Raoult *et al.*, 2004).

There is a corresponding change in the training of medical staff in the United Kingdom, with broader based training across the infection specialities. There is a new cohort of physicians qualified to practice both as infectious disease clinicians and as laboratory-based staff. With possible future centralization and 'factory style' diagnostic testing facilities, laboratory-based specialists need to strengthen their role in clinical consultation and front-line management of patients, for example in hepatitis and HIV clinics and on transplantation ward rounds. For some, this broad-based training in infection demands a radical change in attitude and outlook.

We live in a global village, with rapid international travel and communication and the possibility of major environmental transformation due to climate change. Deforestation and expanding urban development facilitates

epidemic spread of infection by bringing human and animal populations closer together, allowing pathogens to 'jump' species. The threat of bioterrorism must not be neglected. These factors make human and animal populations more vulnerable than ever before to the epidemic spread of novel (as well as well-known) viruses, as seen in recent years with SARS-CoV and avian H5N1 influenza. In recognition of this, the World Health Organization (WHO), within the framework of the International Health Regulations, has developed a vision that '*every country should be able to detect, verify rapidly and respond appropriately to epidemic-prone and emerging disease threats when they arise to minimize their impact on the health and economy of the world's population*'. This should be achieved by focusing on three principles: contain known risks, respond to the unexpected and improve preparedness (www.who.int/csr/about/en/#strategy).

In rural, resource-poor areas of the world such as parts of South East Asia and Africa, little is known about the epidemiology of many viral infections. The burden of viral disease in such areas is likely to be considerable, but the infrastructure to implement the cross-sectional epidemiological studies necessary to define the causes of infectious disease is not available, largely due to economic constraints. Research in such resource-poor areas is fragmentary and often focuses on the interests of a specific individual or group. The developed world has been able to embrace the recent advances in diagnostic virology and improve clinical care, but in resource-poor areas basic diagnostic facilities which could go some way towards delivering the WHO vision are not available. For example, basic laboratory testing to support the roll-out of highly active anti-retroviral therapy (HAART) is often lacking in resource-poor settings. An important challenge for the infection community is to develop cost-effective and robust diagnostic and monitoring assays for use in the developing world. The discipline required to do this may also bring benefits by helping to rationalize the congested work schedules in laboratories in the developed world.

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Viral Transmission: Infection Acquired by the Blood-borne Route

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INTRODUCTION

Blood (or other bodily fluids, Table 2.1) is a potential infection source, mediating transmission of viruses from one individual to another. Although humans are not often exposed to each others' blood, blood-borne transmission can arise in a variety of settings, some perhaps more obvious than others:

- transfusion of blood or blood products from an infected individual;
- organ or tissue transplantation from an infected donor;
- sharing or re-use of blood-contaminated needles and syringes, whether for medicinal or recreational purposes, or through activities such as acupuncture, tattooing or body piercing;
- accidental inoculation injuries with blood-contaminated needles/sharps;
- exposure to blood splashes, or to blood-contaminated objects and instruments; and
- exposure to contaminated maternal blood, either ante- or peri-natally.

Blood-borne transmission of infection is a particular concern in the context of the provision of health care. Blood/blood-product transfusion is an obvious potential hazard for the recipient, but blood sampling or instrumentation of infected individuals is also commonplace in health-care settings, resulting in potential contamination of both sharps and the environment, and thereby creating possibilities for nosocomial spread. The literature is replete with outbreaks of infection with a variety of viruses,

but particularly the 'big-three' blood-borne viruses (BBVs) of human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV), arising from blood-borne transmission in a health-care setting. As these risks have become recognized, so it has become necessary to devise policies and guidelines to prevent, or at least minimize the chances of, such outbreaks occurring.

It is useful to consider three particular circumstances under which BBV transmission may occur within health-care settings:

- patient-to-patient transmission;
- patient-to-health-care worker (HCW) transmission; and
- HCW-to-patient transmission.

Table 2.1 Body fluids which should be handled with the same precautions as blood

Cerebrospinal fluid
Peritoneal fluid
Pleural fluid
Pericardial fluid
Synovial fluid
Amniotic fluid
Semen
Vaginal secretions
Breast milk
Any other bodily fluid containing visible blood, including saliva in association with dentistry

Adapted from (Department of Health, 1998).

Strategies designed to minimize the risks of nosocomial transmission of BBV need to address:

1. prevention of exposure to blood or other bodily fluids through the establishment and maintenance of high standards of infection control; and
2. prevention of infection through the implementation of policies for the pre-exposure prophylaxis (where possible) and post-exposure management of patients and HCWs should a needlestick or other exposure happen.

PREVENTION OF EXPOSURE THROUGH INFECTION CONTROL

Good infection control practice provides a firm basis for limiting all nosocomial BBV transmission. General principles of infection control have been elaborated in detail in UK guidance (Department of Health, 1998). Specific guidance for reducing the risk of percutaneous and mucocutaneous exposure during surgical procedures is also given in this document, including measures to reduce eye and other facial exposure. Other general measures which should be taken to prevent HCW exposure to BBVs include adequate sterilization, disinfection and cleaning of equipment and work surfaces (Department of Health, 1996).

Approaches to reduce hazardous exposures of HCWs to BBVs can be categorized as (i) the taking of special precautions for patients or specimens known or suspected to be infected or (ii) the adoption of universal precautions to cover all patients and specimens, the principle of which is that the level of precautions taken depends on a risk assessment of the procedures being undertaken, rather than on the patient's infection status. In recent years there has been a major shift towards the latter (Department of Health, 1996), one of the main reasons being that for every patient known to be infected with a BBV, there will be many more in routine clinical practice where this status is not known or even suspected. There is also evidence to suggest that knowledge of a patient's high-risk status may not reduce the risk of blood exposures during surgical procedures (Gerberding *et al.*, 1990), whilst practicing universal precautions does reduce the frequency of blood exposures among trained HCWs (Beltrami, 2000; Fahey *et al.*, 1991).

Other possible approaches to prevent the occurrence of inoculation injuries include the development of safer surgical techniques, avoidance of the use of sharp instruments where alternatives exist (e.g. when suturing), better training of junior surgeons using surgical rigs, effective systems for collecting and disposing of used sharps, and the design of safer needle and syringe combinations

which reduce needle handling and automatically protect the needle after use.

PREVENTION OF INFECTION THROUGH SPECIFIC PRE- AND POST-EXPOSURE POLICIES

The nature of pre- and post-exposure interventions differs depending upon which of the three transmission circumstances is being considered.

PATIENT-TO-PATIENT TRANSMISSION

This may arise in several ways.

Transfusion of Contaminated Blood/Blood Products

Blood transfusion services have adopted several measures to protect the blood supply, including rigorous procedures for selection of donors, and appropriate testing of donors for evidence of infection. The importance of donor selection cannot be over-emphasized. The World Health Organization (WHO) asserts that blood donation should be 100% voluntary and free of incentives. Countries which have never used paid donors have a much better record in terms of BBV transmission by blood or blood-product transfusion than those where donors have been paid—the latter process discouraging individuals from declaring risk factors in case of rejection. Anyone wishing to be a donor in the United Kingdom is given an explanatory leaflet which asks them to self-defer if they have identifiable risk factors. In addition, first-time donor interview reduces the infectious risk 100-fold. The safety of repeat as compared with first-time donors shows a significant difference (Table 2.2).

All individual donations should be tested—in the United Kingdom it is mandatory to screen for evidence of HIV, HBV, HCV, human lymphotropic virus (HTLV)-1 and syphilis infection. According to the WHO, failure to introduce donor testing results in up to 16 million HBV, 5 million HCV and 160 000 HIV infections worldwide per year (Editorial, 2007). The modality of testing differs for each organism and between countries. Current practice in the United Kingdom is:

HIV—combination anti-HIV and antigen enzyme immunoassay (EIA) testing of all donors; pooled (48 members) nucleic acid testing (NAT) of all donors.

HBV—hepatitis B surface antigen EIA testing.

HCV—anti-HCV EIA testing of all donors; pooled (48 members) NAT of all donors.

Table 2.2 Risks of BBV transmission by UK blood donors

Incidence	Per million donations			1 per <i>x</i> million donations		
	All	New	Repeat	All	New	Repeat
HIV	0.24	0.58	0.20	4.22	1.72	5.01
HCV	0.03	0.13	0.02	35.52	7.92	57.41
HBV	0.80	2.51	0.61	1.25	0.40	1.63

HTLV—anti-HTLV (I and II) EIA testing on pools of 48 samples.

syphilis—TPHA, TPPA or EIA testing.

Some donations are also tested for antibodies to cytomegalovirus, malaria and *Trypanosoma cruzi*. Donors with a tattoo or skin piercing since their last donation are deferred for six months and then screened for anti-hepatitis B core.

Despite all of the above, BBV transmission by transfusion still occurs. In the United Kingdom, from 1995 to 2006 there were a total of sixty infections and thirteen deaths arising from transfusion. Twelve recipients acquired HBV infection from eleven infected donors, two acquired HCV infection (two donors) and four acquired HIV infection (two donors). In the same period there were thirty-three bacterial transfusion transmitted infections (TTIs), with eight deaths (CDR, 2007). One cause of TTI is the presence of window-period donors; that is, individuals with recent infection, in whom there is not yet a detectable antibody response, or in whom the HBsAg antigen level is below the detection limit of currently available assays. The development of sensitive HIV antigen detection assays resulted in some closure of the window period for this infection. Much attention has been paid recently to the use of NAT as a way of reducing the window period even further. HIV and HCV NAT testing has been introduced in the United States, resulting in an estimated prevention of about five HIV and fifty-six HCV infections per year at an average cost of around \$2 million per infection prevented (Goodman, 2004). Currently, only HCV NAT is used on all donations in the United Kingdom. In the first two years since its introduction in 1998, about 7 million donations were tested and three window-period donors identified, considerably less than anticipated. The estimated cost per quality-adjusted life year (QALY) gained by HCV NAT screening is in excess of £7 million (Simmonds *et al.*, 2002). The cost-effectiveness of NAT is thus the subject of some debate. The development of HCV combined antibody and antigen EIA assays may offer a cheaper alternative to NAT, albeit at the expense of some loss in sensitivity.

Two possible strategies for the reduction of HBV TTIs have been proposed, but both have drawbacks.

Screening for antibodies to HBV core antigen (anti-HBc) may identify donors who are HBsAg negative but have low levels of HBV DNA. This pattern of reactivity may arise after acute infection, when the individual is in the process of clearing infection (i.e. this is not 'window-period' infection). However, anti-HBc assays may yield significant numbers of false-positive reactions (poor specificity), and deferral of all anti-HBc positive donors would also lead to significant losses of safe donations from individuals who had truly cleared infection. HBV NAT testing is also possible, but is likely to be of limited benefit in countries, such as the United Kingdom, which use extremely sensitive HBsAg assays, capable of detecting around 0.1 ng ml^{-1} HBsAg, equivalent to the sensitivity of 24 sample minipool HBV NAT for the identification of window-period infections. Individual NAT may have an increased sensitivity, but would also increase costs considerably.

Infection risks from transfusion may also be reduced by the use of viral inactivation steps such as heat or solvent-detergent treatment. These methods have been used for many years on pooled fractionated products such as factor VIII or immunoglobulin solutions, rendering them safe from HIV, HBV and HCV transmission. Application of these, or other, inactivation procedures to labile blood products, including cellular components, is of interest, but technically difficult (Council of Europe Experts Committee in Blood Transfusion Study Group on Pathogen Inactivation of Labile Blood Components, 2001).

Thus, even with all the above precautions, transfusion of blood or blood products can never be guaranteed to be free of the risk of BBV transmission. For some agents transmitted by this route, there are no appropriate validated screening tests currently available, for example prion diseases. For other infections, it is deemed not to be cost-effective to screen, for example parvovirus B19 and hepatitis A virus. Even for the big-three BBVs, where screening is performed there remains a residual risk of transmission, of the order of 1 in 2 million donations for HIV and HCV in the United States, for example (Stramer *et al.*, 2004). The residual risks of infection from transfusion in the United Kingdom are given in Table 2.2.

Organ/Tissue Transplantation

Prevention of BBV transmission by organ or tissue transplantation is achieved largely through screening organ and tissue donors. Requirements for microbiological testing of donors are set out in official UK guidance (Department of Health, 2000). Selection of organ donors cannot be as rigorous as for blood donors, particularly in near-death settings. Thus, as a generality, organ donors are more likely to have a BBV infection than blood donors. There are also additional difficulties in testing when the only donor samples available for screening are taken post-mortem.

Organs and tissues may also be donated to, or accepted from, countries abroad. In addition to local guidelines, there are also European Union directives covering the technical requirements for the donation, procurement and testing of human tissues and cells (European Union Directive, 2006). These may differ from, and therefore supersede, national guidelines. One example of discrepancy is the need for anti-HBc testing, which in the UK guidance is only necessary where liver transplantation is being considered, but is required for all donated organs and tissues in the EU directive.

Exposure to Blood-contaminated Instruments/Environments

Blood sampling of a BBV-infected patient creates an immediate opportunity for nosocomial transmission of that infection. Common errors in infection control leading to outbreaks of infection include the use of multidose vials, or re-use of improperly sterilized sharps or instruments. The largest recorded nosocomial outbreak of HCV infection was in a haematology/oncology outpatients department, involving 99 transmissions, most probably arising because of the practice of using shared saline bags to flush through intravenous lines (Macedo de Oliveira *et al.*, 2005). However, in many instances, the precise route of transmission in a particular outbreak cannot be identified with certainty.

Nosocomial BBV transmission has been a particular concern in the context of renal haemodialysis units, and thus specific guidelines have been produced and updated to minimize this risk (Department of Health, 2002a). The principles of these are:

- strict adherence to universal precautions (defined above);
- immunization of patients (and staff) against HBV infection;
- regular (e.g. three-monthly) screening of all patients for evidence of HBV, HCV or HIV infection;
- segregation of patients returning to the unit who have been dialysed outside the United Kingdom ('holiday

dialysis'), until such time as they have been shown to be negative for HBsAg, anti-HCV and HCV RNA;

- enhanced surveillance for one or more BBV and/or continued segregation for patients returning from dialysis abroad, the precise details of which will depend on a risk assessment;
- known HBV-infected patients should be dialysed in isolation on dedicated machines; and
- known HCV-infected patients should be segregated from the main unit when undergoing dialysis. Segregation of HIV-infected patients should be considered, based on a local risk assessment.

Exposure to Infected Maternal Blood

Whilst not a cause of nosocomial BBV transmission, this is nevertheless an important route whereby BBV may be transmitted from one individual to another. Detailed discussions of the management of such transmission can be found in the relevant virus-based chapters.

PATIENT-TO-HCW TRANSMISSION

Epidemiology

Blood exposure incidents are, unfortunately, all too common for HCWs in a hospital setting. It is estimated that more than 380 000 needlestick injuries occur in US hospitals each year (Gerberding, 2003). Studies of sharps injuries in operations report rates of 1–7% (Camilleri *et al.*, 1991; Gerberding *et al.*, 1990; Hussain *et al.*, 1988; Williams, 1997). Risk factors include long procedures, high blood loss, major operations and wound closure with staples. Needlestick injuries are also more common after a night on-call (Ayas *et al.*, 2006). By their final year in training, 99% of surgical residents in a survey in the United States had suffered a needlestick injury, and in over half, the injury had involved a high-risk patient (Makary *et al.*, 2007). Other potential exposures include splashing of contaminated blood or bodily fluids onto mucous membranes (conjunctivae, oropharynx) or skin. Whilst intact skin is impermeable to HIV, HBV and HCV, transmission through this route has been reported in individuals with non-intact skin, for example those with chronic dermatitis or with cuts and abrasions.

In the United Kingdom, a surveillance system was established in 1996 for the reporting of significant occupational exposure incidents. The period 2002–2005 saw a 49% increase in such incidents. The greatest proportion of percutaneous exposures was to HCV. Numerically, the largest number of incidents involved nursing staff, but proportionately more injuries were suffered by doctors and dentists. Between 1997 and 2005, over a third

(607/1669) of all incidents were deemed to have been preventable had there been proper adherence to universal precautions and safe disposal of clinical waste (Health Protection Agency, 2006).

Hepatitis B

Serological surveys conducted prior to the introduction of HBV vaccination clearly identified an increased prevalence of HBV markers in HCWs exposed to blood and bodily fluids, for example in the United States the overall risk to persons employed in health-related fields was four times that of the general adult population, with even higher risks in physicians and dentists (5–10-fold), and surgeons and laboratory workers (greater than 10-fold) having frequent contact with blood samples (West, 1984). Similar conclusions can be drawn from surveillance of acute hepatitis B infections in the United Kingdom. Whilst the average annual rate of infection in men in the whole population between 1980 and 1984 was 6 per 100 000, this rose to up to 37 per 100 000 for HCWs (Polakoff, 1986). Again, surgeons and laboratory workers were the subgroups with the highest rates of infection.

The risk of HBV transmission after accidental inoculation of infected blood is 19–31% from HBeAg-positive source patients, compared to 1–6% for HBeAg-negative sources (Grady *et al.*, 1978; Masuko *et al.*, 1985; Seeff *et al.*, 1978; Werner and Grady, 1982). Data to allow estimations of the risk of HBV transmission after other types of exposure are not available, but there must also be at least a theoretical risk of transmission by exposure of mucous membranes to blood splashes. There are no reports of transmission of HBV via blood contact with intact skin. Hepatitis B transmission has been reported following human bites (Cancio-Bello *et al.*, 1982; Hamilton *et al.*, 1976; MacQuarrie *et al.*, 1974), presumably related to inoculation of the biter's blood-stained saliva. This sort of exposure may be experienced by workers with mentally-disturbed or subnormal patients, or by police and prison officers.

Hepatitis C

In contrast to HBV, serosurveys for evidence of HCV infection conducted in a variety of different countries and health-care settings do not consistently show strikingly higher rates of infection amongst HCWs compared to the general population (reviewed in Irving and Harling, 2005). However, there are well-documented examples of acquisition of HCV infection by HCWs through needlestick injury, some of which have been confirmed by molecular evolutionary analysis, and transmission by a blood splash into the conjunctiva has been reported (Sartori

et al., 1993). There are no reports of transmission of HCV via blood contact with intact skin.

An extensive review of published data described 39 acquisitions of HCV infection by HCWs from 2506 needlestick exposures, a rate of 1.6% (Henderson, 2003). The Centers for Disease Control (CDC) guidelines in the United States quote a similar figure of 1.8% (Centers for Disease Control, 2001).

The UK surveillance scheme referred to above has identified 11 HCWs who acquired HCV infection following a needlestick injury. At least five of these exposures occurred through noncompliance with appropriate policies for infection control and disposal of sharps. The first nine of these HCWs are known to have cleared infection, either spontaneously or following therapy.

Human Immunodeficiency Virus

The risk of HIV transmission after a percutaneous exposure to known HIV-infected blood, in the absence of post-exposure prophylaxis, is of the order of 0.3%. Mucocutaneous exposure carries a risk of around 0.09% (95% confidence interval, 0.006–0.5) (Centers for Disease Control, 1991). Table 2.3 shows data relating to factors influencing the risk of needlestick transmission, derived by the CDC from a large retrospective survey (Cardo *et al.*, 1997).

Table 2.4 shows the number of documented and possible transmissions of HIV from patient-to-HCW reported in the world literature to December 2002 (HPA, 2005). These data are derived from countries with established surveillance systems, and therefore there may be significant under-reporting from South East Asia, the Indian subcontinent and Africa. 96/106 documented cases arose as a result of percutaneous exposure. Nurses and clinical laboratory workers account for 69% of documented cases, whilst 13% are doctors (including medical students). Only one case was in a surgeon.

Table 2.3 CDC case-control study of HIV seroconversion in HCWs after percutaneous exposure. Data sourced from Cardo *et al.*, NEJM 1997; 337; 1485–90

Risk depended on	Odds ratio	95% CI
Deep injury	15	6.0–41
Injury with device visibly contaminated with blood	6.2	2.2–21
Needle from vein or artery	4.3	1.7–12
Source patient died within two months	5.6	2.0–16
PEP (zidovudine)	0.19	0.06–0.52

Table 2.4 Documented and possible occupational acquisition of HIV infection

	USA	Europe	(UK)	Rest of world	Total
Documented seroconversion	57	35	(5)	14	106
Possible occupationally-acquired infections	139	85	(14)	14	238

Prevention of BBV Transmission from Patient to HCW

Pre-exposure

The importance of adhering to strict infection-control policies has been referred to above. All HCWs, including students, should be educated about the potential for and the risks of occupational exposure to infectious patient-derived material, and fully trained in the general principles of infection control. They should be aware of institutional protocols and policies for the management of needlestick or other exposure incidents, including the recommended initial first-aid measures to be taken, and to whom any such incidents should be reported for urgent advice and management, both within and outside normal working hours.

There are no prophylactic vaccines available to protect individuals against either HIV or HCV infection. In contrast, there are highly effective vaccines for the prevention of HBV. It is World Health Organization and European Union policy that HBV vaccines should be adopted as universal vaccines of childhood. The United Kingdom is in a decreasing minority of countries which maintain instead a selective vaccination policy, aiming to target vaccine to those subgroups of the population that are identifiably at increased risk of acquisition of HBV infection. The UK Department of Health vaccination guidance (Department of Health, 2006) lists a large number of such subgroups, one of which covers HCWs including students and trainees.

It is important that HBV-vaccinated HCWs should have their anti-HBs response checked, in a sample taken 6–10 weeks after the last dose. About 10% of adults do not mount any anti-HBs response to current vaccines and are therefore not protected against infection. Such individuals can nevertheless be protected against needlestick acquisition of infection by passive immunization with hepatitis B immunoglobulin (HBIG). Definitions of a protective response to vaccination vary—official US guidelines specify >10 IU/L (Centers for Disease Control, 1991). UK guidelines are more conservative, stating that it is preferable to achieve >100 IU/L. HCWs with a sub-optimal response

of 10–100 IU/L should be given a further dose of vaccine (Department of Health, 2006). There is some controversy regarding the need for booster doses of vaccine to maintain protective immunity over long periods of time, as immune memory will persist despite decline of anti-HBs levels below detectable levels (European Consensus Group on Hepatitis B Immunity, 2000). The UK recommendation is for a single booster dose five years after the initial course.

Post-exposure

Risk assessment It is incumbent on any HCW who suffers an exposure to a patient's blood (or blood-stained bodily fluids) to report the incident promptly so that appropriate action may be taken on their behalf. This in turn means that every health-care institution needs to have definitive protocols and easily-identifiable lines of responsibility for the management of such incidents.

The first step in managing an exposure incident is to perform a risk assessment, taking into account a number of relevant factors (Table 2.5). For ALL exposure incidents, it should then be a universal policy to approach the source patient and ask for a blood sample on which to test for markers of HBV, HCV and HIV infection. This normalizes the process, so that individual patients do not feel discriminated against. The approach should be made by a member of the team responsible for the care of the source patient, but NOT the recipient of the exposure incident. It is helpful if there are guidelines available which can help HCWs not familiar with seeking permission for BBV testing to explain to the source patient the reasons for and benefits of being tested. It is unusual for patients to refuse permission for testing if approached in an appropriate manner.

The individual responsible for the post-exposure management of the incident must decide initially on the basis of the risk assessment whether or not any further action should be recommended, and if so, what. This decision may later be reviewed in the light of the results of testing the source patient for evidence of BBV infection.

Table 2.5 Post-exposure risk assessment

Source patient
BBV infection status (including drug therapy)
If status unknown, risk factors for acquisition of BBV infection
The incident
Type of exposure (e.g. percutaneous, mucosal, skin)
Type of needle (blunt or hollow-bore)
Source of needle (intramuscular, parenteral, etc.)
The recipient
Degree of injury
BBV infection status (including HBV vaccination)

All HCWs who report a BBV exposure incident should be appropriately followed up and tested for evidence of infection. In the United Kingdom, only 20% of HCWs reporting an exposure to HCV received the recommended follow-up testing—the biggest default being the absence of anti-HCV and HCV RNA tests taken at six months post-exposure (Health Protection Agency, 2006). It is conceivable that some of those HCWs did indeed acquire HCV infection, and by not being tested, were denied the opportunity of curative early therapy.

Hepatitis B Virus Table 2.6 shows the UK algorithm for management of an HBV-exposed HCW (PHLS Hepatitis Subcommittee, 1992), taking into account the nature of the exposure and the HBV status of both donor and recipient. A significant exposure is defined as one which is percutaneous (needlestick or sharps injury, bite), mucocutaneous (non-intact skin, conjunctiva or mucous membrane) if involving blood, or sexual. The actions to be taken vary from nothing through to administration of HBV vaccine and HBIG. HCWs should be tested for HBV markers at six months after exposure.

Hepatitis C Virus There is no vaccine for either pre- or post-exposure active immunization, nor any preparation approved for passive immunization. Management is therefore based on the observation that treatment of patients with recently-acquired HCV infection is highly successful in achieving sustained virological responses (SVRs) (Jaeckel *et al.*, 2001). At-risk recipients should be tested at 6 weeks for HCV RNA, and 12 and 24 weeks for both anti-HCV and HCV RNA. Early therapy should be considered if there is any evidence of acquisition of infection. For genotype 1 infection, treatment with pegylated interferon alone, for a minimum of six months, should begin as soon as possible, as delay of onset of therapy significantly reduces SVR rates. For genotype 2 or 3 infection, delay of therapy up to 20 weeks does not impact on SVR rates, so it may be sensible initially to see if spontaneous clearance occurs. Treatment of these genotypes may require only 12 weeks of therapy (Kamal *et al.*, 2006a, 2006b).

Human Immunodeficiency Virus The mainstay of management of an HIV-exposure incident is post-

Table 2.6 HBV post-exposure prophylaxis for reported exposure incidents

	Significant exposure			Non-significant exposure		
HBV status of person exposed	HBsAg positive source	Unknown source	HBsAg negative source	Continued risk	No further risk	
≤ 1 dose HB vaccine pre-exposure	Accelerated course of HB vaccine* HBIG × 1	Accelerated course of HB vaccine*	Initiate course of HB vaccine	Initiate course of HB vaccine	No HBV prophylaxis. Reassure	
≥ 2 doses HB vaccine pre-exposure (anti-HBs not known)	One dose of HB vaccine followed by second dose one month later	One dose of HB vaccine	Finish course of HB vaccine	Finish course of HB vaccine	No HBV prophylaxis. Reassure	
Known responder to HB vaccine (anti-HBs > 10mIU/ml)	Consider booster dose of HB vaccine	Consider booster dose of HB vaccine	Consider booster dose of HB vaccine	Consider booster dose of HB vaccine	No HBV prophylaxis. Reassure	
Known non-responder to HB vaccine (anti-HBs < 10mIU/ml 2–4 months post-immunisation)	HBIG × 1 Consider booster dose of HB vaccine A second dose of HBIG should be given at one month	HBIG × 1 Consider booster dose of HB vaccine A second dose of HBIG should be given at one month	No HBIG Consider booster dose of HB vaccine	No HBIG Consider booster dose of HB vaccine	No prophylaxis. Reassure	

*An accelerated course of vaccine consists of doses spaced at zero, one and two months. A booster dose may be given at 12 months to those at continuing risk of exposure to HBV. Source: PHLS Hepatitis Subcommittee (1992).

exposure prophylaxis. This strategy is supported by data from animal models, and also indirectly by the CDC retrospective case-control study of needlestick injuries, which demonstrated an 81% reduction (95% confidence interval (CI) 43–94) in risk in those HCWs given post-exposure prophylaxis (PEP) (at that time, consisting solely of zidovudine monotherapy); see Table 2.3 (Cardo *et al.*, 1997). However, there are no data from a randomized control trial to prove this contention, and nor will there ever be. The protective effect of PEP is less than 100%; at least 24 HCWs have acquired HIV infection following occupational exposure despite appropriate administration of PEP (HPA, 2005).

The taking of PEP is not without risk to the HCW; serious events are rare but can be life-threatening. In particular, nevirapine is not recommended, following reports of serious adverse events, including twelve cases of hepatotoxicity, one of which required liver transplantation. Thus, PEP should only be recommended to HCWs if, after full risk assessment, they have had a significant occupational exposure to blood or other high-risk fluid from a known, or high-risk, HIV-infected source. PEP should be started as soon as possible after an incident, and therefore an emergency supply pack must be available within all health-care settings for out-of-hours access. Source testing of the latter should enable a specific decision to be made as to the need to continue PEP. A particular concern regarding emergency access to PEP relates to medical students going to high HIV-prevalence areas for their elective studies. Most but not all medical schools in the United Kingdom recommend students to take a PEP starter pack with them, which will give them time to seek expert advice and follow-up should an exposure incident occur (Tilzey and Banatvala, 2002).

Current recommendations for PEP in the United Kingdom (Department of Health, 2008) are shown in Table 2.7 (these may be modified in individual circumstances, for example if the source patient is known to have drug-resistant virus). It is to be taken for four weeks, initiated as soon as possible after the exposure, and preferably within one hour (Department of Health, 2004). No antiretroviral drugs are licensed for PEP—their use on an ‘off-label’ basis therefore requires written, informed consent from the HCW.

Table 2.7 UK recommended starter pack for HIV PEP

One Truvada tablet (300 mg tenofovir and 200 mg entricitabine) once a day plus Two Kaletra film-coated tablets (200 mg lopinavir + 50 mg ritonavir) b.d.
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Table 2.8 Summary of patient-to-HCW transmission

Virus	Risk (%)	Pre-exposure	Post-exposure
HBV	19–31	Vaccination	HBIg, vaccination
HCV	1–3	None	Monitor and early therapy
HIV	0.3	None	PEP

All drugs used for PEP have side effects, and HCWs will require support and counselling in order to comply with the full four-week regimen. In the United Kingdom surveillance of occupational exposure scheme, 77% (233/303) of HCWs exposed to an HIV-positive source patient began HIV PEP, 34% within an hour of exposure and 89% within 24 hours. Earlier data from the same scheme showed that where the source was subsequently shown not to be HIV infected, 71/173 HCWs had taken PEP for more than one day, and 17 for more than one week, indicating an unnecessary exposure to potentially toxic drugs with unpleasant side effects, arising from delays in source patient HIV testing (Health Protection Agency, 2006).

Reassurance of the absence of HIV transmission can only be provided once an exposed HCW has been shown to be anti-HIV negative at least six months after cessation of PEP.

Risks and strategies for prevention of patient-to-HCW transmission of BBV are summarized in Table 2.8.

HCW-TO-PATIENT TRANSMISSION

The risks of and policies governing possible HCW-to-patient transmission vary significantly depending on which virus is being considered. However, all prevention policies are based on the concept of ‘exposure-prone procedures’ (EPPs), a term coined in 1991 by the CDC to describe procedures where there is a risk of percutaneous injury to the HCW, and where, if such an injury does occur, the worker’s blood may contact the patient’s tissues or mucous membranes (Centers for Disease Control, 1991). This is more likely when manipulation of needles or other sharp instruments in a body cavity without clear vision or in a restricted space is necessary.

Hepatitis B

Transmission of HBV from Infected Health-care Workers

There are over 45 reports of HCW-to-patient transmission of HBV in the literature, with over 400 infected patients and transmission rates of 6–15% (Mele *et al.*, 2001). In

the United Kingdom, between 1975 and 1990 there were 12 outbreaks of HBV infection associated with infected HCWs, with 91 infections identified (reviewed in Hep-tonstall, 1991). In 11/12 instances, the HCW was HBeAg positive (the status of the 12th was unknown). In five outbreaks where tracing and testing of exposed patients was performed (i.e. lookback), acute icteric HBV infection occurred in 1–2% of patients, with an overall transmission rate (including asymptomatic cases) of 4–9%. In a report of 20 such outbreaks in the United States involving over 300 infected patients, all 17 surgeons who were tested were HBeAg positive (Centers for Disease Control, 1991).

Prevention of Transmission of HBV from Health-care Workers

Guidelines for the prevention of HCW-to-patient transmission of HBV were first introduced in the United Kingdom in 1981. There have been a number of revisions since, each being more restrictive than the last. Guidelines released in 1993 resulted in the banning of HBeAg-positive HBV-carrier HCWs from performing EPPs, a decision based on the extensive evidence available at that time that HBV-transmitting HCWs were all HBeAg positive (where testing had been performed).

Subsequently, there have been 9 HCWs who have transmitted infection to at least 15 patients, 3 of whom died of fulminant HBV infection (reviewed in Irving and Harling, 2005). All anti-HBe positive surgeons harboured pre-core mutants and had high levels of HBV DNA (Incident Investigation Team and Others, 1997). Further restrictions on HBsAg-positive HCWs were therefore introduced in 2000 (Health Service Circular, 2000), specifying that HCWs without HBeAg should be further tested for HBV DNA. A cut-off value of 10^3 copies per ml was set; only HCWs with DNA levels below this level were allowed to continue to perform EPPs, subject to annual testing of their HBV DNA levels. The HBV DNA assays are performed in only two designated laboratories. Two samples from each HCW are required, taken a few days apart.

There are controversial aspects to this policy. The cut-off value was set bearing in mind known HBV DNA levels in anti-HBe-positive HCWs who transmitted infection (Corden *et al.*, 2003), possible variations in viral load within individuals (Tedder *et al.*, 2002) and assay performance characteristics, but is, nevertheless, arbitrary. Other countries have adopted a higher value, for example the Netherlands: $>10^5$ copies per ml (Gunson *et al.*, 2003). With the advent of drugs which can effectively suppress HBV replication, it is possible that an HCW whose HBV DNA level is above the cut-off may wish to take such medication purely for the purposes of allowing them to continue their chosen career. In recognition of this, guidance was released in 2007 which sanctions this course of

action, limited to those HCWs with initial viral loads of 10^3 – 10^5 copies per ml, and subject to stringent controls, including HBV DNA viral load assays every three months (Department of Health, 2007b).

Policies which allow known HBV-infected HCWs to continue to perform EPPs should emphasize that any needlestick or sharps injury to the HCW received whilst performing an EPP must be reported appropriately, regardless of the HCW's HBV DNA level. The transmission risk to the patient is not simply dependent on the HBV DNA load of the HCW—other factors, such as the amount of blood to which the patient is exposed, are important. A risk assessment must therefore be performed to determine whether or not to offer post-exposure prophylaxis (HBIG and/or HBV vaccine) to the patient.

It should be noted that HBsAg-positive individuals may harbour low levels of anti-HBs (Shiels *et al.*, 1987; Tsang *et al.*, 1986). Thus, for HCWs who will perform EPPs, it is preferable to test for HBsAg before vaccination. Otherwise it is possible that HCWs tested post-vaccination and found to be anti-HBs positive will erroneously be allowed to perform EPPs. This has happened in the United Kingdom—one of the nine surgeons to have transmitted since 1993 achieved an anti-HBs level of 252 IU/L after nine doses of vaccine, and ultimately transmitted infection to three patients, one of whom died.

Hepatitis C Virus

Transmission of HCV from Infected Health-care Workers

Several HCW-to-patient transmissions of HCV have been reported (reviewed in Irving and Harling, 2005). In the United Kingdom, at least seven surgeons have transmitted HCV infection to patients. Taking all reports together, the transmission rate per patient operated on by an HCV-infected surgeon is around 1 in 400, or 0.25%.

In addition, there are a number of transmissions associated with anaesthetists. Numerically, these far outweigh the surgeon-to-patient transmissions. Most have arisen through a small number of anaesthetists who have contaminated vials of narcotics intended for patient use with their own blood in the process of illicitly 'sharing' the dose with the patient, although some have undoubtedly been through non-adherence to good infection-control practice, such as the re-use of multi-dose vials.

The magnitude of risk of transmission from infected workers has been estimated in the United Kingdom (Department of Health, 2002b), on the basis that 1 in 200 surgeons currently practising in the United Kingdom is infected with HCV (i.e. the same prevalence as in the general population), and that an infected surgeon transmits infection in 1 in 400 operations. The chances of an

individual patient acquiring infection from a surgeon of unknown status is then 1 in 80 000. Thus, if half a million exposure-prone procedures are performed annually, somewhere between 5 and 10 patients per year will acquire HCV infection through this route.

Prevention of Transmission of HCV from Health-care Workers

Guidelines have been introduced to try to reduce the incidence of HCW-to-patient transmission of HCV (Department of Health, 2002c). Again, there are elements of controversy in these, as they represent a compromise between two extreme strategies—on the one hand, take no action; on the other, screen all HCWs performing EPPs and ban any who are HCV RNA-positive. HCWs who are known to be infected with HCV are banned from performing EPPs. There is no recommendation for blanket screening of all HCWs performing EPPs, although any HCW with reason to suspect they might be infected by way of having a risk factor for infection is encouraged to be tested. However, screening is introduced for all HCWs entering training in a specialty which requires them to perform EPPs. The aim of this is to block the recruitment of HCV-infected individuals into the pool of HCWs who perform EPPs, at a stage in their career when it is reasonable for them to make an informed career choice. Implicit in this policy is the realization that a small number of patients (5–10 per year) will acquire HCV infection from infected HCWs.

A later refinement of the above guidance appeared in 2007, extending the testing of HCWs for HCV infection to ‘new HCWs’ (Department of Health, 2007a). ‘New HCWs’ encompasses those ‘moving into training or posts involving EPPs for the first time’ and those ‘returning to work in the NHS (National Health Service) . . . who may have been exposed to serious communicable diseases while away’. Examples of the latter include those ‘returning from research experience, electives, voluntary service with medical charities, sabbaticals, exchanges, locum and agency work or periods of unemployment spent outside the UK’.

Human Immunodeficiency Virus

Transmission of HIV from Infected Health-care Workers

HCW-to-patient transmission of HIV is exceedingly rare. The first documented cases arose from an HIV-infected dentist in Florida, USA, six of whose patients acquired infection. Despite intensive investigation, the precise route(s) of these transmissions has never been determined (Ciesielski *et al.*, 1994). One French orthopaedic surgeon,

who himself is believed to have acquired HIV infection from a needlestick exposure in 1983, is thought to have transmitted infection to a patient in 1992 (Lot *et al.*, 1999). A Spanish gynaecologist transmitted infection to one patient undergoing Caesarian section (Bosch, 2003). Evidence for HCW-to-patient transmission in both of the latter incidents included detailed molecular epidemiological studies.

Lookback studies performed on patients operated on by surgeons subsequently discovered to have HIV infection confirm the scarcity of transmission events. Lookbacks from at least 44 infected HCWs in the United States and 22 in the United Kingdom, covering 22 759 and 7000 patients respectively, have failed to identify a single additional transmission (Gerberding, 1999).

Prevention of Transmission of HIV from Health-care Workers

Despite the above data indicating that the risk of transmission from a known HIV-infected HCW is extremely low, the guidelines governing this situation are the most draconian of those for any BBV. Known HIV-infected HCWs are not allowed to perform EPPs (Department of Health, 2005). This is irrespective of the disease status, viral load or antiretroviral status of the individual HCW concerned.

Up until 2007 there was no compunction for UK HCWs who performed EPPs, or who were training in specialties involving EPPs, to be tested for HIV infection. However, guidance introduced in March 2007 stipulates that ‘new HCWs’ (explained above) who will perform EPPs should be screened for HIV infection (Department of Health, 2007a). This guidance has, not unexpectedly, attracted controversy, given that it introduces compulsory HIV testing for the first time for certain categories of HCW.

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Viral Transmission: Infection Acquired by All Other Routes (Respiratory, Eye–Nose–Mouth, Inoculation and Faeco-orally)

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INTRODUCTION

This chapter will cover the important clinico-pathological aspects of all the major groups of human viruses, including relevant epidemiological characteristics, with the aim of providing the practical knowledge to enable microbiologists and virologists to prevent and contain the spread of virus infections in hospital. The entire subject of nosocomial virus infection has not had the prominence it deserves. It has been neglected in part because of the preference to measure nosocomial bacterial infection. This is somewhat surprising since bacteria, perhaps with the exception of *Legionella*, do not have the capacity to close wards and entire hospitals that certain viruses have, norovirus and varicella zoster being just two examples. Indeed, an analysis of the literature that exists regarding ward closure (Outbreak database, www.outbreak-database.com) performed in 2005 showed starkly that of the six virus types in the database, four (noroviruses, rotavirus, hepatitis viruses, influenza/parainfluenza virus; 67%) were significantly associated with ward closure. This compares with only 3/14 (*Acinetobacter* and *Streptococci*; 22%) bacterial genera (Hansen *et al.*, 2007). This is due largely to the fact that viruses may be spread quickly among patients and staff by aerosol and droplet routes in addition to direct contamination by unwashed hands, which

is the principal way nosocomial bacterial infections occur.

Hospitals may also act as centres of virus amplification for the further spread of virus in the wider community. This has been exemplified recently in the case of severe acute respiratory syndrome (SARS), and previously with Ebola virus outbreaks centred in hospitals in Africa, and appears to be happening again with measles. There is also an ever-increasing burden of extremely vulnerable patient groups, including pre-term neonates and those with immunodeficiency secondary to human immunodeficiency virus (HIV) or solid organ and bone-marrow transplants (BMTs). Global travel has also meant that the staff who work in health services come from all over the world. This brings with it other unique problems, such as increased susceptibility rates to chickenpox if staff are from equatorial climes.

The prevention of nosocomial virus infections requires a team effort. Very often virology is able only to prevent secondary cases where the incubation period is relatively long, for example 10–14 days for measles, chickenpox and so on. Preventing the nosocomial spread of viral respiratory and gastrointestinal pathogens with very short incubation periods of sometimes only hours to a few days, however, relies heavily on the staff admitting the patient recognizing the potential for transmission and adhering strictly to basic infection-control measures

such as hand washing. The admission to single rooms of patients experiencing viral prodromal illness of, for example, parvovirus or measles during epidemic seasons or local upsurges, along with cohort nursing, is very effective but requires thought from admitting teams. To facilitate this approach, clear, concise guidance from the virology, infection-control and occupational health departments on how to manage individual virus infections and staff exposures to them is required. In other ways, however, the prevention of hospital-acquired virus infection should be considerably easier than it is for the vast majority of multiply-resistant bacteria, because of the success of many viral vaccines. For these preventative measures to work and with regard to vaccine-preventable disease, for example measles, mumps, varicella and influenza, which may easily establish a hospital-based outbreak, it is essential that a robust method for identifying and vaccinating susceptible staff is in place and functioning, as shown by regular audit of the uptake of vaccination within the hospital and across a country. Such information must be prospectively gathered prior to an outbreak in order to prevent transmission of vaccine-preventable infections. Given that it is not acceptable for any patient to acquire such a nosocomial virus infection, it is incumbent on the management teams of hospitals to give strong consideration to making certain immunizations mandatory prior to acceptance of any future student or employee unless there are genuine contraindications to its use in that individual.

Control of virus infections in hospitals must be a proactive process. Although rapid diagnosis and excellent communication with staff in the clinical areas are essential if nosocomial infection is to be prevented, anticipating recurring problems, for example the annual norovirus and respiratory syncytial virus (RSV) winter epidemics, by informing staff through continuous education programmes and proactively if local outbreaks are occurring, is of additional benefit.

We will illustrate the problems and some of the solutions to controlling viruses spread by all routes and means of contact other than blood. Practical measures that should be taken to prevent or control an outbreak including data on virus survival, the activities of different disinfecting agents and additional epidemiological information is provided in Tables 3.1, 3.2 and 3.3 respectively. Guidance on the management of staff susceptible to particular agents will also be given.

MEASLES, MUMPS AND RUBELLA

Measles

The risk for measles infection in medical personnel is estimated to be 13-fold higher than that in the general

population (Atkinson *et al.*, 1991; Davis *et al.*, 1986). Approximately 5–10% of all notified cases of measles are reported to have been acquired in a medical setting and over 33% of cases acquired in hospitals are in health-care workers (HCWs), mostly (>85%) previously unvaccinated (reviewed in Centers for Disease Control and Prevention, 1997; Farizo *et al.*, 1991).

In African countries it is a much more serious issue, as shown in 1989 by Cotton, who examined paediatric patients admitted for their risk of acquiring a nosocomial infection (Cotton *et al.*, 1989). Overall, 193/1350 (14.3%) children admitted to hospital developed a nosocomial infection; of these 28/193 (14.5%) were measles, resulting in seven deaths. Moreover, because already-hospitalized patients have by definition an illness, the mortality rate in nosocomial measles may be considerably higher (50% vs 2%) when compared with community acquisitions (Reynolds and Klein, 1987). Such nosocomial outbreaks are also a threat to the eventual elimination of measles, as hospitals act as amplifiers of infection (Marshall *et al.*, 2003).

Measles virus infection is now a particular problem, partly because of the success of MMR vaccination. Whilst this has dramatically reduced the incidence of infection in the community, the clinical skills required to diagnose measles have also waned. Moreover, as the population of immunosuppressed patients has continued to grow (organ/bone-marrow transplantation, intensive care and HIV), atypical presentations are more likely to occur and the diagnosis is more likely to be missed (Kidd *et al.*, 2003). Thus, as measles virus has a reproduction rate of 14 : 1, it can easily re-emerge in developed countries, due largely to importation of measles from countries with less well-developed measles vaccination programmes (Ramsay *et al.*, 2003). This becomes even more of a problem if the MMR uptake rate falls, as indigenous ongoing transmission will then occur (Thomas *et al.*, 1998).

There are two ways of containing measles virus infection within hospitals. Firstly, it is essential that all staff involved in the care of patients and ideally all staff in a hospital are aware of their measles immune/vaccine status and have taken appropriate steps to ensure that they do not pose a risk to others. Secondly, one must have guidelines for clinically suspected measles. A useful acronym already in use in the United Kingdom, which currently stands for Consultant in Communicable Disease Control (CCDC), may also be used to assist in measles diagnosis by substituting the following words: ‘a febrile patient with two of the following: Cough, Coryza, Diarrhoea and Conjunctivitis’. When after a few days this is accompanied by a rash, the diagnosis is virtually certain, and in the context of a community-wide outbreak or an epidemiological link with other cases, 100% accurate. Sometimes a history of travel abroad or contact with

recently-returned travellers also increases the likelihood of the diagnosis, as does having had no or only one dose of MMR. Other clues to the diagnosis are lymphopenia $<0.8 \times 10^9 \text{ l}^{-1}$, slight thrombocytopenia $<150 \times 10^9 \text{ l}^{-1}$ and biochemical hepatitis (ALT 3–5 \times normal). Patients should be admitted to a single room and be cared for only by staff who know their measles immunity status. A strict gloves, aprons and hand-washing policy must be adhered to if further infection in staff or patients is to be avoided. Rapid diagnosis may be by serum IgM (often positive), serum IgG (often negative) or by monoclonal antibody fluorescence on a urine sample. If real-time polymerase chain reaction (PCR) is available, this may be applied to an oral fluid sample or to urine.

Transmission is by droplet infection, though fortunately the degree of infectivity reduces quickly after the rash appears and lasts for only a few days (4) afterwards. The incubation period may be as short as 7 days, but more typically 10–13 days. However, because it may be as long as 18 days, susceptible staff are excluded from work for 5–21 days after contact.

Problems continue to arise in the United Kingdom as it has only just introduced a formal policy of establishing measles immunity for all new staff (Salisbury *et al.*, 2006). The recommendations are that an individual should have either detectable measles IgG or have received two doses of a measles-containing vaccine after their first birthday. However, these guidelines have been in place in the United States for some time, and despite high levels of vaccine coverage across the US, in 2005 it experienced the largest documented outbreak of measles since 1996, from a single individual who had acquired infection overseas (Parker *et al.*, 2006). As a result of this community-wide outbreak, almost exclusively in unvaccinated individuals, a hospital employee (a phlebotomist) contracted measles. Due to the extensive movements of that member of staff the hospital had to administer 210 doses of immunoglobulin in addition to 317 doses of MMR vaccine; the total accrued costs were \$113 647, spent in efforts to prevent further infection and severe disease. The staff member had only received one dose of measles vaccine after her first birthday and had never had her antibody tested.

It used to be acceptable that a cut-off year of 1957 was used as a proxy for immunity to measles, as people born before this year are very likely to have had measles infection as a child. However, as the susceptibility rate in this age group is still 5%, individuals in this group who cannot provide evidence for physician-diagnosed measles must undergo antibody testing and, if susceptible, given two doses of MMR (Braunstein *et al.*, 1990; Schwarcz *et al.*, 1992; Uckay *et al.*, 2007).

Even pre-exposure vaccination is not always successful, however, as shown in the Netherlands, where there was

measles transmission to susceptible hospital staff where the level of immunity in health-care workers was 98.5% (de Swart *et al.*, 2000). Nevertheless it must be remembered that vaccination is not only highly efficacious, as shown by two primary school outbreaks where the attack rate in unimmunized children ranged from 26 to 46% yet was only 0.4% in children who had had MMR (Richardson and Quigley, 1994), but is also our only defence against infection as measles is untreatable.

It is a responsibility of hospital facilities to ensure that their staff have made all practicable efforts to eliminate the risk of measles transmission to patients. This is because given recent reductions and continued low uptake rates of MMR, we may be creating a cohort of children, at least in the United Kingdom, which will become large enough to not only sustain local virus transmission, but also be a source for susceptible infants who have yet to receive MMR. This is because most mothers now have vaccine-induced immunity and there is a lack of circulating virus to create natural boosters, so it is much more likely that measles antibody will have disappeared in infants by nine months. Infection under a year of life presents a much greater risk of developing SSPE later in life. The absence of natural boosting may also increase the rate of breakthrough infection in adults who have received only one dose of the original measles vaccine in the 1970s–1980s. Indeed, I have seen a case of breakthrough infection recently in an adult aged 45 years who transmitted virus to his son, who was only old enough to have received one dose of MMR.

To contain measles in a hospital the only effective measure is to isolate the patient, but the focus should be on maintaining high uptake rates of primary immunization. Even post-exposure vaccination is of dubious merit. When administered in an epidemic setting the efficacy can be as low as 4% (King *et al.*, 1991). Our experience has been very similar when attempting to prevent an outbreak in infants at a nursery. The same day the case was notified, which was the day of the rash in the index case, five of the six contacts (all aged less than two years and previously unimmunized with MMR) were vaccinated. Ten to fourteen days later all six children developed clinical measles, confirmed by detection of wild-type virus in one of the vaccinees (Rice *et al.*, 2004). Thus it is essential to achieve the highest possible level of staff immunity before an outbreak.

Failure to do so will eventually result in ward closure and death in unimmunized immunocompromised patients (Kidd *et al.*, 2003).

Mumps

Mumps virus infection in the post-vaccine era has until recently not been a problem for most developed countries,

due to the success of MMR immunization programmes. During 2006 in the United States and 2004–2005 in the United Kingdom, extensive community outbreaks of mumps were seen in age-limited cohorts (Dayan *et al.*, 2008; Gupta *et al.*, 2005; Savage *et al.*, 2005). In the pre-vaccine era mumps was an almost universal infection acquired by the end of secondary schooling (16 years). Since the 1970s in the United States and 1988 in the United Kingdom, mumps vaccine has been in widespread use in the childhood vaccination programmes. Dramatic reductions in mumps incidence were seen following vaccination, and these reductions have been maintained for 15 years in the United Kingdom and almost 30 years in the United States.

The United Kingdom has had a particular problem in recent years with individuals born between 1982 and 1990, who are likely to have received at best only one dose of MMR. As the efficacy of single-dose MMR is estimated at 80%, this is not sufficient to contain transmission in closed communities. However, the United States has had about 90% vaccine coverage with two doses. The US outbreak was again age-limited (18–24 years) and geographically localized to the Midwestern states, but there it was thought that multiple factors played a role in generating and sustaining it. These were waning immunity, high population density and contact rates in colleges, and incomplete herd immunity despite the extensive two-dose vaccine coverage. Such herd immunity is estimated to be 88–92%; so individual vaccine efficacy of about 80%, even with exceptional coverage rates of 95% for both doses, will only achieve herd immunity of about 90%. This is only at the limit to prevent widespread epidemics (Dayan *et al.*, 2008).

Whilst these factors might explain outbreaks in closed communities they do not explain why there is a lack of described outbreaks in hospitals. Two factors are likely to play a role. Mumps infection is relatively frequently asymptomatic and as such these patients will not present to health-care services. Secondly, health-care workers aged 30 years or older will very likely be immune from natural childhood infection in the pre-vaccine era. Mumps outbreaks in closed communities including hospitals have been described, especially when there are many opportunities for close contact (Wharton *et al.*, 1990). One such outbreak occurred at trading floors in Chicago in 1987, with over 100 cases (Kaplan *et al.*, 1988). The reason for an increase in cases in young adults is because of the success of MMR vaccination. This has led to fewer exposures for a cohort of young adults who were ineligible for immunization and so have escaped natural infection.

Controlling mumps virus in hospitals is still necessary because of the risk of meningitis and subsequent sensorineural deafness. A high index of clinical suspicion,

hand washing and admission to a single room with droplet precautions remain the main ways that transmission is prevented in hospitals. For staff, it is necessary to test those exposed for mumps IgG and to immunize seronegative individuals. As mumps virus excretion in saliva commences five to six days prior to symptom onset and MMR vaccine or normal immune globulin given post-exposure is entirely ineffective in preventing secondary cases, it will be necessary to furlough from work staff requiring vaccination for a period of 10 days, starting 12 days after the first contact with a case (Aitken and Jeffries, 2001).

Rubella

Outbreaks of rubella in hospitals are now rare, due largely to effective staff and student antibody screening and immunization (Greaves *et al.*, 1982). As a history of rubella infection is not reliable and voluntary immunization has been shown not to prevent outbreaks it is now accepted by staff that they have to be rubella-immune before being allowed to work in high-risk areas, namely obstetrics (Heseltine *et al.*, 1985). The susceptibility rates in staff in the 1970s and 1980s varied from 5 to 18% depending on age (Polk *et al.*, 1980). Thus, with such high rates it was possible for rubella virus to circulate in hospitals. Staff susceptibility rates are now much lower, such that if transmission to a member of staff does occur it is just as likely that this merely reflects a larger community-wide outbreak. However, individuals born in developing countries without rubella vaccination programmes are at increased risk of infection in hospital if a health-care worker develops rubella. They can then pose a risk to those they live with at home. It is essential that staff from such countries are identified by screening procedures, as hospitals with a large number of such staff may become the amplifier of an outbreak that spreads back into the wider community. This has been shown recently in other types of workplace, where attack rates were almost 100-fold greater than the general population (Danovaro-Holliday *et al.*, 2000). In this particular outbreak, though not in a hospital, 6/24 (25%) pregnant women became infected. In health-care settings, of particular concern are women (patients and staff) from south Asia and Africa, where susceptibility rates may be almost 20% in those of childbearing age (Devi *et al.*, 2002; Miller *et al.*, 1990). Indeed, in the last three years I have seen three cases of rubella in the first sixteen weeks of pregnancy, all resulting in fetal loss, where the woman was from a country without well-organized childhood rubella vaccine programmes (Mauritius, Philippines and Russia) and had travelled back to her country of origin for family celebrations.

Furthermore, infants with congenital rubella syndrome (CRS) are a potential source of infection for susceptible staff and other infants due to the excretion of high-titre

virus in urine for prolonged periods of about two years (Aitken and Jeffries, 2001). Indeed, the first demonstration of a baby with CRS infecting another baby was shown in a London hospital, when a baby with CRS infected a baby in a neighbouring cot (Sheridan *et al.*, 2002). The only way that transmission could have taken place is by non-adherence to hand washing by staff after dealing with urine and other bodily fluids from the baby with CRS. The best way to prevent nosocomial rubella is to fully investigate staff members with rash illness, remembering that in the first three days after the rash has appeared they may be IgG and IgM negative. Once identified, colleagues in close daily contact should be tested for rubella IgG, with the focus on pregnant staff <18 weeks gestation. Although post-exposure rubella vaccination is ineffective in preventing infection, any nonpregnant susceptible staff should then be immunized and tested two to three months later for a response.

CYTOMEGALOVIRUS

Cytomegalovirus (CMV) is one of the easiest viruses to control in hospital yet probably causes more anxiety, especially among pregnant staff, than any other virus excluding HIV. The anxiety usually results from the identification of a baby or infant with congenital infection, often on the neonatal unit. As congenitally-infected babies excrete virus at high titre ($>10^6$ ml⁻¹), staff are, understandably, concerned at the risk these babies pose to pregnant members of staff or to other babies on such units. At this stage HCWs are sometimes offered CMV screening, by other staff or occasionally by occupational health departments, principally as a means of providing reassurance. However, such screening tests are offered in ignorance of the data concerning the risks of transmission to staff and are erroneously believed to be capable of determining if a person is 'immune' to CMV infection. Moreover, the potential implications and consequences of commencing CMV antibody testing in pregnant members of staff are never considered by those requesting the tests. Indeed, the first contact between a clinical virologist and the staff member is when the pregnant member of staff contacts them to discuss the results of their CMV serology and its significance. This is where the difficulties arise. As discussed in the chapter on CMV, the concept of CMV immunity, especially in relation to pregnancy, is incorrect as reactivation and re-infection may lead to congenital infection and occasionally disease in newborns.

Though the CMV serology may indicate past infection or susceptibility, occasionally CMV IgM is detected, which produces considerable anxiety. Further tests, such as IgG avidity, are then required, as well as a hunt for

earlier samples to try and determine, as accurately as possible, the timing of any CMV infection. Furthermore, when past infection is shown, the potential for re-infection or reactivation has then to be discussed.

If the member of staff is susceptible, the discussion widens to include the potential benefit and harm of follow-up testing, prenatal diagnosis and the possible outcomes for the baby if a primary infection is demonstrated.

It is obvious from this outline that screening of staff for CMV is a big undertaking and should only be done after a prolonged and informed discussion with the clinical virologist and an obstetrician. The advice that should be given to the staff member is that if they have been adhering to universal precautions and have washed their hands after dealing with patients, serological testing for CMV should not take place. This is because there are several well-conducted studies which all conclude that there is no evidence that CMV excreted by hospitalized patients poses a significant additional risk for pregnant staff (Balcarek *et al.*, 1990; Balfour and Balfour, 1986; Dworsky *et al.*, 1983).

The incidence of CMV infection in hospital staff is approximately 1–2% and there is no difference between various occupational groups within and the general population outside hospital. Balcarek *et al.* (1990) showed, for example, that there was no difference in the rate of infection in staff regardless of area of work, job type (nurse, doctor, support services, administrative staff) or number of patient contact hours of between 10 and 40 hours per week. Moreover, when the rates of CMV excretion by patients are known in different areas of the hospital, this has no effect on the rate of transmission to staff. Demmler *et al.* (1987) demonstrated this when rates of CMV excretion varied from 3% in newborns to 16% in a chronic care unit for children, many with severe neurological and physical handicap, a proportion of whom would have had congenital infection and be excreting high-titre virus in urine.

Further proof that patients are not the source of infection has come from studies that have examined virus strains detected in staff who are shown to seroconvert at work. In these cases either the strains of virus in staff and patient have been different or another source outside of hospital, often a sexual partner or child at home for example, has been shown to be shedding an identical virus (Demmler *et al.*, 1987; Onorato *et al.*, 1985). Such virus sequencing has also been used to disprove suspected nosocomial infection between babies, with staff as the likely vector of infection, on a neonatal unit (Stranska *et al.*, 2006).

The single study which did show an increased incidence of infection among staff demonstrated this only for student nurses on their first placement in paediatrics (Haneberg *et al.*, 1980). Such staff were reportedly

observed to kiss drooling infants while feeding them. Indeed, this paper highlights the fact, as stated by others, that intimate and prolonged contact is required for CMV transmission to occur (Onorato *et al.*, 1985). Hence the increased rate of infection seen in pregnant mothers with a young child at home or in staff employed in child day care, where the transmission rate may be 10–25% per annum (Adler, 1989).

CMV, being an enveloped virus, does not survive well on inanimate surfaces or on hands. This was shown clearly in a study in a paediatric hospital, where the virus was not isolated from any equipment, toys or hard surfaces, and although not isolated from the dry hands of staff members, was removed completely by hand washing with soap and water (Demmler *et al.*, 1987). Simple hygienic measures and washing of surfaces with household detergent are sufficient to remove infectious material from the environment.

It is certainly not advisable to routinely screen staff after a possible exposure occupationally to CMV as considerable anxiety is experienced whilst waiting for the results and there can be difficulties in interpreting them correctly. Indeed, where this was attempted in a hospital in the United Kingdom some years ago, it was a ‘disaster’ and led to ‘public relation, psychological and management problems’ (reviewed in Young *et al.*, 1983). Staff should be educated about how the virus is transmitted (sexually, kissing, lapses in hand washing especially after changing nappies) and about the many pitfalls of antibody testing. If a staff member has requested to be tested after an exposure, ideally the virologist should see them to discuss all of the issues relating to the possible outcomes. The issues to be discussed would be the fact that CMV infection is ubiquitous, staff are at no increased risk of infection, transmission is interrupted easily by hand washing and CMV serology can be fraught with difficulties if low- or even high-level IgM is detected.

VARICELLA ZOSTER VIRUS

Preventing patients and staff from contracting chickenpox in hospitals forms a significant part of the workload of a clinical virologist. Aside from the excess costs that nosocomial varicella zoster virus (VZV) infection imposes (estimated to be £5–10 million in the NHS of England and Wales annually), it causes significant disruption to wards, even occasionally entire hospitals, increases the workload of clinical virology laboratories at very short notice, and leads to considerable disruption when susceptible staff have to be excluded from work after exposure. Chickenpox can also occur at any age; I have witnessed primary infection in two 65-year-olds, a 78-year-old and a man of 92 years!

In recent years, however, and solely as a result of varicella vaccination in susceptible staff, the rate of hospital-acquired VZV has dramatically reduced. Nevertheless, it is still important to understand the peculiar problems that hospitals have hitherto faced with this virus, because the United Kingdom still has not embraced routine childhood vaccination and a 2004 survey of UK clinical virologists revealed a disappointingly low rate of implementation of just 14% of using the vaccine in susceptible staff (Breuer, 2005). Until this rate improves and/or VZV vaccine is made part of childhood immunization schedules, clinical virologists and microbiologists need to be knowledgeable in the peculiarities of VZV infection in hospitals.

When a patient or staff member with VZV infection is identified, all staff in contact must know their immune status. Indeed, knowledge of such status is part of routine occupational health screening. A well-taken history of previous chickenpox correlates very well with immunity (99%) and, even for those who are unsure if they have had chickenpox in the past or claim never to have had chickenpox, 60% will have evidence of past infection. However, a history may be less reliable in those born in countries where varicella is less common in childhood, essentially in the tropics, and some have advocated testing of all staff born in such countries. For reasons currently unknown, individuals born in hotter climates, particularly the Indian subcontinent, Africa and the Caribbean islands are much more likely to be susceptible. Indeed, of the VZV-susceptible staff employed at St George’s who developed chickenpox in the last three years, >50% were born in these countries (Devi and Rice, 2002).

It is thus important for staff to appreciate the problems that VZV transmission may cause and for susceptible staff to report any contact with virus. This is especially so for staff who have children, as approximately 50% of susceptible staff who develop chickenpox do so after a home exposure.

The VZV transmission rate to susceptible staff after contact has been estimated to be between 4.7 and 29% (Langley and Hanakowski, 2000; Myers *et al.*, 1982; Wreghitt *et al.*, 1996). As there are several factors that affect this rate and most are difficult to quantify, guidelines on the degree of exposure can be helpful in deciding to what extent exposure has occurred. Examples of significant exposures have been defined as a face-to-face contact for at least 15 minutes or being present in the same room for 1 hour with an individual with chickenpox or disseminated/uncovered zoster (Salisbury *et al.*, 2006). An intermediate risk occurs when exposure has taken place in a multi-room setting; that is, an area with beds separated by doors, partitions or curtains. In a prospective study by Langley and Hanakowski 2000, they found

that transmission risks of varicella for single-room and multi-room settings were 11.8% (4/34; 95% CI 3.3–27.5) and 0% (4/89; 95% CI 1.2–11.1) respectively.

At the other end of the exposure spectrum, however, the likelihood of transmission taking place from cases of covered zoster, for example thoraco-lumbar, is remote. Although airborne transmission on open wards has been described, the evidence for airborne spread of VZV is very limited, and complicated by the difficulty in reliably excluding droplet transmission.

Although chickenpox is much more infectious than zoster, most of the practical problems involved with control of infection arise from patients who have zoster. This is simply because chickenpox is much more easily recognizable and such patients are admitted immediately into a single room. Zoster, however, often develops in a patient who has been on the ward for several days or even weeks and the rash is often present for several days before the diagnosis is suspected. Thus, many more staff and patients are exposed to VZV after contact with patients who have zoster than those with chickenpox. This was demonstrated clearly in a study from Cambridge, UK. During a five-year period, only 1/28 (3.6%) of susceptible staff developed chickenpox after an exposure to varicella, compared with 5/29 (17.2%) after exposure to zoster (Wreghitt *et al.*, 1996).

It is wise to remember, however, that chickenpox can be mistaken for other vesicular exanthems due to enteroviruses, measles or even allergy, contact dermatitis or eczema. If you are presented with the information that a member of staff, patient, visitor or relative, for example on the neonatal unit, has developed chickenpox, the implications for the unit are considerable. Multiple doses of varicella zoster immune globulin (VZIG) will almost certainly need to be administered, staff will need to be sent home, visiting may become restricted and it is possible that the unit will have to be closed to new admissions. Thus, although it is an extreme example of what might happen, before embarking on the above course of action it is essential that the diagnosis of chickenpox be confirmed by an experienced virologist or microbiologist with access to laboratory facilities capable of providing rapid results either by direct immunofluorescence or by real-time PCR.

If a susceptible member of staff has been exposed, the simplest way to manage the exposure is to exclude them from day 8–10 after contact until day 21 after the first exposure. This is because cases of chickenpox are infectious two days prior to rash onset. Another option used quite commonly is to allow staff to remain at work providing that they feel well and are afebrile (Haiduven, 2003; Josephson *et al.*, 1990; Weber *et al.*, 1996). While at work they should wear a mask and take their temperature twice daily, and they should remain at

home or leave work if they become unwell/febrile as this could be the first sign of a prodromal varicella illness. This measure is used more frequently in key staff whose absence from a clinical area would be detrimental to patient care. Alternatively, the individual could perform duties that do not involve patient contact, though the usefulness of this approach for an experienced ward sister, for example, is questionable.

All of the preceding was much more applicable in the pre-VZV vaccine era. Now, the most cost-effective long-term way of managing such staff is through active vaccination against VZV either before or after an exposure (MMWR, 1999).

Live attenuated varicella vaccine has been in use for more than 20 years in Japan and the Far East and almost a decade in the United States, but only recently have two varicella vaccine products been licensed for susceptible individuals >12 years of age in the United Kingdom.

Both products, one from Glaxo Smith Kline, the other from Aventis-Pasteur, are highly effective at preventing chickenpox prior to exposure, and produce a seroconversion rate of >95% after a two-dose schedule (Sharrar

Table 3.1 Persistence of viruses in the environment

Type of virus	Duration of persistence (range)
Adenovirus	7 d to 3 mo
Astrovirus	7–90 d
Coronavirus	3 h
SARS associated virus 7	2–96 h
Coxsackie/ECHO virus	1 to >2 wk
Cytomegalovirus	8 h
HSV types 1 and 2	4.5 h to 8 wk
Influenza virus	1–2 d
Norovirus	8 h to 7 d
Papillomavirus	16 h to >7 d
Papovavirus	8 d
Poliovirus	4 h to <8 d
RSV	Up to 6 h
Rhinovirus	2 h to 7 d
Rotavirus	6–60 d
Vaccinia	3 to >20 wk

Various factors influence virus survival. Low temperature appears to favour virus persistence, especially for those acquired faeco-orally and HSV. Humidity, however, gives conflicting results. The initial inoculum and the presence of faeces appear also to increase the chances of persistence. The role of environmental contamination appears to be important for rota- and rhinoviruses and probably for noroviruses, especially when vomiting has occurred. In experimental studies using a bacteriophage as a marker of the transferability of infectivity, it has been shown that contaminated hands may pass virus to 5 or more surfaces and are capable of passing to 14 additional people when not disinfected. (Adapted from Kramer *et al.* 2006.)

Table 3.2 Physical characteristics of viruses and resistance and sensitivity patterns to agents of disinfection

Virus family	Nucleic acid	Enveloped: yes/no	Resistant to	Sensitive to
<i>Herpesvirus</i> (HSV, VZV, CMV, EBV)	dsDNA	Yes	No disinfecting agents	Low pH, desiccation, lipid solvents, ^a detergents, chlorine-based agents, alcohols
<i>Adenovirus</i> (all types)	dsDNA	No	Mild acid conditions, lipid solvents	Chlorine-based agents, ^b >56°C for 10 min
<i>Parvovirus</i> (B19)	ssDNA	No	pH 3–9, lipid solvents, 56°C for 10 min	Formalin, β-propiolactone, hydroxylamine, oxidizing agents, for example chlorine-based agents/iodine
<i>Polyomavirus</i> (BK/JC)	dsDNA	No	Lipid solvents, pH 2–3, 50°C for 60 min	Chlorine-based agents
<i>Papillomavirus</i> (all types)	dsDNA	No	Lipid solvents, pH 2–3, 50°C for 60 min	Chlorine-based agents
<i>Rotavirus</i> (all types)	ds-segmented RNA	Partially (mature particles: No)	pH 3–9, relatively stable to 50°C, lipid solvents (absence of envelope in mature particle)	β-propiolactone, formalin, chlorine-based agents, phenol, alcohol solutions NB 95% solution in water
<i>Astrovirus</i> (all types)	ssRNA	No	pH 3, 50°C for 60 min, 60°C for 5 min, lipid solvents, non-ionic detergents	Chlorine-based agents
<i>Calicivirus–Norovirus</i> (all types)	ssRNA	No	pH 3, 50°C for 60 min, 60°C for 5 min, lipid solvents, non-ionic detergents	Chlorine-based agents
<i>Picornavirus</i> (Enterovirus)	ssRNA	No	Stable at acid pH, lipid solvents, non-ionic detergents	Chlorine-based agents
<i>Picornavirus</i> (Rhinovirus)	ssRNA	No	Stable at pH 5–6, lipid solvents, non-ionic detergents	Low pH, chlorine-based agents
<i>Paramyxovirus</i> (Measles, Mumps, RSV, Metapneumovirus, Parainfluenzavirus)	ssRNA	Yes	No disinfecting agents	Heat, lipid solvents, ionic and non-ionic detergents, formaldehyde, oxidizing agents; that is, chlorine-based agents
<i>Orthomyxovirus</i> (Influenza A and B)	ss-segmented RNA	Yes	No disinfecting agents	Heat, lipid solvents, ionic and non-ionic detergents, formaldehyde, oxidizing agents; that is, chlorine-based agents
<i>Coronavirus</i> (SARS and other types)	ssRNA	Yes	No disinfecting agents	Heat, lipid solvents, ionic and non-ionic detergents, formaldehyde, oxidizing agents; that is, chlorine-based agents

Table 3.2 (Continued)

<i>Togavirus</i> (Rubella)	ssRNA	Yes	No disinfecting agents	Heat, lipid solvents, ionic and non-ionic detergents, formaldehyde, oxidizing agents; that is, chlorine-based agents
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ds, double-stranded; ss, single-stranded.

^aExample of lipid solvent possibly in use in hospital setting: acetone.

^bThe effectiveness of chlorine-based disinfectants is compromised by excessive amounts of organic materials.

Alcohol rubs should be used only on visibly clean hands and at a concentration of 70% since the mode of action is to denature proteins, which is best at that ratio of alcohol to water.

et al., 2000). Although pre-exposure vaccination has been shown to be cost-effective, this has been assessed only in mathematical models and there are several important practical considerations before advising a staff-wide vaccination policy (Gray *et al.*, 1997). These include how to manage staff working in high-risk areas, as a small proportion of vaccinees will develop a rash both at the site of vaccination and outside of the injection area, and there is a measurable rate of breakthrough infection after exposure to wild type virus and the potential for ongoing transmission. The arguments for and against use of VZV vaccine in health care are detailed in Table 3.4.

The rate of vesicular rash post-vaccine is about 8% (Arbeter *et al.*, 1986). However, in the majority the rash is only maculo-papular; in only 30% of these is it vesicular and even then the average number of lesions is only 14. The nature of the rash and the lack of evidence for pharyngeal excretion of virus probably explain why transmission of vaccine virus to another healthy susceptible individual occurs only extremely rarely. Nevertheless, to take account of this problem staff employed in high-risk areas should take particular care in the two to four weeks after both doses and report to occupational health immediately a possible varicella-like rash develops. Breakthrough infection, however, is potentially more troublesome as this is a long-term problem and occurs at a rate of 5–8% after an exposure (Arbeter *et al.*, 1986; Watson *et al.*, 1993). The average timing for such breakthrough infections is 30 months post-vaccination (Watson *et al.*, 1993).

Despite breakthrough cases of infection being described after exposure to VZV at work, where presumably the degree of exposure is less intense compared with at home, transmission of wild-type virus from cases of breakthrough infection to susceptible contacts within the home is only 12% (Watson *et al.*, 1993). This should be compared with a household transmission rate of 80–90%. The lower rate of breakthrough infection as a result of work-based exposures was also demonstrated in a prospective study of 120 HCWs, in whom such infection developed 6 months to 8.4 years after vaccination. The attack rates following exposure at home or work were 18% (4/22) and 8%

(6/72) respectively. The illness was universally mild, with a mean of just 40 vesicles. Thus, although this might represent a risk for onward transmission to susceptible patients, it presents a considerably lower risk as the mean number of lesions seen in wild-type varicella is 250–500. Although it is possible for breakthrough infections to occur after a documented seroconversion to VZV vaccine, in a study by Gershon, 9/12 (75%) of staff experiencing breakthrough were shown to be seronegative after their initial vaccination (Gershon *et al.*, 1988). It is possible therefore that this group could be offered a booster dose of vaccine post-exposure and retested in seven days to assess the response. If antibody was detected they could almost certainly continue to work uninterrupted. VZV vaccine as post-exposure measure is also highly effective, provided it is given within three days of contact with infectious material.

A study from Asano *et al.* (1977) examined 26 contacts in 21 families who were immunized within three days of the index case developing chickenpox. None of the vaccinees developed a rash. However, a control group of 19 unimmunized contacts in 15 families were all infected. In the same study Asano also gave vaccine to 34 healthy siblings within three days of the index case rash; 2/34 (5.8%) got varicella (Asano *et al.*, 1977). The protective efficacy of post-exposure vaccination is therefore >90%. Whilst this approach may not necessarily allow the member of staff to remain at work in very critical areas such as bone-marrow transplantation units, the exposure that led to use of VZV vaccine as post-exposure should be the individual's last time, where they need to be sent home from work for two weeks.

For the three years 2000–2002 at St George's Hospital all cases of varicella in staff and students notified to the medical microbiology and occupational health departments have been documented. A total of 25 cases have been notified across all staff/student disciplines. Of these 25 cases, 13/22 (59%) occurred in individuals born outside of the United Kingdom where the country of birth was known. If pre-exposure vaccination had been adopted at SGH even amongst only the nursing and medical staff

Table 3.3 Incubation periods, communicability and transmission modes for common virus infections

Virus	Mode of transmission	Incubation period	Duration of shedding	Risk of transmission in hospitals ^a	Prevention	Adverse outcomes
Rubella	Respiratory droplet	15 to 20 d	-7 before to +2 d after rash Greatest before rash onset	Low	Screening and vaccination Notification	Congenital Rubella Syndrome
Measles	Respiratory droplet	7 to 18 d	-1 before prodrome to +4 d after rash	Low	Screening and vaccination Notification	SSPE Death in I/C pts
Mumps	Respiratory droplet	14 to 19 d	-6 before to + d after symptoms (maybe up to 9 d)	Low (may increase in certain cohorts)	Screening and vaccination Notification	Meningitis Deafness
Chickenpox	Respiratory droplet Physical contact Airborne, possibly	10 to 21 d (normally 14)	-2 to +7 (maximum in children) (or until scabbed)	Moderate	Screening and vaccination Reporting/notification	Congenital chickenpox Death in I/C pts ^b
Parvovirus	Respiratory droplet	13 to 18 d	-6 to -3 d before symptoms	Low/moderate	Reporting	Miscarriage Aplastic crises Chronic anaemia
Enteroviruses	Faecal-oral Fomites Airborne	5 to 7 d	1 to 8 weeks	Low/moderate	Reporting	Severe neonatal disease
Influenza	Respiratory droplet Airborne, possibly	2 to 3 d	-1 to 7 d	High	Reporting vaccination	Death (I/C, elderly)
Parainfluenza	Respiratory droplet	1 to 7 d	7 to 21 d	Very high	Reporting	Ward closure
RSV	Respiratory droplet	1 to 5 d	7 to 21 d	High	Reporting	Death in I/C
HSV	Saliva Physical contact Fomites (mean = 6)	Few d	1 to 8 weeks	High if breakdown in hand washing/ personal hygiene	Reporting	Death in I/C Death in neonates
CMV	Physical contact with virus at mucosal surfaces	1 to 2 wk	week	Low	Hand washing	None
Rotavirus	Faecal-oral Respiratory	2 to 4 d	6 to 10 d	High	Isolation Hand washing	Prolonged stay in hospital
SRSV	Faecal-oral Airborne, via vomiting	1 to 3 d	Up to 2 weeks (allow back to work 48 h after symptoms resolved)	Very high	Reporting Isolation	Ward and hospital closure

^aRefers to overall risk of transmission, modified by level of immunity in the population.^bI/C pts, immunocompromised patients.

Table 3.4 Arguments for and against use of varicella vaccine for health-care workers

For vaccine use	Against vaccine use
Eliminate varicella from staff	Vaccine costs £80 (€/\$150) per person
Of great use, especially in high-risk areas (paediatrics, HIV, transplant, neonatal, Obs and Gyn)	Administration costs and potential for post-vaccine antibody testing
Much lower risk of transmission to pregnant staff and patients in hospital	Vaccine-associated rash
Reduce potential for nosocomial transmission	Durability of immunity, especially after exposure
Very good safety record; in use for about 30 years	Possible need to send off or restrict to nonclinical work after intense exposure
Lower incidence of shingles in vaccines	Inadvertent immunization of immunosuppressed staff
Lower rate of vaccine-related events compared with natural infection	Latent virus reactivation (zoster)
Durable immunity with natural boosters	
Virtually no risk of transmission from one healthy person to another	
Less worry for final exams in students (about 5–10% intake susceptible)	
May be used post-exposure (recent US and UK license)	
Potential case for complaint if transmission of wild-type virus occurred from a susceptible staff member to vulnerable patient	

and students, 85% of chickenpox cases would have been prevented (Devi and Rice, 2002). Since having used the vaccine for all susceptible staff, we have seen a dramatic decline in the number of staff developing varicella, having witnessed just one case of breakthrough infection. At the same time, we have found that about 10% of 130 vaccinated staff in the three years since receiving vaccine have reported exposures to VZV either at home or in the workplace, with only one developing breakthrough disease (Shin, G., personal communication). This represents a considerable financial saving to a hospital with 5000 employees, of whom 3–5% will be susceptible to chickenpox when starting work.

Two other problems with the issue of immunizing staff against chickenpox are that as it is a live attenuated vaccine it may reactivate causing zoster later in life, and that it may be inadvertently given in pregnancy. The issue with accidental use of vaccine in pregnancy is clearly ideally avoided, but the data that exist suggest that it presents a very low/theoretical risk to the developing fetus. In 629 prospectively-enrolled women who received vaccine whilst pregnant, among the 131 babies born to seronegative mothers 3 had major defects (rate, 3.7% (95% CI 0.8–10.7)) yet there was no evidence of congenital varicella syndrome. Though the number of women followed up is too small to exclude a very low risk, the data collected so far do not support a causal role of the vaccine in birth defects (Wilson *et al.*, 2008).

The vaccine probably offers benefit over wild-type infection because the rate of zoster with reactivated vaccine virus is about 50% lower than after wild-type infection, as evidenced by the fact that reactivation of the Oka strain occurs five to seven times less commonly than wild-type virus (Saiman *et al.*, 2001). The key problem with a widespread staff immunization policy, even though it is established that vaccinating susceptible staff will reduce considerably the risk to patients from chickenpox, is that immunity to infection after vaccination is not guaranteed as it is after natural chickenpox, and that it is not possible to identify which staff may be at risk of breakthrough infection after an exposure.

The decision has to be made what to do with immunized staff in these circumstances. The data indicate that whilst transmission of breakthrough wild-type varicella infection may occur, especially in immunosuppressed patients, this is much less likely than after natural varicella. If a staff-wide vaccination policy were to be instituted, it should be accompanied by a concerted effort to immunize simultaneously the most vulnerable patient groups.

HERPES SIMPLEX VIRUS

The prevention of nosocomial herpes simplex virus (HSV) infections relies entirely on good standards of personal hygiene. Though virus may be shed in genital tract and in

saliva without clinical lesions, adhering to hand washing with soap and water or alcohol-based gels is sufficient to destroy infectious virus. The main reason for concern over nosocomial HSV infections is the potential for transmission to neonates born either pre-term or term, as neonatal HSV infection still has extremely high rates of mortality and morbidity even with the prompt use of antivirals (Francis *et al.*, 1975; Hammerberg *et al.*, 1983). Approximately one-third of cases of neonatal HSV infection are thought to be acquired from a nonmaternal source. It is vital therefore that staff employed on delivery suites, post-natal wards and neonatal units understand the importance of hand washing and of the absolute requirement to absent themselves from work if they develop a herpetic whitlow. A herpetic whitlow in a midwife/doctor/nurse working on the delivery suite, post-natal or neonatal units is an extremely serious matter as transmission can be very efficient. An extensive outbreak of herpetic gingivostomatitis with a transmission rate of about 40% was described in a dental practice after the dental hygienist was found to be at work and suffering from a herpetic whitlow (Manzella *et al.*, 1984).

It is important to note, however, that although nosocomial transmission has been proven by restriction enzymatic digestion analysis of cultured isolates, often the identity of the presumed common source has remained undiscovered (Linnemann *et al.*, 1978).

Virus has also survived for more than 60 hours on a cot which had been used for a baby who died from disseminated HSV infection. Further transmission was entirely preventable, since more than 60 hours after this baby died the cot was reused without any form of decontamination. A second neonate was then nursed in it. Twelve days later this infant also died (Sakaoka *et al.*, 1986).

Herpetic whitlow is also an occupational hazard, especially for those working in intensive care when handling parts of respiratory equipment, which may be contaminated with HSV as a result of a silent reactivation secondary to intercurrent illness. In some reports there is circumstantial evidence of HSV nosocomial pneumonia in such patients. Evidence for this has been provided by the presence of typical viral inclusions from the fluid obtained at bronchoscopy and a dramatic response to aciclovir. However, in such reports although the assertion was made that HSV transmission between patients is possible, the same unit was unable to determine which bronchoscope was used on which patient (Mohan *et al.*, 2006). Moreover, isolation of HSV from the respiratory tract of patients in intensive care may be as high as 31%, so its causation in nosocomial pneumonia is uncertain (Daubin and Vincent, 2005).

NOROVIRUSES

Of all the viruses capable of causing nosocomial infection, noroviruses probably present the greatest challenge to clinical virologists and infection-control teams. Though initially termed 'winter vomiting disease' after a school outbreak in Norwalk, Ohio in 1966, these agents are now known to circulate all year round. To an individual they are of little consequence as the illness is short-lived, self-limiting and only in exceptional cases does it lead to any complications. In hospitals, nursing and residential care homes, however, when outbreaks occur, almost without exception, either the ward or occasionally the entire hospital must close. Moreover, fatalities are described. Between 1992 and 2000, a total of 43 fatal cases were seen in 38 such outbreaks in England and Wales (Lopman *et al.*, 2003). Of particular note with these viruses is their propensity for considerable spread to contacts, resulting in an attack rate of more than 50%. There are many factors, both host and viral, that lead to this. Norovirus outbreaks are notoriously disruptive to the normal functioning of the hospital, resulting in closure of key units and cancellation of elective operations and often attracting unwanted media attention. In terms of cost alone it has been estimated that the NHS in England spends £115 million (US\$230 million) on outbreak control of gastroenteritis annually (Lopman *et al.*, 2005).

The highest outbreak rates have been seen in general medical units, followed by geriatric and orthopaedic units, in units with shorter average lengths of stay and in those with high levels of activity. All this points to continued and repeated introductions of susceptible hosts (patients) to fuel transmission. Indeed, acute hospitals almost certainly have a role in providing further impetus to enable virus to be transmitted back into the community from recently discharged patients. Units which have experienced a previous outbreak, especially within the past month, are also more at risk of subsequent outbreaks (Lopman *et al.*, 2005).

It is known from volunteer studies and outbreak investigations that the incubation period is short, often 12–48 hours. Indeed, volunteers exposed to infectious virus orally have been found to be shedding virus in faeces only 15 hours later. The illness commences abruptly with projectile vomiting in over half of cases and profuse watery diarrhoea. However, because the occasional case of vomiting and/or diarrhoea is not unusual among patients, this may be overlooked initially. The environment can then become contaminated quite quickly; as the infectious dose is 10–100 particles it has even been suggested that a single episode of vomiting is sufficient to generate between 300 000 and 3 000 000 infectious doses (Caul, 1995). This is further exacerbated by the fact that

viral particles are capable of resisting low-dose chlorine disinfection and temperatures of 60°C so that any breakdown in hand washing leading to secondary cases occurs via the faeco-oral route. Fomite transmission is also possible because of its environmental stability and it has been suggested that infection may occur via airborne transmission (Marks *et al.*, 2003). Evidence from PCR detection of viral RNA indicates that virus excretion persists for longer than previously thought. Although virus shedding in stool is maximal at 24–72 hours after exposure, virus can be detected for almost two weeks in both symptomatic and asymptomatic persons. The epidemiological significance of these new findings is unclear. Finally, because of the strain diversity among the noroviruses, there is incomplete cross-protection and no long-term immunity (Lopman *et al.*, 2008). Repeated infections throughout life are therefore common.

The main focus of infection control should be that after notification of an outbreak on a ward, whilst attempts can be made to stop the virus from spreading to other patients or staff on that ward, maximum effort must be to stop it affecting other wards in the hospital. An outbreak described in Salford, United Kingdom in 1994 is illustrative. By the time the problem was highlighted to microbiology, over 80% of the eventual total number of cases on the first ward to be affected were either symptomatic or incubating the infection.

Establishing an early aetiological diagnosis of norovirus gastroenteritis with laboratory techniques has been difficult as the sensitivity of the traditional technique of electron microscopy is as low as 20% and decreases rapidly in time from symptom onset as the diarrhoea, which is often short-lived, improves. Whilst use of enzyme immunoassays offers an alternative approach, as they are significantly less sensitive than real-time PCR, they are still useful only in outbreaks when the sensitivity of a clinical diagnosis is already high. However, real-time reverse-transcriptase polymerase chain reaction (RT-PCR) is becoming much more widely available, is extremely sensitive and is applicable to testing of individual specimens. This can be both useful and problematic at the same time because whilst increases in test sensitivity are welcome, asymptomatic infection is being detected (Adamson *et al.*, 2007; Gallimore *et al.*, 2004; Pang *et al.*, 2004). As the significance of such a finding is presently unclear it still presents problems with regard to the clarity of infection-control advice.

With more laboratories being able to confirm suspected norovirus infections in real time, this may have a positive impact on infection control of this virus, but this remains to be seen. Indeed, in a prospective study of the epidemiology and costs of nosocomial gastroenteritis in England

in 2002–2003, the most important act to limit the spread of infection was prompt closure of the affected wards. Outbreaks in which the affected unit was closed within three days of the first case were contained in a mean of 7.9 days compared with 15.4 days for units where closure took place more than three days after the first case was identified (Lopman *et al.*, 2004).

Fortunately, to aid rapid identification a clinical diagnosis of a norovirus outbreak is usually reliable if the following criteria are adhered to: stool samples are negative for bacterial pathogens, the percentage of cases with associated vomiting is >50%, the duration of illness is short (one to three days), the incubation period, if available, is only 24–48 hours and both patients and staff are affected.

All affected patients should either be nursed in single rooms or cohorted. Strict hand washing must be observed and gloves and gowns must be worn. The risk of infection has been shown to increase in a linear fashion with the number of patient contacts or exposure to nearby vomiting. Although there is no evidence that the wearing of masks reduces the risk of infection, they could be considered for individuals who clear up and dispose of vomit and faeces. All dirty linen should be disposed of with minimal agitation (to prevent further environmental contamination), horizontal surfaces and floors should be cleaned with 1000–5000 ppm of available chlorine, approximately equivalent to about a 10% solution of household bleach. It is also important to remove visible faecal matter using a detergent prior to disinfection with hypochlorite solution as norovirus RNA was detected on up to almost a third of surfaces which had been treated with bleach only (Barker *et al.*, 2004). There is no need to clean walls unless visibly contaminated. This environmental cleaning should continue once the outbreak has subsided. As the number of staff affected is often high, personnel shortages may lead naturally to ward closure. If they do not, however, the ward must be closed to new admissions for a period of 72 hours after the last case. Furthermore, the staff on the ward at onset of the outbreak must not work on other wards and neither should patients be transferred to other areas unless it is medically necessary to do so. Even then consultation should take place with the infection-control team. Staff must be made aware of the critical importance of not coming to work unless well. Visitors too must be made aware of the necessity for hand washing before and after entering the ward and non-essential staff should be excluded. To prevent outbreaks it is also worth remembering that regular staff education in a variety of formats (public lecture, e-mail, information with payslips, hospital web site, public notices at hospital entrances) is to be encouraged.

ROTAVIRUS

Rotavirus infection is universal in childhood so it is not surprising that it can be spread in hospital and health-care settings, though the exact burden of nosocomial rotavirus infection remains unknown. The available literature makes it very difficult to give an accurate incidence; the rate varies between studies from 1–2 to as high as 50 per 1000 hospital days (Chandran *et al.*, 2006). Infection in the community is seasonal, with a winter peak normally among children aged three months to three years; in cases of nosocomial infection the same winter seasonality is seen but neonates, older children and the elderly may all be affected, with outbreaks described in hospitals, day-care centres and nursing homes. In neonatal infection the disease may often be mild or asymptomatic and may even protect against symptomatic disease in later childhood. Breast-feeding may offer protection in this particular patient group.

Controlling infection is difficult. Experimental infection in volunteers was shown to require only low virus inocula (via the faeco-oral route) and the mature virus particle is not enveloped and as such is resistant to detergents and many disinfectants (Sattar *et al.*, 2000; Ward *et al.*, 1986). It can survive in the environment for many days or weeks, making fomites such as toys into vehicles for transmission, and transfers by hand to and from inanimate surfaces has been proven (Ansari *et al.*, 1988; Sattar *et al.*, 1986). On hands it can persist for at least four hours and effective removal requires high concentrations of alcohol-based solutions (>80% alcohol) or chlorine-based compounds at 800 ppm. Nevertheless, concerted campaigns can reduce the rate of nosocomial infection (Zerr *et al.*, 2005).

Ultimately the reintroduction of two new rotavirus vaccines into childhood vaccination programmes in some developed countries may lead quickly to the disappearance of severe infection in childhood infection and so should, along with good hand-hygiene practice, lead to a reduction in the nosocomial burden, as children will be much less likely to require hospital admission. Whether or not a reduction in nosocomial rotavirus infection will occur depends on the full potential of the vaccine being demonstrated and the necessary resources and infrastructure in developing countries becoming available.

PARVOVIRUS B19

Infection with human parvovirus B19 occupies a unique place among nosocomial virus infections. This is because once a single case of symptomatic infection has been identified in a member of staff, secondary cases have probably

already been infectious for several days and infected tertiary cases, who, in turn, will be infectious in the next few days. When stated thus, it would appear that infection with parvovirus would be universal in childhood and that the vast majority of adults would be immune. This is not the case. Indeed seroprevalence of parvovirus B19 IgG among adults reaches only 50–60%. For reasons unknown, parvovirus B19 is not as infectious as most other childhood virus infections. This can create problems when patients are admitted to hospital with parvovirus infection or when a member of staff develops infection. However, the problems are largely illusory, as proven nosocomial transmission is in my opinion an uncommon event.

When the index case (patient or staff member) is identified they pose no further risk of infection to others; the potential for spread of infection lies with the contacts (staff and patients) whose infectivity or immunity status is unknown. There are two reasons for this. Firstly, the typical symptoms of rash and arthralgia manifest only when virus antigen–antibody complexes are formed; thus the index case presents when no longer infectious. Secondly, volunteer studies have shown that the period from exposure to such symptoms is normally between 13 and 18 days (maximum 21 days), yet the period from exposure to infectiousness is only 7 days. In addition, as an estimated 20–30% of adult cases are symptomless, it would appear likely that virus transmission could pose significant problems within hospitals.

Such nosocomial transmission may also be especially hazardous as there are particularly vulnerable patient groups at risk of adverse outcomes, namely pregnant women, the immunocompromised and those with reduced red blood cell survival, who may develop a life-threatening transient aplastic crisis.

The available information, however, suggests that whilst hospital outbreaks do occur, they are uncommon. Moreover, before ascribing cases detected within a hospital to be nosocomial, they need to be examined carefully in the context of a wider community epidemic that may be occurring concurrently.

Indeed, a pseudo hospital outbreak was described in a maternity unit after several cases among staff and patients during the preceding weeks had been notified (Dowell *et al.*, 1995). However, when cases of acute infection on other wards, different hospitals and even healthy control subjects were sought for it was demonstrated that the percentage of individuals with recent infection both in and out of hospital was not significantly different; 23–30% in all locations or wards. The most likely explanation put forward was the presence of the largest community-wide parvovirus outbreak for 18 years (Dowell *et al.*, 1995).

Furthermore, a formal evaluation of the risk of nosocomial transmission was performed among 87 HCWs

exposed to two patients admitted with aplastic crises who were not placed in isolation, compared with a control group of 88 HCWs who were unexposed to the cases (Ray *et al.*, 1997). The level of previous infection in the two groups was almost identical, as was the rate of transmission among the non-immune-exposed group and those unexposed; 1/32 (3.1%) and 3/37 (8.1%) respectively. The rates of transmission moved even closer with a subgroup analysis of the most heavily exposed, which showed a transmission rate of 5.8% (1/17) (Ray *et al.*, 1997).

When hospital outbreaks are described they appear initially to be explosive since they are noticed only after many cases in staff and patients have occurred, often over a period of several days. This is demonstrated by the attack rate of 27–50% in susceptible individuals, both staff and patients (Bell *et al.*, 1989; Lui *et al.*, 2001; Miyamoto *et al.*, 2000; Pillay *et al.*, 1992; Seng *et al.*, 1994). What is less clear is whether or not any infection-control measures can prevent further virus transmission. This is important since the measures proposed are potentially very costly in terms of staff absences or redeployment and possible ward closure. Indeed, to limit the spread of infection some authors have proposed ward closure, transfer of only immune staff to any affected ward and preventing the nursing staff from working on other wards. (Pillay *et al.*, 1992). However, by the time an outbreak has been notified it is highly likely that the majority of virus transmissions that are going to occur have already taken place. This was clearly demonstrated in an outbreak at a London, England teaching hospital (Seng *et al.*, 1994). Over a one-month period on a single ward a total of 18 cases of acute parvovirus B19 infection were detected in 15 staff, of whom 12 were symptomatic; 3 patients were also affected. However, of the 12 symptomatic staff and the 3 affected patients, all who had dates of symptom onset, 10 either were or had recently been symptomatic prior to outbreak notification. Moreover, of the 5 further cases that were going to occur, only 4 had developed symptoms within 8 days of notification of the index case. Thus only 1 case was possibly preventable by the institution of infection-control measures (Seng *et al.*, 1994). Another outbreak on a paediatric ward demonstrated, however, that 50% of cases might have been prevented had notification to the infection-control team been made earlier (Pillay *et al.*, 1992). These hospital outbreaks mirror the experience from community outbreaks, for example in schools. Nevertheless, the high transmissibility of parvovirus B19 was shown in an outbreak investigated in a UK primary school for children aged 3–11 years during the epidemic year of 1994 (Rice and Cohen, 1996). This showed that 75% of the total number of cases had already occurred by the time the first few cases had been notified, and of the remaining 25% most had been infected and were

incubating the infection by this time. Any efforts to limit the spread of the infection would thus have had little effect on limiting the outbreak.

The high infectivity of parvovirus in an outbreak setting is also illustrated by demonstrating that after this single outbreak in the school the prevalence of parvovirus IgG rose from 15 to 60–70%, that is to an adult level of immunity (Rice and Cohen, 1996). It is my experience that when single cases of infection are identified among staff, further transmission among staff occurs probably only as a result of social contact. This was also raised as one of the reasons for the extensive ward outbreak referred to earlier (Seng *et al.*, 1994). Moreover, given the reasonably high attack rate in close, social and household contacts of approximately 50% and the long incubation period, it is possible to detect virus in susceptible contacts by PCR or even by using standard electron microscopy. This is an additional measure which may be undertaken to limit the spread of infection.

Nosocomial transmission is a particular problem from patients with aplastic crises, since they present earlier in the course of their illness when they are still infectious. This was clearly demonstrated in a paediatric ward outbreak where 12 health-care workers were infected, most probably as a result of a failure to recognize the infection early enough (Bell *et al.*, 1989). This outbreak was, however, probably made more likely by the fact that in the two outbreaks only 12% and 31% had pre-existing immunity. Transmission has also been described from immunocompromised patients with chronic infection, due to an inability to clear the viraemia and who presumably still shed oro-pharyngeal virus (Lui *et al.*, 2001). However, absence of transmission has also been documented from those with transient aplastic crises and chronically-infected patients whose titres of viraemia are likely to be very significantly different because of the different nature of the two disease syndromes (Koziol *et al.*, 1992; Ray *et al.*, 1997).

Despite the conflicting evidence as to the real risk of nosocomial parvovirus infection, as significant harm to vulnerable patient groups and pregnant staff can occur, the following appear to be reasonable precautions to prevent virus spread:

Patients with reduced red cell survival should have their antibody status assessed and, if non-immune or unknown, should be admitted to a single room if they experience a sudden drop in haemoglobin consistent with a possible transient aplastic crisis. Acute infection should be assumed, especially if the reticulocyte count is normal or low, indicating a shutting down of erythropoiesis. They should not be nursed by pregnant staff and hand washing should be re-emphasized. A case can be made for routine staff screening for those working in particular high-risk

areas, namely paediatric haematology (Bell *et al.*, 1989; Crowcroft *et al.*, 1999; Pillay *et al.*, 1992).

Any staff with rash and especially those with arthralgia should be investigated for parvovirus and rubella, and their household and close social contacts should be offered testing for recent or past infection. However, not all laboratories offer testing and those that do may only perform tests in weekly batches, and it will often be necessary to make a judgement on whether or not staff should be redeployed or furloughed before test results are available. Those who are found to be susceptible, however, can then be tested for the presence of viraemia by real-time PCR. Decisions may then be made about furloughing of such staff.

Pregnant staff who are at less than 20 weeks gestation are at increased risk of miscarriage or fetal hydrops. However, the rate of transmission in the community during an outbreak is likely to be very similar to that seen in hospital and they should be advised accordingly. They should be advised before testing of the low probability of any adverse outcome due to the low transmissibility, moderate levels of adult immunity and low risk of miscarriage. However, advising what an individual staff member may wish to do will also be influenced by other factors, including whether or not the pregnancy was achieved using IVF.

RESPIRATORY VIRUSES

The respiratory viruses are well established as agents capable of nosocomial transmission. They are particularly well suited for many reasons. They are highly infectious with reproduction rates of 5–15 : 1, can spread by both droplet and aerosol routes and have short incubation periods. Explosive outbreaks are thus possible. Furthermore, during the epidemic season high community attack rates of 10% for influenza and 40% for RSV in the groups at highest risk of infection make possible multiple introductions into the hospital. They are capable of survival on surfaces and unwashed hands for hours, and among immunocompromised patients infectious virus may be shed for weeks. Finally, vaccination is available only for influenza and effective antiviral agents are available for only two genera (RSV and influenza). They represent a significant financial burden on hospitals by prolonging hospital stays, increasing mortality in vulnerable patient groups and causing staff absences, with the need to hire of temporary replacements at additional cost. Ward closure may sometimes be necessary to terminate an outbreak.

There are also no tests for checking the immune status of staff. Indeed, for influenza and RSV, re-infection is common. To prevent outbreaks, close cooperation between the microbiology department and the

infection-control team is essential. A key element of this is rapid communication of positive results on all patients in a hospital, especially at the start of the winter season. To facilitate this, rapid diagnostic assays should be employed. A variety of methods are available, but regardless of sensitivity and specificity their proper use is the key to success in controlling infection. Near-patient testing, if used properly, has sensitivity approaching that of conventional immunofluorescent tests, but it still requires knowledgeable staff capable of collecting an adequate specimen. Whilst near-patient tests offer a result within minutes of sample collection, they are less sensitive. Newer PCR protocols involving real-time assays are capable of providing results as fast as IF with enhanced sensitivity. A recent paper demonstrated that real-time PCR using the light cycler could generate a result in less than two hours after specimen receipt and it was significantly more sensitive in older children and adults (Whiley *et al.*, 2002). This study showed that out of 77 samples positive by LC-RT-PCR, 7 were negative by IF testing. Of these 7, however, 6 were from adults or children aged >12 years. This is compatible with lower viral loads in these individuals, most probably as a result of re-infection. Thus PCR offers advantages over IF testing in certain groups, for example less cooperative children, who may be harder to sample, and adults.

Influenza

Influenza is a particular problem in hospitals because of the explosive nature of outbreaks and the especially vulnerable patient groups that are heavily exposed during the epidemic season. During the winter up to 10% of the general population is infected; in hospitals during outbreaks the attack rate may be as high as 50% in patients on affected wards and up to 20% in the general hospital population. All types of ward have been affected and whilst the mortality can be low, in geriatric wards it can be as high as 16% (Gowda, 1979) and in transplant centres mortality due directly to influenza is between 30 and 60% (Weinstock *et al.*, 2000). These figures are particularly worrying since it has been estimated that up to 70% of influenza infections in transplanted patients are nosocomial in origin. Staff are also at high risk of infection, with transmission rates of 11–60% in those caring for patients with influenza (reviewed in Salgado *et al.*, 2002). In hospitals with a particular interest in influenza and a well-developed strategy for preventing nosocomial infection, however, the attack rate in staff is significantly reduced to only 2% (reviewed in Salgado *et al.*, 2002).

Influenza viruses are notable for their antigenic diversity. They are also, however, capable of undergoing sudden shifts in their external glycoproteins, exemplified

by the global spread of two novel avian influenza types (H7N7 but most notably H5N1) which have caused serious infection in humans, with a mortality of >50% in healthy adults. We are currently fortunate in that their person-to-person spread appears to be very inefficient, although it is essential to maintain vigilance since they may become better adapted to the human host and may, like avian influenza viruses, be capable of transmission faeco-orally (Gambotto *et al.*, 2008; Wang *et al.*, 2008).

There is good evidence that influenza viruses are capable of surviving for varying amounts of time in artificially-generated aerosols and that when such aerosols are applied to animals and human volunteers virus transmission is reasonably efficient, with a transmission rate of about 20% (Alford *et al.*, 1966). This would suggest that airborne transmission might be responsible for natural infection. This is an important consideration when advising on infection-control procedures since it would mean that wearing a face mask, and preferably an N95 mask, would be necessary to prevent patient-to-staff transmission.

However, the evidence that airborne spread is important is weak in three key areas. The particle size of the artificial aerosols is significantly greater than that produced naturally by coughing or sneezing; particles are much smaller in experimental conditions, with a diameter of about 5–6 μm or less, with <10% being 8 μm in size. This compares with 99.9% of the naturally-produced particles, being >8 μm in diameter. Particles of the larger size would fall quickly to the ground and so would not be available for aerosol spread. Some of the experimental studies are also flawed in that they have had to use equipment to agitate the ambient air in order to maintain the aerosol. Finally, the infectious dose is considerably lower (10–100 fold) when delivered by artificial aerosol compared with intranasal inoculation. As this does not, in the opinion of certain authors, reflect the natural history of influenza transmission it is uninterpretable with respect to virus transmission in the natural state (reviewed in Brankston *et al.*, 2007).

Although there are nine observational studies reporting on airborne spread, the most frequently quoted paper demonstrating such a transmission route concerned a 72% attack rate in passengers in the cabin of an aeroplane with an inoperative ventilation system (Moser *et al.*, 1979). The four individuals who decided to leave the plane escaped infection. However, those remaining were free to move around the passenger compartment and so large particle droplet infection is very likely to have occurred. Nevertheless, two other studies have suggested that airborne transmission may occur based on a higher rate of infection in unirradiated departments of 19%, compared with a rate of just 2% in those areas that had upper-air UV disinfection, a known virus sterilizer. Additionally, units

with efficient ventilation systems saw that virus transmission was less common (9–16% patients became infected when between 30 and 70% of the room air was replaced with outside air, compared with <2% transmission with 100% such air exchange between the room and outside (reviewed in Brankston *et al.*, 2007).

The six other studies were more consistent with a droplet route or contact transmission. Indeed, the one study that reported on control measures which were not biased by pre- or post-exposure prophylaxis reported control of an outbreak on a neonatal intensive care unit (NNU) by cohorting patients and staff and instituting droplet or contact control measures. Airborne precautions were not applied (Munoz *et al.*, 1999).

The only time that it would be sensible to consider influenza as an airborne pathogen would be if aerosol-generating procedures were going to be used; that is, in the collection of naso-pharyngeal or broncho-alveolar lavage specimens. It would seem sensible to take precautions against both inhalation and droplet spread by the wearing of a facemask and eye protection.

Influenza virus is stable for 24–48 hours after aerosolization and for at least 5 minutes on unwashed hands. Virus shedding begins one day prior to symptom onset and continues for four to seven days in immunocompetent patients, though extended periods of virus excretion of several weeks are seen in the immunocompromised.

Preventing influenza spread in hospitals is difficult because of the multiple routes of entry of virus in staff, patients and visitors. However, low nosocomial transmission rates are achievable through a concerted action by all staff and the infection-control and occupational-health departments. Staff must understand that they should not report for work if they are ill and will be sent home if they develop an influenza-like illness. The ethos should be one of protecting patients as well as working as a team.

There are essentially three major ways by which the incidence and overall disease burden of nosocomial influenza may be brought under control. The first is to immunize children, as they commonly experience infection and are often seen in hospital. Secondly, the groups at high risk of complications should similarly be vaccinated. Lastly, staff should be immunized on an annual basis. However, staff uptake rates are low; in the United States about 10–40%. There are many reasons for this, mostly based around the lack of knowledge concerning the illness itself and the effectiveness/safety of the vaccine.

This is unacceptable as the importance of influenza vaccine in preventing nosocomial transmission has been shown both ways; outbreaks in hospitals and long-term care facilities have been associated with low vaccination rates in staff Cunney *et al.*, 2000; Saito *et al.*,

2002), whilst units with higher uptake rates have demonstrated a lower incidence of nosocomial infection (Carman *et al.*, 2000; Potter *et al.*, 1997; Salgado *et al.*, 2004); in a unit where the vaccine uptake rate increased from 4 to 67% between 1987 and 2000 the proportion of laboratory-confirmed cases of influenza in staff fell from 42 to 9%, whilst in hospitalized patients the rate of nosocomial infection decreased from 32% to zero (Salgado *et al.*, 2004).

The way to increase uptake rates is to educate staff. Since self-protection is an efficient motivator they should be told about the benefits of vaccination and the risk that influenza poses to them, their families and, finally, their patients. This can be achieved, for example, by posters and messages via hospital e-mail or in pay slips. When senior medical and nursing staff act as role models, their staff have higher vaccine acceptance rates.

Improving access to vaccine is seen as a vital step to increase staff coverage. This can be done by offering the vaccine in as convenient a form as possible, for example by immunizing staff in their workplace, during conferences or in out-of-hours clinics at the end of the working day. By adopting such an approach, uptake rates can increase from 10 to 70% (reviewed in Salgado *et al.*, 2002). Finally, the vaccine coverage should be measured regularly and reported, including posting the rates of vaccination in different areas of the hospital and among different occupational groups. This could also be used as a measure of the quality of care an individual hospital provides (Pearson, 2006).

The overall efficacy of vaccine in patients and healthy staff is 70–90%. Vaccination of staff is probably the most effective measure in reducing mortality in hospital from influenza, as shown in a study from Scotland, especially as some studies in elderly institutionalized patients have demonstrated much lower rates of efficacy, of 30–40%, for preventing infection (Carman *et al.*, 2000).

Patients who are admitted with suspected influenza should be placed in a single room and put on respiratory precautions; that is, plastic apron, gloves, hand washing and mask. Specimens for rapid diagnosis should be sent as soon as possible so that cohorting is possible if the demand for single rooms becomes too great. There is no need to place such patients in negative pressure as the experience over a 15-year period from a hospital in Virginia, USA, has been that admitting patients with influenza to private rooms at slight positive pressure has not resulted in temporally-related secondary cases (reviewed in Salgado *et al.*, 2002).

If there appears to be an increase in the number of cases of proven influenza or influenza-like illnesses on a particular ward, consideration should be given to immunizing staff as rapidly as possible. Whilst this takes effect,

antiviral prophylaxis might also be considered. Amantadine, the oldest of the antiviral agents active against influenza, was shown many years ago to be an effective prophylactic regimen. However, it does suffer from three major problems: it is inactive against influenza B, shows rapid development of drug resistance and has significant CNS toxicity. Even during a short treatment course of only two to five days, up to one-third of influenza virus isolates will have developed resistance. These are as transmissible as wild-type virus and have been known to cause fatalities. CNS toxicity is also problematic and 18% of patients suffered from CNS side effects, compared with just 2% taking Rimantadine. The newer neuraminidase inhibitor agents, Zanamivir and Oseltamivir, given by inhalation and orally respectively, have an improved side effect profile and so are better tolerated. Furthermore, when used in the home as post-exposure prophylaxis they are extremely effective in preventing influenza illness, with efficacies of 74 and 90% respectively. Nevertheless, with increasing use it has become apparent that high-level resistance to Oseltamivir has been seen in influenza isolates across Europe and in the United Kingdom. Indeed, I have seen such an occurrence with virus transmission involving an NNU where all four of the five patients with clinical evidence of infection and a sufficient sample had the key mutation (H274Y) in neuraminidase detected.

Parainfluenza Virus

Like other respiratory viruses parainfluenza virus may cause outbreaks on nurseries, neonatal units, long-term care facilities and BMT units. It is important to remember that infection in high-risk patients has considerable mortality, approximately 40–50% in BMT patients (Zambon *et al.*, 1998); specific antiviral compounds do not exist and often the only way to terminate an outbreak is for the affected unit to be closed to admissions. When outbreaks occur it is always in the context of a wider community epidemic, which for parainfluenza virus usually runs from May to September each year. Thus it is possible that what may appear to be an outbreak is merely the result of multiple introductions of virus into the ward by patients, staff and visitors. However, the available data from two large outbreaks, one on a paediatric ward, the other in a BMT unit, showed the introduction of one PIV strain and its spread and propagation from person to person (Karron *et al.*, 1993; Zambon *et al.*, 1998). How virus may be transmitted is not easy to determine with absolute confidence, but transmissions by unwashed hands, contaminated fomites and large droplets are all possible. In a report from the United Kingdom, one of two outbreaks on a BMT unit started nine weeks after the index case had been identified, who was still shedding infectious virus (Zambon *et al.*, 1998). This

emphasizes the absolute necessity for extreme vigilance when adhering to hand washing and for prolonged periods when patients are immunocompromised. Indeed, virus excretion for four months has been demonstrated in this patient group (Zambon *et al.*, 1998). This may be one reason why symptomatic surveillance and patient isolation have been shown not to terminate outbreaks in such units (Nichols *et al.*, 2004). What was also clear from this study and from another in Canada is that parainfluenza virus outbreaks can, unlike influenza virus outbreaks where the median duration is only seven days, be protracted and ultimately only terminated by closure of the unit to new admissions (Moisiuk *et al.*, 1998). When the first case of a parainfluenza virus is discovered on a ward with high-risk patients, full adherence to all necessary infection-control measures (gowns, gloves, patient cohorting and hand washing), including reiterating the standing instructions to staff not to come to work if unwell, must be rigorously enforced. It has now been established that all four types of human parainfluenza virus are capable of causing prolonged outbreaks with nosocomial spread (Lau *et al.*, 2005).

Adenovirus

Adenovirus is a leading cause of epidemic keratoconjunctivitis in adults and conjunctivitis in children as well as community-acquired upper and lower respiratory tract infection and gastroenteritis. Nosocomial transmission has been described in different hospital and care settings, for example paediatric, neonatal, haematology and chronic psychiatric facilities, but most notably in ophthalmic wards and departments (Chaberny *et al.*, 2003; Klinger *et al.*, 1998; Montessori *et al.*, 1998; Warren *et al.*, 1989). Whilst other areas of the hospital may be affected, the original source of virus is often associated with contaminated ophthalmic equipment (Percivalle *et al.*, 2003). Whilst the original route of acquisition may be from improperly disinfected equipment by the use of isopropyl alcohol (Montessori *et al.*, 1998) or from contaminated solutions for instilling into the eye, the propagation of an outbreak is also from inadequately decontaminated hands of hospital staff (Montessori *et al.*, 1998; Percivalle *et al.*, 2003). Large outbreaks may also occur and be problematic to bring under control, especially if there is an upsurge in community-acquired infection. One important aspect of an effective infection-control strategy is to triage and cohort patients with suspected conjunctivitis so that they can be treated by staff who are assigned only such patients (Montessori *et al.*, 1998; Warren *et al.*, 1989).

The outbreaks that cause most concern are those that affect the immune-suppressed, as infection has a significant excess mortality and with the possible exception of cidofovir, no truly specific antiviral agent

is currently available. It is also particularly troublesome since it resists desiccation, especially on nonporous surfaces, surviving for up to 35 days (Nauheim *et al.*, 1990); it is only effectively disinfected by chlorine-based agents and may be shed for prolonged periods from conjunctiva and the gastrointestinal and respiratory tracts. Sharing of toilets and bathrooms may exacerbate virus spread (Jalal *et al.*, 2005).

Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV)

The spread of severe acute respiratory syndrome coronavirus (SARS-CoV), now known to be caused by a previously unknown coronavirus, has highlighted the extreme dangers that are posed by emerging, particularly respiratory, pathogens to virgin-soil populations. During 2003, over 30 countries in 5 continents were affected with great rapidity. Over 8000 cases were diagnosed, resulting in 774 deaths. Though it commenced as a zoonosis, originally from the horseshoe bat (*Rhinolophus* sp.), it became apparent quite quickly that it was capable of person-to-person spread. It also disappeared almost as quickly, only to briefly reappear in late 2003 upon resumption of the wildlife food market, at which time it was found to be present in the horseshoe bat. Thus vigilance is necessary as it could re-emerge again, especially since not only are coronaviruses are capable of genetic recombination but there is a culture of the consumption of exotic animals in southern China.

In man, the virus is present in the respiratory secretions, faeces, urine and tears of affected individuals. It became apparent that nosocomial transmission was facilitated by the use of nebulizers, bronchoscopy and suctioning procedures, and cardiopulmonary resuscitation. All of these activities result in the generation of a large quantity of droplets containing infectious virus particles. It was also unfortunate that hospitals were unwittingly involved in amplifying the spread of the epidemic (Tomlinson and Cockram, 2003). This was seen very clearly in the Prince of Wales Hospital in Hong Kong, China. Within just three weeks of admission of the index case, 156 further cases had been diagnosed, all capable of being traced back to this single case. The major reasons given for the spread of infection in this hospital were failure to apply appropriate isolation precautions to cases not yet identified as SARS-CoV and breaches of such precautions. Staff were also appearing for work with mild fevers, so potentially exposing other patients and staff. Of especial importance in the rapid dissemination of infection in this hospital was the use of a nebulized bronchodilator in the index case, hugely increasing the droplet contamination of the patient's environment.

A case control in five Hong Kong hospitals demonstrated that adhering to such precautions as use of a mask, gloves, gowns and hand washing resulted in no secondary cases in 69 staff who followed these prevention-of-infection measures. However, all of the 13 staff who became infected after caring for SARS-CoV cases reported not adhering to at least one of these measures (Seto *et al.*, 2003). When each of the four infection-control measures was examined, however, only the wearing of N95 masks was shown to be essential for protection against infection. This agrees with the principal mode of spread of SARS being by respiratory droplets. Unlike most of the other respiratory viruses, wearing an N95 mask, capable of trapping 95% of all particles, adds a further level of protection to staff. The wearing of simple paper masks did not afford any protection.

The WHO has since given recommendations to control the spread of SARS-CoV should it reemerge. It is essential that these are adhered to as there is neither vaccine nor antiviral agent; preventing infection is the only option. The recommendations are centred around rapid case identification, triage and immediate isolation, with droplet and if necessary airborne precautions. Thus when a suspected case enters a hospital, these conditions must be strictly enforced, the patient must be isolated in a single room and, if procedures which may generate an aerosol are necessary for patient diagnosis and management, this should be a negative-pressure room. If mechanical ventilation becomes necessary, this must take place in such a room. However, if the only patient requires supplemental oxygen, the use of nasal cannulae is believed to reduce the risk of airborne spread compared with a high-flow face mask. If no additional oxygen is required, patients who are proven or who may represent possible cases of SARS-CoV should be given a face mask to wear, preferably one that filters exhaled air, in order to prevent the spread of SARS-CoV in hospitals. Furthermore, after discharge the patient must be told to adhere to strict personal hygiene. The staff involved in the initial contact must wear a mask, for example N95 with 95% filter efficiency and goggles. They must wash hands before and after contact and wear gloves. Standard disinfectant solutions such as household bleach and alcohol gel preparations should be readily available in the immediate vicinity of the patient as the virus is easily inactivated. Further infection-control measures include restricting visitors and supervising them in the use of protective equipment. It has also been suggested that the wearing of footwear that can be easily decontaminated should be considered. The removal of linen should be done by staff wearing full protective equipment (goggles, N95 or N100 masks, gloves, disposable gowns/aprons). The linen must be placed in biohazard bags and destroyed by incineration. As virus

has been shown to survive in faeces and respiratory secretions for 4 and >7 days respectively, room cleaning should take place using a broad-spectrum disinfectant, for example household bleach.

Respiratory Syncytial Virus

RSV, like all other seasonal pathogens, is problematic for a brief period of time, during which the paediatric wards soon become full because of infants with bronchiolitis. Whilst this is expected and normal for winter, RSV can unexpectedly lead to disastrous outbreaks on paediatric intensive care units (PICU)s and NNUs, and among BMT recipients. In one such NNU, leaving aside potential excess mortality, the cost of an outbreak was estimated at US\$1.15 million (Halasa *et al.*, 2005). It is an especially serious infection in the immune-suppressed and those with chronic cardiac and pulmonary pathology when the mortality is high. The most effective methods for preventing nosocomial infection with RSV are regular hand washing by staff, parents and visitors, the wearing of gloves and gowns, and either the cohorting of infected patients or admission directly into single cubicles. This directly limits virus spread, as it has been shown that direct contact with contaminated secretions plays the key role in transmission (Hall and Douglas, 1981). Hand washing is important because it helps irradiate the culture among all involved in patient care. Indeed, the effectiveness of such a measure has been shown by several studies. Isaacs *et al.* 1991 studied hospital-acquired infection among children with congenital cardiac and lung disease. They showed a nosocomial infection rate of 35% prior to the introduction of the enhanced infection-control measures of hand washing and patient cohorting. Not only were they able to demonstrate a 66% reduction in the rate of nosocomial RSV but among patients admitted for >14 days, the rate was even higher. During the year prior to the intervention of 11 patients staying longer than 14 days in hospital, 8 (73%) developed nosocomial RSV, compared with 2/77 (2.6%) during the period of intervention.

Conversely, when a breakdown in barrier precautions takes place a rapid upsurge in nosocomial infection may result. A large outbreak occurred in a PICU, probably as a result of just such a breakdown in basic droplet precautions and the fact that some children became persistent shedders of infectious virus for as long as 27 days (Thorburn *et al.*, 2004). Indeed, in this outbreak it was demonstrated that droplet precautions (strict hand washing, single-use aprons and education) played a more important role than isolation in a single room, since nosocomial spread was reduced despite all available cubicles

being occupied and infectious cases being treated in open ward areas (Thorburn *et al.*, 2004).

Although others have suggested a role for Palivizumab in controlling neonatal RSV transmission, this is still unclear (Abadesso *et al.*, 2004).

Prevention of infection is paramount on BMT units, where the mortality from LRTI RSV infection may be 30–100% (Harrington *et al.*, 1992). Moreover, since infected patients shed virus for longer they act as potential reservoirs for the maintenance and cascading of infection in the ward.

Rapid virus diagnosis may assist in reducing the rate of nosocomial transmission but the beneficial effect of testing all symptomatic infants for RSV infection remains to be demonstrated (Goldmann, 2001). Indeed, whether cohorting is required on the basis of specific virus type or on respiratory symptoms alone is debatable. It was noteworthy in the study by Madge *et al.* (1992) that where children were initially cohorting on the basis of respiratory symptoms alone, no symptomatic child initially placed in an RSV-infected cohort but subsequently shown to be RSV negative became infected as a result of this contact (Madge *et al.*, 1992). The underlying reason for this must be the protective effect of viral interference in the upper and lower respiratory tracts. Indeed, with the increasing use of real-time multiplex PCR for respiratory virus diagnosis, cohorting patients infected with two or more viruses is, practically speaking, impossible.

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Emerging Virus Infections

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INTRODUCTION

Although infectious disease mortality declined sharply throughout the twentieth century, apart from a dramatic increase following the 1918 pandemic of influenza virus, in the last 20 years a small but noticeable increase in mortality from infectious diseases can be observed (Armstrong *et al.*, 1999; Mahy, 2000; Figure 4.1). The start of this increase roughly coincides with the appearance in the population of human immunodeficiency virus (HIV) infection and associated cases of acquired immune deficiency syndrome (AIDS), but there is good evidence suggesting that many factors contributed to this situation.

In 1991, the Institute of Medicine (IOM) of the US National Academy of Sciences convened a multidisciplinary committee to consider emerging infectious diseases and the factors responsible for them, and to make recommendations for future actions to be taken in response to their threat to public health (Lederberg *et al.*, 1992). In 2001, the IOM convened a new committee on Microbial Threats to Health in the Twenty-First Century (Smolinski *et al.*, 2003), with a similar mandate to review the situation 10 years later. It is noteworthy that during the period covered by the two sets of recommendations more than 60 new viruses were recognized in the human population, and four years later, in 2007, this number reached 80 (Figure 4.2). These range from hepatitis C virus (HCV), first recognized in 1988, to the new human coronavirus, first recognized in 2003, which is responsible for severe acute respiratory syndrome (SARS). As with most emerging virus infections, these are examples of world diseases that threaten public health throughout the world and are not limited in their potential geographic range. As pointed out in the first report on emerging infections, in

the context of infectious diseases, there is nowhere in the world from which we are remote and no one from whom we are disconnected (Lederberg *et al.*, 1992).

FACTORS CONTRIBUTING TO EMERGENCE

Considering infectious diseases as a whole, the committee of the IOM recognized 13 factors contributory to emergence, and these are listed in Table 4.1. Some of these are natural events that can be responded to but not prevented from occurring, such as microbial adaptation and change, and climate and weather. Others are societal and political in origin and could be eliminated, such as the breakdown of public health measures, and poverty and social inequality. Finally, 'intent to harm' refers to acts of bioterrorism, an example of which might be the deliberate release of smallpox (variola) virus (Mahy, 2003).

This chapter reviews the factors responsible for the emergence of new virus infections under four broad headings: (i) virus evolution; (ii) human demographics, susceptibility to infection and behaviour; (iii) improved technology for the detection of virus infection; and (iv) increased contact with vectors of virus infection. By considering examples of virus emergence under each category, the factors responsible for the remarkable global increase in human virus infections over the last 20 years (Figure 4.2) will become clear.

Virus Evolution

There are a number of different mechanisms by which viruses may evolve. In general, RNA viruses are much more likely to evolve by genome changes than DNA

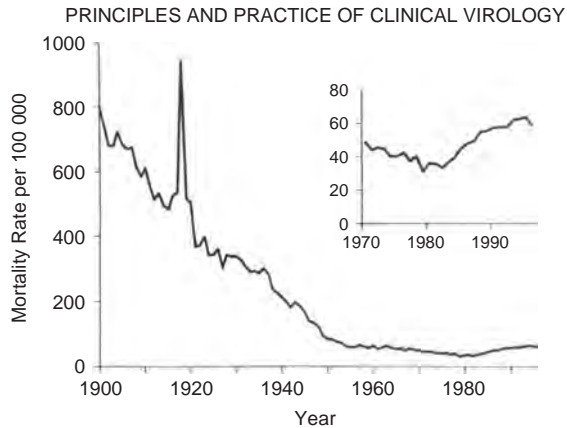


Figure 4.1 Infectious disease mortality in the United States, 1900–1996. (Reproduced from Armstrong, Conn and Pinner (1999) *Journal of the American Medical Association*, 281, 61–66, Copyright © 1999, American Medical Association.)

viruses, and this in part reflects the proofreading functions present in the host cell that preserve the integrity of DNA. Such proofreading mechanisms do not exist for RNA, and so RNA viruses are much more mutable.

Table 4.1 Factors in emergence.

Microbial adaptation and change
Human vulnerability
Climate and weather
Changing ecosystems
Economic development and land use
Human demographics and behaviour
Technology and industry
International travel and commerce
Breakdown of public health measures
Poverty and social inequality
War and famine
Lack of political will
Intent to harm

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Mutation

The most obvious mechanisms for evolution involve point mutations in the genome RNA nucleotide sequence, either random (spontaneous) or in response to a selective pressure, such as inhibition by an antibody or an antiviral drug. For example, influenza viruses undergo frequent

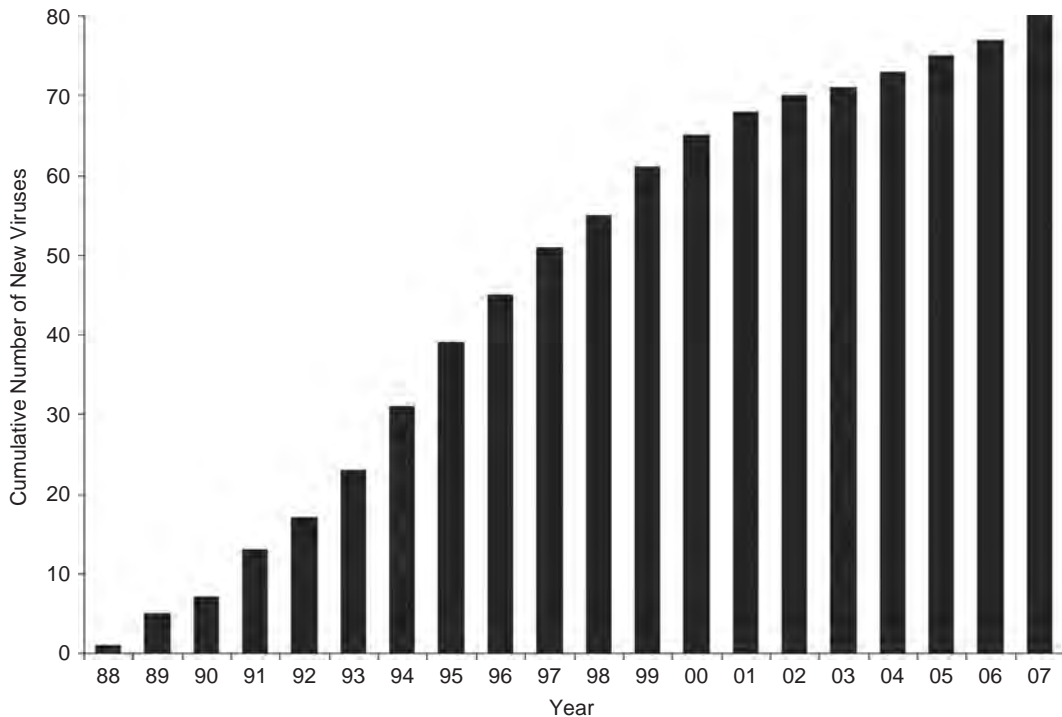


Figure 4.2 Emergence of viruses affecting humans.

mutations in response to humoral antibodies against influenza which exist in the general population; this phenomenon produces 'antigenic drift'.

Another example is HIV, which, in the presence of a drug which prevents DNA replication, such as zidovudine (azidothymidine), generates rapidly mutant viruses that are resistant to the drug.

Reassortment

A second mechanism for genetic change is reassortment, which is a common feature of those viruses that have a segmented genome, such as influenza viruses and rotaviruses. In cases where two viruses of distinct genetic lineage infect the same cell, it is possible for exchange of genome segments to occur, giving rise to a new reassortant virus that may have altered properties of transmission or pathogenesis. This is of particular importance clinically with the influenza viruses, and it has been clearly demonstrated that both the 1957 'Asian' influenza pandemic and the 1968 'Hong Kong' influenza pandemic resulted from viruses that were apparently generated by reassortment between human and avian influenza virus strains (Webster and Kawaoka, 1994).

Recombination

The third most common mechanism of virus evolution is recombination between the genomes of two different viruses. Although not absolutely proven, it seems most likely that rubella virus was originally generated in this way, and we see many examples of recombination in action, especially with the picornaviruses, such as poliovirus. For example, an outbreak of poliomyelitis in Hispaniola in 2001 was caused by a recombinant virus between the live Sabin poliovirus vaccine strain and an enterovirus, which restored the pathogenicity of the vaccine virus (Kew *et al.*, 2002).

As a general rule, recombination events seem to be much more common with viruses having a positive-strand RNA virus genome, and such events are rarely reported for negative-strand viruses, although they certainly can occur.

Human Demographics, Susceptibility to Infection and Behaviour

Changes within host populations have contributed greatly to the emergence of virus diseases during the past 20 years.

Increases in World Population and Global Travel

The rapid increase in the world population has combined with the greatly enhanced opportunity for global travel

to increase the opportunity for persons to come into contact with virus diseases formerly considered to be exotic to the developed world (such as Lassa fever, which causes 5000 deaths per year in West Africa; in 1989 a patient died from Lassa fever in a hospital in Chicago (Holmes *et al.*, 1990). The patient was an American citizen who had visited Nigeria to attend his mother's funeral, and became ill two days before his return to Chicago. Although 102 people had contact with him in the Chicago hospital, fortunately none of these contacts became infected.)

Global travel also provides the opportunity for very rapid movement of a newly emerged virus, as we witnessed when SARS was first recognized in Guangdong Province, China, on 16 November 2002, where it had caused 300 cases of unknown aetiology and 5 deaths. This was reported to the World Health Organization (WHO) on 11 February 2003, and then on 26 February a WHO official working in Hanoi, Vietnam, Dr Carlo Urbani, reported an unusual case of severe acute respiratory disease to the WHO. Other cases were soon identified in Hanoi and in Hong Kong, and many of these were health-care workers. On 12 March 2003 the WHO issued a global alert about these cases, and on 14 March cases of a similar disease syndrome were reported by Canada. Over the next few weeks it became clear that all these cases of severe respiratory disease could be linked to a hotel in Hong Kong (Hotel M), where the index case of the disease, a 65-year-old medical doctor from Guangdong Province in China, had arrived on 21 February. He had noted his onset of symptoms on 15 February, and he was hospitalized on 22 February and died the following day. Although he only stayed in the hotel one night to attend a family wedding, this single individual infected 12 other guests in the hotel, and from them several hundred persons in widely disbursed geographic areas became infected within a few weeks, resulting eventually in more than 300 deaths between February and June 2003. By the end of the epidemic in August 2003, the WHO reported more than 8000 probable cases of SARS worldwide, of whom 10%, some 800 persons, died (World Health Organization, 2003). Most of those who died were elderly patients (Centers for Disease Control and Prevention, 2003).

Increased Numbers of Elderly Persons

This brings us to another demographic change that is affecting the emergence of infectious diseases. The elderly population is growing faster than any other segment of society in most highly-developed countries, but elderly people may have impairment of normal defence mechanisms against infection, resulting in greater susceptibility to disease.

Immunosuppression

Other factors that affect susceptibility to infection include immune suppression, due to genetically-inherited traits, malnutrition, use of immunosuppressive drugs following organ transplantation or as part of cancer treatment, or infection with HIV. Worldwide, many millions of persons are now infected with HIV, and this has created a large segment of the global population in which emerging virus infections may spread and become amplified. In addition, a person infected with HIV may also suffer from reactivation of latent virus infections that are normally held in check by the immune system. These latent infections may include several members of the herpesvirus group, such as cytomegalovirus, or the human polyomavirus JC, which may cause progressive multifocal leukoencephalopathy.

Human Behaviour

Human behaviour is a major factor in the emergence of virus diseases. HIV, for example, would not spread within the human population if obvious precautions were taken by all individuals to prevent transmission. In addition, disease outbreaks have been recognized as resulting from unusual human behaviour. The emergence of monkeypox in the United States in 2003 is an example of a disease that only emerged because of the demand by US citizens for importation of exotic animal pets. In this case, the importer brought in several African rodents—giant pouched rats (*Cricetomys* spp.), rope squirrels (*Funisciuris* spp.) and dormice (*Graphiurus* spp.)—all of which were found to be infected with monkeypox virus (Hutson *et al.*, 2007). These African rodents were housed together with other animals, especially prairie dogs, burrowing rodents of the genus *Cynomys*, which are a popular pet animal in Midwest America. The prairie dogs proved to be highly susceptible to monkeypox virus, and the virus then spread from them to children and adults who handled these pets. This incident raised concern about the possible development of a wild rodent reservoir of monkeypox virus in the United States, but fortunately this did not happen. One or more of the African rodents was infected

with monkeypox virus, which spread to the prairie dogs, and from them to people (Hutson *et al.*, 2007).

A more serious example of virus emergence due to human behaviour occurred in the early 1980s, when a decision was made to alter the production process of bovine meat and bone meal, a dietary supplement for young calves, by omitting a lipid extraction phase. As a result, a new prion disease, bovine spongiform encephalopathy (BSE), became established in the UK cattle population and eventually led to human cases of the disease, called new variant Creutzfeldt–Jakob disease (nvCJD) (Collinge *et al.*, 1996). The exact mechanism by which humans acquire this disease remains uncertain, but it is believed to be the result of eating meat from an infected bovine animal. Certainly the virus found in the brains of humans who have died of nvCJD is identical to the virus causing BSE (Almond and Pattison, 1997).

Improved Technology for the Detection of Virus Infection

In recent years a variety of molecular techniques, especially the polymerase chain reaction (PCR), have been used to identify viruses causing a variety of diseases that were formerly of unknown aetiology. Examples of these are listed in Table 4.2.

Screening cDNA Expression Libraries

One of the most important discoveries that used molecular technology was the identity of the virus causing hepatitis non-A non-B, now known as Hepatitis C virus. It had long been recognized that an important cause of serum-transmitted hepatitis was neither hepatitis A nor hepatitis B virus. In 1988, Bradley at the Centers for Disease Control and Prevention (CDC) collaborated with workers at Chiron Corporation. They used the blood of a chimpanzee that had been experimentally infected with a preparation known to transmit non-A non-B hepatitis. RNA extracted from the chimpanzee's blood was reverse-transcribed using random primers to make cDNAs, which were then cloned into a bacteriophage expression vector. The resultant bacterial colonies were then

Table 4.2 Examples of molecular approaches to pathogen discovery

Virus	Type of approach	Reference
Hepatitis C virus	Expression library	Choo <i>et al.</i> 1989
Sin Nombre virus	Consensus PCR	Nichol <i>et al.</i> 1993
Human herpesvirus 8 and KS	RDA	Chang <i>et al.</i> 1994
GB hepatitis viruses	RDA	Simons <i>et al.</i> 1995
TT virus	RDA	Nishizawa <i>et al.</i> 1997
Human metapneumovirus	RAP-PCR	van den Hoogen <i>et al.</i> 2001
Human bocavirus	DNase-SISPA	Allander <i>et al.</i> 2005

screened using sera from patients with non-A non-B hepatitis. Thousands of clones were screened in this way, until one was found that reacted with antibodies in the sera of infected patients. DNA from the positive clone was then used to screen other clones by DNA hybridization, and eventually the full-length sequence of HCV was elucidated (Choo *et al.*, 1989). This enabled the development of diagnostic tests that are crucial to screening the blood supply, and to preventing transmission of hepatitis C by blood transfusion. A very similar molecular approach was used later to identify hepatitis E virus, responsible for occasional large outbreaks of water-borne viral hepatitis (Purdy and Krawczynski, 1994).

Consensus PCR

Direct application of the PCR technique proved very valuable when an outbreak of cases of acute respiratory disease syndrome occurred in the south-western United States, initially on a Navajo Indian reservation. Initial serological studies suggested that the serum of patients who had experienced the disease contained antibodies that cross-reacted with Hantaan virus, a bunyavirus that was a known pathogen transmitted by rodents and causing haemorrhagic fever with renal syndrome (HFRS) in Asian countries such as Korea, but Hantaan virus was not suspected of causing fatal pulmonary disease in the United States.

Because of the serological cross-reaction, the known nucleotide sequence of Hantaan virus was used to make consensus primers for PCR amplification, and by this means a new group of hantaviruses, causing severe pulmonary distress syndrome (hantavirus pulmonary syndrome (HPS)), was identified (Nichol *et al.*, 1993). Once the consensus primers had amplified the RNA genome of the new hantaviruses, more specific primers could be designed, based upon sequence analysis of the new hantaviruses. The prototype virus of what has proved to be a very large group of viruses present in rodents of the subfamily *Sigmodontinae* was named Sin Nombre virus. So far, rodents transmitting HPS have only been found in the Americas.

Random Primer PCR

In 2001, a Dutch group reported the isolation of a new respiratory virus from young children which had sequences, amplified by random primer PCR, which resembled a pneumovirus of turkeys that differed from respiratory syncytial virus (RSV), and was classified as avian metapneumovirus (van den Hoogen *et al.*, 2001). They named the new virus human metapneumovirus, and serological studies showed that all children tested in the Netherlands were exposed to the virus by five years of age, and that

the virus has been circulating in the population for at least 50 years. The disease caused by this virus is similar to infection by RSV, and it has now been reported throughout the world (van den Hoogen *et al.*, 2004).

Representational Difference Analysis

In addition to PCR, sequence representational difference analysis (RDA) was used to identify human herpesvirus 8 (HHV8), a rhadinovirus found in association with Kaposi's sarcoma (KS), a rare skin tumour found in AIDS patients. In this technique, DNA extracted from KS tissue was compared with DNA extracted from normal tissue, and DNA sequences of a new herpesvirus were only found in the Kaposi's sarcoma tissue (Chang *et al.*, 1994; Moore *et al.*, 1996). Similar virus sequences were subsequently detected in tissue from patients with multicentric Castleman's disease and the rare primary effusion lymphoma, and HHV8 is believed to be associated causally with these tumours.

The RDA approach has also been successfully used in the identification of other viruses which are apparently common human infections but for which no clear association with disease has been found (Mushahwar, 2000). Three viruses, known as GBV-A, GBV-B and GBV-C, were found as a result of studies on the serum from a surgeon (George Barker) with acute non-A non-B hepatitis. When injected into marmosets (tamarins) of *Saguinus* spp., his serum caused hepatitis which could be serially passed in marmosets; the causative virus was distinct immunologically and structurally from the known hepatitis A, B, C and E viruses. Two viruses (GBV-A and GBV-B) were isolated from serum of the infected marmoset using the RDA technique and found to be new members of the family *Flaviviridae*, and associated with GB agent hepatitis.

A third newly-discovered flavivirus, GBV-C, was identified in human sera by reverse transcription and consensus PCR, using primers based upon the GBV-A, GBV-B and HCV helicase gene, and appears to be identical to a virus that was independently isolated and called hepatitis G virus (Linnen *et al.*, 1996; Simons *et al.*, 1995). For this reason, the virus is usually called GB virus C/hepatitis G virus. This latter virus is widely distributed in the human population, but has not been shown to cause hepatitis. It seems able to infect both liver and spleen cells without causing obvious disease symptoms.

The RDA approach was also used to identify a completely different class of viruses in the human population. Once again, the initial discovery of the virus occurred during attempts to isolate a new agent causing transfusion-related hepatitis that was not due to hepatitis viruses A, B, C, D, E or G. Using RDA, DNA clones

were isolated from a patient (initials T.T.) before and after transfusion. The differential DNA analysis revealed a small non-enveloped virus with a DNA genome, and it was named TT virus (TTV) after the initials of the patient (Nishizawa *et al.*, 1997). Although no association of TTV has been found with any human or nonhuman primate disease, the virus appears to be ubiquitous in the healthy human population, with multiple variants (Hino and Miyata, 2007; Khudyakov *et al.*, 2000; Mushahwar *et al.*, 1999; Okamoto *et al.*, 2000). The name *Anellovirus* has been proposed to describe this large genus of viruses that have a circular, single-stranded, negative-sense DNA genome approximately 3850 nucleotides in length. Analysis of a large collection of TTV genomes showed nearly 50% of them to be recombinant (Worobey, 2000).

DNase-SISPA

Recently, a new screening approach was developed that involved treating human samples with DNase to remove any DNA, then amplifying the remaining RNA by sequence-independent single primer amplification PCR (Allander *et al.*, 2001). By this means, RNA sequences of several previously unknown human viruses were found.

The first to be reported was a new human parvovirus, which had a sequence similar to bovine and canine parvoviruses, which belong to the new genus *Bocavirus*. The new human virus was found in respiratory secretions and has been named 'human bocavirus'. As soon as it was reported, virologists worldwide found that it was present in respiratory secretions of children under five years of age in all continents, and appeared mainly to be associated with bronchiolitis, though the exact disease associations are still being examined (Allander *et al.*, 2007b; Sloots *et al.*, 2006).

Subsequently this technique was used in the detection of two new human polyomaviruses, KI and WU, in human respiratory secretions (Allander *et al.*, 2007a; Gaynor *et al.*, 2007). Some 30% of their genome differs from human polyomaviruses BK and JC. Their role in disease, if any, remains unknown.

Conclusions Regarding Molecular Techniques

It seems likely that as molecular techniques continue to be applied in this manner, many more viruses that have no obvious association with pathogenesis will be found in the human population. In addition, it is likely that, using molecular approaches, a number of diseases presently of unknown aetiology will prove to be associated with causative viruses that have yet to be discovered.

Despite the availability of these sophisticated molecular approaches for the identification of emerging viruses, the

most recently identified emerging virus pandemic—a human coronavirus causing SARS—was found by the classical technique of electron microscopy of the virus growing in cell culture (Ksiazek *et al.*, 2003). Only once the virus was identified by electron microscopy were molecular techniques used to establish that it was a newly-identified coronavirus not hitherto seen in the human population.

Increased Contact with Vectors of Virus Infection

As the global human population continues to expand, there is increased opportunity for human contact with natural virus vectors, such as arthropods, birds or rodents, and in recent years we have seen the emergence of a number of new virus diseases, as well as a resurgence of other diseases that were formerly well controlled. Indeed it has been postulated that many major infectious diseases arose in the human population as the result of transfer from animals (Wolfe *et al.*, 2007). Some instances involve the invasion by humans of a new ecological niche which brings them into closer contact with animal or arthropod vectors. As an example, in South America deforestation of large land areas may deprive rodents carrying arenaviruses or hantaviruses of their usual habitat, resulting in infestation of houses and closer contact with the human population. Although there has been no large pandemic of influenza since 1968, it is highly likely that reassortment of an avian influenza virus with a human one, as happened in the last two pandemics, will occur again. There is a vast reservoir of influenza viruses in the avian population (Webster and Kawaoka, 1994) and our ability to control such a pandemic, if it occurs, has not significantly improved in the last 30 years.

Arthropod-borne Viruses

Another important factor that has contributed to the emergence of virus diseases is the vector population density. This is particularly true of mosquito-borne viruses. Once well controlled, the mosquito population, including *Aedes aegypti* and *Aedes albopictus*, has expanded greatly since the cessation of the use of DDT, resulting in increasing cases of dengue fever worldwide (Figure 4.3) and in particular a resurgence of dengue haemorrhagic fever, a severe form of dengue involving infection with two or more serotypes of dengue virus.

Translocation of a vector-borne virus into a new geographical region can result in the emergence of a dramatic disease outbreak. In 1999 there was an outbreak of West Nile viral meningoencephalitis, first detected in New York City. This virus had only been detected previously in Africa and the Middle East, and once the virus found in New York was sequenced, it was found to be identical in sequence to a virus isolated from diseased birds

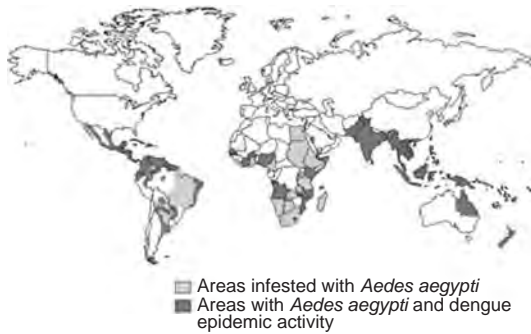


Figure 4.3 World distribution of dengue, 2005.

in Israel. How the virus was imported into the United States is unknown—an infected person, a mosquito or a bird are all possibilities. However, once established in a virgin population with the appropriate vector, the virus soon spread both south and west from New York, and has now moved south into the Caribbean and Mexico and north into Canada. By 2002, West Nile virus had spread to 39 states and DC, and caused 2741 human cases of meningoencephalitis and 263 deaths. More than 124 000 dead birds were reported, as well as more than 12 000 cases in horses. The virus was recovered from 37 species of mosquito, and is now an endemic disease requiring considerable public health efforts for its control.

Rodent-borne Hantaviruses

In 1993 an epidemic of acute respiratory disease occurred in south-western United States, and was identified as HPS, a new clinical disease of high mortality (40%), spread by a virus in the deer mouse (*Peromyscus maniculatus*) population in the area. It soon emerged that climatic conditions in the summer of 1993 had favoured an explosion in the population of deer mice, and this was the major factor which had contributed to a large number of HPS cases from which the epidemic became recognized. Once recognized, other hantaviruses were identified in both North and South America, many causing severe and often fatal HPS in the human population (Figure 4.4).

Although most rodents worldwide appear capable of housing and transmitting hantaviruses, the only cases of severe HPS have been associated with rodents of the subfamily *Sigmodontinae*, which is confined geographically to the western hemisphere, in North and South America. In other parts of the world, hantaviruses cause milder disease, with few fatalities.

Bats as Virus Vectors

Another important group of animals that act as vectors for virus diseases are bats, which make up a fifth of all



Figure 4.4 New world hantaviruses (2007). Dark yellow represents viruses causing hantavirus pulmonary syndrome, and cream represents rodent isolates not associated with disease in humans. (Courtesy of James Mills, CDC, Atlanta, GA, USA.)

living mammalian species (Wilson and Reeder, 2005). In the United States, insectivorous bats are the most important vector species for human rabies, often transmitting the disease silently, by a bite of which the human is unaware. In recent years fruit-eating bats have also been recognized as important disease vectors (Halpin *et al.*, 2007). There are some 170 species of fruit-eating bats (*Megachiroptera*) in the tropical regions of Asia.

In 1994 a horse trainer and a stablehand who worked on a farm in Hendra, a suburb of Brisbane, Australia, both became ill while nursing a sick pregnant mare that had recently been brought on to the property. The disease spread to other horses on the property, and 14/21 infected horses died of pulmonary disease with haemorrhagic manifestations. Of the two infected humans, the trainer died, but the stablehand survived the infection after a lengthy illness. One year later, another horse farmer died 600 miles away in Mackay, from encephalitis caused by a similar virus. The virus, named Hendra virus, was found to be a new paramyxovirus with a large genome, about 19 kb in length, morphologically similar to other paramyxoviruses but with a very long nucleocapsid. Subsequently, this virus was recognized as causing disease again in 1997, when it caused the death of a horse in Cairns, in the far north of Australia, and in Queensland in 2007, when another veterinarian became ill after performing an autopsy on a thoroughbred horse that died of Hendra virus infection. However, in the meantime work carried out in the Australian Animal Health Laboratory in Geelong had identified the virus in fruit bats, and a survey revealed that a high proportion of fruit bats had antibodies against the virus.

Beginning in 1997, an outbreak of a new respiratory disease in pigs was noticed by farmers in Malaysia. In late 1998 and 1999 many farmers and other persons having close contact with pigs developed severe symptoms of encephalitis. In all there were 265 human cases, of whom 105 persons died. The disease did not appear to be transmissible between humans, and was stopped in May 1999 by the slaughter of more than a million pigs, causing great economic loss to the Malaysian pig industry. In March 1999 a virus was isolated in cell culture from the brain of a patient in Sg Nipah village, Bukit Pelandok, Malaysia. The virus caused syncytia during growth in Vero cells, and gave a positive test by immunofluorescence assay carried out at CDC using an antiserum made against Hendra virus. By thin-section electron microscopy of infected Vero cells, a paramyxovirus with a long nucleocapsid was found. Complete sequencing of the virus genome showed that it was a large negative-stranded RNA molecule, 18 246 nucleotides in length, with about 80% homology to the genome of Hendra virus. The new paramyxovirus, called Nipah virus (Chua *et al.*, 2000), was subsequently found in large fruit-eating bats, and it was from these that the virus is presumed to have spread first to pigs, and then to humans in close contact with infected pigs. Curiously, the mortality rate in pigs was less than 5%, much less than was seen amongst the human cases. Pigs develop rapid, laboured breathing and an explosive nonproductive cough as the main symptoms, with occasional neurological changes, such as lethargy or aggressive behaviour. Humans develop a febrile encephalitis which rapidly progresses to multisystem involvement with vasculitis and syncytial giant cell formation at various sites. Spread to the brain is by the vascular route and leads to discrete small foci of necrosis and neuronal degeneration.

In 2001 an outbreak of Nipah virus infection occurred in Siliguri, West Bengal, involving eight human cases, and in nearby Bangladesh outbreaks involving humans who had contact with bats occurred in 2001 and 2004. These outbreaks were caused by a virus slightly different in genome sequence from the Malaysian Nipah virus (Harcourt *et al.*, 2005). Hendra and Nipah virus have now been classified as the members of a new genus of the *Paramyxoviridae* called the *Henipavirus* genus (Eaton *et al.*, 2006).

Nonhuman Primates as Virus Vectors

It is now recognized that HIV-1 and HIV-2 have their origins in chimpanzees and sooty mangabey monkeys, respectively (Holmes, 2001), which can both be infected with these viruses but do not themselves routinely develop AIDS. The transmission of these zoonotic infections has been ascribed to the practice of killing and eating the primates (as bushmeat), and it is clear that

other viruses can emerge in the same way. Viruses causing severe haemorrhagic fever, such as Ebola virus and Marburg virus, have certainly passed from nonhuman primates to man by this means on several occasions, but it is generally believed that another natural reservoir exists for these viruses, since, like man, nonhuman primates are highly susceptible to both these filoviruses. Because they cause extremely high mortality in man (up to 90% of infected persons), a great deal of concern is generated when an outbreak occurs. The original outbreak of Marburg virus was caused by direct transmission from infected monkeys by persons handling fresh monkey tissues, but in general filovirus outbreaks are largely amplified by person-to-person transmission, usually in a hospital setting, as occurred in the last major outbreaks caused by Ebola virus in Kikwit, Democratic Republic of the Congo, in 1995, and by Marburg virus in Angola in 2004–2005. Recent evidence suggests that the natural reservoir for Marburg and Ebola hemorrhagic fever viruses is a common African fruit bat (*Rousettus aegyptiacus*) (Towner *et al.*, 2007), and it is from this species that apes and humans become infected in the first instance.

Other viruses which have long been known to have their origin in nonhuman primates are human T-lymphotropic viruses (HTLVs) types 1 and 2, which originated independently and are related to distinct lineages of simian T-lymphotropic viruses (STLV-1 and STLV-2, respectively). These viruses belong to the genus *Deltaretrovirus*. It has been known for some time that HTLV-1 causes adult T-cell leukaemia and HTLV-associated myelopathy/tropical spastic paraparesis (HAM-TSP) in about 2–5% of those infected. HTLV has spread worldwide to more than 20 million people, sexually, from mother to child, and by exposure to contaminated blood through transfusion and injection drug use. HTLV-2 appears to be less pathogenic than HTLV-1. The butchering and eating of primates is a common occurrence in parts of Africa, and recently several new HTLVs have emerged from studies of persons in Africa involved in the preparation of 'bushmeat' from nonhuman primates (Switzer *et al.*, 2006; Wolfe *et al.*, 2005). These include HTLV-3, which appears to be related to STLV-3, a virus not previously seen in humans, and HTLV-4, which is not related phylogenetically to any previously isolated T-lymphotropic virus. It is not yet known whether these newly-discovered retroviruses are important causes of disease in humans.

Conclusions Regarding Contact with Vectors

It is clear from the experience gained over the past 20 years that animal, bird or arthropod reservoirs are extremely important sources for the emergence of new diseases. This has led to an assumption that most

newly-discovered virus diseases have their origin in another species. The outbreak of SARS coronavirus may be a case in point, and suspicion has been placed on various exotic animal species that are eaten by the Chinese and may have started the epidemic in Guangdong, although the reservoir species for the SARS coronavirus appears again to be a bat (Shi and Hu, 2008; Wang *et al.*, 2006). This remains to be proven, but it is widely believed that the next pandemic of human influenza will occur as a result of reassortment between an avian and a human influenza virus. Only by continued global surveillance and prompt reporting to the WHO can we hope to control the outcome of such an event.

FUTURE DIRECTIONS

It is clear that a large number of new viruses have emerged in recent years, and in many cases it is still unclear what role, if any, they play in human disease. This is in contrast to the original development of virology, when viruses were discovered by observing them or isolating them from diseased tissue. Recently a return to these classical observations has been the identification of a novel gammaretrovirus in human prostate tumours of patients homozygous for a reduced interferon response involving the activity of RNase L (Urisman *et al.*, 2006). The virus was isolated from human prostate tumours and found to be a novel gammaretrovirus, xenotropic murine leukaemia related virus (XMRV). XMRV provirus integration sites have been mapped in human prostate tumour tissue, and it is clear that this virus has infected at least a subset of human prostate cancer cases, though it is not yet clear whether the virus or its integration site have any influence on the aetiology or progression of prostate cancer (Dong *et al.*, 2007).

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Vaccinology

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An ounce of prevention is worth a pound of cure
BENJAMIN FRANKLIN

INTRODUCTION

The science of vaccinology encompasses many disciplines and activities. They converge in making the best possible use of the discoveries of Edward Jenner, a country doctor in Gloucestershire, and his successors, who have shown that infectious diseases can be prevented by artificially inducing immunity through the administration of vaccines. Historically, a farmer from Yetminster in Dorset named Benjamin Jesty was the first known person to have experimentally tested, in 1774, the prevalent rumour that milkmaids had beautiful facial skin because they contracted cowpox during their work and were then spared the disfigurement caused by smallpox. However, Jesty only ‘vaccinated’ his wife and two of his children and did not pursue that bold experiment further. It was left to Jenner to provide convincing support for the hypothesis that cowpox protected against smallpox, get it generally accepted and popularize the practice of vaccination (Pead, 2006).

Around 1768, a milkmaid had told Jenner about cowpox, a rare bovine disease sometimes transmitted to humans which according to countryside folklore protected against smallpox. He thought about this over many years and discussed the hypothesis with many others before doing the crucial experiment. Finally, on 14 May 1796, Jenner scraped pus from a lesion caused by cowpox, from the hand of a milkmaid named Sarah Nelmes, and scratched it into the arm of an eight-year-old boy called James Phipps. This was done in the hope of protecting the boy against

smallpox, a contagious disease that had caused endless death and suffering among humans since antiquity. It is not known for certain whether Jenner had heard about Jesty before performing that experiment, although there are strong suspicions that he had (Pead, 2006). Much later, Jenner acknowledged the contribution of Jesty (Plotkin *et al.*, 2008). To prove that James had been rendered resistant to smallpox he underwent on 1 July of that year ‘variolation’, the then widely used, though risky, practice of inoculating dried material from smallpox lesions by various means in order to try to protect them against smallpox. James was found to be ‘secure’ (i.e. immune) to smallpox! Jenner and his followers then proceeded to generalize the inoculation of pus from cowpox lesions, from person to person, with great success, though also with some major disasters due to contamination of his inoculum with other pathogens. There was, however, no doubt that Jenner’s invention protected against smallpox. He even predicted that it would ‘annihilate’ (i.e. eradicate) the disease, something that was only achieved nearly 200 years later.

To honour the memory of Jenner, the word ‘vaccine’ (from ‘*vacca*’, ‘cow’ in Latin) was coined by Louis Pasteur many decades later. Pasteur, who established that ‘spontaneous generation’ did not occur and that transmissible diseases were caused by microorganisms, also developed the second vaccine against a human disease—rabies. This was subsequently shown to be caused by a virus, as was the case for smallpox. The past tense can now be used for smallpox because that bane of mankind (credited with being the natural main brake on population growth in Europe until the general use of vaccination) (Bonanni, 1998) has been eradicated (Fenner *et al.*, 1988). Smallpox eradication has been hailed, with good reason,

as Medicine's greatest triumph. However, since smallpox virus was not totally destroyed and has officially been kept in two laboratories (in the United States and Russia), and, unfortunately, may have been (unofficially) transferred to a small number of other countries, there remains the fear that bioterrorists may malevolently reintroduce it (Henderson, 1998).

The best weapon against viral diseases, in spite of the development of several efficacious antivirals, is still vaccination (Plotkin *et al.*, 2008). The purpose here is not to describe every viral vaccine that has been developed (they are, in any case, covered in the chapters on the individual viruses) but to describe the principles and practice of vaccinology. A non-exhaustive list of the disciplines and activities involved is:

1. Burden of viral diseases and their reproductive rates.
2. The immune system and its role in natural and artificially-induced immunity.
3. Discovery of protective antigens in pathogens.
4. Presentation of protective antigens through vaccines and types of vaccines.
5. Research and development on vaccines and their commercial introduction.
6. Social marketing of introduced vaccines.
7. Planning and implementation of vaccination programmes.
8. Surveillance of disease incidence and adverse events before and after implementation of vaccination.
9. Rectification of publicized falsehoods and maintenance of vaccination coverage.

The first nine sections of this chapter are therefore devoted to those aspects of vaccinology, while the final section, 'Viral Vaccines on the Horizon and Roadblocks to Future Vaccine Development', is a brief survey of the viral vaccines that may become available in the foreseeable future and of the hurdles to be cleared (Andre, 2003).

BURDEN OF VIRAL DISEASES AND THEIR REPRODUCTIVE RATES

Once the viral aetiology of a disease has been established, and before there is any discussion about the need to develop a vaccine, it is important to study its epidemiology and its burden on society in terms of its incidence, morbidity (range of severity and frequency of complications) and mortality. This is usually done by calculating the number of disability-adjusted life years (DALYs) that are lost as a result of the disease, at the local, country, regional and global levels (Murray and Lopez, 1996). In addition, the

financial cost of the disease (both direct medical and indirect societal costs) should be taken into consideration to get a total picture of the importance of an infection to human societies. The burden of the same disease on different societies may be different, greatly due to epidemiological and economic circumstances (Drummond *et al.*, 2001). The need for a vaccine will be determined by the DALYs lost and the economic burden inflicted worldwide by the disease it is meant to protect against.

The mode of transmission of the virus must be studied and it is also important to know whether the infection occurs in epidemic waves or is endemic. Seasonality of outbreaks will become obvious if precise diagnosis and recording of cases are in place. In order to design an optimal vaccination programme, the infectiousness and contagiousness of the virus should be known. This is expressed as the basic reproduction rate (R_0), defined as 'the average number of individuals infected by an infectious case during their infectious period, while in a totally susceptible population'. R_0 is a parameter that allows the calculation of the proportion of persons (P_c) that must be immune (either naturally or by vaccination) to reduce effective R_0 to below 1 (thus halting transmission in the population). The relation between P_c and R_0 is $P_c = 1 - 1/R_0$. When the actual P_c in a 'closed' population is high enough, herd immunity results. Attainment of herd immunity is the main reason, apart from the obvious one of protecting as many people as possible, for advocating high vaccine coverage rate, because when that is achieved, even the unvaccinated minority in a population is protected (Anderson and May, 1991). Table 5.1 shows the minimum vaccination coverage (taking into account vaccine efficacy) required to stop transmission of measles, mumps, rubella and poliomyelitis (Anderson and May, 1990; Nokes and Anderson, 1988). For varicella the required vaccination coverage is even higher, as >95% of susceptible close contacts of a case in the infectious phase will get infected.

What can be achieved by vaccination is illustrated in Table 5.2. The figures speak for themselves.

Table 5.1 Minimum vaccination coverage required to stop transmission of several viruses

Virus	Infectiousness (R_0)	Vaccination coverage needed (%)
Measles	15–17	92–95
Mumps	10–12	90–92
Rubella	7–8	85–87
Poliomyelitis	5–6	80–85

Adapted from Anderson and May, 1990.

Table 5.2 Vaccine-preventable diseases: reduction in annual prevalence in the United States after introduction of routine vaccination

Disease	Peak prevalence year	Number of cases	Cases in 1999	Percentage reduction
Diphtheria	1921	206 939	1	99.99
Measles	1941	894 134	86	99.99
Mumps	1968	152 209	352	99.76
Pertussis	1934	265 269	6031	97.63
Polio (wild)	1952	21 269	0	100.00
Rubella	1969	57 686	238	99.58
Congenital Rubella	1964–1965	20 000+	3	99.98
Tetanus+	1923	1 560	33	97.88
Invasive Hib Disease (>5 yr)	1984	20 000+	33	99.83

Based on Centers for Disease Control (1999, 2001).

THE IMMUNE SYSTEM AND ITS ROLE IN NATURAL AND ARTIFICIALLY-INDUCED IMMUNITY

The immune system is a collection of organs, tissues, cells and molecules that work together to defend the body against attacks by pathogens and other foreign and potentially harmful substances. Its mission is to restrict entry of, neutralize and eliminate potentially harmful microorganisms and agents. It must recognize molecules as causing damage to tissues and take action to destroy or remove them, thus conferring protection. Damaged tissues liberate damage-associated molecular patterns (DAMPs), which are signals of danger. Danger signals are detected by the immune system through its recognition of pathogen-associated molecular patterns (PAMPs) on pathogens. DAMPs and PAMPs bind to pattern recognition receptors (PRRs) like toll-like receptors (TLRs) on dendritic cells and macrophages (Matzinger, 2007).

Two parts in the immune system, the innate and the adaptive, have been recognized.

The innate immune system provides the first line of defence against infectious or other attack; it is present from birth and is nonspecific. It does not generate a memory against the invading organism or other substance, since it is not more efficient in dealing with it on re-exposure. Innate immunity is, in the first instance, mediated by physical, chemical and biological barriers provided by the skin and mucous membranes of the gastro-intestinal, respiratory and genito-urinary tracts, and also by genetic attributes like the absence of a cellular receptor for a particular virus. For example, mice are genetically not susceptible to infection by polioviruses because they do not have a poliovirus receptor (PVR). However, if they are made transgenic for PVR, they can be infected and paralysed by virulent poliovirus (Levenbook and Nomura, 1997).

Unless the integrity of the skin and mucous membranes is breached by chemical or physical trauma, most microorganisms cannot cross them and invade the body. Protection by chemicals is conferred by acidic secretions (e.g. in the stomach and vagina) or enzymes like lysozyme secreted onto the surface of physical barriers. In addition, a biological protection is offered by the resident bacterial flora on these surfaces. The bacteria produce antimicrobial substances that attack potential invaders that land on them. Should the physical, chemical and biological barriers not suffice to prevent entry into the *milieu intérieur*, roaming cells (mainly macrophages and neutrophils) constitute a second line of defence. They phagocytose the invader and neutralize it by producing molecules like interferon that inhibit the replication of viruses. The innate system is also responsible for detecting DAMPs and PAMPs and presenting them through PRRs to antigen-presenting cells (APCs) in the adaptive system, in a way that will prompt it to respond most efficiently to the infection. The innate immune system is permanently present and acts on the pathogen immediately after invasion, while at the same time informing the adaptive immune system about the invasion (Medzhitov and Janeway, 1997; Trinchieri and Sher, 2007).

The adaptive immune system comes into play if innate immunity cannot stop the further spread of the infection. It is mediated by lymphocytes of two types: B lymphocytes (derived from the bone marrow), which secrete antibodies of various types (IgA, IgD, IgE, IgG, IgM) to antigens, and T lymphocytes (derived from the thymus), which react to the same antigens and inactivate the virus that carried them into the body. In cases where this does not occur (as for example in human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) or hepatitis C) a chronic infection is established. Sometimes the infection becomes 'latent', as

with herpes viruses. Nevertheless, infections sometimes result in death, either acute or delayed.

Immunity mediated by antibodies is called ‘humoral’, whereas that conferred through lymphocytes (such as killer T cells or specific cytotoxic lymphocytes) is said to be ‘cell-mediated’. Humoral and cell-mediated immunity (CMI) constitute the effector arm of the adaptive immune system.

In order to be brought into action, the effector arm needs information about the antigens that it must deal with and the microorganisms that carry them. This information is provided by the afferent arm of the system. That arm is mainly constituted by ‘professional’ APCs, such as ubiquitous macrophages and dendritic cells such as Langerhan’s cells in the skin. Some antigens—the T-independent ones like polysaccharides—can directly instruct B lymphocytes to synthesize specific antibodies that recognize them, but most antigens require the help of T cells to elicit an immune response. Antigens are molecules that stimulate the immune system to develop a specific response (either humoral, cell-mediated or both) to them. The adaptive immune system’s responses are ‘learned’ and antigen-specific; they rely on the ability of the system to recognize an antigen as ‘foreign’ or one (in the case of T-dependent antigens) that has been seen before (which implies the previous acquisition of a memory for that particular antigen). The memory for an antigen acquired by the adaptive system is manifested by an accelerated and enhanced humoral and/or cellular immune response when it again encounters the antigen. This response is called ‘secondary’ to differentiate it from the ‘primary’ response elicited on the first encounter. Figure 5.1 shows the serum antibody kinetics in the primary and secondary responses. The ability to mount a secondary response often

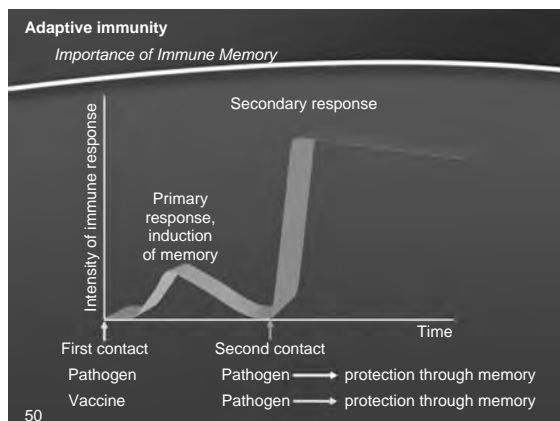


Figure 5.1 Kinetics of primary and secondary antibody responses and acquisition of immunological memory induction.

persists for life and in many cases ensures lifelong immunity to disease (although not necessarily to re-infection). T-independent antigens elicit only primary-type responses and therefore confer relatively short-lived protection.

In some diseases the incubation period is longer than the time required for the secondary response to reach protective levels. Detectable vaccine-induced serum antibody may be lost (e.g. following hepatitis B vaccination) but the infection that may result is subclinical and does not develop into a chronic one (the medically most important one).

Antibodies protect against viral infection and disease by at least two mechanisms:

1. Neutralization of the infectivity of viruses due to binding of a (or several) viral surface antigen(s) to a cellular receptor (steric hindrance). Antibodies can also hinder infection by preventing cell fusion and uncoating of viruses after they have entered a cell, thus conferring what has been called ‘sterilizing immunity’.
2. Destruction of infected cells by the mechanism of antibody-dependent cellular cytotoxicity (ADCC). Natural killer cells and macrophages carrying Fc receptors bind to the antibodies, reacting with the epitope of a viral antigen on the surface of the infected cells.

Also, specifically activated CD8 cells that liberate interferon-gamma and tumour necrosis factor-alpha destroy virus-infected (as well as cancer) cells, as well as having a direct cytolytic action. Perforins and granzymes mediate apoptosis of cells, which has been described as ‘pre-programmed cellular suicide’.

Until fairly recently, it was thought that the innate and adaptive immune systems were independent. But, with the discovery of TLRs (found on cells of both the innate and adaptive parts of the immune system), it is now known that they collaborate by secreting molecules like cytokines in order to ensure the best possible protective response (known as natural immunity) to a potentially dangerous invasion in nature. However, natural immunity sometimes fails against pathogens that have evolved escape mechanisms, for example, human papilloma virus (HPV). For such pathogens, vaccines that perform better than nature are needed.

TLRs recognize PAMPs and DAMPs and transfer information about them to professional APCs that have PRRs (Pattern Recognition Receptors). These APCs then present them to the effector arm of the adaptive system. Knowledge about TLRs is progressing fast and already contributes to a better understanding of natural and artificial immunity. Thus it is known that MPL (3-*O*-desacyl-4'-monophosphoryl lipid A), an adjuvant

used in some licensed vaccines, binds to toll-like receptor No 4 (TL-4) and so enhances the production of antibodies. Also, CpG motifs bind to the TL-9 that triggers CMI. Adjuvants are substances of various kinds that, alone or mixed, enhance an immune response.

A thorough understanding of the workings of both the innate and the adaptive immune systems and their interaction is very helpful in the development of new vaccines. Modern vaccine technology provides new means to modulate the magnitude and specificity of immune responses following vaccination. Matching the right adjuvant with the right antigen can improve immune responses in target populations and prevention of specific diseases. The use of novel adjuvant systems has shown encouraging preliminary results in preventing diseases such as malaria and (pre)pandemic influenza that had long been considered beyond the reach of conventional vaccines. Potential benefits from new adjuvants are:

- More appropriate direction of humoral and/or cellular immune responses.
- Induction of long-term protection through:
 - stronger immune responses
 - improved immunological memory
 - broader protection against related clones (cross-protection).
- Reduction of antigen dose.

‘Artificially-induced immunity’ is that resulting from vaccination (providing active immunity), or from the iatrogenic administration of protective antibodies or their transfer from a pregnant woman to her fetus via the placenta (conferring passive immunity). Passive immunity is short-lived as it will last only as long as the acquired antibodies, which are catabolized and not replenished, persist above the protective level, whereas active immunity is long-lived due to persistence of vaccine-induced antibodies at protective levels and immunological memory. The art of inducing artificial active immunity consists in matching the right adjuvant with a (some) protective antigen(s) to obtain a vaccine that will induce optimal protection in the target population (Aguilar and Rodriguez, 2007; Fraser *et al.*, 2007; Garçon *et al.*, 2007).

DISCOVERY OF PROTECTIVE ANTIGENS IN PATHOGENS

Before embarking upon the discovery of protective antigens, a crucial question that must be answered is: ‘do those who survive a viral disease develop immunity against further attacks of disease when exposed and infected with the same virus?’ In other words, does natural immunity occur after infection? If the answer is ‘yes’

then ‘protective antigen(s)’ exist(s) in the pathogen and it should therefore be possible to develop a vaccine against it. For viral diseases like AIDS and hepatitis C where natural immunity does not exist or is at best suboptimal, the problem of developing a vaccine becomes more complex, though hopefully not impossible.

The concept of protective antigens is fundamental to the development of modern vaccines. Historically, vaccines were developed by empirical hit-and-miss methods based on observation, hypothesis generation and confirmatory experimentation. The obvious example was the confirmation by Jenner that cowpox could protect against smallpox; a belief widespread among farmers at the time he undertook his experiment on James Phipps. A more recent example, in which the authors were personally involved, was the demonstration that a vaccine based on an attenuated bovine rotavirus could protect against human infantile rotavirus gastroenteritis. This was based on the hope that the Jennerian approach might again work—and it did (Vesikari *et al.*, 1983, 1984, 1985). Although the initial candidate vaccine derived from cattle was not further developed, that early proof of concept led to the commercial introduction of several rotavirus vaccines. Modern vaccines are being and will be developed by more rational scientific approaches but it must be recognized that traditional methods and serendipity may still sometimes yield valuable products.

A ‘protective antigen’ is one that can (but not necessarily will) stimulate an immune response, whether of the humoral or cellular type, and prevent or alleviate the clinically-important consequences of infection with a pathogen. The active principle(s) of future vaccines should only be the protective antigen(s), if necessary rendered more immunogenic by the addition of adjuvants. The art consists of choosing an adjuvant or mixture of adjuvants that will stimulate the relevant arm of the adaptive immune system through the help of the innate system and give optimal and long-lasting protection against the disease in question.

In the past, the discovery of protective antigens relied on the identification of presumed protective antibodies. Thus, the immunizing antigen elicited production of antibodies that would neutralize the infectivity of a virus in tissue culture or in an animal-challenge model. Nowadays, with our knowledge of the coding sequence of the genome of microorganisms it is possible to perform what has been called ‘reverse vaccinology’ (i.e. starting from the proteins that the genome can synthesize, finding those that are protective in an animal model (if it exists)). The putative protective (in man) antigen(s) can thus be identified and developed further (Mora *et al.*, 2006). Sometimes the protective antigen is not found on a particular molecule but is formed by the juxtaposition of several proteins on the

surface of the virus, as is the case for hepatitis A. An efficacious vaccine can then only be envisaged by presenting that epitope to the immune system through whole-virion vaccines (either inactivated or live) or nucleotide immunization (André, 1995).

However, the identification of a (some) protective antigen(s) is only the first step towards developing a vaccine. The following sections will make it clear that there is a very long road to follow before a vaccine is made commercially available and used efficiently for disease prevention, even after protective antigens have been identified and isolated from the pathogen, produced by genetic engineering or introduced into the body through nucleic acids that code for the antigen or protective epitope.

PRESENTATION OF PROTECTIVE ANTIGENS THROUGH VACCINES AND TYPES OF VACCINE

Once the protective antigen(s) is/are known, a decision must be made on how best to present them to the immune system.

In the field of viral vaccines, the classical methods of producing protective antigens were to either culture, harvest and then inactivate (chemically or physically), or to attenuate (by repeated passages in tissue culture) the virus. The resulting product was a 'killed' whole-virion vaccine or a live attenuated vaccine. Nowadays, more purified vaccines (like subunit influenza vaccine) and pure antigen vaccines produced by genetic engineering (like yeast-derived hepatitis B vaccine, produced in transformed *Saccharomyces cereviceae* cells that express the surface (protective) antigen of the virus) have been developed (André, 1990; Emini *et al.*, 1986; Stephenne, 1990). Attenuation of viral pathogens can now be obtained by genetic reassortment between wild and attenuated 'master viruses' for influenza (Maassab and Bryant, 1999) and rotavirus vaccines (Clark *et al.*, 1996), deletion of genes that code for virulence factors, or the directed mutagenesis of these genes to render them inoperative (Cohen, 2001).

There are many routes to developing vaccines that present the protective antigens they contain to the immune system in an appropriate way. The types of viral vaccine that can currently be envisioned are:

- Non-replicative (inactivated):
 - whole-virion, split, subunit, pure antigen(s) with or without adjuvant(s).
- Replicative:
 - live attenuated viruses
 - vectorized (gene of protective antigen introduced in a vector)
 - DNA-based (nucleotide or genetic vaccine).

The type of vaccine that will be considered for further development will depend upon considerations of feasibility and cost (not forgetting commercial viability), as the financial and human resources required for the research, development and introduction of a new vaccine in the current politico-economic world system are only likely to be available to large corporations (André, 2002).

The possible routes of administration of vaccines are multifarious. Traditionally, vaccines have been administered by scarification of the skin (e.g. smallpox), injection with needle and syringe (most vaccines), orally (e.g. oral polio vaccine (OPV)) or intranasally for the live influenza vaccine. There are many other possibilities, however, as listed in Table 5.3. These routes are being explored to achieve a more user-friendly administration of vaccines.

RESEARCH AND DEVELOPMENT ON VACCINES AND THEIR COMMERCIAL INTRODUCTION

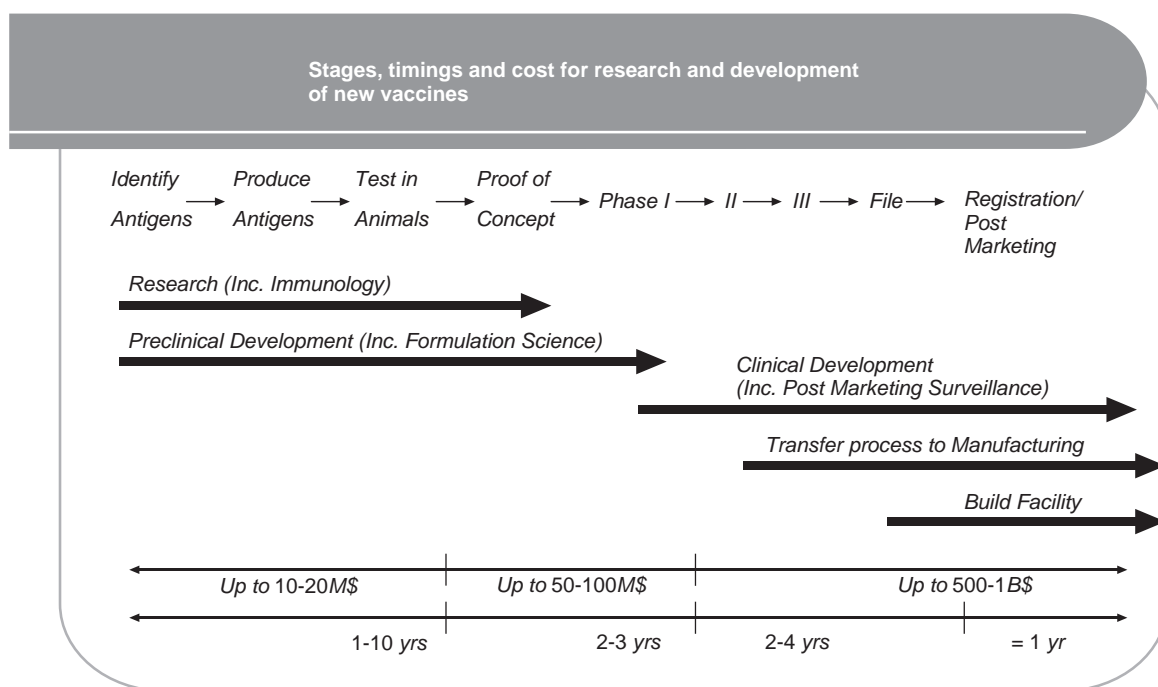
Whereas in the past single individuals or small research institutes could develop new vaccines, there are nowadays only a handful of vaccine manufacturers that have the resources and know-how to bring a new vaccine to market. More and more, the early research phases on new vaccines are done by biotechnology companies, research institutes and academia. Discoveries made in these institutions are then outlicensed to the few major manufacturers who can afford to bring them to market. Indeed, it now costs up to a billion pounds and 20 years of research and development to produce, register and launch a new vaccine. The phases of R&D and associated costs are shown in Figure 5.2.

The vaccine manufacturer, almost always a division of a major pharmaceutical company, must show to the satisfaction of regulatory authorities that the vaccine to be introduced is safe, efficacious and can be produced with consistent quality. This involves extensive characterization of the product to be tested, provision of a suitable antigen dose and administration schedule, and the application of good manufacturing practices to show it can be manufactured in a reproducible way. Antigen dose is based on relevant immune responses in preliminary (Phases I and II) clinical studies, which also monitor the reactogenicity and safety of the candidate vaccine. This stage of development may require the use of the vaccine in hundreds of volunteers of various ages (starting with healthy young volunteers) to determine an acceptable dose and administration schedule for the chosen vaccine formulation. In order to establish the safety, efficacy and consistency of the 'final' product (the commercial product may be produced by slightly different manufacturing

Table 5.3 Possible routes of administration of viral vaccines

Classical	New
By scarification (smallpox)	IM, SC, ID with needleless multidose jet-guns or unidose disposable devices
IM, SC, ID with needle and syringe	Transcutaneous, through antigen-loaded skin patches
Orally (OPV)	In sugar-glass needles
	With 'gene-guns'
	Mucosal (intranasal, respiratory, vaginal, rectal, sublingual)
	As combined vaccines

IM, intramuscular; SC, subcutaneous; ID, intradermal.



The Research & Development pipeline for new vaccines

Figure 5.2 Stages, timings and cost for research, development and introduction of new vaccines.

methods, in which case bridging studies are required), studies in greater numbers of subjects using at least three vaccine lots are carried out in Phase III. Up to 100 000 study participants may be needed to demonstrate that the vaccine is safe and efficacious. ‘Safety’, always a relative concept, is shown by the absence of unacceptable reactions attributable to the vaccine, while efficacy will be demonstrated in statistically valid controlled randomized prospective double-blind field trials. At this point the vaccine developer and manufacturer will have to collate

a voluminous dossier to submit to competent national and/or supranational regulatory authorities, which will describe, in great detail, the work that has been done to substantiate the claim that the candidate vaccine is safe and efficacious and can be produced at a consistent quality in required quantities for the vaccination of humans. To have any chance of getting marketing authorization, a candidate vaccine must have been developed according to the strict guidelines of regulatory authorities (European Medicines Agency, 2006).

SOCIAL MARKETING OF INTRODUCED VACCINES

It is illusory to think that every newly-introduced vaccine will be readily adopted by health authorities, governments and the general public (rarely, they are!), even if it has been shown to be safe and efficacious. It must be marketed just like any other new product in the national and international markets. The major client groups must be informed about the availability of the vaccine and the fact that the disease that it protects against is serious enough to cause a significant disease burden, and shown that it is 'safe' and efficacious. Furthermore, it must be marketed at a cost-effective price; in a growing number of countries this must be shown by pharmaco-economic studies. The best way to achieve acceptance will vary in different countries but usually consists of publishing scientific articles in medical journals and organizing symposia to inform the medical profession and the press.

Governments have to be persuaded that the vaccination is justified as they will need the support of the electorate

to spend taxpayers' money on any nationally-financed vaccination programme. The lay public must also be educated by the mass media through newspapers, magazine articles and radio and television programmes, not forgetting the powerful Internet. Getting vaccinated needs to be seen as being for personal benefit as well as for that of the whole community. It would be a waste of time and money to develop and introduce a new vaccine that is not used to best effect to prevent disease, disability and deaths. However, the successful introduction of a new vaccine requires meticulous planning and the coordinated deployment of the skills and efforts of many enthusiastic believers in it in order for it to gain acceptance by all stakeholders in both the public and private institutional sectors, as well as the general public. The studies and activities required to reach this goal, particularly in developing countries, are summarized in Figure 5.3. In developed countries, the goal can, in general, be achieved by a well-thought-out, well-prepared, widely-publicized, competently-implemented, closely-monitored and adequately-financed national vaccination programme led by dedicated individuals (Miller *et al.*, 2002).

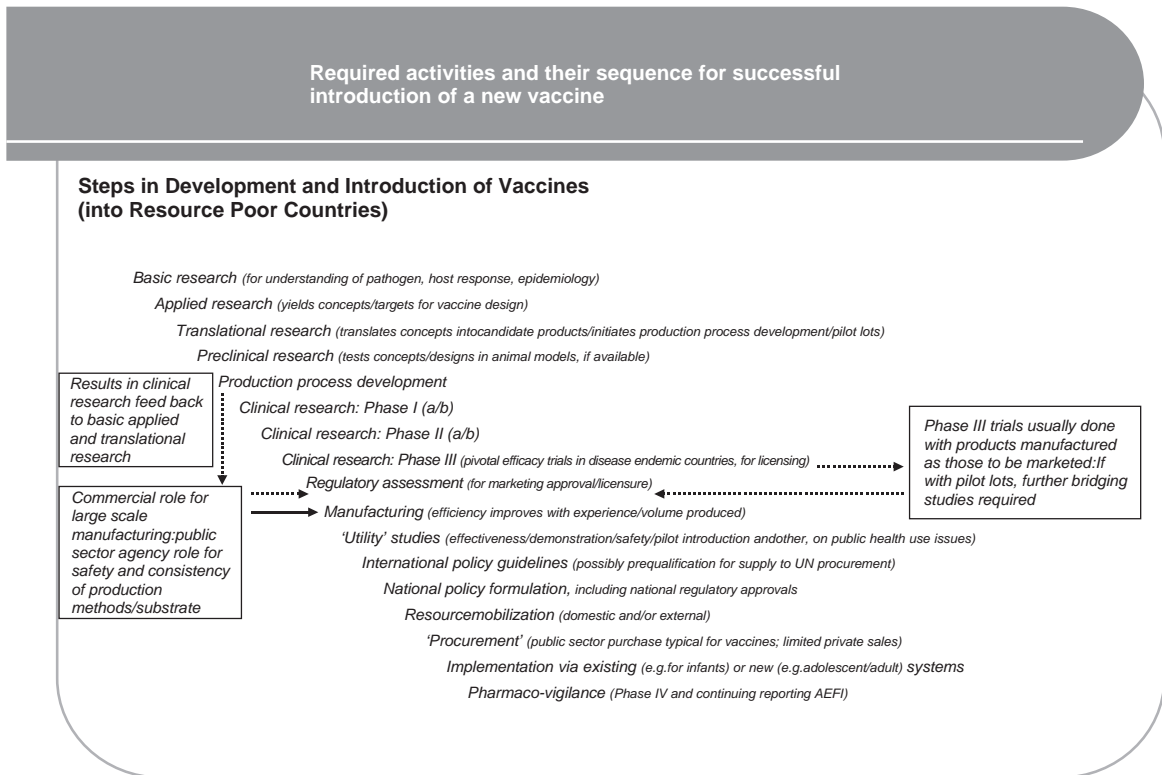


Figure 5.3 Required activities and their sequence for the successful introduction of a new vaccine. (Source: Amfo, K., May 2008 (personal communication).)

PLANNING AND IMPLEMENTATION OF VACCINATION PROGRAMMES

At the outset of the planning process for a vaccination programme, it is important to review thoroughly what is known about the epidemiology of the disease the vaccine is targeting. The strategy that is likely to have a maximum impact on its incidence can then be arrived at. Obviously, the vaccine should be administered before the age-related peak incidence of the disease, taking into account practical logistic considerations, such as ease of access to potential vaccinees, and whether the vaccine is efficacious in the age group being considered for vaccination. In the case of live vaccines, such as measles, maternally-acquired antibodies may prevent a 'take' of the vaccine and vaccination must be delayed until they have been catabolized. A fundamental issue is whether all at risk (potentially everybody except those known to be immune) will receive vaccine or only those at high risk of infection (because their environment, occupation or lifestyle might expose them to the pathogen). For relatively rare diseases that affect well-defined risk groups, it is conceivable that targeting these groups for vaccination (assuming they can be reached) might reduce disease incidence in the population to an acceptable level (i.e. control it). However, for prevalent diseases, targeted vaccination will almost certainly not have a significant impact on incidence and more widespread vaccination of the population will be required to achieve disease control. If the aim is to eliminate the infection, the effective reproduction factor will need to be reduced to <1 . Mathematical modelling of the levels of immunity in various age groups, obtained through serological surveys, can predict what would be the most efficient strategy to attain herd immunity (Nokes and Anderson, 1988). In most cases, systematic vaccination (at a coverage level determined by R_0 and the protective efficacy of the vaccine) of all future birth cohorts will be needed, augmented as required by a (periodic) catch-up campaign(s) in older age groups. Occasionally, the effectiveness of a vaccine in reducing disease incidence is much higher than could have been predicted from its protective efficacy and vaccine coverage. In Israel, for example, hepatitis A was controlled in the whole population within a few years by vaccinating only toddlers, since they were the spreaders of the virus in the community through their attendance at day-care centres (Dagan *et al.*, 2005). The capacity to protect the entire population by targeted vaccination of spreaders of the pathogen has recently been referred to as 'source drying' (André *et al.*, 2008).

Once a strategy has been decided upon and the budget to implement it has been made available, all those involved in the acquisition, storage, distribution, transport and administration of the vaccine must be adequately

trained to avoid mistakes. Nothing must be left to chance otherwise things will go wrong, discrediting all involved. The population will then continue to suffer unnecessarily the pain and misery of preventable diseases.

SURVEILLANCE OF DISEASE INCIDENCE AND ADVERSE EVENTS BEFORE AND AFTER IMPLEMENTATION OF VACCINATION

Good planning of a vaccination programme involves putting in place a surveillance system for disease incidence in order to monitor the impact of the programme on the outcome that matters, namely a reduction of the targeted disease following its implementation. Findings in surveillance systems that reveal changes in disease epidemiology (such as changing age-related disease incidence, immunity gaps detected in serological surveys and more precise information on duration of vaccine-induced protection) may require periodical adjustment of vaccination programmes.

Another surveillance system that must be available is one for adverse events following immunization (AEFI) so as to measure if some AEFIs occur more frequently after vaccination than could be expected from the background incidence of such events. This has become very important because there are many antivaccine lobbyists actively trying to discredit vaccines. The only rational way to deal with this issue (which is consuming much of the time of pro-vaccine scientists) is to have epidemiological studies that show whether AEFIs are causally linked or not, bearing in mind that science cannot prove for certain the absence of a link (Stratton *et al.*, 1994; Varricchio *et al.*, 2004).

RECTIFICATION OF PUBLICIZED FALSEHOODS AND MAINTENANCE OF VACCINATION COVERAGE

Currently the vaccination service in the United Kingdom is recognized worldwide as being one of the best since it incorporates the activities and skills that should guarantee success (Miller *et al.*, 2002). The British mass media have traditionally been fond of giving vaccines adverse publicity, thus encouraging the general public to refuse vaccination for nonscientific reasons, though more recently things have improved. The Internet, where unsubstantiated data can be posted by anybody and which is avidly read by the general public, is also not helpful in this regard. This is not a new phenomenon; vaccinologists have had to deal with antivaccination sentiment since the time of Jenner.

When Jenner's method of protecting against smallpox became generally known, critics predicted that inoculated

persons would develop bovine characteristics because the inoculum had been obtained (albeit indirectly) from a cow. The medical fraternity of the time was also against Jenner because he damaged their business by offering protection against smallpox free of charge; the technique of variolation (affordable only by the well-to-do) was a source of revenue for its practitioners. Laws were passed during the nineteenth century to make vaccination against smallpox mandatory, but sometimes this led to riots because it went against the political principle of free choice in democracies. In the twentieth century the undeniable effectiveness of vaccines to control infectious diseases like diphtheria, tetanus, whooping cough, poliomyelitis and measles led to a general acceptance that vaccines were desirable, but they became the victims of their own success; in the early seventies, for example, a Glasgow professor became convinced (erroneously as it turned out) that whooping cough vaccine was not only unnecessary—since the disease had almost disappeared because of vaccination—but also dangerous. According to him, it caused permanent neurological damage and he campaigned diligently to persuade families in the United Kingdom to refuse vaccination against whooping cough for their children. This had catastrophic consequences (Gangarosa *et al.*, 1998). The anti-whooping-cough-vaccine movement spread to other countries like Japan, Sweden, Italy and West Germany with the same effects. In the United Kingdom, vaccination against mumps, measles and rubella (MMR) has recently also been seriously damaged by a single doctor's unwarranted assertion that the MMR vaccine causes autism. For no good reason vaccines have a bad name in the mass media. However, to the credit of the general public, the great majority of children are still vaccinated; at least in developed countries. On the Internet, however, most comments about vaccines are negative (Wolfe *et al.*, 2002) and the general public invariably reads antivaccine Web sites. Furthermore, there is a general tendency to believe that decision makers and 'the authorities' seek to persecute the underdog, whereas the truth is that both those for and those against vaccination have their own reasons to believe that they are right. Objective facts support vaccination but personal decisions are often taken on subjective preferences.

How to deal with this dilemma? Clearly, scientific 'truth' can only be obtained through observation and experimentation. In Denmark it has been shown that allegations that childhood vaccines are responsible for autism (MMR vaccine and mercury in vaccines), type 1 diabetes and nonspecific infections due to 'overload of the immune system' are not supported by fact (Hvaid, 2008). The publicity in France about hepatitis B vaccine being causally responsible for multiple sclerosis has also been

scientifically demolished but lingers in the national psyche (Balinska, 2001; Mikaeloff *et al.*, 2007). In fact, scientific evidence supports the notion that vaccines are among the safest efficacious medical interventions. Experimentation to test the 'truth' of hypotheses about the safety of vaccines is made impossible by ethical considerations. It must not be forgotten that the scientific method can only disprove a hypothesis; it can never 'prove' anything.

It is now obvious that a major international campaign should be orchestrated to give vaccines the place that they deserve in the medical armamentarium (André, 2005). This has already begun but the process needs to be accelerated. The purpose is to make global opinion aware of the fact that vaccination is not the enemy of the populace but one of its best friends. Vaccinologists and other scientists have a duty to vigorously defend and propagate this undeniable truth in order to defend against the increasingly successful attacks of the antivaccine lobby (Cooper *et al.*, 2008).

VIRAL VACCINES ON THE HORIZON AND THE ROADBLOCKS TO FUTURE VACCINE DEVELOPMENT

Vaccines against practically all known human pathogenic viruses are in various stages of development. In the 2007 Jordan Report of the National Institute of Allergy and Infectious Diseases of the United States (US Department of Health and Human Services, 2007), 26 viruses are listed in an eight-page table as being targeted by vaccines in various stages of development. The many tens of projects for an AIDS vaccine were not even listed in that table. For many of the viruses there are several approaches towards developing a vaccine listed in the report. As an example, the projects for a cytomegalovirus (CMV) vaccine are shown in Table 5.4. For dengue there are no less than 15 different approaches. The 2007 Jordan Report lists 41 viral vaccine projects in basic R&D, 40 in preclinical development, and 19 in Phase I, 23 in Phase II and 3 in Phase III clinical studies. Among the 3 vaccines in Phase III clinical testing in 2006, 2 HPV vaccines (marketed by Merck and GlaxoSmithKline) have since been introduced. It is clear that only a few of the others will reach the market in the foreseeable future, not necessarily because their development is technically and scientifically unfeasible, but because the vast resources required are not available. Many of the projects have been in gestation for decades since they are not attractive enough for research-based vaccine manufacturers to work on them as a priority (André, 2002). Unfortunately, many potentially useful vaccines may not become available because of economic considerations.

Table 5.4 Status of cytomegalovirus vaccine research and development in 2006

Cytomegalovirus (CMV)	Basic R&D	Pre-clinical	Phase I	Phase II
Live attenuated strains (conventional)	+	+	+	+
Live attenuated strains (engineered)	+	+	+	
Glycoprotein subunit vaccine	+	+	+	+
Multiprotein subunit vaccine	+			
Nucleic acid (DNA) vaccines	+	+		
Canarypox vectored	+	+	+	
VEE-vectored	+	+		
Peptide	+			
DNA prime + inactivated boost	+			

VEE, Venezuelan equine encephalitis.

(Source: US Department of Health and Human Services, 2007.)

CLOSING COMMENTS

It is abundantly clear to those who have taken the time to seriously examine the evidence that vaccination is, across the board, one of the best and most cost-effective tools in health care worldwide. This conclusion was reached by the World Bank in 1993 (World Bank, 1993) and acknowledged again at the end of the last century (Centers for Disease Control, 1999). In many cases vaccination is even cost-saving. In the United States, for example, vaccination against diphtheria, tetanus, whooping cough,

polio, measles, mumps, rubella, varicella, hepatitis B and *Haemophilus influenzae b* are all cost-saving, as illustrated in Figure 5.4.

In 1967, when the global eradication programme started, smallpox was still annually killing 1.5–2 million people in developing countries. The programme was completed in 10 years at a total cost of US\$100 million. It led to annual global savings of US\$1.35 billion (Barrett, 2004). In 1998, former US President Bill Clinton stated that the United States was, every 26 days, getting back (from not having to vaccinate against smallpox) what it

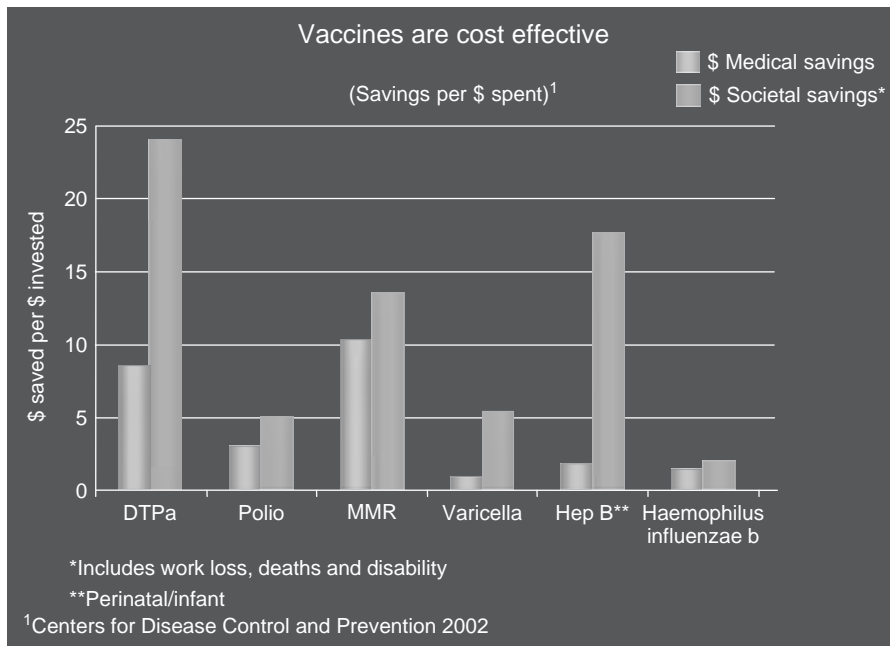


Figure 5.4 Some vaccines are not only cost-effective but also cost-saving: savings per unit of expenditure, medical and societal, in the United States.

spent on smallpox eradication. Other than for HIV, the contribution of antivirals towards alleviating the impact of viral diseases on human health pales into insignificance compared with what has been, is being and will be achieved with existing and future viral vaccines through prevention. Preventive medicine (as illustrated by the achievements of vaccinology) is far superior to curative medicine, though, unaccountably, the latter continues to get 98% of the health-care budget worldwide.

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Herpes Simplex Virus Type 1 and Type 2

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Human herpesvirus 1 and human herpesvirus 2 are more commonly known as herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2). They are both members of the order *Herpesvirales*, a large group of enveloped double-stranded DNA viruses. More than 150 members of the *Herpesvirales* have been identified and the viruses have been found in almost every species in which they have been actively sought, including both warm- and cold-blooded species, vertebrate and invertebrate. There are presently nine members that are known to infect man (Table 6.1): eight whose natural host is man and one transmitted as a zoonotic infection from monkeys that can cause fatal encephalomyelitis in humans (Huff and Barry, 2003).

Viruses included in the order *Herpesvirales* have a double-stranded DNA genome of between 80 and 150 million Da molecular weight (Table 6.2). Following infection of their natural host the viruses establish a latent infection that persists for the life of the host. In the latent state only a small subset of the viral genes are expressed. Reactivation, with expression of viral proteins (VPs) and production of progeny virus, may occur at intervals to produce recurrent infection. This allows virus transmission to new, susceptible hosts. Individual members are well adapted to their natural host and exhibit little or no ability to cause cross-species infection.

The order *Herpesvirales* has three families:- the *Herpesviridae* containing mammalian, bird and reptile viruses (Davison et al., 2008); the *Alloherpesviridae* containing

fish and frog viruses; and the *Malacoherpesviridae* containing an oyster virus. The Herpesvirus Study Group of the International Committee on the Taxonomy of Viruses divided the *Herpesviridae* family into three subfamilies, the *Alpha-*, *Beta-* and *Gamma-herpesvirinae* (Fauquet et al., 2005), broadly on the basis of differences in the biological properties of the various viruses (Table 6.3). The subfamily *Alphaherpesvirinae* contains four genera—*Simplexvirus*, exemplified by human herpesvirus 1; *Varicellovirus*, exemplified by human herpesvirus 3; *Mardivirus*, exemplified by Gallid herpesvirus 2 - Marek's disease virus type 1; and *Iltovirus*, exemplified by Gallid herpesvirus 1 - infectious laryngotracheitis virus.

MORPHOLOGY

The morphology of the herpes simplex viruses (HSVs) as visualized by transmission electron microscopy (TEM) is the same as for all members of the *Herpesviridae* (Figure 6.1). With negative staining the virus particle often appears to be pleomorphic. However, the pseudoreplica electron microscopy (EM) technique reveals a spherical virus particle 150–200 nm in diameter with four structural elements:

- an electron opaque core;
- a protein capsid, surrounding the virus core, comprising 162 capsomeres;

Table 6.1 Classification of the human herpesviruses

Official name	Subfamily	Genus	Trivial name and abbreviation
Human herpesvirus 1	<i>Alphaherpesvirinae</i>	<i>Simplexvirus</i>	Herpes simplex virus type 1 (HSV-1)
Human herpesvirus 2	<i>Alphaherpesvirinae</i>	<i>Simplexvirus</i>	Herpes simplex virus type 2 (HSV-2)
Human herpesvirus 3	<i>Alphaherpesvirinae</i>	<i>Varicellovirus</i>	Herpes varicella-zoster virus (VZV)
Human herpesvirus 4	<i>Gammapherpesvirinae</i>	<i>Lymphocryptovirus</i>	Epstein-Barr virus (EBV)
Human herpesvirus 5	<i>Betaherpesvirinae</i>	<i>Cytomegalovirus</i>	Human cytomegalovirus (CMV)
Human herpesvirus 6	<i>Betaherpesvirinae</i>	<i>Roseolovirus</i>	HHV-6
Human herpesvirus 7	<i>Betaherpesvirinae</i>	<i>Roseolovirus</i>	HHV-7
Human herpesvirus 8	<i>Gammapherpesvirinae</i>	<i>Rhadinovirus</i>	Kaposi's sarcoma associated herpesvirus (KSHV)
<i>Macacine</i> herpesvirus 1	<i>Alphaherpesvirinae</i>	<i>Simplexvirus</i>	'B' virus

Table 6.2 Properties of the human herpesviruses

Virus	Site of latency	G + C (moles%)	Genome (kb pairs)	Sequence ^a
Human herpesvirus 1	Sensory nerve ganglia	68	152	X14112
Human herpesvirus 2	Sensory nerve ganglia	69	154	Z86099
Human herpesvirus 3	Sensory nerve ganglia	46	125	X04370
Human herpesvirus 4	Leukocytes, epithelial cells	60	172	V01555
Human herpesvirus 5	B lymphocytes	57	229	X17403
Human herpesvirus 6A	T lymphocytes (CD4 ⁺), epithelial cells	43	159	X83413
Human herpesvirus 6B	T lymphocytes (CD4 ⁺), epithelial cells	43	162	AF157706
Human herpesvirus 7	T lymphocytes (CD4 ⁺)	36	153	AF037218
Human herpesvirus 8	B lymphocytes, epithelial cells	59	170	AF402655

^aEuropean Molecular Biology Laboratory Accession Numbers (www.embl-heidelberg.de).

- an amorphous tegument surrounding the capsid; and
- an outer envelope with spikes on its surface.

The core is composed of linear double-stranded DNA packaged in the form of a torus. Data derived from electron micrographs suggests that the DNA is physically stabilized within the capsid by a series of protein fibrils embedded on the inner surface of the capsid and passing through the central hole of the torus. The ends of the genome are probably held in close proximity within the capsid since the DNA rapidly circularizes in the absence of protein synthesis soon after it enters the host-cell nucleus. The complete sequence of HSV-1 and HSV-2 is known (Dolan *et al.*, 1998; McGeoch *et al.*, 1988). The genome of each virus can be viewed as consisting of two covalently-linked components, L (long) and S (short), each comprising unique sequences flanked by inverted repeat sequence. These components can invert relative to one another and equimolar concentrations of the four possible isomers of the DNA molecules formed by this inversion can be found in wild-type virus (Roizman *et al.*, 2004).

The viral capsid (100–110 nm in diameter) is a closed shell in the form of an icosahedron (T = 16) with

162 capsomers arranged as 12 pentamers (vertices) and 150 hexamers (face and edges). Using high-resolution cryo-EM and computer-image reconstruction techniques the three-dimensional structure of empty capsids of HSV-1 has been determined to a resolution of approximately 8.5 Å (Baker *et al.*, 2003; Zhou *et al.*, 2000). There appear to be four VPs (5, 19c, 23 and 26) aggregated as pentons and hexons that are associated with polypeptide triplexes to form the nucleocapsid. The interior of the capsid is accessible via transcapsomeric channels ('tubes') formed by the polypeptide arrangement of the pentons, hexons and holes at the base of each triplex. These openings are postulated to play a role in the transport of genomic DNA and scaffolding proteins during capsid morphogenesis.

Between the capsid and envelope is the tegument. Visualized in cryo-electron tomograms of isolated virions, the tegument appears to form an asymmetric cap: on one side, the capsid closely approaches the envelope; on the other side, it is separated by about 35 nm of tegument. The tegument substructure appears particulate, with some short actin-like filaments (Grünwald *et al.*, 2003). The tegument contains at least 20 proteins that are believed to

Table 6.3 Biological properties of *Herpesviridae***Common properties**

Large, linear, double-stranded DNA genome
 Synthesis of DNA and assembly of capsid within the nucleus, acquire envelope by budding through nuclear membrane
 Specify a large array of enzymes involved in nucleic acid metabolism and synthesis
 Production of progeny virus results in destruction of the host cell
 Establish latency in their natural host

Alphaherpesvirinae

Variable host range
 Short reproductive cycle
 Rapid spread in cell culture
 Efficient destruction of infected cells
 Establish latency primarily but not exclusively in sensory ganglia

Betaherpesvirinae

Restricted host range (a non-exclusive property of this subfamily)
 Long reproductive cycle
 Infection progresses slowly in culture, frequently forming enlarged (cytomegalia) cells
 Latency in secretory glands, lymphoreticular cells, kidneys and other tissues

Gammapherpesvirinae

Experimental host range limited to family or order of natural host
In vitro replication in lymphoblastoid cells
In vivo replication and latency in either T or B lymphocytes

have important functions in the early stages of virus replication following penetration of the host cell by the virion.

The virus envelope has a typical trilaminar appearance and is thought to be derived from patches of host-cell membrane modified by the insertion of virus glycoprotein spikes. Using cryo-electron tomograms of isolated virions the envelope can be seen to contain 600–750 glycoprotein spikes, which vary in length, spacing and the angles at which they emerge from the membrane. Their distribution appears to be nonrandom, suggesting functional clustering (Grünewald *et al.*, 2003).

REPLICATION

HSV-1 and HSV-2 are characterized by a short (18–24 hour) replicative cycle that is cytolytic. Initial attachment and penetration of the host cell is mediated via the 11

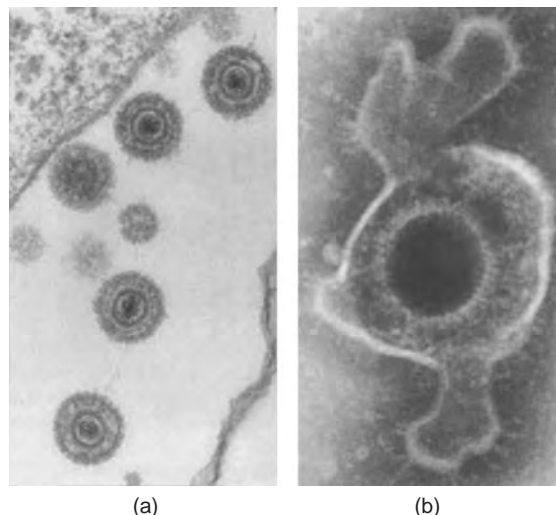


Figure 6.1 Enveloped herpesvirus particle. (a) Thin section. (b) Negative staining (Magnification $\times 140\,000$) (Courtesy of Hans Gelderblom, reproduced with permission from Van Loon *et al.*, (2004).

types of glycoprotein spike found on the virus envelope (Table 6.4). These spikes are responsible for the major antigenic differences between HSV-1 and HSV-2 and relate to the type-specific epitopes found on certain of the glycoproteins (Bergström and Trybala, 1996).

Initial attachment of HSV-1 appears to involve attachment to cell-surface glycosaminoglycans on heparan sulphate mediated via glycoprotein C and glycoprotein B, or, on cells devoid of heparan sulphate, equivalent glycosaminoglycan moieties of other cell-surface proteoglycans, such as chondroitin sulphate. Glycoprotein D then interacts with one of three alternative entry receptors: nectin 1, a member of a family of intracellular adhesion molecules; herpesvirus entry mediator (HVEM), a member of the tumour necrosis factor (TNF) receptor family; or 3-*O*-sulphated heparin sulphate (Campadelli-Fiume *et al.*, 2007; Reske *et al.*, 2007). The binding of glycoprotein D triggers a process of fusion of the virion envelope and the plasma membrane (or endosome membrane) through glycoprotein D working in concert with glycoprotein B and a heterodimer of glycoproteins H and L. The major pathway for virus entry is fusion of the envelope with the plasma membrane and transport of the released nucleocapsid to the nuclear pore. Alternative, ancillary, pathways have also been identified in cell culture, including fusion within an acidic endosome and fusion within a neutral endosome (Reske *et al.*, 2007).

Fusion results in the introduction of tegument proteins and viral nucleocapsid into the cell cytoplasm. Subsequently, host macromolecular metabolism is rapidly

Table 6.4 Herpes simplex virus glycoproteins

Glycoprotein nomenclature ¹	Gene ²	Essential for virus infectivity? ³	Function
gB	U _L 27	Yes	Essential for viral entry. Induces neutralizing antibody.
gC	U _L 44	No	Mediates cell attachment of virion to the cell. Modulates complement activation by binding C3 and blocking C5 and properidin binding to C3.
gD	U _S 6	Yes	Required after attachment to the cell. Interacts with the three cellular receptors for virus entry to the cell and thereby determines viral tropism. Enables fusion of the virion envelope with the plasma membrane through fusion mediated by gB, gH and gL.
gE	U _S 8	No	Forms a heterodimer with gI. The gE–gI complex constitutes an Fc receptor which is implicated in disruption of host immune defence. The gE–gI also facilitates basolateral spread of progeny viruses in polarized cells, suggesting a role for it in virion transport.
gG	U _S 4	No	Function not known. gG2 of HSV-2 is larger than gG1 of HSV-1 and the different antigenic determinants can be used to provide a means of differentiation of HSV-1 and HSV-2 immune responses.
gH	U _L 22	Yes	Essential for virion infectivity and for cell–cell fusion of infected cells. Transport of gH from the Golgi apparatus requires interaction with soluble gL. gH induces neutralizing antibodies.
gI	U _S 7	No	See gE.
gJ	U _S 5	No	Shown to block apoptosis.
gK	U _L 53	No	Found in low abundance. May have a role in preventing infected cells fusing with one another.
gL	U _L 1	Yes	Interacts with gH as detailed above. Probably acts to regulate the fusogenic activity of gH.
gM	U _L 10	No	Interacts with the product of U _L 49.5. May be required for the packaging of U _L 49.5 in virions.

¹Viral glycoproteins are named sequentially by letter as glycoprotein A, B etc. The missing sequence letters (e.g. glycoprotein A, glycoprotein F) reflect earlier misidentification of precursors of glycoprotein species as the actual virion glycoprotein. There are a further two (the products of genes U_L20 and U_L34), and possibly more, non-glycosylated viral proteins inserted in the virion envelope.

²Gene or transcriptional unit: U_S = unique short sequence of the genome; U_L = unique long sequence

³Information from *in vitro* experimentation with HSV-1. All glycoproteins are essential in wild-type virus. In cell culture only, some functionality can be dispensed with. Requirement for glycoprotein that is non-essential in routine cell culture can be demonstrated under specialised conditions, e.g. gC is essential for attachment to the apical surface of polarised MDCK cells; gI is essential for basolateral spread of virus in polarised cells.

and efficiently shut down. Host DNA synthesis ceases, protein synthesis declines rapidly, ribosomal RNA synthesis is reduced and host protein glycosylation ceases. There are at least 20 tegument proteins, and whilst the function of a number of these is not understood, they are all believed to act directly or indirectly to produce early shut-off of host macromolecular synthesis and to contribute to the early events of replication. The virion-associated host shut-off protein (vhs) appears to remain in the cytoplasm, where it causes the disaggregation of polyribosomes and degradation of cellular and viral RNA. Conversely, some tegument

proteins are believed to facilitate attachment of the nucleocapsid to nuclear pores and release of viral DNA.

The nucleocapsid is transported through the cytoplasm to a nuclear pore via microtubules of the cell cytoskeleton. On arrival at a nuclear pore, virus DNA is released from the capsid into the nucleus of the cell, where it immediately circularizes. To initiate transcription, the circularized DNA binds a host-cell protein Octamer binding protein 1 (OCT-1), the tegument protein α -trans-inducing factor (α -TIF), an additional host-cell factor designated C1, and other transcriptional factors to promote the expression of

α (or 'immediate-early') genes—the first set of viral genes to be transcribed (Roizman *et al.*, 2004). Viral-gene expression is regulated and sequentially ordered as a cascade ($\alpha \rightarrow \beta \rightarrow \gamma$). The five α -gene products are regulatory proteins; their expression is required for the production of all subsequent polypeptide groups. The proteins serve to *trans-activate* β and γ gene expression and to turn off both α - and early γ -gene expression (other domains of the viral genome are transcribed under ' α ' conditions, including the 'latency-associated transcript' LAT-1—see below). Viral DNA is transcribed throughout the replicative cycle by host RNA polymerase II.

The expression of the β genes results in the production of enzymes involved in nucleic acid metabolism (ribonucleotide reductase, thymidine kinase (TK), thymidilate synthetase, alkaline DNase, dUTPase, etc.) and in DNA synthesis (DNA polymerase, helicase, primase, etc.). Peak rates of synthesis of β -gene products are observed 5–7 hours post-infection and when sufficient levels of these proteins have accumulated within the infected cell, viral DNA replication commences. Early (α) gene expression is significantly reduced following the start of DNA replication, while late genes begin to be expressed at high levels. HSV DNA is believed to replicate by a rolling-circle mechanism, yielding a concatomer that must be subsequently cleaved to package genome lengths of DNA within the nucleocapsid.

The synthesis of nucleocapsid and all other structural proteins occurs when γ -gene expression is induced by β -gene products. HSV capsids are assembled around viral scaffolding proteins in the nucleus, and other VPs then interact with replicated viral DNA to allow DNA encapsidation. The tegument proteins also migrate to the cell nucleus and form patches underneath the modified nuclear membrane. The DNA-filled capsids proceed to associate with tegument (matrix) proteins near the nuclear membrane. Virus glycoproteins undergo extensive post-translational modification during transit through the golgi apparatus; they then become inserted in the nuclear and other cellular membranes. Three different models for the release of mature nucleocapsids from the cells have been proposed. In the first model capsids are enveloped at the inner nuclear membrane and transported within vesicles that fuse with the plasma membrane to release the enveloped capsids into the extracellular milieu; in the second model capsids are enveloped at the inner nuclear membrane and undergo de-envelopment at the outer nuclear membrane and re-envelopment at cytoplasmic membranes; and in the third model nuclear pores become enlarged sufficiently to allow egress of naked capsids and the capsids then become enveloped at cytoplasmic membranes. The evidence for and against each of the models is the subject of current debate. Whichever

method the virus uses for its release, productive infection of a cell results in the destruction of the host cell through the major structural and biochemical changes induced by virus replication.

Latency

Latency is central to the success of HSV as a human pathogen; it permits persistence of the virus in the presence of a fully-developed immune response and life-long infection of the host. As a result of periodic reactivation of latent virus and the production of recurrent infection, virus shedding and transmission of infection to susceptible individuals occurs at intervals throughout life, allowing the virus to persist in populations with high levels of herd immunity.

During the primary or initial infection, virus comes into contact with the cutaneous receptors of local sensory nerves. Virus attaches to and penetrates the nerve cell via these receptors (Figure 6.2). In oro-facial HSV infection this is predominantly the trigeminal nerve innervating the face, in genital HSV infection lumbro-sacral nerves innervating the genitalia. The unenveloped nucleocapsid and tegument proteins of the virus are transported within the nerve cell by retrograde transport along the microtubules of the axon (Diefenbach *et al.*, 2008). In animal experiments, viral-gene expression can be demonstrated within the nerve ganglion 24–72 post-infection. Several components of the innate immune response are then thought to interact to control early viral replication. Cytokine and chemokine expression increases within the infected ganglia and CD8⁺ T cells infiltrate the ganglion; the amount of virus decreases around day 7. CD8⁺ cells are thought to have a key role in controlling viral replication during the acute infection of the ganglia and within nervous tissue. At the level of virus replication a key factor in this initial phase of the establishment of latent infection appears to be the absence of expression of α (immediate-early) proteins. It has been shown that the cellular cofactor C1 that is required with Oct-1 and α -TIF for α gene expression is only found in the cell cytoplasm and is only transported to the nucleus when the virus reactivates. It is possible that the initial innate immune response within the ganglion is responsible for blocking the recruitment of C1 from the cytoplasm to its site of action within the cell nucleus. However, this is but one of several possible explanations of the process (Valyi-Nagy *et al.*, 2007).

A latent infection of the nerve cell is established. In the latent state the virus is believed to exist as extrachromosomal circularized DNA (analogous to plasmids). No virions can be detected and no viral antigens appear to be expressed on or within the latently-infected cell. The host immune response to infection rapidly eliminates virus and virus-infected cells from peripheral sites, but does not

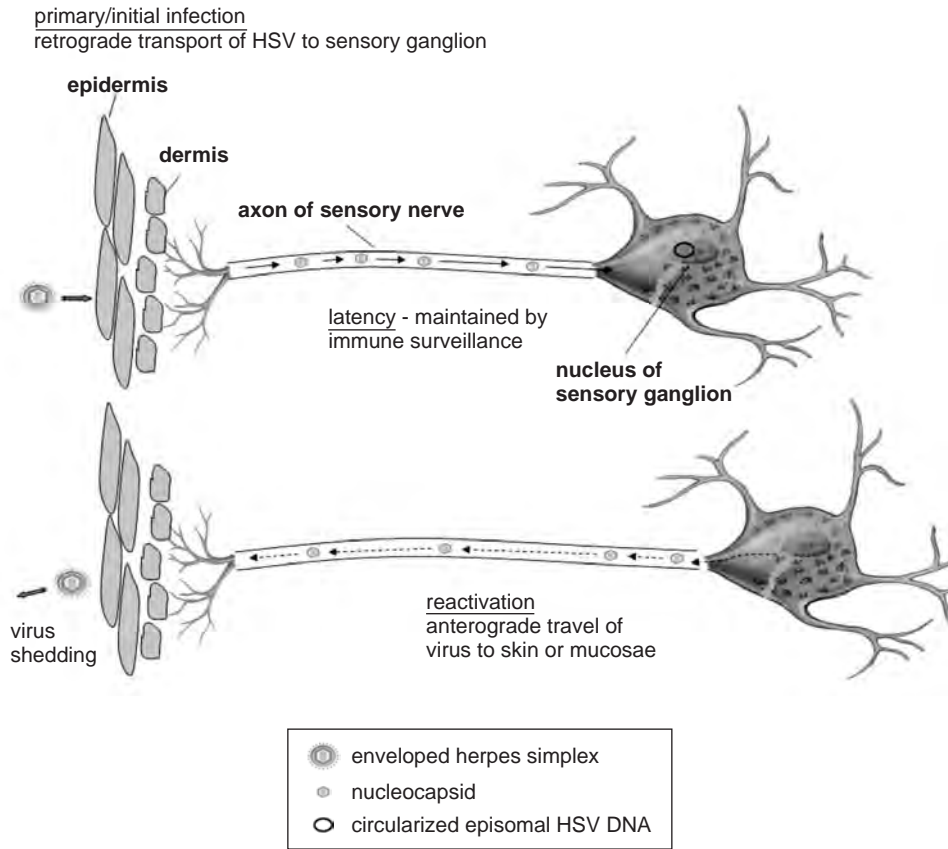


Figure 6.2 The life-cycle of herpes simplex viruses.

recognize latently-infected nervous tissue as harbouring virus since no viral antigens are expressed.

The site of virus latency is related to the site of primary infection; for HSV-1 the trigeminal ganglia and for HSV-2 the sacral ganglia are the most common sites. Other dorsal root ganglia, including the superior cervical, vagal and geniculate ganglia may also harbour virus. Although primary HSV-1 infection of the genitalia and primary HSV-2 infection of the oropharynx are often reported, recurrent infection of the genitalia by HSV-1 and recurrent oro-facial infection by HSV-2 are uncommon. Infrequent reactivation is not thought to be due to a failure by HSV-1 to establish latent infection in cells of the sacral ganglia or of HSV-2 to fail to establish latent infection in cells of the trigeminal ganglion, since the respective genomes of the viruses have been detected at these sites. The inhibition seems to be at the level of effective viral-gene expression in relation to the particular environment of these sites.

A latently-infected neurone shows no evidence of viral-gene expression. A viral transcript—the LAT—is

expressed within the latently-infected ganglion but no VP expression is observed. Prolonged expression of cytokines and chemokines are observed in the latently-infected ganglion, and in murine experiments $CD8^+$ T-cell infiltration can be demonstrated. About 10% of the cells in a latently-infected ganglion are thought to contain at least one viral genome. Laser-capture microdissection of human trigeminal tissue latently infected with HSV suggests that there are an average of 11 genomes per cell of the HSV genome. This could be because multiple viruses infect an individual cell during primary infection, or because the viral DNA is replicated at a low level by viral or cellular mechanisms. The maintenance of the HSV genome in latently-infected neurones appears to be passive in that it does not require any other viral-gene expression or gene product. It is possible that low levels of replication of the viral genome occur since in animal experiments the numbers of copies per cell appears to increase with time. Such low-level replication may be important for the maintenance of latency and to allow

reactivation. A host-dependent origin of DNA replication has been identified within the viral genome (Sears and Roizman, 1990) and thus host-cell enzymes could effect VP-expression-independent replication of the viral DNA in latently-infected neurones.

The LAT intron is stable within the latently-infected cell nucleus and whilst viruses where the LAT gene is deleted are capable of establishing latency, viruses which lack LAT, a LAT promoter or a 348bp sequence in the 5' end of LAT exhibit reduced efficiency of reactivation. However, a full understanding of the role of LATs and of other molecular events involved in the latency remains elusive (Bloom, 2006). Among the many functions ascribed to the LAT introns are down-regulation of genes that are lytic, blocking of the transcription of the gene-encoding ICP0 (a potent gene transactivator), enabling of reactivation from the latent state, maintenance of virus in the latent state and protection of neurones from apoptosis.

Reactivation of latent virus produces recurrent infection. A variety of nonspecific 'triggers' for this process have been described, for example injury to tissue innervated by the latently-infected neurones, emotional or physical stress, menstruation, ultraviolet light and hormone imbalance. It is assumed that in reactivation the cascade of viral-gene expression ($\alpha \rightarrow \beta \rightarrow \gamma$) occurs in the ganglion and leads to the production of progeny virus that are released from the axonal tip after anterograde transport down the axonal microtubule system. The fate of the cell post viral reactivation is a subject of debate. If the usual process of virus replication were followed in nervous tissue then an individual experiencing frequent reactivation over a period of years might be expected to experience local loss of sensation through the progressive depletion of sensory nerves. The damage might also be compounded through the action of the immune system, because in a productive infection virus antigens are expressed on the infected cell surface, rendering the infected cell 'visible' to the immune system. Various models have been proposed to explain this process, though none are entirely satisfactory. It seems likely that upon reactivation, virus replication is so thoroughly controlled that only a few virions are produced, with no expression of virion glycoproteins on the cell surface (thereby permitting the cell to escape immune-mediated cytolysis). Viral genes interfere with apoptosis to prevent cell death and the cytopathology associated with normal productive infection is thereby prevented. In this way the neurone may be able to survive repeated episodes of reactivation.

The Immune Response to Infection

Data derived from human observation and animal experimentation suggests that the host genetic make-up has

an important role in determining immune responsiveness and disease pathogenesis. In man, numerous reports have (presumably because of population selection bias) alternately suggested or denied human leukocyte antigen (HLA) associations with HSV infection. In consequence there is no firm evidence to either confirm or refute HLA involvement in disease pathogenesis. Recovery from HSV infection and the control of reactivated virus infection involves all components of the immune system acting in concert. Macrophages, specific T-cell populations, complement, specific antibodies and lymphokine and cytokine responses all have important roles in the immune response to HSV infection.

Innate and Adaptive Immunity

The innate immune defence system acts to provide a formidable defence against infection and involves both intracellular and extracellular defence mechanisms (Pack and Rouse, 2006). Dendritic cells with natural killer cells, macrophages and neutrophils act with complement, natural antitoxin and interferons to provide an immediate defence against infection. HSV has evolved a number of strategies to overcome both intracellular and extracellular innate immunity to allow the virus to gain access to sensory nerve endings and establish a latent infection during a primary or initial infection.

The adaptive immune response then plays a crucial role in the resolution of primary or initial infection. T-cell-mediated immunity is of major importance. Host T-cell deficits result in prolonged and severe infections. Defence against HSV is believed to depend upon the development of cytotoxic CD8⁺ and CD4⁺ T cells and the products of Th1-dependent CD4⁺ cells (Jones and Cunningham, 2004; Koelle and Corey, 2003). Effector T-cell recognition of virus-infected cells or viral-antigen-expressing cells will result in both the destruction of the target cell and a local inflammatory reaction mediated by inflammatory cytokines that can cause ancillary tissue damage resulting in viral-mediated immunopathology.

Humoral Immunity

Western-blot and radio-immune-precipitation experiments show that serum from patients recovering from severe HSV infection may contain antibody to all the structural (virion) proteins of HSV. The actual range and the amount of antibodies detected correlate with the severity of infection; in mild infection only a restricted number of antibodies may be detectable.

The immune response is principally directed to the glycoprotein spikes (Table 6.4) found on the virion envelope and on the surface of virus-infected cells. Neutralizing

and cytolytic antibodies may be detected, glycoprotein D being the most potent inducer of neutralizing antibody. In a primary infection, an IgM subclass response is detected just before or at the same time as an IgG and IgA immune response. The response is relatively short-lived but IgM antibody may also be detected during recurrence, rendering serological differentiation of primary from recurrent infection difficult. Both IgG and IgA antibody persist, although the IgG antibody response is of greater magnitude. Repeated HSV recurrences lead to a gradual boosting of antibody levels but individual reactivation events or even re-infection may not result in a significant increase in circulating antibody. The presence of serum antibody may not protect from re-infection and neither the quantity nor the range of reactivity (i.e. the lack of antibody to a particular virus protein) appears directly to influence either the frequency or severity of reactivation events.

The early immune response to HSV is relatively type-specific but 'later' antibody is more broadly cross-reacting. Monoclonal antibody studies show that both type-common and type-specific epitopes may be found on virion glycoproteins. As a major part of the humoral immune response is directed to virion glycoproteins the change from type-specificity with time presumably reflects temporal changes in the relative preponderance of antibodies to type-specific and subsequently type-common epitopes on the virion glycoproteins.

Cell-mediated Immunity

The cellular immune response to infection involves a complex interaction of natural killer cells, macrophages, T lymphocytes and associated cytokines. During initial or primary infection the appearance of nonspecific inflammatory changes coincides with the peak of viral replication. Delayed type-hypersensitivity reactions that are followed by a cytotoxic T lymphocyte (CTL) response and the appearance of IgM- and IgG-specific antibodies may be detected. When virus reactivates from latency and produces lesions there is a swift infiltration of natural killer cells and CD4⁺ T cells, followed by CD8⁺ T cells. High levels of β chemokines and T-cell cytokines are also found. CTL activity peaks with CD4 activity and correlates with viral clearance (Koelle and Corey, 2003).

Although HSV-seropositive individuals are presumed to harbour latent virus, less than half the members of an HSV-seropositive population give a history of herpes labialis. Some may recall only one recurrence whilst others report frequent episodes. If seropositive persons with no history of herpes labialis are monitored, silent virus shedding may be shown to occur at intervals. Differences in humoral immune status fail to explain these observations. In a comparison of persons with infrequent recrudescence of oro-facial herpes and those with frequent

recurrence (10 or more episodes per year), differences in only HSV-specific T-cell proliferation, interferon- γ production and levels of HSV-specific IgE could be demonstrated between the two groups (McKenna *et al.*, 2001). In those suffering frequent recurrence the immune response is apparently both delayed and less effective than in those with infrequent recurrences, and is manifest as less effective control of HSV in the periphery following reactivation from latency. The reasons for the appearance or nonappearance of clinical symptoms and the severity of these symptoms are therefore probably explained in terms of an early and effective local cellular immune response. Support for this hypothesis is found in patients with known cellular immune deficiencies where herpes labialis occurs frequently and is a very much more severe and prolonged disease.

Immune Modulation

HSV infection triggers a complex series of cellular events to overcome the host innate immune response (Hook and Friedman, 2007; Roizman *et al.*, 2004). Infection leads to blockage of type 1 interferon effects and interferon-stimulated gene expression. Whilst infection activates toll-like receptors and toll-like receptor signalling with the production of pro-inflammatory cytokines and of type 1 interferon, both of these signalling pathways can be reduced by expression of the virus protein ICP0. Infection can also block dendritic cell maturation, thereby interfering with dendritic cell antigen presentation, and glycoprotein C has been shown to bind complement C3b and prevent complement activation.

Infection also blocks aspects of both the humoral- and the cell-mediated responses (Gewurz *et al.*, 2007). The product of HSV gene ICP47 binds to the transporter protein and blocks peptide transport in the endoplasmic reticulum for loading on to major histocompatibility complex (MHC) class I. This allows evasion of CD8⁺ T-cell effector mechanisms. HSV-infected cells can also inactivate cytotoxic T-cell function. The HSV gene product ICP22 inhibits the ability of B cells to present MHC class II epitopes to CD4⁺ T cells. Finally, the HSV gE and gI complex forms an Fc receptor of high affinity. The binding of the Fc portion of IgG-class antibodies prevents virus neutralization by these antibodies and also blocks antibody-dependent cytotoxicity.

Pathogenesis in Immunocompromised Patients

Congenital deficiencies in humoral immunity (hypogammaglobulinaemia or even agammaglobulinaemia) do not appear to be significant risk factors for serious primary or recurrent HSV disease. Congenital deficiencies in cell mediated immunity (CMI) may, however, be associated

with severe HSV disease. The risk of serious disease varies with the particular immune deficit; for example, patients with congenital athymic aplasia (Di George syndrome) do not appear to be at particular risk, whilst those with severe combined immune deficiency such as the Wiskott-Aldrich syndrome are. These differences emphasize the complex interplay of the immune network in controlling HSV infection. Severe HSV disease is also observed in infants, children and adults with deficiencies in CMI induced by 'therapy'. These include recipients of cytotoxic chemotherapy (e.g. for cancer) and recipients of major organ grafts. The severity of HSV disease in these patients is related to the type and degree of immunosuppression. Severe primary and recurrent HSV infections also occur in patients with acquired immune deficiency syndrome (AIDS).

EPIDEMIOLOGY

HSV has a worldwide distribution, and is endemic in all human population groups examined. There do not appear to be any animal reservoirs for the infection. The rate of infection and the timing of primary infection differ for HSV-1 and HSV-2, reflecting the differences in the major modes of transmission of the two viruses. Whilst both virus types may infect all bodily sites, and an increasing rate of primary HSV-1 genital herpes has been observed in certain populations, genital spread remains the principal route for transmission of HSV-2.

Primary HSV-1 Infection

Primary HSV-1 infection usually occurs when a susceptible individual comes into close and intimate contact with an individual who is actively shedding the virus in, for example, saliva. Thus infants become infected when their parents or relatives kiss them; adolescents who escape infection in infancy are usually infected later by kissing. Infection in an infant is likely to be missed, or dismissed as 'teething'. In adolescents, infection is more commonly symptomatic, but rarely severe. Because the majority of primary infections are asymptomatic, epidemiological data collected by observation of clinically apparent disease provide only a partial measure of its true incidence. For accurate data collection, serological studies must be employed.

The broad principles of the seroepidemiology of primary HSV-1 infection were established in the classic study of Burnet and Williams (1939) and have since been confirmed in numerous studies and, during the last decades, with type-specific serology. In Burnet and Williams' study, during the first six to nine months of

life infants were shown to escape infection by virtue of passively-transferred maternal immunity. Later, during the following five years, infants became infected and developed HSV IgG antibody, and those who escaped underwent seroconversion during adolescence or early adulthood; 90–95% infection rates were then observed by early adulthood and primary HSV-1 infection was a rare event in those of >30 years of age. A relationship between the age of acquisition of the virus and socio-economic status was also found. Populations associated with a low socio-economic environment collectively exhibited earlier acquisition of HSV infection than more affluent populations.

In recent years seroepidemiological studies have shown that, whilst there are large inter-country and intra-country differences, there has been a lowering in the prevalence of HSV-1 antibody in highly-hygienic young adult populations in Europe (e.g. in the United Kingdom; Pebody *et al.*, 2004), and in North America and Japan, where HSV-1 antibody prevalences of <40% have been recorded in young adults of <30 years (Smith and Robinson, 2002).

The major mediator of seroprevalence is the frequency of direct person-to-person contact. In crowded areas (e.g. disadvantaged inner-city areas with abundant opportunities for exposure in crowded households with high rate of family members with prior oro-labial infection) seroprevalence is and remains highest; in affluent and rural areas seroprevalence is lowest. Interestingly, despite the increased mixing which occurs as a result of increased day-care attendance of children in a country like Sweden, there has been no increase in the prevalence of HSV-1 infection in this age group (Svahn *et al.*, 2006). On a worldwide basis, however, overall rates of 90–95% seroprevalence are still commonplace in many adult populations, as judged by investigations of 20–40-year-old individuals in several cities in Asia, Central America and Africa (Nahmias *et al.*, 2006; Paz-Bailey *et al.*, 2007; Smith and Robinson, 2002).

Primary HSV-2 Infection

HSV-2 transmission is apparently less efficient than that observed for HSV-1. The principal route of transmission is sexual activity. Although a few infants acquire HSV-2 infection, in most cases primary infection is delayed until the onset of sexual activity in adolescence and early adulthood. At this time many persons will have already experienced primary infection with HSV-1 (i.e. infection with HSV-2 often causes an *initial* rather than a *primary* infection). A prior HSV-1 infection will not prevent a genital HSV-2 but will reduce the likelihood of HSV-2 acquisition. Because of the shared antigenicity of HSV-1 and HSV-2, HSV-1 immunity may be partially protective and the initial (first) HSV-2 infection is associated

with lesser clinical manifestations than are observed in primary HSV-2 infections. In contrast, a prior HSV-2 infection seems to provide some protection against HSV-1 acquisition.

The majority (80% or more) of those found to be HSV-2-antibody positive cannot recall signs of primary infection and are unaware of recurrent genital herpes, emphasizing that like HSV-1 the majority of HSV-2 infections are clinically 'silent'.

A major problem in determining the seroepidemiology of HSV-2 infections has been the lack of well-characterized methods to differentiate HSV-1 and HSV-2 antibody. Only during the last few years have relevant assays become available on a commercial basis. Not all those exposed to HSV-2 will necessarily become infected but women are generally more easily infected than men. The major influence on acquisition of HSV-2 infections is, as might be expected for a sexually-transmitted virus, the number of sexual partners (Smith and Robinson, 2002). Rates of up to 95% seroprevalence have been reported in some female commercial sex workers. In the general population there are wide differences in seroprevalence between different patient groups and even between apparently similar social groups in different cities. Women are generally infected at an earlier age than men and rates of infection in women are higher for all age groups up to and including those of 45 or more years of age. Studies among genito-urinary-medicine clinic attendees suggest that only in homosexual men do the rates of infection with HSV-2 match those found in women, although even in this context it is not until age 40–45 years that equivalent rates are observed.

HSV-2 and HSV-1 prevalence, overall and by age, varies markedly by country, region within country, and population subgroup, and in populations with higher-risk sexual behaviour (Pebody *et al.*, 2004). Available data (although incomplete) on HSV-2 prevalence in non-high-risk populations suggest that rates in general are highest in Africa and the Americas, lower in western and southern Europe than in northern Europe and North America, and lowest in Asia.

The Changing Seroepidemiology of HSV Infections

The changing seroepidemiology of HSV-1 infections in developed countries has meant that primary eye infections with HSV-1 are now infrequent in children but much more frequent in young adults. Young adults entering the world of work are also more frequently susceptible to primary herpes, as manifest by an increase in incidence of occupationally-acquired herpes simplex infection of the hand (see 'Herpetic Whitlows; Traumatic Herpes', below). In some populations followed over decades

(e.g. in the United States, Sweden, Hong Kong), HSV-2 seroprevalence rates showed an increase over time—with marked increases occurring during the 1970s, when oral contraceptives came into general use—and plateauing thereafter (see, for example, Forsgren *et al.*, 1994). In sub-Saharan Africa striking increases have been observed, especially among young women aged 15–24 years. In other populations the rates have remained stable or even decreased, although in most regions relevant data are lacking.

HSV-1 is now much more commonly seen in association with primary genital herpes. This trend was first noticed in Japan in the mid 1970s. During the last decade, 40 to more than 60% of isolates of HSV from first-episode genital herpes have been found to be due to HSV-1 in high socio-economic populations in studies from the United Kingdom, Scandinavia and the United States. A similar change has not been observed in populations of low socio-economic status.

Recurrent HSV-1 and HSV-2 Infection

Both silent and overt (i.e. symptomatic) recurrences of HSV-1 and HSV-2 infection occur, thus without continuous monitoring of a cohort for viral shedding, accurate data on rates of recurrence cannot be obtained. Only 38–45% of an adult population (in whom seroprevalence rates of 90–95% are reported) will give a history of recurrent herpes labialis.

The frequency of recurrence of genital HSV-2 infection (up to 60%) is higher than that observed in HSV-1 herpes labialis, although the number of lesions produced per episode and their duration are generally shorter. The rate of recurrence is believed to be slightly higher in men than in women, with rates of up to 2.7 and 1.9 episodes per 100 patient days, respectively.

Although primary HSV-1 genital herpes infection is now more commonly seen, this has not been accompanied by a corresponding shift in the type of virus involved in recurrent infection. This is because of the less common reactivation of genital HSV-1 and of oro-facial HSV-2. The majority of clinical and subclinical recurrences in the oro-facial region are HSV-1, whereas genital recurrences are still dominated by HSV-2.

Endogenous and Exogenous Re-infections

Re-infection, recognized by the appearance of lesions at another bodily site, for example infection of the finger (herpetic whitlow), can occur at any age. Exogenous re-infection of all types occurs more often in the immunocompromised than in the immunocompetent. Differentiation of endogenous and exogenous re-infection can only be accomplished by examination of viruses obtained from

distinct sites and demonstration of genetic polymorphism by restriction enzyme analysis or sequencing of a region of their respective DNAs.

VIRAL DIAGNOSIS

The ability of HSV to establish latent infection inevitably complicates diagnosis. A clinical role for the virus in causation of disease is not established simply because it is recovered from a patient. To achieve *meaningful* diagnosis a close collaboration between clinic and laboratory is always necessary, for interpretation of findings against clinical data. Moreover, in many clinical situations rapid diagnosis is a challenge for the viral laboratory as serious HSV disease may not be clinically distinguishable from other conditions and there is pressure for antiviral treatment to be started early (if possible within 24 hours after onset) to achieve maximal effect. Adequate preventive measures may also be dependent on the immediate aid of the laboratory.

Light and UV Microscopy

In diagnostic virology, direct-light microscopic examination of clinical material is now seldom used to provide a diagnosis of HSV infection, although histopathological examination remains an important technique in the differential diagnosis of infection. Staining of relevant tissue sections with specific antibodies tagged with enzymes provides rapid and specific localization of virus. Rapid detection of HSV in clinical material may be achieved by direct or indirect immunofluorescence (IF) microscopy. Scrapings from the bottom of vesicles before crusting, impression smears and cryostat sections of tissue biopsies all provide suitable specimens. Since virus localization within cells can be directly observed, the technique allows prompt diagnosis with 'in-built' specificity control, as opposed to the enzyme-linked immunosorbent assay (ELISA) techniques (see below). The drawbacks of these procedures are the requirement for experience in sampling and the special expertise in IF microscopy to achieve high test sensitivity and specificity.

Electron Microscopy

Transmission electron microscopic examination of negatively-stained vesicle fluid presents one of the most rapid methods for detection of HSV. Although now only available in few diagnostic laboratories, this procedure can be very helpful in establishing a rapid, early diagnosis. Collection of specimens for electron microscopy is a skilled procedure and several methods are available (Hazelton and Gelderblom, 2006). The

morphology of HSV is characteristic, although HSV-1, HSV-2 and herpes varicella-zoster virus (VZV) cannot be differentiated by such direct examination. The technique is relatively insensitive and a specimen must contain at least 10^6 or more particles per millilitre to allow detection of virus. In practice, vesicle fluid from primary infections will usually yield sufficient virus. Thin-section EM, sometimes combined with immunological staining using gold-labelled specific monoclonal antibody, has a role in examination of histological material.

Virus Culture

While the use of virus culture has declined in many diagnostic virus laboratories, virus culture remains an indispensable tool for the specialized laboratory when live virus is required for detailed analysis. HSV-1 and HSV-2 are among the easiest of viruses to cultivate and propagate in the laboratory. A wide range of both primary and continuous monolayer cell cultures can be infected with HSV. Cytopathic effect (CPE) develops as a rule within one to seven days of inoculation. Both ballooning degenerating cells and polykaryocytes may be observed. The CPE of HSV-1 and HSV-2 may be rapidly differentiated by IF staining of infected cells with type-specific monoclonal antibodies.

Virus isolation in cell culture provides a highly sensitive method for the detection of HSV but its efficiency depends on the method of specimen collection and the preservation of virus infectivity between the patient and the laboratory. Vesicle fluid from a 'fresh' vesicle is usually rich in virus. Virus is rarely isolated from crusted vesicles. A virus collection swab is used to swab ulcers or mucous membranes in order to detach virus-containing cells. To reduce loss of infectious virus between the patient and laboratory the swab is then placed in a suitable transport medium to stabilize HSV infectivity. If there is delay in transportation, the material should be maintained at $+4^{\circ}\text{C}$ or immersed in liquid nitrogen. Freeze-thawing should be avoided. Cerebrospinal fluid (CSF), biopsy or necropsy specimens are collected into dry sterile containers and require no special transport media. Such specimens should be transported and maintained at $+4^{\circ}\text{C}$; they should not be frozen. On arrival in the laboratory, specimens are inoculated on to at least two different cell types. Biopsy or necropsy specimens are homogenized in a small amount of transport medium prior to their inoculation on to cell cultures. As for human cytomegalovirus (CMV) (detection of early antigen fluorescent foci (DEAFF) test), detection of virus growth in tissue culture can be accelerated if specimens are centrifuged on to cells cultivated on small cover slips and the cells are stained after 24 hours with a monoclonal antibody directed to HSV early antigen. Isolates of virus may be readily typed as HSV-1

or HSV-2 by the use of specific monoclonal antibodies tagged with fluorescein isothiocyanate.

Enzyme-linked Immunosorbent Assay (ELISA)

A number of ELISA procedures are available for the rapid detection of HSV. Whilst the specificity of these procedures is high (about 98%), their sensitivity for the detection of virus in acute vesicular lesions is only about 80%, and with material from crusting lesions may reduce to less than 60%. In comparison to culture, immunoassays, which are not reliant upon the detection of infectious virus, may offer an advantage where suboptimal transportation of specimens to the laboratory has resulted in loss of virus infectivity.

Nucleic Acid Detection

Direct hybridization of clinical samples with radio- or enzyme-labelled oligonucleotides has been used for the direct detection of HSV but these techniques are now seldom utilized apart from in the examination of histopathological materials (see '*In Situ* Hybridization and *In Situ* PCR' below). A number of alternative nucleic-acid amplification or signal amplification techniques have been applied to the diagnosis of HSV infection but it is nucleic acid amplification, in particular the polymerase chain reaction (PCR), that is most widely applied. A wide variety of PCR techniques have been used, including single, semi-nested and nested PCR techniques, with product detection via gel electrophoresis, Southern blotting, ELISA-like hybridization, microarray or bead-based arrays such as the 'Luminex' procedure. The real-time PCR procedure is now most commonly used; this offers testing of high sensitivity, with reduced risk of intra-laboratory cross-contamination of samples from amplicon release and reduced test turnaround times, achieved by combining the detection of amplification with the thermal amplification process. The test allows direct detection of the products of amplification in real time, is quantitative and usually allows typing of HSV-1 and HSV-2 within the same test. Multi-target or 'multiplex' PCR procedures are also available in both real-time and conventional PCR formats. Careful optimization of these test procedures is necessary to ensure that the sensitivity of detection of each of the individual target viruses within the test is maintained when targets are combined. In special situations, for example the detection of HSV and VZV in vesicle fluids, preferential amplification of a target virus present in high concentration may prevent the detection of a second virus present in low concentration. Nevertheless, the advantage of multi-target detection is an immediate attraction for a diagnostic laboratory, and several successful procedures have been

described in the literature and are available as commercial assays.

Rigorous quality control and attention to detail to ensure adequate sensitivity and specificity are essential for routine application of any PCR technique. Many types of clinical sample contain substances that prove inhibitory to the PCR reaction, which, if not efficiently removed, will result in the production of false-negative test results. Internal control molecules within PCR tests are used to monitor for test failure through test-sample inhibition. Where internal molecules are not available the same check may be performed by 'spiking' a sample with a known amount of HSV or by checking for an alternative human gene expected always to be present within the clinical sample.

Extra- or intra-laboratory contamination of samples with virus or amplicons may give rise to false-positive PCR results. This represents a significant practical problem and requires careful consideration in the design of PCR protocols. A particular consideration in herpesvirus PCR is the possible detection of DNA from (asymptomatic) recurrent herpesvirus infections of no relevance for the current disease. This may create difficulties in defining the clinical significance of a test result.

Sequencing of products of PCR is a useful method for the comparison of strains of virus detected, for example, from different bodily sites or from different persons, and provides a more rapid method for epidemiological and population diversity investigation (Bowden *et al.*, 2006) than the technically demanding restriction-fragment polymorphic analyses previously applied in such studies.

PCR has been most widely applied in the diagnosis of herpes encephalitis and of herpes meningitis. While all types of PCR have been found to be capable of detecting HSV DNA in CSF, rigorous quality control and assay standardization are essential (Linde *et al.*, 1997). The application of real-time PCR for the diagnosis of herpes infection of the central nervous system (CNS) has shown that a sensitivity of between 10 and 25 genome equivalents (copies) per millilitre of CSF is necessary for adequate detection of HSV infection of the CSF. In HSV-1 infection of the CNS, initial CSF viral load has been found to vary between 2×10^2 and 2×10^7 ml⁻¹ for HSV-1 (Hjalmarsson *et al.*, 2007) and between 7×10^2 and 6×10^5 ml⁻¹ for HSV-2 (Franzen-Röhl *et al.*, 2007). The level of HSV DNA in the CSF should decline with treatment, and long-term persistence of HSV DNA in the CSF correlates with a bad prognosis. This further emphasizes the role of PCR in the diagnosis and management of HSV infection of the CNS.

The availability of simplified and automated methods for sample extraction and assay set-up and of improved assays employing internal control molecules to monitor possible sample-induced inhibition of PCR has

led to widespread uptake of PCR in diagnosis of other HSV infections. The use of automation coupled with real-time PCR procedures has allowed PCR to become price-competitive with virus culture. One of the major uses of viral isolation in cell culture was for confirmation of a diagnosis of genital HSV infection. Increasingly, PCR is being utilized to supplant culture, providing more rapid and more sensitive detection and typing of virus than can be achieved by cell culture.

***In Situ* Hybridization and *In Situ* PCR**

HSV nucleic acids may be detected in biopsy or necropsy material by *in situ* hybridization. Paraffin-embedded tissue sections are de-waxed, rehydrated and digested with proteinase-K. After a denaturing step, a labelled DNA fragment or 'probe' (an oligonucleotide produced synthetically or a cloned (plasmid-amplified) fragment of HSV DNA) is incubated with the tissue section. The section is then washed to remove unhybridized probe DNA. The probe may be detected by radiological, enzymatic or chemiluminescent methods appropriate to the label utilized. Where particularly small amounts of viral DNA are present in a clinical sample, *in situ* PCR methods are available to allow detection. The technical complexities of this procedure currently preclude its application except in research settings.

Assays for Antiviral Drug Resistance

Determination of phenotypic expression of antiviral drug resistance depends upon *in vitro* culture of the virus in cell culture. The increasing use of PCR for detection of virus in routine diagnostic laboratories therefore presents a problem if virus is no longer routinely cultured in cell culture. Genotypic analysis involving analysis of regions of the genome with known mutations leading to antiviral resistance is possible. The drawback is that the mutations resulting in resistance have not all been identified and that interpretation of the effects of other mutations that sequencing may identify may be difficult. Phenotypic tests are thus easier to interpret and may detect resistance that genotypic analysis cannot yet discriminate. Maintenance of the capability of cell culture in diagnostic laboratories is thus important.

Serology

Peripheral Blood

A large number of techniques are available to detect and quantify the humoral immune response to HSV infection, including complement fixation tests (CFTs), IF tests and tests for neutralizing antibody. Differences in specificity and in sensitivity are observed between the different

techniques and different assays. These tests have now been largely replaced by commercially-available ELISA tests. In most clinical situations assay of HSV-specific IgG antibody is sufficient. In primary infection seroconversion is readily detected in adequately-spaced samples (14 or more days). In a primary infection IgG production can be detected at or around the same time as an IgM response. Differentiation of primary and reactivated infection may be possible by measurement of HSV-specific IgG avidity. In recent infection IgG avidity will be low, while in reactivated infection IgG avidity will usually be high.

IgM antibody to HSV can be detected using a suitable enzyme-labelled antiglobulin in the standard indirect ELISA technique or, with superior specificity, by IgM 'capture' immunoassay. Detection of HSV IgM in HSV IgG-negative patients or in patients with very low levels of specific HSV IgG is suggestive of a primary infection. False-positive IgM reactions are known to occur, however, and follow-up serology to confirm a positive IgM antibody detection is therefore necessary. During reactivation and in initial infection an IgM response can often be detected. The detection of IgM antibody—especially low-level activity—does not always allow differentiation of a primary infection and a reactivation event. The magnitude of the response can vary from individual to individual. Thus the results of IgM immunoassays must be interpreted with caution.

The type-common epitopes of HSV-1 and HSV-2 ensure that most conventional ELISA techniques are adequate for the detection of seroconversion to either virus in a person without previous experience of HSV infection. Coincident seroconversions to herpes VZV are occasionally observed and may be due to an anamnestic immune response or possibly because of synchronous reactivation ('dual reactivation') or synchronous infection ('dual infection') of HSV and VZV.

ELISA procedures utilizing 'whole virus'- or membrane-associated glycoprotein preparations representative of the complete antigenic spectrum of HSV provide the most sensitive assays for HSV antibody. However, to differentiate HSV-1 and HSV-2 serological responses, ELISA procedures that utilize type-specific purified virion glycoprotein G, peptides and/or recombinant antigens to distinguish HSV-1 specific and HSV-2 specific antibodies are necessary. Such assays are commercially available and are useful in epidemiological studies and in specialized situations such as determination of the serostatus of the partner of a newly diagnosed case of genital herpes or characterization of the first episode of herpes in a pregnant woman. In low-risk populations their positive predictive value is not yet satisfactory. The sensitivity

and specificity of the commercial assays vary but in the best-performing assays are high. In general the sensitivity of these assays has been better for detection of HSV-2 antibodies and the specificity better for HSV-1, although this continues to improve. A prerequisite for the use of these assays is a knowledge of the performance and limitations of the test in use, and access to a reference test for equivocal results (e.g. western blot, Ashley *et al.*, 1988) as well as experience in validation and interpretation of the results (Ryan and Kinghorn, 2006).

Type-common serology remains the basis for the diagnosis of primary infections. Type-specific assays only provide a complement in this situation as the type-specific antibody response may be very late and take up to several months to appear.

Cerebrospinal Fluid

Demonstration of a peripheral blood HSV antibody response is not in itself diagnostic of CNS infection. Such a response may merely reflect reactivation of latent HSV secondary to neurological disease of unrelated aetiology. Serological diagnosis of CNS infection requires proof of intrathecal synthesis of specific antibody. To determine whether specific antibodies are produced intrathecally, and not passively transferred from serum, the integrity of the blood–CSF barrier (BCB) must be assessed. The most accepted methods in general use are those in which the ratio of antibody quantitated in both serum and CSF is compared to the distribution of a reference protein such as albumin (Cinque *et al.*, 1996). The use of total IgG as the reference protein or calculation of the herpes-specific fraction of the intrathecally-produced antibody however provides certain advantages in differentiation between polyclonal stimulation in neurological disease (Jacobi *et al.*, 2007). A technically simpler and rapid diagnostic procedure to attain this goal is λ -capture assays (for references see Aurelius *et al.*, 1989; Fomsgaard *et al.*, 1998). However, the requirement of labelled HSV antigen or specific antibody has hampered their general use due to a lack of commercial tests.

Quantitative and qualitative differences between CSF and serum antibody responses, for example in patterns after isoelectric focusing or western blot, provide additional information (Monteyne *et al.*, 1997). The antigen-mediated capillary-blot technique, performed after isoelectric focusing of serum and unconcentrated CSF, provides an alternative and sensitive method for the qualitative detection of specific intrathecal synthesis of antibody. The presence of two or more anti-HSV oligoclonal IgG bands present only in CSF is considered to provide a definite diagnosis.

ANTIVIRAL CHEMOTHERAPY

A large number of compounds with *in vitro* anti-HSV activity have been described. Few have progressed to clinical trial and fewer still have gained a place in clinical practice. The major ‘anti-herpetics’ in current use, aciclovir and penciclovir, are nucleoside analogues. Prodrugs of aciclovir and penciclovir, valaciclovir and famciclovir, are also available and offer better systemic bioavailability following oral administration (Waugh *et al.*, 2002). These antiviral drugs can suppress symptomatic disease and asymptomatic shedding, and prevent transmission and HSV infection, but they neither cure nor alter the natural history of HSV infections. The nucleoside analogues are virostatic and it is of crucial importance that therapy is initiated very early in HSV infection. Their efficacy in the very common mucocutaneous manifestations of HSV infection in immunocompetent individuals is moderate.

A second-generation nucleoside analogue, brivudin and derivatives, encountered serious, even fatal, drug–drug interaction with fluorouracil used for cancer therapy. Promising drugs in the pipeline (Biron, 2007) include compounds targeting the HSV helicase–primase and appear to be good candidates for combination therapy in the immunosuppressed. Resistance problems have been encountered with some HSV isolates but these were fortunately fully sensitive to aciclovir.

First-line or Standard Therapy

Aciclovir (Zovirax)

The structure of aciclovir is shown in Figure 6.3a and the mode of action of aciclovir is illustrated in Figure 6.4. The compound is an acyclic nucleoside analogue. Virus-infected cells appear to be slightly more permeable to aciclovir than noninfected cells but the compound is only entrapped and selectively concentrated within virus-infected cells. Within the infected cell, a virus-specified enzyme, thymidine kinase, TK, effects the monophosphorylation of aciclovir. The resulting aciclovir monophosphate cannot traverse cellular membranes and is consequently localized within the virus-infected cell. Host-cell kinases (including host-cell-derived TK) do not appear capable of catalyzing this reaction to any significant degree.

The active antiviral drug is aciclovir triphosphate and conversion of aciclovir monophosphate to the active triphosphate form is accomplished by host-cell kinases. The triphosphate form of aciclovir has much greater affinity for virus-specified, as opposed to host-cell-derived, DNA polymerase. Aciclovir triphosphate binds to virus-specified DNA polymerase, leading to the inactivation of this enzyme’s activity. Thus, viral

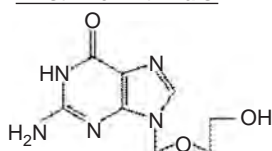
DNA replication is inhibited whilst normal host-cell DNA metabolism remains virtually unaffected. An additional antiviral action is via chain termination through incorporation into the growing viral DNA chain (absence of 2' and 3' carbons of guanosine).

Valaciclovir (Valtrex)

The oral bioavailability of aciclovir is relatively low and for this reason a prodrug—the L-valyl ester of aciclovir—valaciclovir—was developed (Figure 6.3b). Valaciclovir hydrochloride is rapidly adsorbed from the gastrointestinal tract and rapidly and almost completely

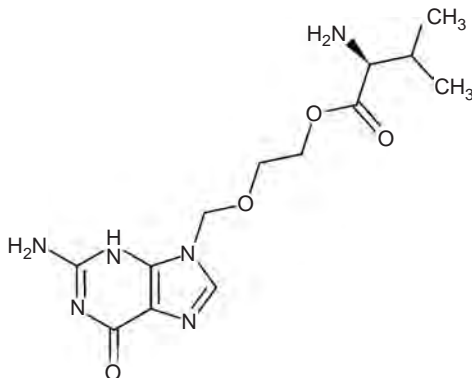
converted to aciclovir and L-valine by first-pass intestinal and/or hepatic metabolism. The mode of action, safety profile and clinical spectrum of activity of valaciclovir are believed to be identical to those of aciclovir. The plasma concentrations of aciclovir achieved after oral administration of valaciclovir are equivalent to those achieved by intravenous administration of aciclovir and are three to fivefold greater than are achieved with oral administration of aciclovir. Valaciclovir can therefore provide a much more convenient dosing regimen of once, twice or three times daily, in comparison to the five times daily for aciclovir.

First-line Antivirals



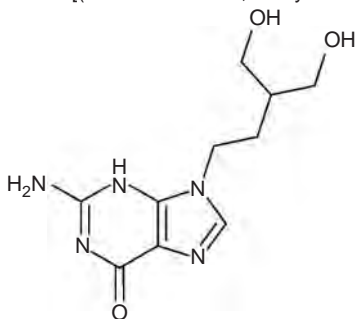
(a) **Aciclovir**

2-amino-9-((2-hydroxyethoxy)methyl)-1*H*-purin-6(9*H*)-one



(b) **Valaciclovir**

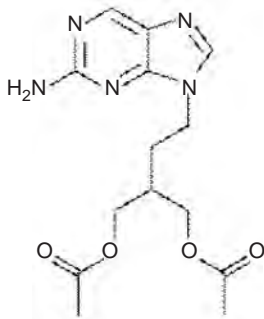
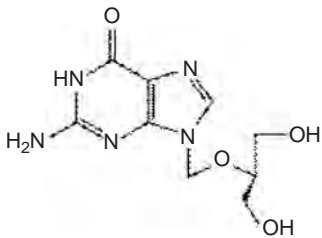
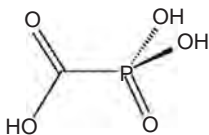
2-[(2-amino-6-oxo-6,9-dihydro-3*H*-purin-9-yl)methoxy]ethyl(2*R*)-2-amino-3-methylbutanoate



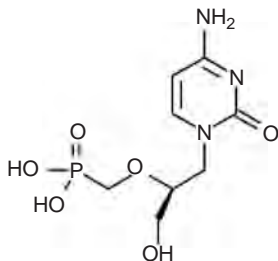
(c) **Penciclovir**

(2-amino-9-[4-hydroxy-3-(hydroxymethyl)butyl]-3*H*-purin-6-one)

Figure 6.3 First- and second-line antivirals.

**(d) Famciclovir**2-[(acetyloxy)methyl]-4-(2-amino-9*H*-purin-9-yl)-butyl acetate**Second-line Antivirals****(e) Ganciclovir**2-amino-9-[[1,3-dihydroxypropan-2-yl]oxy]methyl]-6,9-dihydro-3*H*-purin-6-one**(f) Foscarnet**

(phosphonomethanoic acid)

**(g) Cidofovir**(((2*S*)-1-(4-amino-2-oxo-1,2-dihydropyrimidin-1-yl)-3-hydroxypropan-2-yl]oxy)methyl)phosphonic acid**Figure 6.3** (continued)

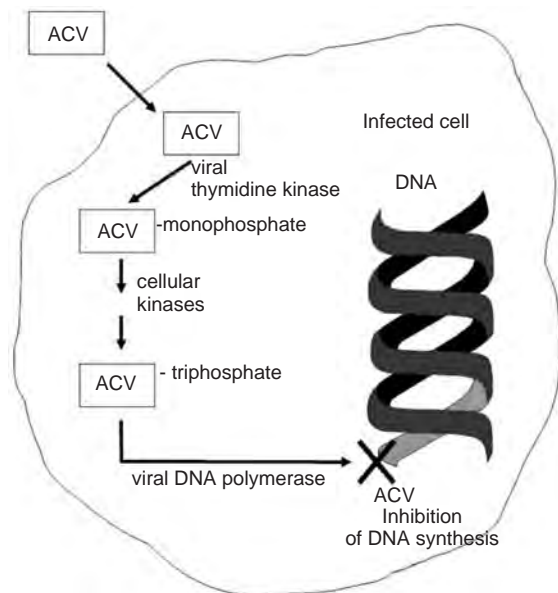


Figure 6.4 Mode of action of aciclovir.

Penciclovir (Denavir)

Penciclovir is an acyclic nucleoside analogue (Figure 6.3c) whose mode of action and safety profile are essentially identical to those of aciclovir. However, the drugs differ in their rate of cellular uptake, phosphorylation rate, stability of the intracellular triphosphate and inhibitory concentration for HSV-DNA polymerase (100-fold higher for penciclovir triphosphate than for aciclovir triphosphate). The intracellular half-life of the active antiviral (penciclovir triphosphate) is substantially longer (7–20 hours) than that of aciclovir triphosphate (0.7–1 hour), which compensates for the slightly lower activity of the drug.

Famciclovir (Famvir)

Famciclovir (Figure 6.3d) is the diacetyl 6-deoxy pro-drug of penciclovir. Famciclovir achieves high levels of systemic bioavailability following oral administration, after which the drug is deacetylated and oxidized to form penciclovir. The mode of action and clinical spectrum of activity are thus identical to those of penciclovir.

Drug Toxicity

The safety profiles of aciclovir, valaciclovir and famciclovir are very favourable due to the selective monophosphorylation of the compounds by viral-specified TK. The risk of adverse reaction is very low; renal dysfunction and CNS neurotoxicity correlate with serum concentrations of

>100 μM (22.5 $\mu\text{g l}^{-1}$), which may be reached only in rapid infusion of large doses. Due to their low bioavailability there is no risk of toxicity with oral aciclovir or penciclovir administration, but toxicity may be seen if overdoses of valaciclovir or famciclovir are given. Safety and resistance data on patients on long-term therapy with aciclovir now extend to over 13 years of continuous surveillance. No long-term toxicity has been noted after suppressive oral aciclovir therapy.

Second-line Antivirals

Ganciclovir ([9-(1,3-dihydroxy-2-propoxy)methyl] guanine) (Figure 6.3e) has good activity against HSV-1 and HSV-2. It has higher toxicity than the aforementioned first-line anti-HSV drugs, its main side-effect being myelosuppression. The L-valyl ester valganciclovir allows oral administration of the drug (see also chapter 8 CMV page 181).

The pyrophosphate analogue foscarnet (trisodium phosphonoformate) (Figure 6.3f), an inhibitor of DNA polymerase, is utilized in severe HSV infections refractory to aciclovir therapy. Slow intravenous infusion of the drug is necessary because of its poor oral bioavailability and potential for nephrotoxicity. An alternative is the nucleotide analogue cidofovir (Figure 6.3g). This compound has, like foscarnet, dose-dependent nephrotoxicity and must be given with oral probenecid to reduce cidofovir accumulation in the renal tubules. The drug's extraordinary intracellular half-life means that only one intravenous infusion is required every one to two weeks.

Drug Resistance

Prolonged use of aciclovir can result in the development of drug-resistant strains of HSV. TK mutants have been reported, with either altered substrate specificity (i.e. aciclovir is no longer recognized as a substrate for virus-specified enzyme) or loss of TK activity. These mutants arise through non-sense, frame shift or missense mutations in the TK gene and are thus readily generated both *in vivo* and *in vitro*. A third type of mutant has altered DNA polymerase such that aciclovir triphosphate no longer binds, with high affinity, to the virus-encoded enzyme (altered substrate specificity). The consequence of alteration in TK is a failure of the virus-infected cell to phosphorylate and thereby selectively concentrate aciclovir. Alteration in DNA polymerase results in a much-reduced antiviral activity since the only antiviral action of the drug resides in the ability of aciclovir triphosphate to induce chain termination.

The emergence of drug resistance gives rise to concern but is currently limited to patients who are immunocompromised and on prolonged treatment; about 4% of HSV

isolates from patients with AIDS and up to 18% of isolates from recipients of bone-marrow transplants are resistant to aciclovir. Among immunocompetent individuals a very low rate (<0.5%) of resistance to aciclovir is observed. Most aciclovir-resistant strains of virus are also likely to be found to be resistant to penciclovir, valaciclovir and famciclovir. The majority of drug-resistant isolates have an altered or deleted TK and thus drugs which act directly upon the viral DNA polymerase, such as foscarnet or cidofovir, are effective as second-line treatments. Multiple drug resistance has, however, been documented. Drug susceptibility assays should be performed in immunocompromised patients with unresponsive herpes simplex infection in order to distinguish drug resistance from problems such as poor compliance or malabsorption of drug.

Transmission of drug-resistant HSV seems to date to be rare. This may be related to a requirement of TK for replication of virus within nervous tissue. Resistant virus shed in cold sores will revert back to wild type in subsequent reactivations.

Immunization

Active Immunization

The short time interval between loss of passively-acquired maternal immunity and acquisition of primary infection, together with the high level of HSV infection in the general population, means that effective prevention of HSV-1 infection by vaccination is problematic. However, the changing seroepidemiology of HSV-1 infections suggests that, at least in developed countries, application of a vaccine against HSV-1 is becoming a feasible possibility. Due to the psychosocial problems of HSV-2 infection and the increase in acquisition risk of human immunodeficiency virus (HIV) infection associated with HSV-2 infection, immunization against this virus has been much more actively sought. At the present time, two strategies are being investigated: classical immunization, which aims to produce so-called 'sterilizing immunity' (i.e. a broad and durable immune response which will be effective in preventing HSV entry via genital mucosa, facial mucosa or the eye) prior to virus exposure; and immunotherapy—modification of the immune response by vaccine administration designed to potentiate immunity and improve control of, or prevent or modify, recurrent infection.

Research on the production of effective vaccines against HSV has been actively pursued since the 1920s. A very wide range of vaccines have been explored, both as 'sterilizing' and immunotherapeutic vaccines. The evidence from the many studies performed suggests that cellular rather than humoral immune responses are important in protecting against both infection and

recurrence. Improved knowledge of the immune-evading mechanisms of HSV and viral determinants necessary to stimulate protective cellular immunity appears to be key to the future development of immunization against HSV (Koelle, 2006).

Two HSV-2 glycoprotein adjuvant vaccines (gB2 + gD2 and gD2) have reached phase III clinical evaluation. The overall protection rates were not significant. However, a gD2 vaccine formulated with an adjuvant alum and 3-deacylated monophosphoryl-lipid A showed a 73/74% protection from disease—but not infection—in women seronegative to HSV-1 and HSV-2. Gender differences and pre-existing HSV-1 serological status influence vaccine efficacy in these trials. Whilst the goal of an effective HSV-2 vaccine has not yet been achieved, the studies have helped define some of the complexity of HSV-1 and HSV-2 disease and shown some of the issues that will need to be addressed if an effective vaccine is to be developed (Kemble and Spaete, 2007).

Passive Immunization

Passive immunization using hyperimmune HSV-specific immunoglobulin has not been widely utilized; normal immune globulin is ineffective. In neonatal disease and possibly in immunocompromised patients, trials of therapy using human recombinant monoclonal antibodies are being contemplated as they may have a role in helping to prevent virus dissemination by viraemia.

CLINICAL FEATURES, DIAGNOSIS AND MANAGEMENT

Oropharyngeal and Oro-Facial Infection

Primary Infection

In a symptomatic, primary HSV infection of the oropharynx, gingivostomatitis is the most common symptom. The incubation period ranges from 2 to 12 days with a median of 4 days and the duration of clinical illness is 2–3 weeks. The spectrum of severity ranges from the trivial, involving the buccal and gingival mucosa, to severe, painful ulceration of the mouth, tongue, gingivae and fauces (Figure 6.5). In severe disease, shallow ulcers on an erythematous base evolve from vesicles and often coalesce, particularly on the mucosa of the cheeks and under the tongue. The ulcers are observed on the hard rather than the soft palate—a feature that may help differentiate herpetic ulcerations from those caused by the *Coxsackie* viruses ('herpangina'). In young children the skin around the mouth is frequently involved. Sub-mandibular lymphadenopathy and fever (39–40°C) are



Figure 6.5 Primary oro-facial herpes, severe ulceration of the mouth.

common and may produce febrile convulsions in children. A moderate lymphocytosis (up to 7000 mm^{-3}) and mild neutropenia are frequently observed. Elevated liver enzymes are noted in occasional cases. Acute gingivostomatitis is a self-limiting disease and resolution begins abruptly. The lesions become painless and inflammation subsides. Intraoral ulceration progresses to healing, although crusting of lesions is not usually observed. The patient becomes afebrile and symptoms regress rapidly, although lymphadenopathy may persist for several weeks.

Other symptoms that may be associated with primary infection include sore throat, mild conjunctivitis, nausea and vomiting, myalgia and abdominal discomfort. Dehydration and anorexia may result through the pain and oedema of the mucosal membrane infection, with its associated mouth discomfort and difficulty in swallowing. Older patients may experience a pharyngitis associated with a mononucleosis syndrome (up to 20% atypical lymphocytes) and submandibular lymphadenopathy. The tonsils are also frequently found to be ulcerated during this pharyngitis.

The oral disease can be associated with lesions elsewhere. Herpetic dermatitis, nasal herpes, ocular herpes, herpetic whitlows and even genital herpes are not infrequent complications of primary HSV infection of the mouth. These may represent viraemic spread of virus or endogenous re-infections caused by autoinoculation from the site of primary infection.

Diagnosis Clinical diagnosis is usually straightforward. The major differential diagnoses are herpangina and hand, foot and mouth disease, both usually caused by *Coxsackie* viruses. Oral vesicles in these conditions are found on the posterior part of the oropharynx and not outside the mouth. Demonstration of virus, viral antigen or viral DNA in saliva/lesions easily confirms clinical suspicion. If the

causative role of herpes is in doubt, seroconversion to HSV confirms the diagnosis of primary infection.

Management Primary or initial infection with HSV is, in the majority of cases, inapparent or only associated with mild symptoms. As the aetiological diagnosis may not be clear, antiviral therapy is not given and is usually not warranted. However, in severe cases and where the diagnosis is clear, oral antiviral therapy can be given. There is evidence of shortening of duration of pain and time to healing in children with gingivostomatitis with oral administration of aciclovir (children 15 mg kg^{-1} , max 200 mg; adults 200 mg doses five times daily (or valaciclovir (500 mg twice daily; not used in children) or famciclovir (250 mg, three times daily)). Intravenous therapy is indicated if the patient cannot swallow or is vomiting.

Secondary bacterial and fungal infection is common in severe symptomatic cases and may require appropriate antimicrobial therapy.

Steroid treatment in general increases the severity of disease and where patients are receiving high doses of steroids (e.g. in treatment of eczema) the appearance of HSV infection is an indication for temporary cessation or reduction of steroid dosage.

Recurrent Infection

Recurrent infection, triggered by a variety of apparently nonspecific stimuli (such as fever, stress, cold, menstruation or ultraviolet radiation), appears as a fresh vesicular eruption termed 'herpes labialis', 'herpes febrilis', 'fever blisters' or 'cold sores'. The most frequent site is at the border of the lips but lesions may appear elsewhere, for example on the chin or cheek and on or inside the nose. Their appearance is usually preceded by a prodrome of tingling or itching at the site where recurrence will occur. In the immunocompetent, the area of involvement is usually small and vesicles progress to the pustular and crusting stage within three to four days. The time for complete healing is variable (5–12 days, mean 7.5 days).

Asymptomatic oral shedding of HSV can occur and is occasionally preceded by a prodrome similar to that associated with overt herpes labialis. Recurrent intraoral ulcers are only rarely caused by HSV. In the few instances where reactivation of HSV is responsible, lesions are limited to the gingivae and the hard palate.

Diagnosis Herpes labialis is easy to diagnose clinically. Virus detection is helpful to confirm the herpes aetiology in minor ulcerative lesions, for example when misdiagnosed as sunburn or similar.

Management A number of household remedies, not subjected to controlled studies, are believed to have effect,

illustrating an apparent placebo effect in healing time. Episodic treatment with antivirals as well as frequent local applications of penciclovir 1% or aciclovir 5% cream have a very modest effect and require a very early start during the prodromal phase; that is, if and when there is an itching or slight burning sensation. The effect of antivirals in combination with topical steroids is under investigation.

Prevention No data are available to support the use of oral antiviral medication for the prevention of oral recurrences. As for recurrent genital herpes (see below), suppressive therapy may be used in frequent and severe recurrence (Gilbert *et al.*, 2007).

Genital Infection

Primary Infection

The clinical features of primary HSV-1 or HSV-2 genital infection are indistinguishable and without treatment primary genital disease may last for up to three weeks. Fever, dysuria associated with urethritis and cystitis (with urinary retention in a proportion of cases with or without apparent vesicles), localized inguinal adenopathy and malaise are common. Onset is usually four to seven days after sexual exposure. A prominent feature is pain, which, especially in women, may be severe. In women the lesions are typically bilateral and located principally on the vulva, but the vagina and cervix are almost always involved. The vesicles on the mucocutaneous surfaces soon ulcerate and coalesce and may extend to the perineum, upper thigh and buttocks. In men, vesicular lesions with an erythematous base are observed on the glans of the penis or on the penile shank. Perianal and anal infections producing proctitis are common in homosexual men. Secondary microbial infections often follow primary genital HSV infection and their occurrence necessitates appropriate antimicrobial therapy.

Complications of genital HSV infection include aseptic meningitis and sacral radiculomyelitis with urinary retention and attendant neuralgia; these may occur in the absence of apparent genital lesions. Primary (maternal) genital herpes occurring at or around the time of birth may produce severe neonatal infection (see below).

Diagnosis Detection and typing of HSV in material from fresh lesions or secretions in the genital tract by cultivation, antigen detection, ELISA, indirect immunofluorescence or molecular techniques provides diagnosis. PCR is the most sensitive procedure and allows typing, and may be considered the new gold standard for detection of HSV in genital secretions.

An analysis of the HSV-specific antibody response reveals the nature of the infection. In a primary infection no HSV IgG antibodies are demonstrable in acute

serum. Seroconversion to type-common HSV antigens confirms the diagnosis. In initial infection (prior infection with the heterologous, noninfecting, type of HSV), seroconversion is seen only to the type-specific antigen of the infecting virus type. It is, however, noteworthy that this type-specific antibody response may take up to 8–12 weeks to appear. If antibodies to the infecting virus are already in acute serum, the episode, which clinically may appear to be the first episode, is in fact a recurrence.

Treatment In practice, the large majority of primary/initial infections are undiagnosed (Figure 6.6), and in those that are, the peak of viral replication may already have passed before antiviral therapy can be commenced. If the diagnosis is apparent, as it often but not always is in severe cases, oral antiviral therapy can be given. There is evidence of shortening of duration of pain and time to healing provided patients are treated early—within five days of the start of disease (or later if lesions are still appearing). Aciclovir (200 mg doses, five times daily), valaciclovir (500–1000 mg twice daily), or famciclovir (250 mg, three times daily) are given for five days unless new lesions continue to appear, when extended therapy should be contemplated. In severe cases intravenous antiviral medication may be required, as well as procedures to control the pain and relieve any urinary retention.

Although severe primary or initial infections are associated with a higher frequency of symptomatic reactivation there is no clear evidence to suggest that treatment can alter the natural course of disease.

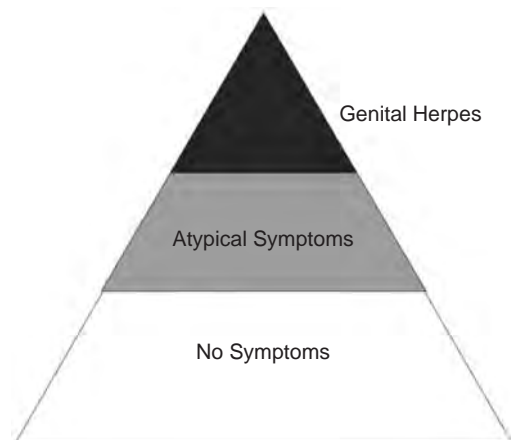


Figure 6.6 The majority of genital herpes infections produce either atypical or no symptoms. Only a minority of cases are recognized.

Prevention Effective preventive strategies are badly needed. Vaccines are not available to provide protection pre-exposure. Present strategies include education of the public to increase awareness about genital herpes; education concerning the substantial protection of consistent condom use in both sexes; education of health-care professionals and patients concerning recognition of vague clinical signs for increased detection; testing of asymptomatic partners; and counselling the seronegative. Abstaining from sex during clinically-overt outbreaks and episodic treatment may be of some protective value, although incomplete, as silent excretion of virus is the major transmission factor. Suppressive therapy of the infected partner (e.g. aciclovir or valaciclovir 500 mg once daily) reduces, but does not eliminate, transmission to the non-infected partner (Corey *et al.*, 2004). Consistent use of condoms significantly reduces transmission of HSV (Wald *et al.*, 2005).

The influence of HSV-2 infection on HIV acquisition and disease has resulted in increased efforts to find strategies appropriate to populations in the developing countries. HSV-2 is the most frequent cause of genital ulcers facilitating HIV transmission (Gupta *et al.*, 2007). Research on topical microbiocides is directed towards HIV prevention as well as to the feasibility of simultaneous prevention of HSV transmission.

Recurrent Infection

In recurrent genital herpes a limited number of localized vesicles are produced, often in unilateral sites anywhere in the anogenital region, the buttocks or the natal cleft (Figure 6.7). Their appearance is usually associated with irritation rather than with significant pain. Prodromes of pain and/or itching may precede the vesicles. New vesicles often appear during the course of a recurrence and delay healing. Complications (neurological or systemic) associated with recurrent infections are less common. Although HSV-1 is increasingly found as a cause of primary genital infection, recurrence of genital HSV-1 is infrequent, averaging about one episode per year in the first year and reducing thereafter (Gupta *et al.*, 2007). Asymptomatic shedding with HSV-1 is of short duration and occurs infrequently. This contrasts with HSV-2 genital herpes, where calculated rates of recurrence reach three to nine episodes per year, with frequent silent shedding in between (Gupta *et al.*, 2007).

Overall about 60% of patients known to have had primary infection will report recurrence, although many HSV-2 seropositives are not aware of their infection. Triggering factors for recurrence are nonspecific and may be menstruation, sexual intercourse or psychological stress, but in most instances the triggering factor is not known.



Figure 6.7 Genital herpes.

Diagnosis As for diagnosis of primary infection, the detection of virus or viral DNA from fresh vesicles provides the mainstay of diagnosis. Atypical recurrences are often misinterpreted by patients as well as by physicians; the recurrent infection may mimic a number of other conditions or appear as very discrete skin fissures or erosions. Demonstration of type-specific antibody to the infecting virus type confirms the recurrent nature of the episode.

In the absence of symptoms the finding of HSV-2-specific antibody is indicative of prior genital HSV-2 infection; in contrast recurrent genital HSV-1 can only be confirmed by virus detection.

Management Recurrent HSV infections inconvenience the host but are rarely serious in the immunocompetent individual. Frequent or severe recurrences may require oral or systemic antiviral chemotherapy. The most effective episodic treatment requires (self-) initiation of antiviral therapy as soon as prodromal symptoms are noticed or the debut of erythema/vesicles is observed. This reduces the severity and duration of the recurrent episode (by on

average two days). A five-day course of aciclovir, valaciclovir or famciclovir has been considered optimal. Data on the comparative efficacy of the drugs used in this way are not available. Recently, the same healing rates and reduced duration have been found with aciclovir (three day), aciclovir (two day) and famciclovir (one to two day). These short-term courses are well tolerated, with the added advantage of being more convenient and economical than standard regimens (Corey *et al.*, 2007).

Prevention Since the maintenance of latent virus infection appears to be independent of virus replication, present antiviral chemotherapy cannot ‘cure’ recurrent virus infection. Patients with frequent or severe recurrences (defined as more than six episodes per year) should be considered for continuous suppressive therapy. The decision has to be made on an individual basis. The optimal dose of aciclovir is 200 mg four times daily or 400 mg twice daily; of valaciclovir 500 mg daily or 250 mg twice daily; of famciclovir 250 mg twice daily. The treatments have been found to reduce (to about 20%) but not eliminate recurrent episodes. Silent episodes of virus excretion also occur. As the recurrence rate and severity may decrease with time after the acquisition of HSV-2, a six- to twelve-month period is usually chosen for continuous therapy and the necessity for therapy is then reassessed over a period of at least two episodes.

Herpesvirus Infections of the Skin

Herpes Simplex Dermatitis

Herpetic dermatitis is a complication of primary infection. Perioral or periorbital herpes simplex regularly accompanies more severe primary gingivostomatitis or primary/initial herpes keratitis. In babies, seeding of the virus can involve large areas of the face, and when transferred to the anogenital area by scratching may involve the whole napkin area. In primary HSV vulvovaginitis, vesicles readily appear on the perineum and thigh.

A distinct form of cutaneous infection ‘zosteriform herpes simplex’ is an infrequent presentation of herpes simplex, but is recognized when the distribution of HSV lesions accords with a dermatome and otherwise resembles zoster. Unlike zoster, recurrences are common and nerve root pain is not a feature.

Eczema Herpeticum, Thermal Burns and Other Conditions

In patients with atopic eczema the normal resistance of the skin to HSV infection is reduced. Primary, recurrent and possibly both endogenous and exogenous re-infection may all produce eczema herpeticum. Eczema herpeticum

as a primary infection carries a small but significant mortality through progression to severe generalized disease. In thermal burns, mycosis fungoides, pemphigus, or with follicular dermatosis (Darier’s disease) and other conditions where the defence of the skin is impaired, severe progression may also be seen.

Herpes Gladiatorum

Herpes gladiatorum, ‘scrum pox’ and ‘mat burns’ are conditions spread among wrestlers and rugby players through bites and facial scraping (Turbeville *et al.*, 2006). The appearance of herpetic vesicles at ‘unusual’ sites can sometimes be explained by an inquiry into the individual’s athletic pursuits!

Herpetic Whitlows; Traumatic Herpes

Under normal circumstances HSV does not readily penetrate healthy skin, but when the dermis is breached a portal of entry is created. Health-care personnel are at particular risk of perforation injuries. Direct inoculation of virus into the fingers of those who constantly manipulate in the oral cavity (dentists, dental nurses, intensive-care doctors, nurses and anaesthetists) may lead to herpetic whitlows, an inflammation of the nail folds (Figure 6.8). This treatable/preventable condition, which is often associated with painful recurrences, is frequently misdiagnosed as bacterial infection, especially if the clinical presentation is devoid of vesicles. A similar condition is seen in children and adolescents, who transfer their own oral virus through nail biting. In other situations, workers such as hairdressers, dressmakers and laboratory personnel can be infected by accidental stab injuries with contaminated needles or broken glassware (traumatic herpes).



Figure 6.8 Paronychia; ‘herpetic whitlow’.

Surgically-induced Reactivation

HSV-1 seropositive individuals undergoing surgery in the trigeminal nerve area (e.g. trigeminal nerve decompression, oral surgery or extensive facial cosmetic procedures) can suffer outbreaks of herpes, which may occur anywhere on the face, as a result of reactivation from the sensory branch of the nerve.

Reactivation of HSV-1 has been implicated in the pathogenesis of idiopathic peripheral facial palsy (Bell's palsy) (see Furuta, 2006 for a review). In a study from Japan (Murakami *et al.*, 1996) HSV-1 DNA was demonstrated in endoneural fluid from the facial nerve of 79% of facial palsy patients and also in 57% of tissues from the posterior auricular muscle innervated by the facial nerve. In a Scandinavian study (Stjernquist-Desatnik *et al.*, 2006) of 20 milder cases of idiopathic facial palsy, HSV DNA was, however, found only in 1 posterior auricular muscle biopsy.

Diagnosis Demonstration of virus/viral DNA in lesions easily confirms the clinical picture of cutaneous manifestations. The definition of a herpes aetiology in facial palsy is more difficult; serology is not useful (except in demonstration of seropositivity to herpes) and the demonstration of virus in, for example, saliva is not of diagnostic value. HSV DNA is not found in CSF (Kanerva *et al.*, 2007; Stjernquist-Desatnik *et al.*, 2006).

Management and Prevention Whilst many of these conditions are self-limiting, intravenous or oral antiviral therapy must be instituted as early as possible in vulnerable patients at risk of severe HSV (e.g. eczema herpeticum and/or similar conditions associated with impaired skin resistance). Steroid treatment in general increases the severity of disease and where patients are receiving high doses of steroids (e.g. in treatment of eczema) the appearance of HSV infection is an indication for temporary cessation or reduction of steroid dosage. Secondary bacterial and fungal infection is common in severe symptomatic primary and initial HSV infection and requires appropriate antimicrobial therapy.

Education on HSV and instruction in basic hygiene should be given to persons in close contact with individuals with known risk of severe herpes infection and also to risk groups such as wrestlers. In the health-care setting, the risk of herpetic whitlow is reduced by use of gloves and universal precautions to prevent penetrating injuries and contact with secretions. Preventative antiviral medication may be indicated before surgical intervention in the trigeminal nerve region and episodic or suppressive therapy may be required in some cases of frequent and painful recurrence of herpetic whitlow.

Prevention of facial palsy is not possible. Whereas there has been consensus on treatment with corticosteroids, the role of antiviral therapy has been less clear. Benefits of treatment with valciclovir plus prednisolone versus placebo have been demonstrated, but the role of valciclovir has been under debate and multicentre studies are ongoing. In a recent Japanese report (Hato *et al.*, 2007) a significantly better treatment effect in patients with facial palsy (excluding that caused by zoster *sine herpette*) was found with valciclovir (1000 mg day⁻¹ for five days) in combination with prednisolone than with prednisolone alone. Treatment was started within seven days. Recovery of complete or severe palsy was seen in 95.7% ($n = 92$) versus 86.6% ($n = 82$) and the recovery rate was more rapid in the valciclovir group.

Erythema Multiforme

Erythema multiforme is regarded as a hypersensitivity phenomenon precipitated by a variety of infectious agents, immunizations or drugs. The clinical lesions are characteristic: acraly distributed erythematous papules which evolve into concentric 'target' lesions; annular plaques and bullae may also be produced. Although usually mild and self-limiting the disease can be recurrent and progress to toxic epidermal necrolysis or to severe mucous membrane involvement (Stevens-Johnson syndrome). *Mycoplasma pneumoniae* and HSV are the infectious agents most commonly associated as precipitants. Up to 65% of patients with recurrent erythema multiforme give a preceding history of herpes labialis, on average 11 days before the lesions of the disease appear. Infectious virus cannot be cultured from the lesions but IF studies have shown HSV glycoprotein B located around keratinocytes in the viable epidermis, PCR studies have detected HSV DNA in cutaneous lesions and *in situ* hybridization has been used to demonstrate HSV nucleic acids within the epidermis.

Management and Prevention As HSV replication is apparently a precipitant of the disease but not a direct cause of the pathology, erythema multiforme does not respond directly to antiviral chemotherapy. However, cases of recurrent HSV-associated erythema multiforme can be prevented by prophylactic use of antivirals. Some cases of idiopathic-recurrent erythema multiforme (i.e. where antecedent herpes labialis is not suspected) also respond to prophylactic use of antivirals. This is perhaps consistent with a preceding 'silent' recurrence of HSV.

Ocular Infection

The early symptoms of herpes keratitis are of a unilateral or bilateral conjunctivitis, with pre-auricular lymphadenopathy. The most common first presentation is,

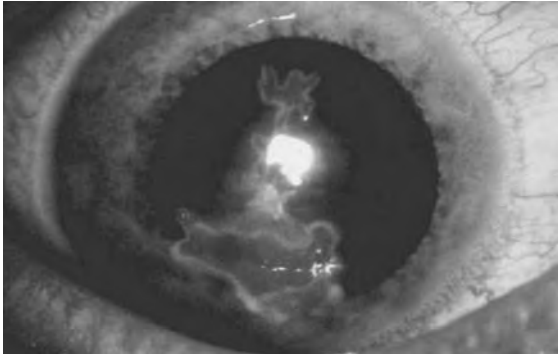


Figure 6.9 Herpes simplex keratoconjunctivitis.

however, of a unilateral, characteristic, dendritic (branching) ulcer (Figure 6.9). The normal course of the disease is three weeks but if ulcers are large, healing can be slow. Mild systemic disturbances, blepharitis and circumocular herpetic dermatitis are commonly present during the primary infection. Infection may occur as a part of a primary infection or by autoinoculation from the site of a recurrent infection. Most cases are due to HSV-1.

New HSV infections of the eye are estimated to occur at a rate of 8.4 per 100 000 person years in the United States (Mayo Clinic data) and the overall incidence of new and recurrent ocular HSV is calculated to be 20.7 per 100 000 person years. Lower rates are reported from Europe, with a rate of 4 per 100 000 population in Croatia and 6–12 per 100 000 population in Denmark (for references see Liesegang, 2006).

A single recurrence of ophthalmic infection is observed in between 20 and 30% of patients within two years of the first infection. Subsequent recurrences are less common but if a second recurrence does occur then further recurrences will be observed in 40–45% of these cases. There are several forms of recurrent ophthalmic infection and these may occur in combination. Dendritic or larger ‘geographic’ ulcers are usually the first manifestation of recurrence, with the patient complaining of ocular irritation, lacrimation, photophobia and sometimes blurring of vision. Infection is usually confined to the superficial layers of the cornea and stromal involvement is absent or only relatively mild.

In some cases days or weeks after the recurrent infection a new disease manifestation may appear where the corneal epithelium ulcerates, to form a nondescript ovoid ulcer known as the post-infectious or metaherpetic keratitis. Virus replication does not appear to be directly responsible for the production of this ulcer. If the ulceration is prolonged (weeks and months) collagenolytic activity may appear, leading to stromal melting and perforation.

Repeated and severe epithelial disease recurrence or chronic epithelial keratitis leads to involvement of the deeper layers of the cornea, producing interstitial or disciform keratitis. Neovascularization and scarring may ultimately lead to loss of vision. In developed countries ocular HSV disease is a major cause of corneal blindness (Liesegang, 2006).

The observation of recurrent infection of the cornea raises questions as to extraneuronal sites of latency of HSV within cornea. Using PCR, HSV DNA has been demonstrated within corneal cells of both normal and diseased corneal tissues obtained at keratoplasty. The incidence of HSV epithelial keratitis is six times higher in patients who have undergone corneal transplantation for nonherpetic corneal disease than in nontransplanted individuals. Moreover, infectious virus has been isolated using cocultivation techniques from corneas obtained from patients with stromal herpes keratitis. However, these observations do not prove that corneal latency of HSV occurs, as it remains difficult to distinguish this from low-grade virus persistence and low-level subclinical reactivation within the trigeminal ganglion.

The influence of the immune response in control of ocular infection is an additional area of interest in the pathogenesis of ocular HSV infection. Whereas superficial corneal epithelial disease is primarily a viral infection, recurrent disease keratitis and the other forms of ocular HSV are more complex, with viral infection, immune and inflammatory responses combining to attack ocular structures. Because of the unique anatomy and physiology of the eye, the local immune response to infection may differ from that occurring at, for example, cutaneous sites. The lack of, and/or relative overproduction of, individual components of the immune response undoubtedly influences disease pathogenesis at this site. Whether viral-strain specificity and genetic susceptibility, as has been demonstrated in animal experimentation, is of importance in human ocular disease is not yet clear.

Recurrent iridocyclitis and occasionally panuveitis may be caused by HSV. Iridocyclitis, produced by intraocular inflammation, is often observed in association with active herpes keratitis. It may be due to direct involvement of the virus or may be secondary to the irritative effects of keratitis. Also, structures behind the lens may be involved. Acute retinal necrosis is a rare, severe ocular inflammatory syndrome which if untreated is associated with a poor visual prognosis. The condition is caused by infection with VZV, HSV-1 (in older patients) or HSV-2 (in younger patients). An association with a history of prior or concomitant HSV encephalitis and meningitis has been reported (Ganatra *et al.*, 2000; Lau *et al.*, 2007). The presentation is usually unilateral but may progress to involve the fellow eye even after extended periods of time.

Ophthalmic disease, associated with intrauterine and neonatal infection with HSV, can present as keratoconjunctivitis or later as chorioretinitis. However, cataract, corneal ulceration, anterior uveitis, vitritis, optic atrophy, nystagmus, strabismus, microphthalmia and retinal dysplasia have all been reported in association with such infection.

Diagnosis HSV keratitis can usually be recognized by its appearance in the ophthalmoscope (Figure 6.9), and specific laboratory tests are as a rule not performed. However, in doubtful cases specific tests are highly recommended. Demonstration of HSV DNA by PCR is more sensitive than virus isolation or antigen detection. Intraocular HSV disease is demonstrable by the detection of HSV DNA in anterior chamber or vitreous fluids and/or locally produced antibody production (Ganatra *et al.*, 2000; Lau *et al.*, 2007). Simultaneously-taken serum is required for parallel analyses, including albumin or total IgG, to demonstrate intraorbital antibody production. HSV DNA and/or herpes-specific antibody production may be found in CSF (El Azazi *et al.*, 1991).

Management In conjunctivitis and corneal epithelial disease conservative treatment is warranted with application of specific antiviral chemotherapy (aciclovir ophthalmic ointment) and possibly debridement of the cornea (Wilhelmus, 2007). Misdiagnosis of the condition has frequently led to the administration of corticosteroids to the eye. As the innate immune response limits the spread of virus and hastens resolution, such treatment may exacerbate and prolong the infection. Steroids should not be given. Oral or systemic antiviral therapy should be given in severe disease. Secondary bacterial infection may occur, necessitating appropriate antimicrobial therapy.

Recurrent ocular HSV infections are managed in a similar fashion, except that the indication for antiviral chemotherapy is more pronounced.

In stromal herpes keratitis, and possibly also in iridocyclitis, steroid therapy is almost certainly indicated, since most damage is believed to result from an inflammatory reaction rather than the direct action of virus replication in corneal tissues. Severe stromal scarring necessitates corneal transplantation.

Prompt antiviral therapy should be given in posterior ocular infections, along with preventive and reparative measures for retinal detachment (Lau *et al.*, 2007).

Prevention As ocular HSV infection may result from an endogenous re-infection (i.e. transfer of virus from the site of a recurrent infection to the eye), education on the need for hand hygiene when overt recurrent herpes lesions are present may be of value in prevention of transmission.

Long-term suppressive oral aciclovir therapy (400 mg twice daily) has been shown to be of benefit in patients with prior HSV stromal keratitis (Herpetic Eye Study Group, 2000) but of lower benefit in preventing recurrence of epithelial keratitis. Antiviral prophylaxis should follow corneal surgery in patients with a history of ocular HSV. Suppressant antiviral therapy is warranted for patients with acute retinal necrosis infection of one eye, to prevent progression of the disease to involve the fellow eye.

Herpes Simplex Encephalitis

The most well-known presentation of herpes simplex encephalitis (HSE) is of a focal encephalopathic process with signs and symptoms that localize to the fronto-temporal and parietal areas of the brain. As the disease progresses, symptoms increase in severity and there is progressive decrease in consciousness, leading in severe cases to coma and death. Herpes encephalitis occurs in all age groups, with one half of cases occurring in individuals older than 50 years and one third in those of less than 20 years of age. The same frequency is found in both men and women; among children there is a slight preponderance of boys affected by the condition (De Tiège *et al.*, 2008). There is no seasonal variation in incidence and cases occur sporadically. An estimate of the nationwide incidence 1990–2001 in Sweden was 2.2 cases of HSV-1 per million population per year; there was a median age of 62 years in male patients and 66 in female patients (Hjalmarsson *et al.*, 2007). Most cases are caused by HSV-1, with HSV-2 causing between 2 and 6.5% of cases. In about two thirds of cases the illness appears to be due to reactivation of the patient's own virus or to re-infection (Studahl and Sköldenberg, 2006; Whitley, 2006). In paediatric HSE patients, especially in infants, HSE is more often (but not always) the consequence of primary HSV infection (De Tiège *et al.*, 2008). In the mildly-to-moderately immunocompromised, 'classical' herpes encephalitis appears to occur at the same rate as in the immunocompetent, although in AIDS patients an atypical, subacute encephalitis associated with either HSV-1 or 2 has been identified in 2% of cases studied at autopsy, usually in association with a concurrent CMV infection of the CNS (Cinque *et al.*, 1998).

The onset of disease may be either abrupt, with altered consciousness and focal neurologic symptoms, or insidious, especially in children. Early signs and symptoms of the infection may not be distinctive. In about half of patients there is a history of a 'flu-like' illness occurring less than a week before the appearance of neurological symptoms. In children seizures are more common than in adults.

Electroencephalographic (EEG) examination is a relatively sensitive indicator of disease process but is of

low aetiologic specificity. EEG will usually reveal focal or diffuse nonspecific slow-wave activity during the first five to seven days of illness. Later, more characteristic paroxysmal sharp waves or triphasic complexes with a temporal predominance may be found. Low-density lesions are demonstrable by X-ray computed axial tomography (CT) within five days of the onset of neurological illness in about 70% of cases. Single photon emission computed tomography (SPECT) may demonstrate hyperperfusion at an early stage in the temporal lobes in HSE, a feature not observed in other encephalitides. Proton magnetic resonance imaging (MRI) has the potential to detect abnormalities that may not be revealed by routine CT. MRI may show fronto-basal and temporal lesions as hypointense lesions on T1-weighted images and hyperintense lesions on proton-density- and T2-weighted images at an earlier stage than changes can be detected by CT. In children, involvement of particularly the parietal lobe and opercular region is seen; thalamic lesions are also much more common than in adults (De Tiège *et al.*, 2008).

The histopathological changes associated with severe HSE consist of acute inflammation that evolves to produce haemorrhagic and necrotising lesions. The lesions are characteristically located in the temporal lobes and the orbital surface of the frontal lobes, but adjacent frontal, parietal and occipital lobes and the cingulate gyri may also be involved. In children the cortical and subcortical regions may be affected. Clinically, herpes encephalitis ranges in severity from a mild encephalitis of low mortality and morbidity to severe necrotizing encephalitis. In the period before specific antiviral chemotherapy and expert neurological care were available, HSE was associated with both high mortality (70–90%) and high morbidity (with <10% of survivors returning to normal neurological function). Prompt initiation of chemotherapy using the antiviral drug aciclovir, combined with intensive neurological nursing care, has resulted in a reduction of mortality to less than 20%, with up to 40% of survivors returning to apparently normal neurological function. However, there may be residual neurological deficit in a considerable proportion. This may be severe, with impairment or complete loss of short-term memory being the most commonly observed sequelae.

In contrast to several other enveloped viruses (including varicella-zoster, Epstein–Barr virus (EBV) and CMV), HSV does not seem to be a significant cause of perivascular leukoencephalopathy ('post-infectious encephalitis'). However, a similar picture has been reported to occur in relapse in patients recovering from acute herpes encephalitis. Five days to many years after apparent recovery from acute encephalitis the patient suddenly develops a 'relapse' and a further bout of encephalitic illness. In some cases HSV may again be demonstrated in CSF,

especially in children, but in other cases a secondary immune-mediated process is more likely as viral DNA is not detected in the CSF or brain biopsy when performed. Most patients survive but neurological deterioration is seen in children (De Tiège *et al.*, 2008), whereas adults may recover to at least their level of disability following acute herpes encephalitis (Sköldenberg *et al.*, 2006; Whitley, 2006).

The pathogenesis of herpes encephalitis remains incompletely understood. The structure of the vasculature within the brain together with the meninges surrounding the brain represents, in the physiologically-normal host, a significant barrier to virus entry to brain parenchyma. Haematogenous spread of virus to the brain is usually prevented by these blood–brain and blood–CSF barriers. To gain access to the brain the virus must circumvent the physiological/anatomic barriers. It may achieve this by transiting from the periphery within nerve cells. Another possibility is reactivation of virus within the brain, as HSV genomes—but not infectious virus—have been demonstrated in normal brain (medulla, pons and olfactory bulb) from apparently normal individuals (Baringer and Pisani, 1994). Various pathways have been proposed, although in all probability no one route explains all cases of herpes encephalitis.

During the early stages, infection is principally of neurones, with only occasional involvement of astrocyte and glial cells. The frontal and temporal lobes, together with the associated limbic structures of the brain, appear to be the primary targets of the infectious process (Esiri, 1982) and this has led to the suggestion that this localization reflects the particular neurochemical and perhaps local neuroimmunological environment of the limbic region (Damasio and van Hoesen, 1985). However, such localization is not observed in all cases and virus may spread to involve other regions of the brain. Host immunity plays an essential role, and genetic susceptibility due to monogenic mutation resulting in impairment of the production of α/β and λ interferon was recently described in children (Sancho-Shimizu *et al.*, 2007).

Diagnosis In retrospective studies conducted using stored samples of CSF from proven cases of herpes encephalitis, with samples taken during the acute stages of illness (less than 10 days after onset of neurological illness), PCR was found to have a sensitivity of between 95 and 100% and a similarly high specificity of between 98 and 100% (Studahl and Sköldenberg 2006, Whitley 2006). In extended prospective studies it is recognized that PCR is not infallible, and especially in children (De Tiège *et al.*, 2003) negative results may be obtained with samples taken early in the disease course (days 1–3 after onset of neurological illness) or where aciclovir therapy

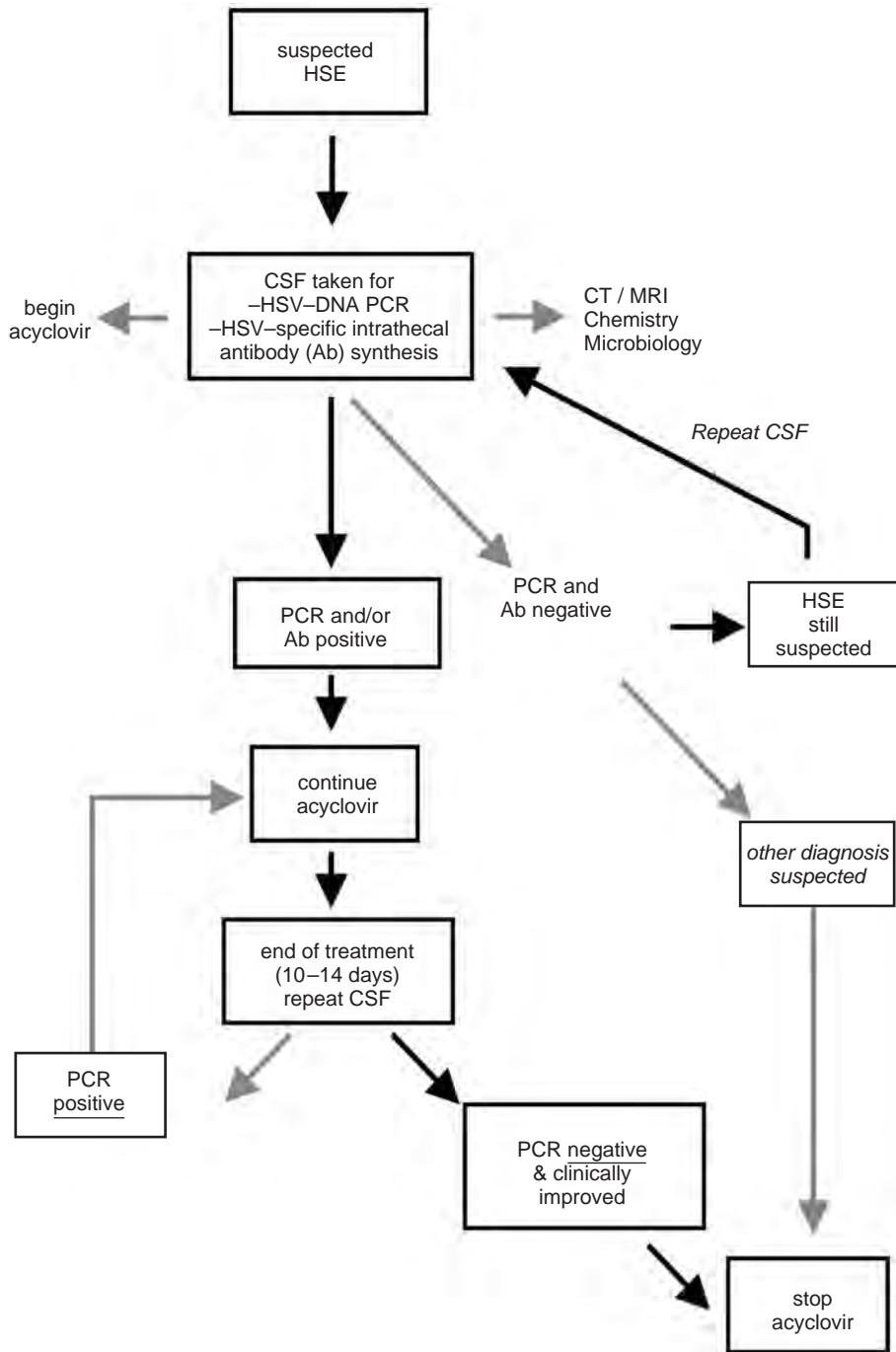


Figure 6.10 Diagnostic algorithm for the management of patients with suspected HSE. (Reproduced, with permission, from Cinque *et al.*, 1996) As patients may present with raised intracranial pressure, immediate lumbar puncture may not be possible. In these circumstances commencement of aciclovir therapy prior to lumbar puncture is warranted.

has been instituted very early in acute infection. Caution is therefore warranted in the interpretation of results, and an algorithm (Cinque *et al.*, 1996; reproduced as Figure 6.10) emphasizes the necessity of repeating the lumbar puncture wherever negative test results are obtained and the diagnosis is still in doubt. It has been reported that initial CSF viral load has prognostic value (Domingues *et al.*, 1998), a finding not confirmed by all investigators (De Tiège *et al.*, 2008; Hjalmarsson *et al.*, personal communication, 2007; Muñoz-Almagro *et al.*, 2008). Quantification is, however, beneficial for follow-up of treatment effect in the individual patient.

The consensus algorithm (Cinque *et al.*, 1996) further mentions the necessity of repeating the lumbar puncture at the end of therapy to ensure full clearance of viral nucleic acid from the CSF. It is important to emphasize that antiviral chemotherapy and/or an intrathecal immune response may clear viral DNA from CSF, and the reliability of PCR in diagnosis of herpes encephalitis declines rapidly in the second week of diagnosis (Figure 6.11). PCR is thus a valuable method during the acute stages of illness but its usefulness reduces 10–12 days after onset, when measurement of specific intrathecal antibody responsiveness is a more appropriate diagnostic procedure, which also—in the majority of cases—allows retrospective diagnosis years to decades later (Aurelius *et al.*, 1991). At the same time, it is a

means of quality control of the routine performance of PCR and antibody techniques. In primary infections, seroconversion and an HSV-specific IgM response are demonstrable in serum/CSF.

Treatment Treatment of herpes encephalitis requires the prompt administration of antiviral chemotherapy. Initiation of treatment should not await firm diagnosis as herpes viral DNA may not be present in the first CSF sample (Cinque *et al.*, 1996; Studahl and Sköldenberg, 2006; Whitley, 2006). Previous trials of antiviral treatment for herpes encephalitis used a 10-day intravenous course of aciclovir at 10 mg kg⁻¹ given every 8 hours. However, due to the high rate of neurologic sequelae and relapses, treatment with 10–15 mg every 8 hours for 14–21 days, and in neonates 15–20 mg every 8 hours for 21 days, are recommended and used in clinical practice (De Tiège *et al.*, 2008; Studahl and Sköldenberg, 2006). The value of at least a further three months of suppression with oral valciclovir is being addressed in ongoing trials. Aciclovir therapy should be approached with caution in patients with impaired renal function, and the dose of aciclovir should be adjusted appropriately since build-up of excessive serum concentrations of aciclovir has been associated with (reversible) neurotoxicity.

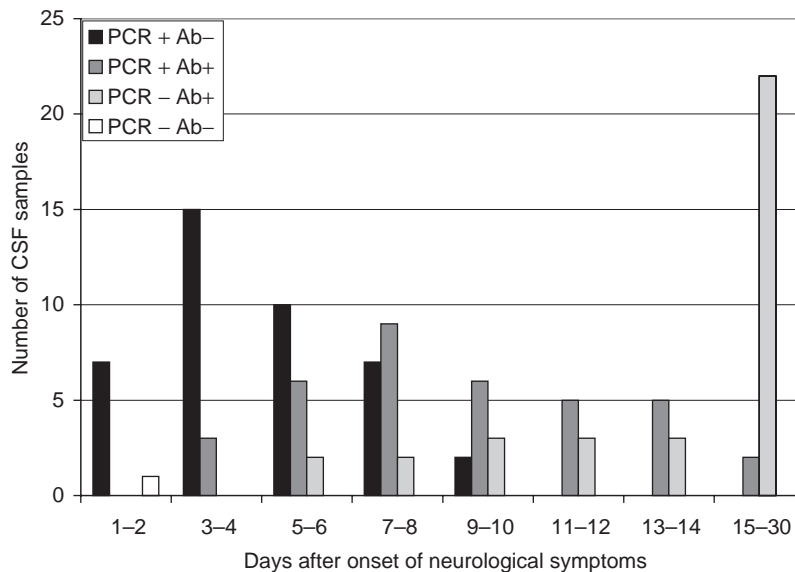


Figure 6.11 HSV-DNA and HSV-specific intrathecal antibody production in relation to time after onset of neurological symptoms in CSF samples from 43 consecutive patients with confirmed herpes simplex encephalitis. None of 36 additional CSF samples taken at 30 days or more of illness were HSV-DNA positive; all 36 had intrathecal antibody production. For details see Aurelius *et al.*, 1991.

Specific antiviral chemotherapy is not the only important factor in treatment of CNS infection. Careful management in close collaboration with several clinical disciplines and the clinical virology laboratory is essential (Cinque *et al.*, 1996; Studahl and Sköldenberg, 2006). Brain oedema is believed to represent the major cause of mortality in herpes encephalitis, hence reduction in intracranial pressure is an important consideration in the overall treatment regime. There is certainly a need for a better understanding of the different pathophysiology and immunological effects of HSV infections in the brains of children and adults, to form more adequate therapeutic strategies in HSE.

Herpes Simplex Meningitis and Other Neurological Disease Caused by Herpes Simplex Virus Type 2

Symptoms of herpes meningitis include headache, stiff neck, vomiting and photophobia and are largely the same as in aseptic meningitis caused by other viruses. Fever is a frequent but not obligatory finding. Mild, mainly monocytic, pleocytosis is found in the CSF. The meningitis often resolves without complications within two to seven days. However, not infrequently, symptoms are protracted, with neurasthenic symptoms persisting for several weeks (Aurelius, 2006). Neurological symptoms due to sacral myeloradiculitis, such as urinary retention, radiating pain in the lumbosacral area, are recorded in about half of the cases of herpes meningitis. Herpes simplex meningitis is a complication of primary genital infection and occurs whether apparent mucocutaneous symptoms are present or not, or have preceded the meningitis by one to two weeks. Herpes meningitis often follows subclinical acquisition of genital herpes HSV-2. Apparent genital or lumbosacral lesions may occur later. Although occasional cases of HSV-1 are reported in association with primary genital HSV-1 infection the increase of genital HSV-1 primary infections has not been accompanied by higher incidence of HSV-1 meningitis.

The prognosis of HSV meningitis is generally good, although in about 20–30% of cases 1, 2 or up to more than 20 new episodes of meningitis may occur after an interval of months to years or even decades—often without mucocutaneous signs of herpes. Improvement in diagnosis through the use of PCR has shown that HSV-2 is the major cause of benign recurrent meningitis, including most cases of so-called Mollaret's meningitis (benign recurrent aseptic meningitis of previously unknown aetiology characterized by 3–10 episodes of fever and meningeal irritation occurring over a period of years). In addition to this well-recognized complication of HSV meningitis, it is becoming clear that HSV-2 may cause a wider

spectrum of neurologic disease (Aurelius, 2006). In patients presenting with episodes of acute headache and malaise interfering with everyday life but without clear-cut symptoms of meningitis, an HSV-2 aetiology should be kept in mind. Urinary retention and other signs of sacral radiculomyopathy or neuritis may occur without obvious signs of genital herpes, and in rare cases HSV-2 has been found as the aetiology to serious or even fatal ascending myelitis or transverse myelitis. Classical HSE localized to the brainstem may also be caused by HSV-2.

The age of acquisition and geographical distribution of herpes meningitis follow those of primary genital infection, with a peak incidence in early adulthood. Herpes meningitis is more common in women than in men. Risk factors other than female gender and a lack of prior HSV-1 antibodies have not been identified. Meningitis has been calculated to occur in at least 4–8% of cases of primary genital herpes; in United States studies this translates into about 20–30% of patients seeking medical assistance for primary genital infection. A study from Denmark suggests an annual crude incidence rate of HSV-2 CNS disease of 0.26 per 100 000 population, of whom 88% have meningitis, 19% of which results in recurring lymphocytic meningitis (Omland *et al.*, 2008). The proportion of aseptic meningitis caused by HSV-2 is dependent on the local epidemiology of both HSV types as well as other causative agents. In a recent Swedish study an HSV-2 aetiology was confirmed in 16% and suspected in another 3% of aseptic meningitis (Franzen-Röhl *et al.*, 2007) in an area with a young adult seropositivity of 20–30% seropositive to HSV-2 and about 70% to HSV-1.

Diagnosis Virus isolation from CSF has a low sensitivity for diagnosis of HSV meningitis. HSV-DNA PCR analysis (of well-controlled sensitivity) of CSF sampled during the first week of illness has a high diagnostic sensitivity (87%) in primary herpes meningitis and a somewhat lower sensitivity (75%) in recurrent meningitis (due to the lower viral load; Franzen-Röhl *et al.*, 2007). Recovery of HSV-2 from peripheral lesions preceding or at the same time as an attack of meningitis may support an HSV-2 diagnosis, although caution is warranted as the peripheral appearance may merely indicate concomitant reactivation of HSV and be of no causative relevance.

Demonstration of seroconversion of HSV-2-specific activity may add diagnostic evidence. Intrathecal antibody production has not been studied to the same extent as in encephalitis. Immunoblotting of oligoclonal bands has been found to be of value in the diagnosis of recurrent meningitis and may motivate repeat lumbar puncture in the diagnosis of HSV-2-seropositive but HSV-DNA-negative cases where the aetiology of the meningitis remains unclear.

Whilst neuroradiological imaging is not often helpful in cases of meningitis, MRI is an important procedure in the diagnosis of serious cases of myelitis.

Management In immunocompetent individuals HSV-2 meningitis is a self-limiting disease. The role of antiviral therapy is less well-defined than in genital infection and controlled studies are in progress. Until more data are available intravenous antiviral therapy should be promptly instituted in a confirmed or suspected case of meningitis with serious neurological complications, such as myelitis and/or encephalitis. Antiviral medication may be justified in cases of meningitis where symptoms are still progressing, in order to shorten the period of convalescence. In patients with nausea and vomiting intravenous aciclovir will have to be given until oral medication can be tolerated. Valaciclovir 1000 mg tid has been found to yield the CSF concentrations required for HSV treatment. In infrequent attacks of recurrent meningitis, prompt episodic treatment may be beneficial.

Prevention Prevention of genital herpes is the major means of preventing meningitis. The value of short- or long-term antiviral therapy to prevent an attack of meningitis after first-episode genital herpes is presently not known. In patients with frequent recurrences of herpes meningitis, long-term prophylaxis with aciclovir, valaciclovir or famciclovir in a dosage at a minimum level to prevent clinical breakthrough is beneficial. The medication should be stopped at intervals to test if the need still persists. In cases of debilitating recurrent attacks of neurologic symptoms of suspected HSV-2 origin (HSV-2 antibodies demonstrable but no firm aetiological diagnosis) a trial of the effect of HSV antiviral treatment or prophylaxis may be worthwhile.

Neonatal Herpes

A child with neonatal herpes may be born with overt disease due to primary maternal infection in pregnancy, or may be infected through premature rupture of membranes and ascending infection from an infected birth canal. Most commonly, infection is acquired during passage through an infected birth canal, causing disease presenting at age three days up to six weeks. Primary oral herpes in the mother or ongoing herpes among relatives or hospital staff in close contact may also infect a baby not protected by maternal antibody.

HSV-1 and HSV-2 can both cause devastating neonatal infection, with symptoms ranging from the very severe to the relatively mild. Even mild disease can, however, be a cause of significant residual morbidity. Three major presentations are seen: lesions of the skin, eye and mouth (SEM, evident age 7–12 days); neurological symptoms

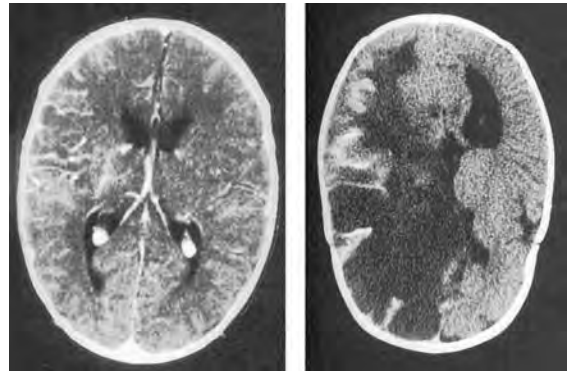


Figure 6.12 Neonatal herpes. Left picture: CT of the brain 8 days after first neurologic symptoms (seizures) in a 20-day-old girl. Increased attenuation is seen in the whole of the left hemisphere. Right picture: 3.5 months of age, widespread destruction of brain parenchyma.

with/without SEM (appearing by age 2–6 weeks); and disseminated disease with/without vesicles, often with involvement of the brain (apparent age 4–11 days). There is an overlap between the types, for example the CNS may be involved in presentations seemingly limited to skin manifestations or may become disseminated. The disseminated disease carries the highest mortality, sometimes presenting as a picture of fulminant liver failure or as multiorgan failure. If untreated, mortality in disseminated disease exceeds 80%, and is >50% in those with CNS symptoms alone. Severe neurological and ocular morbidity is seen in survivors of CNS disease (Figure 6.12). Babies exhibiting only SEM may suffer permanent ocular damage and/or sign(s) of neurological impairment may appear later.

The incidence of neonatal herpes varies with the epidemiology of HSV and may differ in subpopulations from the same area. The magnitude of the problem is reflected in reports of extremes of 1 case in 2500 live births (Alabama, USA) to 1.65 per 100 000 live births (United Kingdom) but the incidence is not known in many areas of the world. Genital herpes and neonatal herpes have in many populations been predominantly caused by HSV-2. In the largest US systematic study of neonatal herpes (over a 17 year period to 1999; Brown *et al.*, 2003), 10/18 were due to HSV-2. In Sweden (Stockholm area; Forsgren and Malm, unpublished data) 29/36 cases were due to HSV-2 over a 23 year period to 1996. However, in Japan and parts of Europe, and associated with a shift in the cause of primary genital herpes to HSV-1, 70% or more of neonatal cases have been reported to be caused by HSV-1. The frequency of neonatal herpes—as reported from the Stockholm area and the Netherlands (van Everdingen *et al.*,

1993)—did not increase over an observation period of 17 years when rates of genital herpes simplex infection were increasing. Under-reporting of cases was thought an unlikely explanation as clinical awareness has increased and the diagnostic tools improved over the period. Factors such as improving hygiene standards, changing sexual behaviour and women postponing childbearing to a higher age come into play.

Clinical awareness and prompt initiation of antiviral treatment are the most important factors for a favourable outcome. The difficulty in early recognition of infection has been identified as a major problem. Obvious skin lesions, facilitating the diagnosis, may be lacking in half of the cases. A history of herpes in the mother and father may give some hint but most mothers transmitting herpes are not aware of herpes-like symptoms. The diagnosis of neonatal herpes must be considered along with other pathogens in septicaemia in the first weeks of life. If there are seizures with no obvious cause starting two to six weeks after birth, or signs of encephalitis, a herpes aetiology must be actively excluded before a clinical suspicion is abandoned. In encephalitis pleocytosis, erythrocytes and increase in protein content are regular findings in the CSF. Abnormal EEG patterns appear two to three days after the first CNS symptoms; lesions are evident on MRI on day 2–3 and on CT scan at days 4–6 (see Forsgren and Malm, 2000).

Diagnosis Appropriate investigation of a potential case of neonatal herpes entails the examination of blood, urine, nasopharyngeal specimens, skin vesicle swabs and scrapings, as well as CSF for viral culture, DNA detection and (if available) detection of antigen by IF in vesicle material. Samples should be taken on wide indications and contact between the clinician and a colleague in a specialized laboratory is highly recommended.

PCR examination of CSF is essential and should be performed in all cases and also when infection is seemingly localized to the skin. However, diagnosis must not rely solely upon examination of CSF and a negative finding does not exclude the diagnosis. In SEM, HSV DNA is found in the CSF in only 24% of cases and even in overt CNS disease only in 76–78% of cases, although a higher diagnostic yield (about 90%) may be achieved if PCR analyses include blood (Kimberlin, 2007; Malm and Forsgren, 1999). HSV findings in blood and CSF are diagnostic of active herpes infection, as is recovery of virus from any other bodily location, provided that sampling is undertaken more than 48 hours after birth. The nonspecific ‘colonization’ of a baby from a mother with herpes virus in the birth canal may then no longer be detectable. Determination of the infecting herpes virus is useful for assessing the prognosis and quantitation, and

follow-up of HSV viral load in serum and CSF provides additional information for the management and prognosis of the herpes-infected child (Kimura *et al.*, 2002).

Serological investigation in the child and mother has a limited place in the acute phase of diagnosis. However, higher levels of HSV type-common and/or specific antibody in the mother compared to simultaneously-taken serum from the child may give a first hint of a possible diagnosis. The demonstration of an HSV-specific IgM in serum and/or CSF from the child may be of value, especially in environmentally-acquired infections.

Retrospective diagnosis of HSV-2 infection may be achieved by the demonstration of HSV-2 recurrences in an older child (which appear in the majority of children with neonatal herpes), or the demonstration of HSV-2-specific antibodies when maternal antibodies no longer persist (>12–18 months after birth). A finding of HSV-1 recurrence or of HSV-1-specific antibody is less conclusive as postnatally-acquired HSV-1 infections are not uncommon in early childhood. If dried blood spots, taken from the child for metabolic testing in the neonatal period, have been stored and are available, the demonstration of HSV DNA in blood extraction may contribute to diagnosis in some—although not all—cases (Lewensohn-Fuchs *et al.*, 2003).

Management Initiation of antiviral therapy should not await a confirmed diagnosis if there is a significant clinical suspicion of infection. If vesicles are present, the suspicion of herpes disease may rapidly be verified in the laboratory. Much more difficult are cases without vesicles, those that mimic septicaemia or those starting with a fit without other obvious cause (often with onset in week 2–6). In such cases therapy must be started as soon as there is reasonable suspicion of a herpes diagnosis—when sampling for herpes and other pathogens has been secured—and not abandoned until an alternative diagnosis is obtained or the clinical course and several laboratory results contradict herpes disease.

Studies have demonstrated the benefit of high-dose aciclovir therapy. Current recommendations (Buckley *et al.*, 2006; Kimberlin, 2007) are to administer 60 mg kg day⁻¹ intravenously in 3 divided doses for a period of 14 days in SEM disease (no HSV DNA demonstrable in CSF) and for at least 21 days in disseminated and CNS forms (defined as positive HSV DNA finding in CSF or CNS symptoms). Lumbar puncture should be repeated near the end of therapy. Those who remain positive should be given intravenous antiviral therapy until the PCR becomes negative. Infants with ocular disease are also given antiviral topical treatment. The prognosis in SEM disease after treatment is very favourable, although neurological sequelae appear in 5–10% of cases. In the disseminated form of

the disease high mortality still occurs despite high-dose aciclovir therapy. The mortality is lower in CNS disease, but unacceptably high morbidity rates remain. Only one third of babies with CNS disease have normal development and more than half of the children are left with severe to moderate neurologic and ocular sequelae. Infections with HSV-2 have a worse prognosis than for HSV-1. Virus is probably already spread via neuronal pathways to the brain at the onset of symptoms. Progressive disease can occur with recurrence of herpetic lesions and relapse of neurological or retinal disease. Acute retinal necrosis 20 years after infection has been observed (El Azazi *et al.*, 1991). Long-term suppressive oral antiviral therapy is often arbitrarily given pending the results of two phase III placebo-controlled trials to examine whether oral suppressive therapy (six months) is of benefit in prevention of the long-term neurologic sequelae in children treated for SEM disease or CNS disease (Collaborative Antiviral Study Group, www.casg.uab.edu).

A child with neonatal herpes should be carefully followed up to assess their special needs for early intervention programmes.

Prevention Primary/initial maternal genital or oral herpes at delivery or in the weeks before and after delivery poses the greatest threat of the baby being unprotected by maternal antibody from the infecting HSV type (Brown *et al.*, 2003, 2007). In recurrent maternal HSV infections maternal antibodies seem to protect a child in the post-natal period but may be insufficient to prevent transmission of the virus to the neonate if exposure to virus in the birth canal is intense. Invasive obstetrical procedures, prolonged rupture of the membranes and low levels of neutralizing antibodies are risk factors for transmission.

The main obstacle to the prevention of neonatal herpes is the frequent lack of clinically-recognizable signs of herpes in the mother in primary as well as recurrent infection. Silent excretion is frequent in pregnant women with genital herpes. The patient's history of previous attacks is also unreliable: a supposed first episode of genital herpes may more often be a secondary infection than a primary infection.

Regular antenatal cultures have no predictive value; not even culture at term is an absolute predictor of risk of neonatal herpes. Current options for prevention of vertical transmission are therefore based on clinical recognition of herpes in pregnant women and a growing insight into the course of HSV infections. However, evidence for the efficacy of intervention practices is still insufficient. Preventive measures include antiviral treatment of overt maternal disease, suppression therapy to decrease the risk of shedding and Caesarean section to decrease apparent exposure of the child. No side effects on the neonate

occasioned by aciclovir treatment in early pregnancy have been reported, and there have been no reports of maternal or fetal toxicity caused by suppressive therapy before delivery. Suppressive therapy before delivery reduces but does not eliminate recurrence episodes and shedding, and there are some reports of neonatal herpes in spite of maternal suppressive therapy or Caesarean section.

Most guidelines agree that primary oral or primary or initial genital herpes acquired at any time in pregnancy should be treated with oral or intravenous aciclovir as appropriate to the clinical condition. Treatment is imperative in infection occurring in the weeks just before and after delivery. Where overt genital lesions are present at term, Caesarean section should be considered. Suppressive therapy before birth should be given after a primary or initial genital infection occurring at any time in pregnancy due to the increased risk of viral shedding and the likelihood of a low level of protective (neutralizing) antibodies. It is recommended that whenever possible, specific diagnostic tools including HSV type-specific antibody tests are used to optimize the management of first-episode herpes. It is important to verify the diagnosis by virus detection if the clinical picture is unclear because of the need to follow up an exposed child.

In mothers suffering recurrent genital herpes the transmission risk is very limited, even in those with active lesions at birth (<1–2%). If noncrusted recurrent maternal lesions within the birth canal are evident at the time of delivery the current guidelines in many countries recommend Caesarean section, provided that the membranes have not been ruptured for more than four to six hours. This recommendation is under debate (for references see Forsgren and Malm, 2000) since protection afforded by Caesarean section is not absolute and Caesarean section is associated with some risk to the mother and, to a lesser extent, to the baby. Also, antiviral suppression with aciclovir or valciclovir before birth, widely applied in pregnant women with frequent or late recurrences in pregnancy, has been questioned in populations with low rates of neonatal herpes (e.g. van Everdingen *et al.*, 1993). It is clear that antiviral suppression may reduce the rate of Caesarean section, but given the low rate of neonatal herpes there have not been randomized controlled trials large enough to demonstrate a reduction in the risk of this disease. Scalp electrodes and instrumental delivery should only be used when strictly necessary. In a high-risk area it has been suggested that type-specific serological assays for HSV-1 and HSV-2 could be used to identify mothers who are seronegative for HSV-1, HSV-2 or both, as well as their discordant partners. Appropriate counselling could then be given to avoid risk during the third trimester. Such a strategy may, however, be difficult to implement and would not prevent all cases of neonatal

herpes. Improved sexual-health counselling with respect to the risk of acquiring genital herpes infection during pregnancy, coupled with heightening of clinical awareness about the condition, remains the current mainstay for prevention of this infection.

Disease in the Immunocompromised

Patients who are immunocompromised through deficits in cell-mediated immunity (for example transplant and oncology patients, patients with congenital or acquired immunodeficiency) are at risk of severe HSV infection with the possibility of life-threatening, disseminated infection (Crippa and Cinque, 2006). Primary, recurrent and exogenous reinfections all pose a significant threat to such patients, the severity of disease observed in them being linked with the degree of immunosuppression. Oral HSV infection can progress to extensive necrotic mouth ulceration and involve the pharynx and oesophagus, with bacterial superinfection or haemorrhage in those with thrombocytopenia. Equally severe, progressive genital HSV infections may occur. Autoinoculation may result in initial infection at sites distant from the original focus. Cutaneous dissemination can take place and may be clinically indistinguishable from varicella (Figure 6.13). Visceral dissemination is also possible in haematopoietic stem cell and organ transplant recipients; this can involve the oesophagus, respiratory tract, liver and even the gastrointestinal tract. In HIV patients with moderate CD4⁺ counts (above 200/ μ l), both typical and atypical mucocutaneous oral, genital or perianal manifestations are frequent. In more advanced HIV infections, severe recurrent HSV infection may occur with high frequency at multiple sites; there may be widespread cutaneous distribution and markedly prolonged times to healing of lesions. Prolonged viraemia leads to multiorgan distribution with extensive



Figure 6.13 Cutaneous dissemination of herpes simplex in an organ transplant recipient (Kaposi's varicelliform eruption).

visceral involvement, also affecting the eye (severe keratitis, progressive or acute necrotic retinitis) and CNS (sub-acute diffuse periventricular encephalitis, and concomitant CMV infection is common).

Diagnosis Demonstration and quantitation of virus by isolation, immunochemistry or molecular methods are the main tools. In cases where the diagnosis is unclear a search for viraemia may be of value. Where there is a suspicion of treatment failure, susceptibility testing of a viral isolate is beneficial. Serological tests are of very limited value. To confirm a causal role for HSV, for example in oesophagitis or colitis, a tissue biopsy for histopathological examination/viral demonstration *in situ* is often required. The diagnosis of disease affecting the eye requires corneal scrapings, biopsy and aqueous or vitreous fluid for virus culture/HSV DNA detection. In CNS infections examination of CSF is an essential diagnostic tool.

Treatment HSV infection in the immunocompromised host always gives cause for concern because of the possibility of the development of generalized multiorgan infection. In cases of iatrogenic immunosuppression, HSV infection is an indicator for temporary reassessment of the dosage of the immunosuppressive therapy. Oral medication with aciclovir, valaciclovir, famciclovir or intravenously with aciclovir has documented effect. As antiviral drug resistance has emerged as a significant difficulty, more often in haematopoietic stem cell recipients (10–18%; 2% in autologous and 19% in allogenic transplant patients) than in solid organ recipients (2–3%) and most frequently in HIV patients with low CD4⁺ counts, therapy may have to be switched to intravenous foscarnet (or cidofovir, although cross-resistance with aciclovir may occur). Serious HSV infections require not only the prompt initiation of aggressive specific antiviral chemotherapy but also full medical and nursing intervention. Generalized infection in the immunocompromised patient produces osmotic imbalances, endocrine dysfunction, and circulatory and respiratory failure, which necessitate an aggressive approach to intensive care.

Prevention HSV has effectively ceased to be a major problem in transplant patients since prophylactic antiviral regimens have become routine. Antiviral prophylaxis is given to those HSV-seropositive recipients receiving haematopoietic stem cells over the aplastic phase (or longer in the case of graft-versus-host reaction) and to organ transplant recipients for four to eight weeks. HSV-seronegative recipients and HIV-positive patients should be counselled on prevention of HSV exposure. In patients with HIV, combination anti-HIV therapy has

significantly reduced the prevalence of active herpes. For those patients with HIV suffering frequent recurrence of infection, suppressive antiviral therapy is used selectively as there is some evidence that active HSV infection, in common with other herpes viruses, can act as a cofactor in AIDS, activating HIV and making it easier for HIV to infect certain cells. Herpes infections remain a frequent complication where antiviral therapy is not available.

CONCLUDING REMARKS

Whereas most HSV-1 and HSV-2 infections are silent or harmless, knowledge of serious disease caused by the two viruses has widened through the application of modern molecular diagnostics. Rapid diagnosis enables early therapy, but the presently available antiviral agents are not in themselves adequate. The crucial viral and host-factor determinants of HSV pathogenicity in man remain largely unknown and cannot be targeted. Devastating sequelae and chronic infection frequently remain after, for example, HSV disease of the CNS. Prevention of disease through vaccination, urgently needed in the face of the worldwide problems of the growing number of immunocompromised patients and of the role of HSV in the HIV epidemic, is not yet attainable. It is to be hoped that research into the genomics and proteomics of HSV and the immunopathology of serious HSV disease will result in steps forward in the field.

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Varicella Zoster

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INTRODUCTION

Two common diseases are caused by human herpesvirus 3, often called varicella zoster virus (VZV): chickenpox (varicella) and shingles (herpes zoster). Nobody appears to know for sure what the word 'chicken' has to do with chickenpox but one possible derivation is from the Old English 'gican' (itch), designating it the 'itchy' pox to differentiate it from diseases such as smallpox. The words 'zoster' and 'shingles' are derived from Greek and Latin words, respectively, both meaning a belt or girdle, and are obviously descriptive of the characteristic distribution of the rash.

Chickenpox is the manifestation of primary infection with VZV and is one of the commonest communicable diseases worldwide. Its characteristic presentation in the majority of cases is as familiar to laymen as it is to doctors and is usually of little concern to either. However, it has also been long recognized that chickenpox can have serious consequences in adults and in immunosuppressed individuals. Shingles is the manifestation of VZV reactivation and although rarely a life-threatening disease, it is perhaps of more concern community-wide because of the pain, not only of the acute lesion but also of the frequent post-herpetic neuralgia (PHN), which can be very debilitating and is notoriously difficult to treat.

The common aetiology of varicella and herpes zoster was first recognized at the beginning of the twentieth century by clinicians who noticed that a case of zoster in a household was often followed by an outbreak of varicella in younger members of the family. Furthermore, it was shown that vesicle fluid taken from lesions of herpes zoster could induce chickenpox when inoculated into young volunteers. Virus particles were first observed in vesicle fluids by electron microscopy (EM) in 1943 and

definitive evidence that the two diseases are due to the same virus came with the isolation of the virus in cell culture by Weller in 1953 (Weller *et al.*, 1958). Edgar Hope Simpson, a GP in Cirencester, first hypothesized that VZV persisted latently following chickenpox and reactivated to cause shingles (Hope Simpson, 1965). Analysis of viral DNA from lesions of a patient with chickenpox who later developed zoster confirmed that the virus was the same in both cases (Straus *et al.*, 1984).

VZV is the first human herpesvirus for which successful vaccines have been licensed. Thus, the increasing number of patients at risk of severe varicella and zoster, as a result of the growth in the size of the elderly population, the success of treatment regimens that are immunosuppressive in the fields of oncology and transplantation surgery, and the increasing number of individuals who are infected with human immunodeficiency virus (HIV), has been matched by the introduction of VZV vaccines for prevention of chickenpox and zoster.

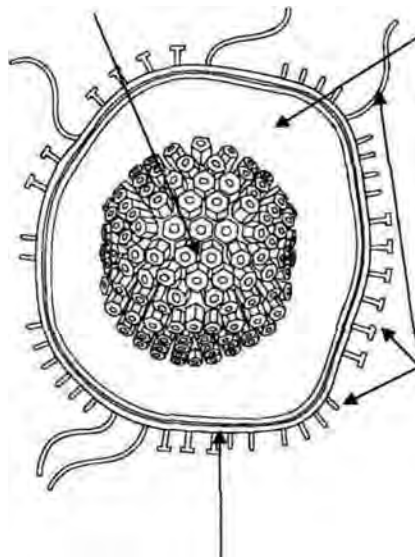
THE VIRUS

Structure

VZV has the characteristic morphological appearance of a herpesvirus (Figure 7.1), which has been described in detail in Chapter 6. However, because VZV is quite difficult to grow and particularly difficult to purify, less is known about its proteins and genome organization than is known about those of herpes simplex virus (HSV). In fact, much of the knowledge about VZV has been obtained by parallels with HSV. The complete DNA sequence of VZV was published in 1986 by Davison and Scott. A general review of the molecular biology of VZV is given

Capsid Three forms exist: A (empty), B (intermediate) and C (mature). Assembly proteins present in the B form are lost during DNA insertion to produce the C form

Tegument A complex mass of proteins surrounding the capsid. Contains enzymes controlling virus replication and regulating cell function



Glycoprotein 'spikes'
Glycoproteins gE, gB, gH, gI, gC and gL project through the lipid envelope, allowing the virus to interact with its environment. gE/gI and gH/gL are present as complexes

Envelope A complex membranous structure derived from cellular membranes of the trans-Golgi network

Figure 7.1 Structure of the VZV particle (virion). When intact, the particle is spherical and approximately 200 nm in diameter. Amorphous forms may be seen using traditional transmission electron microscopy. (Source: *The Sourcebook of Medical Illustration*, Parthenon Press.)

by Davison 1991. The genome is a linear double-stranded DNA molecule with a molecular weight of 80×10^6 (approximately 125 kilobase pairs (bp)) and is thus among the smallest of all herpesviruses studied. Buoyant density estimations, as well as the sequence data, show a (G + C) content of 46%, which is much lower than in most herpesviruses, for example 67% in HSV-1 and 58% in cytomegalovirus (CMV) DNA. Within the genome there are, however, (G + C)-rich regions, notably the repeat regions. The organization of the VZV genome (summarized in Figure 7.2) shows distinct similarities with HSV

DNA and the two viruses have sufficient sequence homology to permit hybridization under nonstringent conditions. However, there is substantial local variation in the extent of this homology, most notably in the almost complete loss of the repeats around the long unique sequence in VZV, which accounts for much of the size difference between the genomes of these two viruses. The similarities between the HSV and VZV genomes would be compatible with a common ancestry, and a model for this has been proposed (Davison and McGeoch, 1986). The VZV genome is divided into two main coding regions:

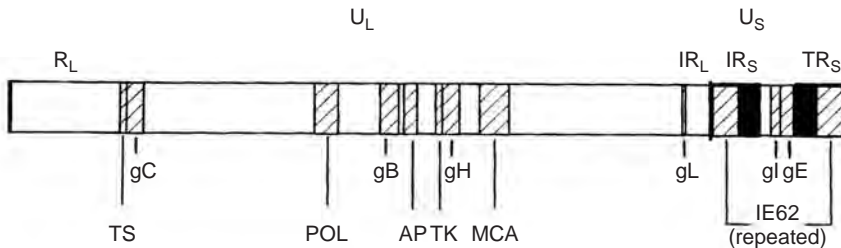


Figure 7.2 Organization of the VZV genome.

a unique long region (U_L) (about 105 000 bp), which is flanked by inverted repeat (IRs) elements (at 88 bp far shorter than those of HSV), and a unique short region (U_S) (about 5200 bp), also flanked by inverted repeat sequences (7300 bp). The short region can be found in either of two orientations relative to the long region, producing two isomeric forms of the genome, which occur in equal proportion. Inversion of the long region is rare. By contrast, in HSV and CMV both regions invert at equal frequency, resulting in four isomeric forms. An inherent size variation of the genome of approximately 2% (2500 bp) has been demonstrated in clinical isolates, concentrated in five variable regions. Restriction endonuclease cleavage patterns show some DNA polymorphism between clinical isolates, and strain differences in circulating viruses are

now becoming apparent, which may make such analyses a useful tool in epidemiological studies (see below).

Seventy unique open reading frames (ORFs) have been identified on the VZV genome, of which three are repeated, two are spliced and two are located entirely within other genes. Over seventy RNA transcripts have been demonstrated in VZV-infected cells but the majority of these appear to contain more than one ORF. The genes that have been identified to date account for virtually the whole genome, although the presence of overlapping genes does allow for further ORFs to be identified. The list of genes that have been identified, mostly by analogy with HSV, is given in Table 7.1. Five of the VZV genes, including a gene for a thymidylate synthetase, are not found in HSV, while three HSV genes have no VZV

Table 7.1 VZV genes for which functional products have been identified

Gene	HSV homologue	Protein identity (%)	Translation product (kDa)	Function
1	No homologue	—	12.1	Membrane protein
4	U _L 54	29	51.5	Transcriptional activator (IE protein)
5	U _L 53 (gK)	28	40	gK, E
6	U _L 52	37	122.5	DNA helicase/primase component
8	U _L 50	25	44.8	Deoxyuridine triphosphatase
9A	U _L 49.5	Low	9.8	Stearoylated membrane protein
10	U _L 48 (VP16)	60	50	Transactivator, tegument protein, NE
13	No homologue	—	34.5	Thymidylate synthetase (TS)
14	U _L 44	23	61.4	Membrane glycoprotein gC (gp V)
16	U _L 42	21	46.1	Associated with DNA polymerase
17	U _L 41	32	51.4	Host shut-off
18	U _L 40	54	35.4	Ribonucleotide reductase small subunit
19	U _L 39	30	86.8	Ribonucleotide reductase large subunit
28	U _L 30	52	134.0	DNA polymerase
29	U _L 29	50	132.1	Binds single-stranded DNA
31	U _L 27	45	98.1	Membrane glycoprotein gB (gpII)
33	U _L 26	34	66.0	Assembly protease
33.5	U _L 26.5	24	32.8	Assembly protein (AP)
36	U _L 23	28	37.8	Pyrimidine deoxyribonucleotide kinase
37	U _L 22	25	93.6	Membrane glycoprotein gH (gpIII)
40	U _L 19	52	155.0	Major capsid antigen (MCA)
47	U _L 13	33	54.3	Protein kinase
48	U _L 12	29	61.3	Deoxyribonuclease
49	U _L 11	26	8.9	Myristoylated protein
51	U _L 9	44	94.4	ori _s -binding protein
52	U _L 8	28	86.3	DNA helicase/primase component
55	U _L 5	56	98.8	DNA helicase/primase component
59	U _L 2	39	34.4	Uracil-DNA glycosylase
60	U _L 1	19	17.6	Membrane glycoprotein gL (gpVI)
61	IE110	Local only	50.9	Transcriptional repressor (IE protein)
62/71	IE175	Complex	140.0	Transcriptional activator (IE protein)
63/70	U _s 1	Local only	30.5	Transcriptional activator (IE protein)
66	U _s	33	43.7	Protein kinase
67	U _s 7	23	39.4	Membrane glycoprotein gI (gpIV)
68	U _s 8	22	70.0	Membrane glycoprotein gE (gpI)

equivalent. In common with other herpesviruses, the synthesis of VZV proteins broadly involves three phases, designated immediate-early (IE) (α), early (E) (β) and late (L) (δ). More than 30 virus-coded polypeptides can normally be detected in VZV-infected cells, ranging in molecular weight from 7 kDa to over 200 kDa. Among these, at least six groups of glycoproteins can be identified, with molecular weights ranging from 3.8 to 118 kDa, which correspond to the gene products of VZV glycoproteins E, B, H, I, C and L. VZV gE is the major glycoprotein expressed on VZV-infected cells and mediates IgG Fc-binding in a complex with VZV gL. Two signal sequences within the gE cytoplasmic domain interact with cellular proteins to transport gE complexed with gL to the *trans*-Golgi network (TGN), where post-translational processing occurs before expression on the cell surface. The cellular receptor for gE has recently been found to be insulin-degrading enzyme (Li *et al.*, 2006). Reduction in IDE leads to reduced cell-to-cell spread of virus. The other glycoproteins, including gB, which is present in virions as a disulfide-linked complex of 140 kDa molecular weight, and gH, which is chaperoned by gL, are also directed to the TGN of membranes to be processed before expression at the cell surface. Glycoproteins C and I have been shown in the SCID-hu mouse animal model (see below) to be important for infection of skin cells (Mortal *et al.*, 2002). In addition to the glycoproteins, a range of nonglycosylated proteins have been identified in the virion. These include the main protein component of the nucleocapsid (major capsid antigen (MCA), 155 kDa), an assembling proteinase/assembly protein complex produced from gene 33 and the IE62 protein (175 kDa), an IE translational activator analogous to the HSV ICP4 protein, which is present in the virions. Many of the other proteins of VZV are involved in virus replication. These include the thymidine kinase and thymidylate synthetase enzymes, several virus-specified protein kinases, a range of DNA-binding proteins, including the viral DNA polymerase, and five transcriptional regulators produced from ORFs 4, 10 and 61–63 (see Table 7.1).

Replication

VZV attaches to the outer membrane of the cell, and this is followed by membrane fusion and entry of the viral core into the cell. This process is mediated primarily by the glycoproteins projecting from the virus particles, although some tegument proteins may also play a role in viral penetration. Attachment involves binding, predominantly of gB to heparan sulphate proteoglycan on the cell surface. This binding is followed by attachment of mannose-6-phosphate (M-6-P) oligosaccharides, present on the surface of VZV particles, to M-6-P-specific receptors on the host cell (Zhu *et al.*, 1995). Glycoprotein

E attachment to insulin-degrading enzyme also mediates virus entry and is important for cell-to-cell spread.

The tegument proteins prepare the cell to produce virus and separate from the nucleocapsid during transportation of virus particles to the nucleus. Once within the nucleus, the linear DNA genome circularises.

In common with other herpesviruses, the replication cycle is coordinately regulated. There are three basic stages of gene expression and protein synthesis: IE, E and L. The first viral proteins (IE) can be detected 4–6 hours post-infection. Structural proteins (E) such as the major capsid protein and viral glycoproteins are not detected until 18 hours post-infection. Viral protein synthesis appears to reach maximum levels at 46–48 hours post-infection in some cell culture systems.

IE transcripts are translated in the cytoplasm and the IE (regulatory) proteins are then transported back into the nucleus, where they induce the early gene expression and downregulate further IE gene transcription. The E proteins include those that are required for VZV DNA replication, such as viral DNA polymerase, viral thymidine kinase and protein kinases. Among the late gene products are the nucleocapsid structural and glycoproteins. DNA synthesis occurs following synthesis of E proteins and involves seven viral gene products, including the DNA polymerase (encoded by ORFs 28 and 16), the origin-binding protein (OBP) (ORF51) and the helicase primase complex (ORFs 6, 55 and 52). DNA synthesis is initiated when the OBP binds to the viral origin of replication, OriS, two copies of which are located in inverted Us repeat (Stow and Davison, 1986). This is followed by the movement of the DNA polymerase around the circularized genomic DNA, producing head-to-tail polymers of the viral genome (concatamers). This is referred to as a 'rolling circle' mechanism. After translation of late mRNA, the structural proteins that will form the viral capsid are transported back to the nucleus and assembled around a core of the viral assembly protein. The newly transcribed DNA genome is inserted into the assembled capsid, concurrent with the loss of at least some of the assembly proteins (Harper *et al.*, 1995). Capsids acquire a 'temporary envelope' from the inner nuclear membrane and move to the TGN, where the final stages of assembly take place. The nucleocapsid acquires an envelope containing the viral glycoproteins, to which the tegument proteins are bound. In the normal course of events it appears that a second membrane derived from TGN forms a transport vesicle around the mature virions, which then leave the infected cell by a process of exocytosis. In some cells, however, the virions appear to be aberrantly processed and are transported to digestive lysosomes, which possibly accounts for the very low level of cell-free infectious virus found in VZV-infected cell cultures (see below).

Growth in Cell Culture

VZV replicates, with varying degrees of success, in cultures of many cells of human and several of simian origin. Cells from non-primates are generally resistant to infection but the virus has been adapted to embryonic guinea-pig cells, and passage in these cells was an early stage of the attenuation of the virus used in the current live vaccine. The behaviour of VZV in cell culture can be regarded as being intermediate between that of HSV, which will grow in nearly all cell cultures, and CMV, which will grow only in a few cell types of human origin. In other respects, VZV behaves more like CMV, for example it grows slowly even in the most sensitive cell systems, with the cytopathic effect (Figure 7.3) taking from three days to over two weeks to appear. This process may be mediated by apoptosis rather than direct cell killing. VZV remains even more strongly cell-associated than CMV, and with most systems passage can be achieved only by transfer of infected cells. Cell-free virus can be obtained by a number of procedures, which typically include sonication of the infected cells. Additionally, protective storage media may be used to increase the yield of infectious virus. Higher yields of infectious cell-free virus can be obtained from the media of infected cultures of human thyroid or malignant melanoma cells, which may reflect the release of a high proportion of defective virus particles in other cell culture systems, as noted above. An M-6-P knockdown MeWo cell line is also reputed to yield higher titres of cell-free virus (Chen *et al.*, 2004).

The cytopathic effect in most cell systems is so characteristic that no further means of identification may be

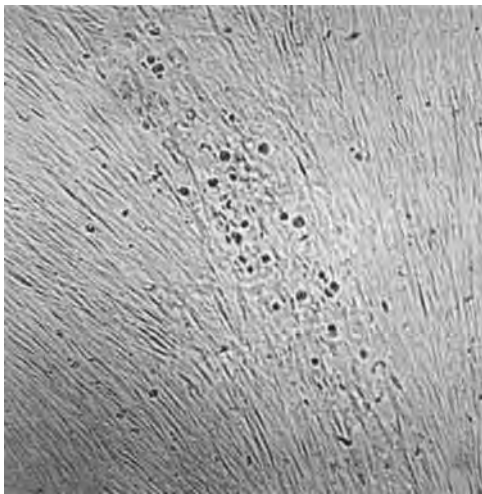


Figure 7.3 The typical early ovoid cytopathic effect of VZV seen in unstained human embryo lung cells.

required. Because of the strong cell association of the virus, the lesion it produces is typically focal and slowly extends into the surrounding cell sheet, due to the spread of virus between contiguous cells. Viral antigen can be detected much more rapidly than cytopathic effect. Typically, antigens can be detected in the cytoplasm and nuclei of infected cells within two to four hours after infection and in neighbouring cells after eight to eighteen hours, depending on cell type and the type of antigen targeted. The shape of the lesion is dependent on the architecture of the cell sheet. The typical elongated ovoid shape of the lesion in human embryo lung fibroblasts is shown in Figure 7.3. Enlarged cells can frequently be seen in the lesion and staining reveals that these are multinucleated, resulting from virus-induced cell fusion. Staining of the affected cell sheet reveals another feature of VZV cytopathology, namely that many of both the mononuclear and the multinucleated cells have irregularly-shaped intranuclear inclusions (Figure 7.4). With time, the infection ultimately spreads to involve the whole cell sheet.

Strain Variation and Antigenic Properties

The nucleotide diversity of the VZV genome as a whole has been estimated at 0.063%, which is comparable to that of human DNA but may be less than that of HSV (0.2–0.5%). As with all herpesviruses, the VZV genome contains blocks of repeated nucleotide sequences, in this case five (R1–5). R1, R2 and R3 are GC-rich and are located in the coding sequences of genes 11, 44 and 22 respectively. R4 (which is present in both of the inverted repeat regions (TRL and IRL) flanking the unique long region) and R5 are noncoding. Variation in the number of repeat elements within these regions has been used to distinguish one strain of virus from another, either by means of restriction endonuclease sites generated or lost, or by variations in the length of the cleaved fragments (Hawrami *et al.*, 1996). Other restriction sites and single nucleotide polymorphisms (SNPs) throughout the genome have also proved useful for typing of clinical isolates and this has led recently to the identification of at least five main genotypes (Muir *et al.*, 2002; Peters, 2006; Norberg, 2006; Loparev, 2007) which are to some extent geographically segregated. There is evidence that the many genotypes have arisen as a result of recombination events occurring in the distant past (Norberg, 2006). Even today, recombinant genotypes are described particularly in areas where migration has occurred, for example in London and Rio de Janeiro. SNPs have been used to distinguish the Oka vaccine virus from wild-type strains. A Pst-1 restriction site in gene 38 and a Bgl-1 restriction site in gene 54 have been used to distinguish most UK and US wild-type viruses from the Oka vaccine strain, but in 30% of cases were unable to discriminate between

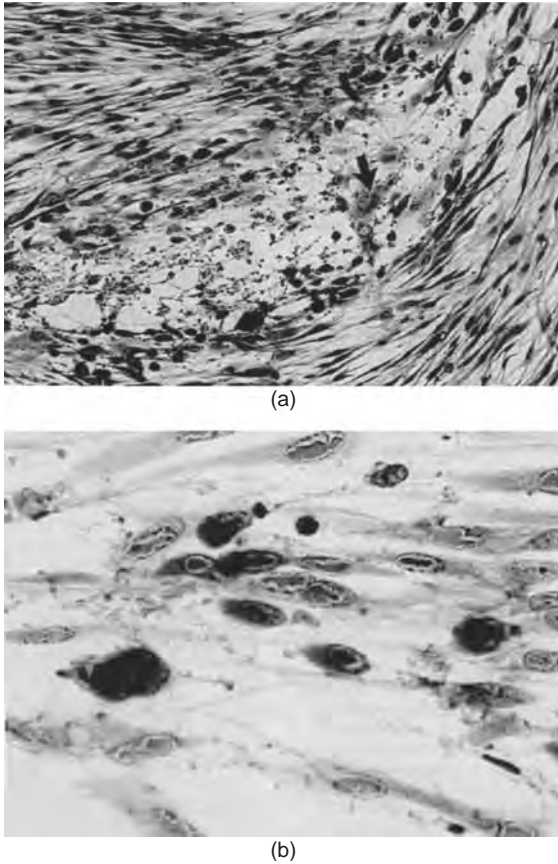


Figure 7.4 (a) Stained VZV-infected human embryo lung fibroblasts. Note the rounded and multinucleated cells (arrow) (H&E stain). (b) High-power view with many affected cells showing typical intranuclear inclusions.

wild-type Japanese strain and Oka. More recently, three fixed mutations in the vaccine strain, each of which generate restriction sites, have been used to distinguish vaccine virus from wild type in cases of chickenpox and zoster occurring in Oka vaccinees (Loparev, 2000; Quinlivan, 2005). One of these hitherto unique vaccine mutations has now been described in a US strain, although whether it is a recombinant between wild type and vaccine remains to be seen.

Worldwide, experience suggests that antibody produced against any strain of VZV is protective against clinical disease and this is the basis for immunization with the live attenuated Oka vaccine. However, well-documented clinical re-infection has been described in up to 13% of children (Hall *et al.*, 2002) and rarely among pregnant women with low antibodies (Martin *et al.*, 1994). A case of recurrent zoster in the same, immunocompetent, person due to different genotypes provides further evidence for

re-infection (Taha *et al.*, 2006). A number of wild-type strains of VZV in which mutations in IgE abrogate binding of the 3B3 monoclonal antibody, which is commonly used for diagnostic immunofluorescence, have been isolated. One of these MSP-VZVs has been shown to replicate and spread more aggressively in tissue culture and animal models, such as the SCID-hu epithelial mouse (Santos *et al.*, 2000).

Some serological cross-reaction with HSV does occur, indicating that these two viruses share common antigens. It has not been shown conclusively on which of the viral proteins the responsible epitope (or epitopes) is situated. The gB glycoproteins of VZV and HSV have cross-reading epitopes but it has been difficult to demonstrate any significant cross-reactivity with other proteins, in spite of the sequence homologies between the two viruses. More likely is that infection with one virus boosts the levels of antibody to any different but antigenically-related virus strains previously encountered—so-called ‘original antigenic sin’. This theory cannot entirely explain the heterologous reactions between VZV and HSV, since it is known that a small number of children with no previous exposure to VZV but who are experiencing primary HSV infection go on to develop low levels of antibody transiently which react with VZV. The converse has also occasionally been observed with patients experiencing primary VZV infection. It is not known whether this cross-reactivity extends to cellular immune responses or whether it confers any cross-protection between the two viruses.

Host Response to Infection

Following infection with VZV, antibodies are produced to the various structural and nonstructural proteins of VZV. Up to 30 protein bands can be detected with convalescent sera by radioimmunoprecipitation or immunoblotting. The predominant immunogenic components of the virus appear to be the glycoproteins, the major capsid protein and the assembly protein complex (Harper *et al.*, 1988). Both IgG and IgM antibodies react with these proteins (Figure 7.5). Typically, sera from cases of zoster react more strongly and reveal a wider range of proteins compared to varicella. gE, gH and the tegument protein IE62 are the major targets for the cell-mediated immune response against VZV, although T-lymphocyte responses are also measured against epitopes in gL, gB and gC, as well as the transcriptional regulator IE63, and the products of ORF4, ORF10 and ORF29 genes (Arvin *et al.*, 1991). Although specific antibody against VZV may protect against or attenuate infection, control of primary infection and clearance of virus appears to depend on cellular immunity. The incidences of complications and

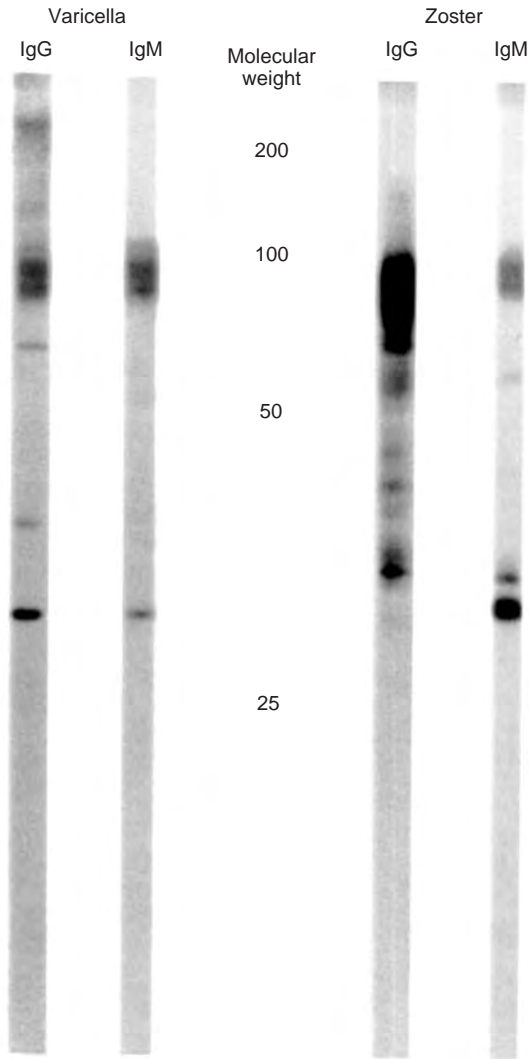


Figure 7.5 Typical immunoblot patterns for IgG and IgM antibodies obtained with early convalescent sera from varicella and zoster cases. The viral proteins were separated on a 9% polyacrylamide gel, electroblotted on to nitrocellulose and reacted with the sera. IgG and IgM antibodies were detected with ¹²⁵I-labelled sheep anti-human IgG or IgM. The bands revealed by zoster sera are more numerous and more intense.

death from primary infection are higher if there is underlying impairment of cell-mediated immunity.

Pathogenicity for Animals

Animals other than man are reputed not to be susceptible to VZV but there are reports of successful infection

of primates, such as gorillas and chimpanzees, as well as marmosets. It has also been shown that guinea pigs can be infected with virus which has been passaged in embryonic tissue obtained from these animals, leading to virus replication in the nasopharynx, viraemia and an immune response, but without a rash. Hairless guinea pigs have been reported to frequently develop a papular rash after inoculation but this model remains challenging to use. Inoculation of the rat hind limb produces infection of the dorsal root ganglia, and similarities in the pattern of gene expression with that seen in latent VZV in humans has led to its use as a model of latent infection (Sadzot Delvaux, 1995). However, no *in vivo* reactivation is observed in this model. Moreover, unlike VZV latent infection in humans, the rats experience neuropathic symptoms. As a result this is also useful as a model for the persistent pain, known as PHN, which can occur following zoster (Fleetwood Walker, 1999). In the past 10 years, the use of SCID-hu mice with thymus/liver and epithelial implants has greatly facilitated studies of virus tropism for and replication in T cells and skin, respectively (Moffat, 1995). Studies of simian varicella virus continue to identify similarities to human VZV (Pumphrey and Gray, 1995) but the applicability of data obtained from such a model directly to man remains to be seen.

Pathogenesis

Knowledge about the pathogenesis of VZV-induced diseases, particularly the primary infection, is still limited due to the difficulty in growing the virus and the paucity of suitable animal models.

Surprisingly little is known about the source and route of transmission of the virus. The skin lesions are certainly teeming with infectious virus, even at the maculopapular stage. Airborne transmission from skin lesions is therefore highly likely, especially since VZV DNA is readily detected by polymerase chain reaction (PCR) in the air surrounding patients with VZV infection. It is virtually impossible to isolate infectious virus at any stage from the upper respiratory tract of cases of varicella, although viral DNA may be detectable by PCR. Nevertheless, it is widely believed that transmission occurs from this site, probably from asymptomatic oral lesions which are present before the skin eruption appears. Whatever the case, VZV undoubtedly is transmissible via an airborne route and does not require close personal contact. The clinical attack rate of varicella in outbreaks is typically around 70% in susceptible individuals, which is slightly less than for other viruses transmitted via the respiratory route, for example 90% for measles.

For many years the model for the pathogenesis of varicella was extrapolated from the Fenner and Grose mousepox model (Figure 7.6). Virus was hypothesized to enter

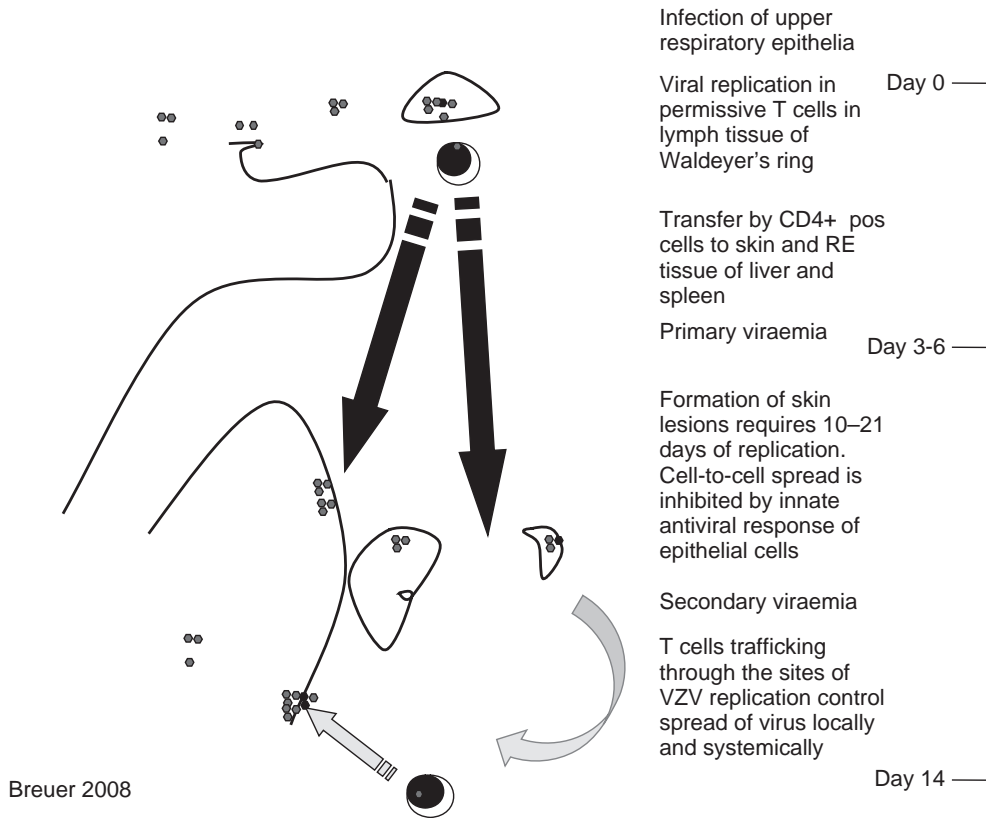


Figure 7.6 Proposed models for the pathogenesis of varicella. (Reproduced from Grose 1981.)

the respiratory tract and replicate in the nasopharyngeal lymphoid tissue. After two to three days the virus spread in lymphocytes to reticuloendothelial tissue, hence after a further period of replication a second viraemic phase resulted in the virus reacting with the end organs, including the skin, lungs, gut and sometimes brain.

Using data from the SCID-hu mouse model, an alternative model of pathogenesis is gaining acceptance. Following replication in the respiratory lymphoid tissue, the virus infects specialized skin-homing CD4-positive T lymphocytes, which are abundant in tonsillar tissue. Virus may be carried directly to the skin within two to three days of infection. The fact that lesions do not form for another 10 days is thought to be due to interferon α production by surrounding uninfected epithelial cells, resulting in barriers to cell-to-cell spread of the virus (Ku *et al.*, 2004). Eventually, the virus is able to downregulate MHC and other cell-signalling molecules, overcoming innate defences and leading to the development of virus-filled vesicular lesions. Further spread occurs by infected T cells trafficking through infected skin. In healthy individuals, primed T

lymphocytes act to limit the severity of infection (Zerboni *et al.*, 2005).

Histological examination of the skin lesions of both varicella and zoster shows focal degenerative changes in the epidermis. In both cases, virus seems to infect the cells in the isthmus of the hair follicle, an area rich in stem cells. From there the virus spreads to infect the epithelium. Affected cells are swollen and many of these contain well-defined eosinophilic intranuclear inclusions. Multinucleated cells, also with intranuclear inclusions, are characteristically seen at the base of the lesion (Figure 7.7). The histology of the skin lesions is thus essentially similar to the cytopathology seen in cell culture. The lesion extends and its centre fills at first with clear fluid, which then becomes cloudy due to the influx of inflammatory cells.

Termination of the infection at this site is indicated by the drying up of the pustules, which is followed by separation of the scabs and regeneration of the epithelium. Termination must be brought about mainly by cell-mediated immunity. The presence of large amounts

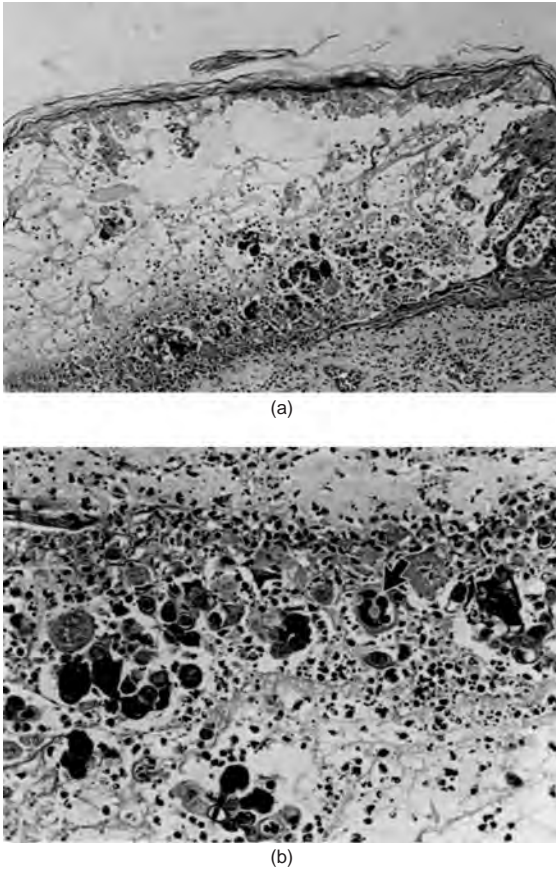


Figure 7.7 Multinucleated cells.

of free and cell-associated virus in the lesion, as well as degenerating cells showing all the characteristic features of VZV infection, strongly suggests that the damage results from a lytic effect of the virus. The lesions in the immunocompromised are not modified in any way other than being more extensive and this makes it unlikely that immunopathology is playing an important part in their genesis.

Recovery from the primary infection results, in most people, in lifelong immunity to exogenous infection, although clinical re-infection has been described in up to 13% of children and in some pregnant women with low antibody titres (Hall *et al.*, 2002; Martin *et al.*, 1994). Subclinical re-infection may be even more common, since it has been demonstrated that seropositive individuals who are in contact with varicella can occasionally show significant rises in the level of specific antibodies and boosting of cell-mediated responses (Arvin *et al.*, 1983; Gershon *et al.*, 1996). Furthermore, viral DNA can be detected by PCR in nasopharyngeal secretions of immune individuals

who are in close contact with chickenpox, suggesting that localized re-infections may occur (Connelly *et al.*, 1993). Asymptomatic systemic spread of virus is also suggested by reactivation of UK viral strains in patients whose primary infections occurred in Africa (Quinlivan *et al.*, 2002). More recently, a case where two episodes of zoster were caused by different strains of VZV re-inforced the likelihood that, as with other herpesviruses, re-infection can occur (Taha *et al.*, 2006). Hope Simpson in 1965 hypothesized that reexposures to VZV throughout life help to maintain effective immunity to varicella, and this has been substantiated by more recent epidemiological data (Brisson *et al.*, 2002; Thomas *et al.*, 2002).

Latency

Following the primary infection, the virus remains latent in one or more posterior root ganglia. The predominant route for infection of nerve ganglia is thought to be by retrograde spread from the skin. Only cell-free virus, which is abundant in vesicular fluid, is able to infect the ganglia and establish latency. Infection with cell-associated virus results in lytic destruction of the ganglion, and this is thought to be because it contains ORF61 protein, which is absent in cell-free virions (Chen, 2003). Production of cell-free virions in vesicle fluid is possible because differentiated keratinocytes lose the M-6-P receptor which normally binds M-6-P present in virion envelope and sequesters the virus into cell-associated endosomes (Chen, 2004).

The trigeminal and thoracic ganglia are most frequently infected, but ganglia at multiple sites may contain VZV DNA, including ganglia not directly connected with the skin (Furuta *et al.*, 1997). VZV can also infect T lymphocytes and this is likely to be the route of infection in cases of asymptomatic re-infection. In up to 25% of individuals, a single reactivation of the virus, known as herpes zoster, occurs usually several decades after the primary infection. The virus reactivates in the ganglion and then progresses peripherally down the sensory nerve to produce the typical skin lesions of herpes zoster, which are restricted to the dermatome supplied by the nerve.

Less is known about how VZV maintains latency and what triggers reactivation than about the corresponding processes with HSV. During latency the viral DNA appears to exist in episomal form, with fused genome termini. Latent virus is located predominantly in the neurons within the ganglia (2 – 5%), with a much smaller proportion (less than 0.1%) of nonneuronal satellite cells being infected. Unlike HSV, there is no evidence that VZV encodes latency-associated transcripts. There is evidence that, unlike HSV, protein products of ORFs 4, 21, 29, 62 and 63 are produced during latency (Mahalingam *et al.*, 1996; Lungu *et al.*, 1995) and are present in the cytoplasm

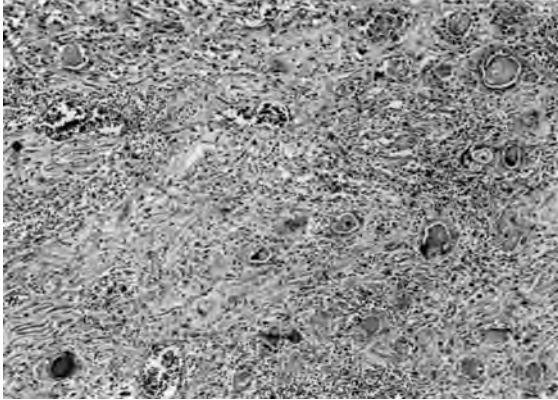


Figure 7.8 Destructive inflammatory changes in a ganglion.

as well as the nucleus, as expected, of both neuronal and nonneuronal cells (Lungu *et al.*, 1995).

Reactivation of virus is associated with intense destructive inflammatory changes in the involved ganglion (Figure 7.8) and this may be reflected in the severe pain which is frequently experienced in association with zoster. The failure of the host defence mechanisms to contain the virus in the ganglia after such prolonged periods of time is not understood. In immunocompetent individuals it is probably due to the decline of the effectiveness of previously acquired ‘cell-mediated’ immunity to VZV. In particular, T-cell responses to IE63 have been proposed to protect against reactivation. Inadequacy of cell-mediated immunity is likely to be of critical importance, since not only the elderly but patients with Hodgkin’s lymphoma, HIV and similar diseases are more likely to experience zoster. These patients are also more likely to experience more than one attack of the disease, or they may develop the ‘disseminated’ form of the disease. The term ‘disseminated’ implies that the virus spreads through viraemia from the affected dermatome to infect the skin or other organ at some distal site, producing lesions that are similar to those of varicella in their appearance and distribution. Most cases of zoster occur spontaneously but trauma and stress have also been proposed as triggers of reactivation.

EPIDEMIOLOGY

Cases of varicella are seen throughout the year but in temperate climates they occur more frequently in the spring months. Annual variation in incidence also occurs, with higher than average incidence in three-to-four-year cycles. Herpes zoster, in contrast, occurs sporadically and evenly throughout the year.

In tropical climates, varicella occurs during the dry seasons and outbreaks are curtailed by the advent of the rains. Different parts of the world can show significant variations in the age distribution of infection. In ‘temperate areas’ it is one of the classic diseases of childhood. In the past the highest incidence of varicella occurred in the age group 4–10 years (Figure 7.9). Recent serological surveys and data from surveillance general practices (Royal College of General Practitioners) have shown that the peak age of primary infection in Western countries occurs in children aged under five years, which is presumably related to an increased use of day-care and playgroup facilities, leading to greater exposure at a younger age. In general, varicella is highly communicable in temperate countries, with a reported attack rate of up to 96% in close contacts (i.e. household or playfriend), and therefore most people become infected before adulthood. Seroprevalence studies generally show that fewer than 10% of young adults are still susceptible to varicella (Figure 7.9) and despite a temporary increase in the incidence and overall proportion of cases in adults (aged over 15 years) (Fairley and Miller, 1996), this remains the case today.

In many tropical countries, varicella is predominantly a disease of adults, with a mean age of 20–25 years, or even as high as 38 years reported from St Lucia. Studies from the Indian subcontinent, South East Asia and the Caribbean have shown that 25–60% of adults aged over 15 years are susceptible to varicella. It appears that less transmission occurs amongst the young children in these areas and this may reflect patterns of social mixing between infected cases and susceptible contacts. The effects of temperature and humidity at these latitudes on a naturally labile virus have also been proposed to explain lower rates of infection, but are partially discounted by evidence of a childhood pattern of infection in countries with similar climatic conditions, such as Hong Kong and northern Australia. The effect of low population density in a rural setting leading to lower rates of transmission is supported by the lower rates of VZV seropositivity for subjects living in rural Bengal and rural Brazil, as compared with their age-matched urban counterparts (Mandal *et al.*, 1998; Yu *et al.*, 2001). Other theories include competition with other viruses infecting the respiratory tract. A study in Thailand showed the interaction of both mixing and temperature, with urban areas which experience cooler temperatures showing earlier infection (Lohlekha *et al.*, 2001). However, little or no evidence for ethnic differences influencing age of infection have been found (Talukder *et al.*, 2007). Asymptomatic infection occurring in infants protected by maternally-transferred antibodies is also possible, with the virus failing to establish latency and some individuals thus remaining susceptible later in life.

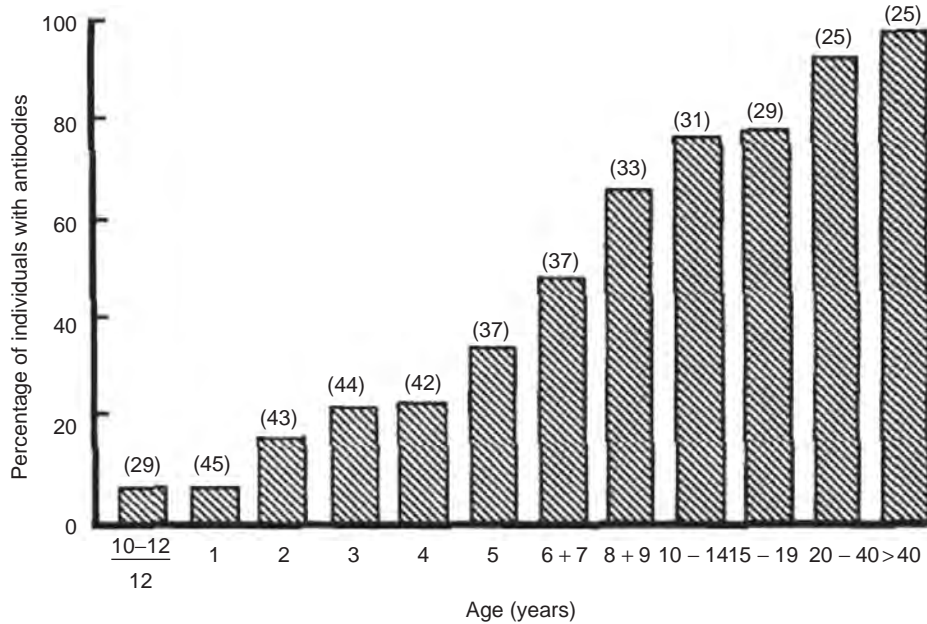


Figure 7.9 Age prevalence of antibodies to VZV. The antibodies were detected by both RIA and indirect immunofluorescence procedures. The numbers of each age group tested are given at the end of the columns. These results were obtained in a collaborative study with Dr J. Cradock-Watson, Withington Hospital, Manchester.

It is important in hospital practice in temperate climates to be aware of the fact that staff who were born in tropical countries are more likely to be susceptible to varicella.

Molecular Epidemiology

The VZV genome is 124 000–125 000 bases long and different viruses have been found to vary by 40–200 bases (Peters *et al.*, 2006). Phylogenetic analysis has shown five distinct genotypes, the nomenclatures of which still have to be agreed. At least three of these strains have arisen by recombination in the past (Norberg *et al.*, 2006). Geographical segregation of strains occurs. Two strains are endemic in countries with populations of European origin and one is endemic in Japan (Muir *et al.*, 2002; Sengupta *et al.*, 2007). There is also evidence that recombination is occurring in areas of population mixing (Barrett-Muir *et al.*, 2002). Genetic typing has been used to prove that the virus causing chickenpox in an individual is the same as that reactivating as zoster some years later, and more recently that re-infection with different strains may occur (Adams *et al.*, 1989; Taha *et al.*, 2006). Genotyping of the Oka vaccine strain has identified changes which distinguish the virus from all wild-type strains. As yet there has not been any evidence for recombination between the vaccine and wild-type strains.

CLINICAL FEATURES

Varicella, the Primary Infection

The incubation period of varicella is approximately 2 weeks but a range of 7–23 days has been quoted. A shortened incubation period occurs particularly in immunocompromised patients. The illness usually commences with the appearance of the rash but occasionally there are prodromal symptoms that resemble an influenza-like illness. These symptoms appear a few days before the rash and are seen more frequently in adults than in children.

The rash is characteristically centripetal in distribution and is seen mainly in areas that are not exposed to pressure, such as the flanks, between the shoulder blades and in the axillae. It is generally sparse in the antecubital and popliteal fossae and is rarely seen on the palms or the soles. This distribution is markedly different from the more centrifugal distribution of the smallpox rash, a distinction which used to be of considerable diagnostic importance. Other differentiating features are that the lesions of smallpox are rounder and deeper than the more superficial and irregular-shaped lesions of varicella.

The skin lesions progress fairly rapidly through the stages of macules and papules to vesicles, which rapidly break down with crust formation. The vesicle with its surrounding area of erythema is the most characteristic

feature of the rash. The lesions appear in a series of crops, so that all stages in their genesis can be seen at any one time. This is very different from smallpox, where the lesions are always at the same stage. Patients with varicella are generally considered to be infectious from a couple of days before the rash until new vesicle formation has ceased and existing vesicles have crusted. This usually occurs five to seven days after onset but may be longer in the immunocompromised. The crusts separate usually within 10 days, revealing healthy skin, but a minor degree of scarring is common. The general constitutional symptoms of the illness are typically mild, particularly in children.

Complications of Varicella

Sepsis Secondary bacterial infection is by far the most common complication of varicella, especially in children, and causes increased scarring of the skin. Streptococci group A and staphylococci are most frequently involved and may be life-threatening in the immunocompromised. Of those children requiring hospitalization for pneumonia associated with VZV, over 30% may have bacterial pathogens.

Viral Pneumonia Symptomatic varicella pneumonia occurs in 1 in 200 000 cases of varicella in children, rising to 1 in 200 in immunocompetent adults, although radiological changes may be found in 10 times this number. In pregnant women the incidence of pneumonitis is increased to 9%, whilst 10% of smokers develop this complication (Mohsen *et al.*, 2001; Parayni *et al.*, 1986). Those most at risk are the immunocompromised, including children with leukaemia, in whom up to 32% develop pneumonitis, with a mortality of up to 25% (Feldmann and Lott, 1987). Adults with malignant disease of the lymphoreticular system who have received organ transplants and patients taking systemic steroids are also at increased risk.

Pneumonia complicating varicella begins one to three (range 1–6) days after the onset of rash and the clinical features include dry cough, dyspnoea and tachypnoea, with chest pain, haemoptysis and cyanosis occurring less frequently. Over 90% of patients with chest symptoms will develop pneumonitis and all such patients should be admitted to hospital for antiviral therapy. Histological examination of an area of lung affected by VZV shows alveoli filled with oedema fluid, a few foamy macrophages and other round cells. The absence of an extensive outpouring of polymorphonuclear leucocytes is a feature that distinguishes this disease from bacterial pneumonia. The presence of multinucleated giant cells with intranuclear inclusions (Figure 7.10) is pathognomonic, although somewhat similar cells are seen in measles pneumonia. It is important to realize that these multinucleated cells are not

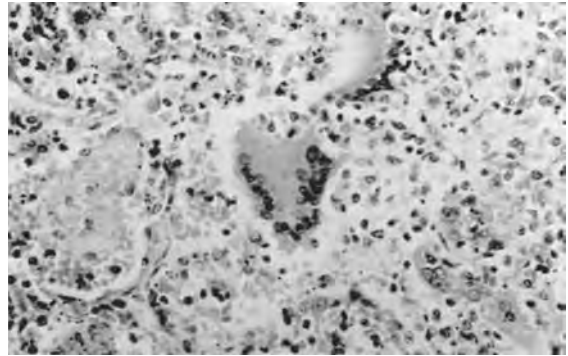


Figure 7.10 Varicella pneumonia. Area of consolidated lung with typical multinucleated cells (H&E stain).

always seen, but large swollen mononuclear cells, similar to cytomegalic cells but without the dominant intranuclear inclusion, are always present. Clinical diagnosis is confirmed by chest X-ray changes and direct immunofluorescence (IF) of vesicle fluid and nasopharyngeal secretions, using a fluorescein-conjugated monoclonal antibody. PCR of vesicle fluid and respiratory secretions and specific IgM antibodies in the serum are also useful.

The illness runs a fulminating course if not treated early with high-dose intravenous aciclovir and is the single most common cause of varicella-associated death in the immunocompromised. Surviving patients may recover completely but others develop fibrosis of the lungs, with permanent respiratory impairment.

Haemorrhagic Chickenpox Haemorrhagic symptoms sometimes occur during the course of varicella and usually make their appearance on the second or third day of the rash. Haemorrhage typically occurs into the skin, but epistaxis, malaena or haematuria may be additional presenting features. The haemorrhage may be so severe as to be life-threatening. This complication is more commonly seen in immunocompromised patients such as renal transplant recipients, in whom a thrombocytopenia and/or consumptive coagulopathy may also develop. Other clinical features in these cases often include hepatitis and gastrointestinal bleeding or distention. Treatment of all serious cases of chickenpox is with high-dose intravenous antivirals (aciclovir or foscarnet, if resistance occurs) and intensive care. Intravenous immunoglobulin, although it has not been formally evaluated, is also commonly used. Steroids may be added in cases of respiratory distress and some benefit may be seen with extracorporeal membrane oxygenation (ECMO).

Encephalitis Varicella meningoencephalitis necessitating admission to hospital occurs in 3–4 cases per 100 000

children and more commonly in adults. Cerebral symptoms are more common in adults, whilst cerebellar ataxia occurs in children. In the majority of cases symptoms begin four to eight days after onset of rash. CSF examination may be normal or reveal a lymphocytosis, with a high cell count and protein level being more likely in encephalitis than in cerebellar disease. Typical cases of encephalitis presenting with headache and vomiting and proceeding to coma are rarely seen but carry a high mortality. By contrast, most patients with cerebellar disease recover fully, although it may take some months. Other neurological disorders, including meningitis, transverse myelitis and Guillain–Barré syndrome, have also been associated with the disease.

As with varicella pneumonia, central nervous system (CNS) involvement occurs much more frequently in immunocompromised patients and is another important factor contributing to the increased mortality of the disease in these patients.

Other Unusual Manifestations and Complications of Varicella As mentioned in the Section “Pathogenesis” above, VZV infection may involve virtually any organ of the body but it is unusual for infection at sites other than the skin and occasionally the lung and brain to manifest clinically. Nevertheless, myocarditis, arthritis, hepatitis and both renal and ureteric damage associated with varicella have been reported.

Reye’s syndrome is a serious and frequently fatal form of encephalopathy which is secondary to liver damage occurring in children with varicella or influenza and is associated with ingestion of aspirin. The incidence of this has fallen with strictures on the use of salicylates in children.

Varicella in Pregnancy

From Figure 7.9 it can be seen that approximately 10% of women of childbearing age are still susceptible to varicella, but this figure may be as high as 20% in communities with a high proportion of immigrants from India, the West Indies, the Far East and Africa. The incidence of varicella in adults aged 15–44 years has been estimated at 3 cases per 1000, which would result in about 2000 cases per year in pregnant women in England and Wales. In areas with immigrant populations from India and Africa, the incidence may be over twice as high. Varicella in pregnancy can present two quite distinct problems, depending on whether the infection is contracted in the early or in the very late stages of the pregnancy. For a review of varicella in pregnancy, see Hanshaw *et al.* (1985).

Varicella in the Early Stages of Pregnancy Congenital varicella syndrome occurs in fewer than 1% of fetuses

Table 7.2 Clinical features of the congenital varicella syndrome

Scarring of the skin
Hypoplasia of the limbs, muscular atrophy, rudimentary digits
Cortical atrophy, psychomotor retardation, choreoretinitis, cataracts

where maternal infection occurs before 13 weeks of gestation, but this rises to 2% if maternal infection occurs between weeks 13 and 20 (Enders *et al.*, 1994; Pastusak *et al.*, 1994). The main features of this syndrome are shown in Table 7.2. Certain defects, such as those involving the brain and eye, are similar to those seen in congenital infections caused by rubella virus and CMV, but other features are quite different. Scarring of the skin, which can be extensive, is a unique feature of the varicella syndrome and indicates that VZV is dermatotropic even *in utero*. Other unique features are hypoplasia of the limbs and rudimentary or even missing digits, indicating that VZV intrauterine infection has a marked propensity for the musculoskeletal system. Gastrointestinal abnormalities are also described. The pathogenesis of the unique lesions of the congenital varicella syndrome is not fully understood but it is probable that they result from fetal zoster following the initial VZV infection. The very short latency period is explained by poorly-developed cell-mediated immunity in the fetus.

All of the reported cases in the literature were seriously affected and less than half of them survived beyond 20 months. In addition, a few cases have been reported in the literature of babies born with severe disseminated varicella acquired as late as the twenty-fifth week of gestation. One case report of congenital varicella following maternal herpes zoster did not exclude the possibility of damage arising as a result of asymptomatic primary maternal infection early in pregnancy.

Varicella in the Late Stages of Pregnancy In common with other viruses (e.g. poliovirus), varicella is known to cross the placenta in the late stages of pregnancy, causing congenital infection of the fetus, and infection acquired in this way can result in the child developing varicella. Varicella occurring within 10 days of delivery is evidence of intrauterine infection. The risk of a child acquiring varicella in this way is dependent on the rapidity with which the mother develops and transfers humoral immunity across the placenta and also the time interval between the date of onset of the rash in the mother and the date of delivery. This period is important because it affects the management of these cases. From numerous clinical observations it has now become consistently apparent that

if the onset of the rash in the mother occurs seven days or more before delivery, sufficient immunity will have been transferred so that, even if infection has occurred *in utero*, it will subsequently be mild or even inapparent. In contrast, if the onset of the mother's rash is six days or less before delivery, the child will be exposed to infection without the protection of maternally-transferred antibody. Young children infected in this way may experience severe fatal disseminated forms of the disease. Case fatality rates of approximately 30% have been reported for untreated neonatal varicella. Also at risk of severe infection are babies born to seronegative mothers who contract varicella in the neonatal period, as no passive immunity will have been transferred from the mother. The administration of varicella zoster immune globulin (VZIG) to attenuate infection (see below) is therefore recommended for all children in contact with varicella within the first 28 days of birth where the mother is seronegative. Infected children born to seropositive mothers are usually protected, although it is worth noting that this protection is not complete, since cases of mild varicella have been reported in babies born to seropositive mothers. The protective efficacy of the maternal antibodies is maximal during the first two months of life. Children born before 32 weeks gestation may not have acquired maternal immunity, even if the mother has antibodies to VZV. In such cases, and indeed in all cases where a prematurely-born infant is exposed to varicella whilst in hospital, it is advisable to test the infant for varicella antibody and administer prophylactic VZIG if it is absent. As stated above, the pathogenesis of varicella is not fully understood. The behaviour of VZV in pregnancy does, however, shed some light on this. First, the fact that intrauterine infection occurs at all provides further evidence that primary infection must have a viraemic phase. Of greater significance is the observation that serious neonatal varicella will occur only if the onset of the maternal rash is within seven days of delivery. This clearly indicates that humoral immunity can affect the course of infection.

Herpes Zoster, the Reactivation Infection

Herpes zoster results from reactivation of latent VZV, which typically affects a single dermatome of the skin. The average annual incidence is 0.2% and it can occur at any age. Over 50% of cases occur in individuals aged over 50 years but the incidence rises sharply from far fewer than 1 per 1000 in the under-50s to 3 per 1000 patient years in those aged over 50 and 8–10 per 1000 in those aged 65 years and above. Zoster is rare in children, although maternal varicella and varicella acquired during infancy have been identified as predisposing factors.

The disease is a result of the virus reactivating in a single sensory ganglion and then proceeding peripherally

down the associated sensory nerve to infect the skin supplied by that nerve. The location of the skin lesions is therefore dependent on the ganglion involved. The ophthalmic branch of the trigeminal nerve, as well as the lower cervical (in children especially), thoracic and lumbar (T5–L2) posterior root ganglia are those that are most frequently involved. The eruption is usually preceded by abnormal sensations and, perhaps, by burning or shooting pains in the involved segment, and the skin may be exquisitely tender to the touch (hyperaesthesia). This is followed by the familiar unilateral 'strap' of vesicles on the trunk. This can be accompanied by muscle weakness in the affected or adjoining limb. The density of the vesicles is very variable, being often sparse in children but densely packed in adults. Definitive involvement of the eye in ophthalmic shingles is signalled by vesicles appearing at the tip of the nose, which is supplied by the nasopharyngeal branch of the nerve (Hutchinson's sign). Where the sacral ganglia may be involved, the skin lesions might be associated with retention of urine or symptoms suggestive of urinary-tract infection, and sometimes there is frank haemorrhagic cystitis. Facial palsy associated with vesicles in the external auditory meatus is known as the Ramsay–Hunt syndrome and is thought to be a form of zoster involving the geniculate ganglion of the seventh nerve. It is often accompanied by hearing loss and vertigo. However, the pathology of this form of zoster is disputed and it is preferable to refer to this disease as 'aural zoster' rather than by its eponymous title or even 'geniculate herpes'. Although VZV reactivation has also been implicated as a cause of facial Bell's-type palsy without vesicles or other symptoms, this association is difficult to prove. A double-blind randomized controlled clinical trial failed to show benefit from aciclovir treatment added to prednisolone (Sullivan *et al.*, 2007), although a study of valaciclovir and prednisolone did improve outcome (Hato *et al.*, 2007). A form-fruste of herpes zoster (*zoster sine herpette*), in which dermatomal pain is present without lesions, has been shown to be associated with high IgG antibody titres to VZV, and VZV DNA is detectable by PCR in circulating lymphocytes (Gilden *et al.*, 1994) and in the nasopharyngeal secretions (Furuta *et al.*, 2001).

As with primary VZV infection, herpes zoster is a far greater problem in immunocompromised patients. In these individuals it frequently occurs earlier in life, and second attacks, which are virtually unknown in the immunocompetent, are seen in up to 30% of HIV-positive patients. Moreover, the disease process is frequently prolonged in immunocompromised patients and they are most likely to experience the 'disseminated' form of zoster. If any patient with zoster is examined carefully it is not uncommon to find isolated vesicles in areas of the skin far away from the main lesion. This means that a minor

degree of dissemination must occur quite frequently. In one study, asymptomatic VZV viraemia was detected in 19% of bone-marrow transplant recipients, using PCR (Wilson *et al.*, 1992). In the immunocompromised patient, however, extensive di-semination of the virus can occur. In some cases visceral involvement without lesions may present as fever with abdominal pain and distention. Diagnosis requires a high index of suspicion and PCR of blood (de Jong *et al.*, 2001). Mortality can be high because treatment with intravenous aciclovir is delayed.

Herpes zoster, unlike varicella, does not usually present a problem during pregnancy. The disease is typically mild in pregnant women. One case of transmission to the fetus has been described; however, from the details, it is not possible to exclude asymptomatic primary infection in the mother earlier on in her pregnancy, with reactivation of virus in both mother and child later on in the pregnancy (West *et al.*, 2006). Herpes zoster near term is not associated with serious neonatal infection, presumably because of the protection afforded by maternal antibodies.

Complications of Herpes Zoster

Infection As with varicella, secondary bacterial infection of the skin can occur and occasionally can lead to impetigo or cellulitis, but with appropriate antibiotic therapy this is rarely a serious problem.

Neuralgia Pain lasting more than four weeks after the onset of the rash has been termed PHN. Since the pain is usually a continuum of the pain occurring during the acute phase, a more modern terminology is zoster-associated pain (ZAP). Prolonged ZAP is the most common complication of zoster, occurring in 15% of cases overall and up to 40% of those aged 60 years and over. Nearly all patients experience severe acute pain at the site of the lesion, although the severity and duration increase with age. The pain may be present before the onset of the rash but in just over 85% of cases it remits within two to three weeks. It may consist of a constant pain, an intermittent stabbing pain or paraesthesia. Worsening of the pain on touch, allodynia, is also characteristic. In some patients severe disabling chronic pain occurs. The cause of the pain is not clear but is associated with ganglionic destruction and scarring with perturbation of type C nociceptor function (Rowbotham and Fields, 1996). Symptoms may be precipitated by temperature change and are often worse at night. Age and, when that is controlled for, severity of pain at the onset of zoster are the factors which most strongly predict severe ZAP. Female gender and ophthalmic location of the rash are also associated with more severe and prolonged pain. Treatment with antiviral drugs will reduce the incidence, duration and severity of ZAP if started within 72 hours of the onset of rash, especially in

those aged over 50 years. The level of active drug, both peak values and total dose (area under the curve), have also been shown to be inversely correlated with severity and duration of ZAP (Beutner *et al.*, 1995) and more recently the presence of virus in the blood at presentation was found to correlate with prolonged ZAP (Quinlivan *et al.*, 2007). PHN is rarely seen in children.

Meningitis Encephalitis and Myelitis Neurological complications due to VZV may be more common than hitherto thought. PCR of CSF has determined that VZV is amongst the most frequently detected viruses in patients with aseptic meningitis. VZV may also cause encephalitis, usually in the presence of lesions but sometimes, in immunosuppressed patients, in the absence of visible rash. Typically the rash, if present, involves the cranial or upper cervical nerves. It is fortunately very rare and little is known about its pathogenesis.

A number of clinical observations have strongly suggested that motor as well as sensory neurons may be involved in cases of herpes zoster. Ptosis associated with ophthalmic zoster and paralysis of the intrinsic muscles of the hand associated with skin lesions of the deltoid region are examples of this. The phenomenon is thought to be due to centripetal spread of the virus from a ganglion into the CNS and thence into a motor neuron. It is possible that facial palsy with aural herpes, referred to above, may be mediated in this way rather than through involvement of the geniculate ganglion. Most of the motor neuropathies are fortunately transient and serious sequelae are rarely seen. Guillain-Barre syndrome and transverse myelitis with ascending paralysis have been reported in small numbers of cases and appear to be more common in HIV-positive patients. A rare but serious complication, particularly associated with ophthalmic zoster, is contralateral hemiparesis. This is caused by a granulomatous inflammatory process in the brain, with infarction of the cerebral arteries.

Ocular The presentation of ophthalmic zoster is complex because of the many structures of the eye and its surrounds which can be involved, such as the eyelid, conjunctiva, sclera, cornea and iris. Consequently the risk of complications is high, even in immunocompetent individuals. The risk of complications is particularly high if the nasociliary branch of the fifth cranial nerve is involved. Iritis and keratitis are the most common complications. Blindness following ophthalmic zoster is, however, rare.

Varicella Retinitis Acute retinal necrosis due to reactivation of VZV has been described, and is characterized by focal, well-demarcated necrotizing retinitis occurring predominantly unilaterally. Treatment with intravenous

aciclovir produces improvement within 48–72 hours and prevents the development of ragged retinal holes and retinal detachment. A similar picture can be produced by HSV, usually in association with encephalitis. The clinical complexities of ophthalmic zoster have been reviewed by Marsh (1976) and Culbertson *et al.*, (1986).

In patients with acquired immunodeficiency syndrome (AIDS), rapidly progressive herpetic retinal necrosis due to VZV infection has been recognized. First described as progressive outer retinal necrosis (PORN), the condition is characterized by outer retinal opacification and absence of inflammatory changes in the eye, bilaterality and multifocality. The signs are rapidly progressive and are distinct from the retinal infiltration and haemorrhages seen in CMV retinitis. Untreated, PORN quickly progresses to bilateral total blindness. VZV is the most common cause of this condition and is diagnosed by PCR of fluid from the anterior chamber. Treatment with intravenous aciclovir or ganciclovir may halt the progression of the lesions but will not usually affect the loss of visual acuity. Foscarnet has also been used, both alone and in combination with a nucleoside analogue, either aciclovir or ganciclovir. Foscarnet and ganciclovir have been given as intravitreal injections.

Zoster and HIV Zoster occurs in up to 30% of patients with HIV infection, and in parts of Africa 85% of patients with zoster aged less than 45 years are HIV-1 positive. Most episodes of zoster occur early in the HIV disease process, before the CD4 cell count has fallen, and tend to be unidermatomal. Zoster occurring after the onset of AIDS is associated with disseminated and multidermatomal zoster. Recurrent zoster is common as the CD4 cell count falls, occurring in up to 30% of patients. Up to 10% of patients will also experience an episode of zoster following the start of antiretroviral therapy. This reactivation can be triggered by immune reconstitution. Some HIV-positive patients may develop a chronic form of zoster with atypical verrucous-like skin lesions, which have been associated with decreased expression of VZV gE and gB (Nikkels *et al.*, 1997).

DIAGNOSIS OF VZV INFECTION

The clinical presentations of both varicella and herpes zoster are usually so typical that laboratory confirmation is rarely required. That notwithstanding, in one series of shingles diagnosed clinically in the community 15% turned out to be due to HSV infection or nonherpetic (Breuer *et al.*, 2001). Where the distinction between HSV infection and herpes zoster is difficult, such as in a generalized vesicular rash occurring in an immunocompromised

patient or where atypical lesions occur, laboratory confirmation should be sought. Similarly, laboratory diagnosis may also be useful for some of the less common CNS and ocular complications affecting immunocompromised patients. The VZV antibody status of an individual is also commonly required, now that treatment and prophylactic measures are readily available and also because of the increasing problem of nosocomial varicella outbreaks which require prompt intervention.

Direct Demonstration Techniques

The main advantage of these techniques is that they are rapid, usually giving results on the same day that the specimen is received.

Electron Microscopy (EM)

Typical herpesvirus particles can be seen in profusion in fluid taken from early vesicles of either varicella or zoster. The particles can also be seen in emulsions of material scraped from the base of lesions. The particles are more difficult to see when the specimens are taken late in the disease. EM unfortunately will not distinguish between VZV and HSV infection unless combined with immunological techniques.

Cytology

Smears or scrapings of the base of the lesions, stained by Papanicolaou's method or, for quickness, methylene blue, will reveal characteristic multinucleated giant cells (Figure 7.11), also known as Tzanck cells. Although not a routine procedure, microscopic examination of biopsies of the lesion will also reveal these giant cells, as well as the other characteristics of the histology which have been described above. Again, neither cytology nor histology will distinguish between HSV- and VZV-induced lesions.

Immunofluorescence Cytology

A more specific diagnosis can be made if IF or immunoperoxidase examination of the smears is carried out. Even cells from crusted lesions contain viral antigen in abundance, allowing easy detection. This method is therefore particularly useful at a time when EM or virus isolation (see below) may not be reliable. Direct detection of VZV in cells scraped from the base of a vesicle is now easily achieved using the FJTC-conjugated monoclonal 3B3 antibody, which is directed against an epitope in gE. This simple method has replaced indirect immunofluorescence.

Detection of Viral DNA

The PCR is more sensitive than IF, EM and culture for the detection of VZV in vesicle fluid. For this reason it is the

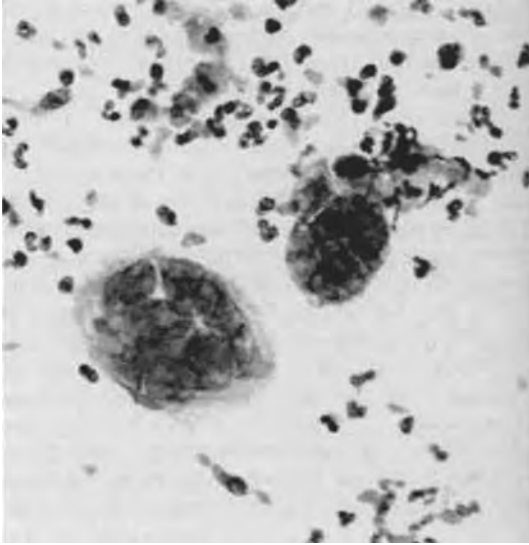


Figure 7.11 Scraping of the base of a lesion of herpes zoster. Note the typical multinucleated cells (Papanicolaou stain).

method of choice where the vesicular viral load is low and a diagnosis is required, for example where the rash is old, following antiviral treatment or where recurrent varicella infection is suspected. PCR of vesicle fluid is also used to confirm verruciform zoster occurring in HIV-positive patients, and has been shown to detect VZV in up to 10% of simplex-like rashes. PCR is most extensively used for the diagnosis of VZV CNS and ocular disease. Using PCR, VZV was the commonest virus (29%) detected in 3231 Finnish patients with suspected meningoencephalitis. This result is borne out by other studies, confirming that VZV is a significant cause of viral meningitis and encephalitis in both immunocompetent and immunocompromised patients. VZV viral load in the blood may be useful for the detection of visceral zoster infection, which may present in immunocompromised patients as abdominal pain in the absence of rash. PCR of vitreous fluid is also used to confirm the diagnosis of ARN and PORN.

More recently, PCR has been used to demonstrate the presence of viral DNA in throat swabs from patients with zoster and zoster *sine herpette* as well as asymptomatic astronauts (Mehta *et al.*, 2004). PCR of vesicle fluid is also the method of choice for differentiating the Oka vaccine virus from wild-type strains in rashes occurring in subjects who have been immunized (Loparev, 2000; Quinlivan, 2005).

Virus Isolation

This remains the gold standard for diagnosing VZV infections; however, isolation of virus is difficult and recovery

rates in most laboratories are generally less than 50%. Human fibroblast cultures are used in most laboratories as they can be maintained for the 21 days required for some isolation.

Vesicle fluids or material swabbed from the base of fresh lesions are the most suitable for isolation attempts. Virus has also been isolated from the respiratory tract of patients with herpes zoster and of asymptomatic astronauts. The most likely explanation for this is that VZV, in common with other herpesviruses, is shed asymptotically, although whether it is infectious remains to be seen.

With virus-containing specimens, the characteristic cytopathic effect of VZV will ultimately be produced (see Figure 7.3). Occasionally, clinical isolates only show a reduced CPE on primary isolation and require 'blind passage' on to fresh fibroblast cultures before the classical CPE develops.

It is possible to increase the speed of isolation by centrifugation-enhanced infection ('shell vial' culture). In addition, immunological staining techniques utilizing monoclonal antibodies can be used to detect viral antigens in cell cultures within 24–48 hours after inoculation, before a cytopathic effect becomes apparent (Figure 7.12).

Identification of Isolates

VZV in tissue culture can be identified by its characteristic CPE and by staining with VZV monoclonal antibodies, which recognize the 3B3 epitope in glycoprotein E (Figure 7.12). There are at least four reports of VZV isolates in which the 3B3 epitope has mutated, abrogating binding of the monoclonal antibody. In each case, the change in the epitope appears to have arisen as an antibody escape mutation. Detection of DNA by PCR can also be used to confirm the identity of VZV.

Serological Diagnosis

A number of different methods are currently available for the serological diagnosis of VZV infection, but perhaps the most important use of this technology is the determination of the immune status of patients prior to the administration of prophylaxis.

The serological diagnosis of varicella using acute and convalescent sera is easily accomplished but is less reliable for herpes zoster. Sera obtained in the early stages of varicella are either devoid of or contain only low levels of specific antibody, whereas these antibodies are present in high titre in sera taken in the convalescent period. Whilst significant rises in antibody titre in paired sera can be demonstrated in cases of zoster, this is generally only possible if the first serum is taken soon after the onset of the rash, for the reason that pre-eruption sera will always contain some specific antibodies and the titres rise very

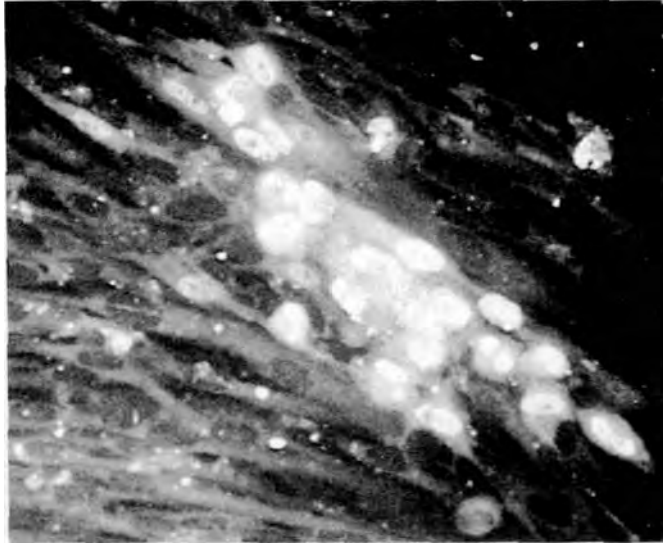


Figure 7.12 Immunological staining technique showing viral antigens in a cell culture.

rapidly after onset. In cases of zoster it is not uncommon to see a drop in the pre-eruption antibody level around the time of onset, so that the antibodies may barely be detectable in sera taken within the first two days after appearance of the rash. Testing for avidity of IgG antibodies provides a means of distinguishing between primary and secondary antibody responses.

The sharing of antigens between VZV and HSV (discussed above) sometimes makes the interpretation of serological results difficult. It is quite frequently found that levels of antibodies to both VZV and HSV will have shown significant rises in association with a particular illness; however, without additional information, such as virus isolation data, it may be impossible to determine which of these viruses was responsible for the infection.

Complement Fixation (CF)

This test is now all but obsolete for the diagnosis of VZV as it is too slow, not sensitive enough for the determination of immune status and more susceptible to cross-reaction between VZV and HSV than other tests.

Immunofluorescence (IF)

This method provides a sensitive determination of serological status to VZV but has now been superseded by enzyme immunoassay (EIA) in most diagnostic laboratories. In IF tests, serial dilutions of sera are reacted with VZV-infected culture cells, and any specific antibodies attaching to these cells are detected with a fluorescein-conjugated anti-human IgG serum. There are

two variants of this basic test. One is the standard IF procedure, in which sera react with acetone-treated culture cells, which is therefore capable, at least theoretically, of detecting antibodies to all virus-induced proteins. This method is no more sensitive than most commercially-produced EIAs. The other is the fluorescent antibody-to-membrane antigen (FAMA) technique, which uses unfixed or glutaraldehyde-fixed cells and is designed to detect only antibodies to viral antigens that appear on the surface of infected cells. FAMA results correlate with the presence of neutralizing antibodies. Moreover, a FAMA titre of >2 has been shown to correlate with both natural and vaccine-induced protection against varicella.

Enzyme Immunoassays (EIAs)

In general, the commercially-available assays for varicella zoster antibodies are less sensitive than FAMA. Currently, commercial tests may fail to detect protective vaccine-induced antibodies in up to 30% of 'immunized' individuals. The gpELISA, developed by Merck, which uses VZV glycoproteins as antigen, is highly sensitive. A level of 5 units or above in the gpELISA at 6 weeks was correlated in one study with protection against breakthrough infection following vaccination. Over 95% of children in this study achieved 5 gpELISA units. In contrast, FAMA testing of vaccinees shows only 78% with levels of >2 . Since post-immunization re-infection occurs in up to 25% of children in the 5 years following immunization, it is likely that FAMA is a better test of protective antibody than a gpELISA reading of 5.

Latex agglutination is more sensitive than most commercially-available EIAs but can be difficult to interpret. Although assays of the indirect solid-phase kind are used most frequently, a competitive-type assay has also been described, which should not, theoretically, be affected by cross-reacting antibodies to HSV.

Neutralization

Theoretically, this should be the method of choice for determining VZV immune status, since it measures antibodies concerned with protection. However, current procedures are technically very difficult to carry out, due mainly to the difficulty of obtaining a consistent supply of challenge virus, since virus infectivity is so highly cell-associated. It is also an insensitive test. For these reasons, it has no role in routine diagnosis. Antibody detected by FAMA correlates well with neutralizing antibody (Grose *et al.*, 1979).

Detection of VZV-specific IgM-class Antibodies

This class of antibody can be detected using the indirect IF or immunoassay procedures, in which the labelled antibody used in the system is directed against human-IgM-class antibodies. Procedures in which the IgM antibodies are captured on to the solid phase (or MACEIA) can also be used. VZV IgM may not initially be detectable in sera from patients with varicella but is present in 100% of convalescent sera, and it can be detected for about three months from the onset of the illness.

Unfortunately it is not possible to use these tests to distinguish reliably between primary and recurrent VZV infection, since specific IgM antibodies are also induced in most cases of herpes zoster. In these patients, however, the amount of IgM antibody is generally lower than that found in varicella cases and is also of shorter duration.

VZV IgM can also be detected in some congenital varicella infections, whereas in others only IgG is detected.

TREATMENT

Varicella

Varicella in healthy individuals is generally mild and complications are rare. Where treatment is indicated, the drug of choice is aciclovir, a nucleoside analogue that blocks viral replication. VZV is less susceptible to aciclovir than is HSV and requires approximately a 10-fold higher concentration of the drug for effective inhibition. The inhibitory concentration (ID50) of aciclovir for VZV in cell

culture is usually in the range of 2–20 μ m, depending on the cell type and virus strain used. It is possible to obtain adequate inhibitory concentrations in the blood if a dose of 10 mg kg⁻¹ (or 500 mg mg⁻² for children aged < 12 years) is given intravenously over a 1 hour period every 8 hours. This dosage maintains plasma levels in the range 10–90 mM. Duration of treatment is normally 5–10 days, depending on the severity and progression of the disease, but a minimum of 7 days is recommended for immunocompromised patients and adults with visceral complications. Oral aciclovir is poorly absorbed and the recommended dose of 800 mg, 5 times a day, will give blood levels of 4 μ m, which are only just at the ID50 concentration for VZV. Studies in children (Dunkle *et al.*, 1991) and adults (Wallace *et al.*, 1992) have shown that antiviral treatment must be started within 24 hours of the onset of rash to be effective. In immunocompetent patients, the duration of rash and fever was reduced by treatment with aciclovir but none of the studies have had the power to show whether treatment reduces the risk of complications. Although in the United Kingdom it is not the practice to treat all healthy adults, this is different from the United States where treatment is advised for adults and adolescents.

The nucleoside analogues famciclovir (Famvir) and valaciclovir (Valtrex) are both better absorbed orally (50–70%) than aciclovir (20%) and are metabolized to produce blood levels of active drug equivalent to intravenous aciclovir. Although not licensed for the treatment of varicella, both are in widespread use for this indication.

A detailed review and recommendations for the management of varicella in different patient groups have recently been prepared by the UK Advisory Group on Chickenpox for the British Society for the Study of Infection (Carrington and McKendrick, 1998).

Varicella in Children

For the typical childhood case no treatment is required apart, perhaps, from soothing lotions for itching and antibiotics if there is any question of secondary infection. A study in Guinea Bissau confirmed anecdotal evidence for more severe infection occurring in second cases (Poulsen *et al.*, 2006) and treatment of such cases, particularly adolescents, is advocated by some, although the current UK consensus is not to use oral aciclovir routinely in healthy children. Children on inhaled or intranasal steroids are not considered to be at special risk but such cases should be considered individually. No great pressure is usually exerted on parents even to quarantine affected children and, indeed, there are some who advocate that it is preferable for children to contract the disease to ensure that immunity is acquired at an early age.

Varicella in Adults

One in 200 adults will develop clinical pneumonitis and approximately 1 in 2000 will require intensive care. Those most at risk include smokers, patients with severe chronic lung conditions and those with more dense rashes. Aciclovir commenced within 24 hours of the onset of symptoms does reduce viral shedding and new lesion formation by 0.5 days, and accelerates rash healing by 1–2 days (Wallace *et al.*, 1992). Pregnant women are also at increased risk of pneumonitis but aciclovir is currently not licensed for use in pregnancy. However, it has been used successfully in numerous pregnant women to treat serious VZV disease without ill effect and there is no evidence to date that it is teratogenic, although it is known to cross the placenta and it can be detected in the urine from infants of mothers who have been treated. It is not known at present whether treatment with aciclovir has any beneficial effect on fetal varicella syndrome. Current UK recommendations are therefore to treat varicella presenting within 24 hours in otherwise healthy adult smokers, patients with chronic lung conditions (including adults on inhaled steroids) and pregnant women, particularly in the second half of pregnancy (Carrington and McKendrick, 1998). Adults presenting more than 24 hours after the onset of the rash should have their clinical progress assessed. Those who appear to be deteriorating, for example with recurrent fever or progressive rash, or who develop chest symptoms or signs, should be admitted for chest X-ray, gases and assessment as to whether they need intravenous aciclovir and antibiotics. For detailed algorithms for the management of varicella in adults, see Wilkins *et al.*, (1998).

Varicella in the Immunocompromised

Varicella in the immunocompromised can be a serious, even fatal, illness and consequently its management in these patients is different from that in a previously healthy individual. Immunocompromised patients, including those on systemic steroids (including for up to three months previously), should be aware of their immune status, and seronegative patients, where possible (that is, in all except those with lymphoreticular malignancies) should be immunized with live attenuated Oka vaccine. Where this is not possible, seronegative patients should be counselled against contact with patients with varicella or zoster as well as being advised to seek medical help immediately if contact occurs. Where the patient has no immunity and significant exposure has occurred, measures should be taken to prevent or attenuate the infection (see below). Significant exposure has been defined arbitrarily by the American Academy of Paediatrics and by the UK Joint Committee on Vaccination and Immunization. Broadly,

there is consensus that ‘significant contact’ constitutes living in the same house as a case of zoster or chickenpox, indoor contact with a case of zoster or chickenpox for a period of time (15 minutes or more in the United Kingdom) and face-to-face contact with a case of chickenpox.

Aciclovir has been shown to be effective in preventing varicella in the immunocompetent if given between seven and nine days post-exposure. Post-exposure aciclovir prophylaxis is not effective if given earlier than seven days, presumably because it interferes with the primary viraemic phase, which primes the specific T-cell responses.

Herpes Zoster

The main aims of therapy in acute herpes zoster occurring in a previously healthy individual are to heal the rash rapidly, alleviate acute pain, prevent PHN and reduce the risks of ophthalmic and neurological complications.

Antiviral Drugs

Several drugs are currently licensed for use in the United Kingdom for treatment of acute shingles, including topical idoxuridine (which is no longer used), aciclovir, famciclovir, valaciclovir and foscarnet. Patients given high-dose aciclovir (800 mg orally, five times day) for acute shingles have been shown, in placebo-controlled studies, to have faster resolution of the rash (by up to two days), less acute pain, reduced viral shedding (by one to three days) and fewer ophthalmic complications. Several studies have also shown a reduction in the incidence, severity and duration of persistent ZAP in those most at risk, that is those over the age of 50 years, when aciclovir is given within 72 hours of the onset of rash. Valaciclovir (a prodrug of aciclovir) and famciclovir (a prodrug of penciclovir) give higher blood levels of the active drug and thus allow easier treatment regimens on account of their improved oral bioavailability. In addition, penciclovir and its derivative, famciclovir, have a significantly longer intracellular half-life compared to aciclovir. A dosing schedule of famciclovir of 250 mg three times per day or valaciclovir 1000 mg t.d.s. are as effective as high-dose aciclovir for treatment of zoster. The newer prodrugs also have the potential to prove more effective than aciclovir in reducing the severity and duration of ZAP (Beutner *et al.*, 1995; Cirelli *et al.*, 1996). Valaciclovir has also been shown to have an effect if given later than 72 hours after the onset of rash.

Other new drugs that have been developed for the treatment of zoster include sorivudine (BVaraU) and brivudin (BVDU). Both these agents are particularly active against VZV when taken orally, and result in accelerated rash healing. BVDU has been licensed in Germany. However,

when administered in combination with 5-fluorouracil, BVaraU has caused the deaths of a number of patients. BVDU is also contraindicated in this situation.

Adjunctive Treatment

Oral prednisolone in addition to aciclovir slightly reduces acute symptoms but does not protect against prolonged ZAP more than aciclovir alone. Oral steroids are generally not used, as the risks are felt to outweigh the benefits. A retrospective case-control study showed that amitriptyline acutely reduced the severity and duration of pain. Other treatments, such as sympathetic nerve blocks, have been reported anecdotally to reduce pain, and require proper trials to assess adequately.

Treatment of Established ZAP

During the acute attack of zoster, paracetamol, with or without mild opiate analgesics, is recommended. Nonsteroidal analgesics are less effective. Low-dose tricyclic antidepressants such as amitriptyline, the dose of which is titrated against side effects and pain relief, can also be given for four to eight weeks, particularly if sleep loss is a problem. The anticonvulsants gabapentin and pregabalin have also been shown to be effective in patients with pain of three months duration or more. Other recommendations for pain relief include ice packs, topical local anaesthetic (e.g. lidocaine patches), capsaicin and, if necessary, stronger opiates. Patients with intractable pain should be referred for specialist pain-management advice.

Ophthalmic Zoster

Management of patients with ophthalmic zoster should include topical aciclovir applied to the eye and oral aciclovir (800 mg five times per day) or valaciclovir (1 g t.d.s.), whatever the patient's age. Treatment should, however, be started as soon as possible to be effective and preferably within 72 hours of onset. Early referral of patients with ophthalmic zoster to an ophthalmologist is desirable and topical steroids should in no case be administered without specialist consultation.

Herpes Zoster in Immunocompromised Patients

The more severe, and particularly the disseminated, forms of herpes zoster are seen in those who are immunocompromised, and may be life- or sight-threatening. Patients at high risk should be educated to recognize shingles and to seek medical advice early. Highly immunocompromised patients should initially receive intravenous aciclovir followed by oral valaciclovir or famciclovir if necessary. Treatment should be continued until the lesions crust,

approximately five to seven days later. Less severely immunocompromised patients with localized shingles can be given oral valaciclovir or famciclovir. Serial measurement of viral load in the blood has proved useful in monitoring response to treatment, although anecdotal evidence suggests that even after clinical response, a low-level viraemia can persist.

Antiviral Drug Resistance

VZV resistance to aciclovir has been described in immunocompromised patients, particularly those infected with HIV. There is currently no evidence that such resistant virus strains are transmissible but they can present a considerable challenge to treatment and are therefore of much concern. Almost all the resistant strains that have been characterized to date have had reduced TK function as a result of mutations in the thymidine kinase gene (Talarico *et al.*, 1993), although some DNA-polymerase mutants have also been found (Kamiyama *et al.*, 2001). Mutations in the TK gene have been identified as either (i) deletions or point mutations leading to a truncated protein, or (ii) single point mutations leading to amino acid substitutions. Mutations involved in resistance to aciclovir have been demonstrated in different positions in the gene and are not restricted to the ABP or nucleotide binding sites on the protein. Some aciclovir-resistant strains have also been shown to be cross-resistant to other TK-dependent drugs, including penciclovir and BVaraU. However, such resistant strains are generally found to be sensitive to foscarnet, a broad-spectrum antiviral drug which directly inhibits viral DNA polymerase and thus provides an alternative treatment. AIDS patients with aciclovir-resistant VZV infections have been successfully treated with foscarnet (40 mg kg⁻¹ every 8 hours in a 1-hour infusion over 10 days) in an open study (Safrin *et al.*, 1991). The nucleotide analogue, cidofovir, is also effective against varicella zoster.

PREVENTION

Prevention of VZV is important in those who are at risk of contracting the severe forms of the disease. An increase in adult susceptibility to varicella (see Section 7.0 above) also has serious implications for hospital infection control, since medical staff without immunity may become infected following contact with zoster patients and can, sometimes with disastrous consequences, transmit the infection to patients who are immunocompromised.

Many immunocompromised patients without pre-existing immunity to VZV are at risk of severe infection

and should be offered prophylactic treatment if in contact with VZV.

Antiviral Drugs

Prophylactic acyclovir or valaciclovir has been shown to reduce the risk of herpes zoster by over 90% in both recipients of solid organ transplant and haemopoietic stem-cell transplants. Oral acyclovir has been shown to prevent or modify varicella in young immunocompetent household contacts given a seven-day course (40 mg kg⁻¹ in divided doses), beginning seven to nine days after the contact with the index case; that is, during the presumed phase of secondary viraemia. Administration within seven days following contact—that is, during primary viraemia—was not as effective, possibly because early priming of T cells is reduced.

Passive Immunization

Human immunoglobulin preparations with high titres of antibody to VZV are an established means of preventing or ameliorating varicella. Such preparations were originally made by cold ethanol precipitation from the blood of patients recovering from shingles and were consequently designated 'zoster immunoglobulin' (ZIG). Current preparations are obtained by processing preselected sera from blood donors with high titres of antibody to VZV. These preparations are referred to as 'varicella zoster immune globulin' (VZIG) or 'human antiviral zoster immunoglobulin'. VZIG preparations are frequently in short supply but antibody concentrations in the blood as high as those with VZIG have been obtained with intravenous normal human immunoglobulin (iv-NHIG) preparations and these may be used empirically as an alternative when VZIG is not available. NHIG given intramuscularly has been shown not to be effective and plays no role in the prevention of varicella.

VZIG is recommended for any susceptible 'at-risk' individual (Table 7.3) who has 'significant' exposure to varicella or herpes zoster. It should be administered as soon as possible after contact, preferably within 96 hours, although some studies have shown VZIG to have a beneficial effect when given up to 10 days after contact. The immune status of the patient should be assessed by testing for specific antibodies in the serum before administration of VZIG whenever possible. Since tests with the appropriate sensitivity (see Section "Diagnosis of VZV Infection" above) are generally only available in specialist laboratories, the administration of VZIG should not be delayed past seven days after the initial contact while waiting for the test result. A convincing history of varicella or zoster is a reasonably reliable indicator of immunity and usually obviates the need to administer prophylactic agents.

Table 7.3 Underlying or associated conditions which place patients at risk of contracting the severe forms of varicella

Leukaemias, Hodgkin's disease and other neoplasms of the lymphoreticular system, whether or not treatment is being given
Other cancers that are being treated with cytotoxic drugs or other regimes that are immunosuppressive
Primary immunodeficiency syndromes
Bone-marrow transplant recipients, irrespective of their own or the donor's VZV status
Diseases requiring systemic steroids at a dosage equivalent to at least 2 mg prednisone/kg/day
Susceptible pregnant women in close contact with VZV
Newborn infants of women who contracted varicella less than seven days before or after delivery
Premature infants whose mothers have no history of varicella or any infant whose birth weight was <1000 g

Nevertheless, it is recommended that an antibody test is performed to confirm the immune status in immunocompromised patients, even if a past history of VZV infection is given. The true efficacy of VZIG has not been established in well-controlled trials and differences in the results of different studies may reflect different potencies of the preparations used. It is known that VZIG gives incomplete protection against infection. In a study carried out in the United Kingdom it was shown that of 27 seronegative children who were in contact at home, 18 (67%) became infected—14 (52%) with symptoms—in spite of receiving VZIG. Therefore, the rationale for administering VZIG to those at risk is not so much to prevent infection but to prevent the serious forms of the disease with visceral involvement. There are numerous studies, based on case series, showing the beneficial effects of VZIG in reducing morbidity and mortality compared to historic controls or untreated patients. Unfortunately, there are also reports of correctly administered VZIG failing to prevent fatal varicella in immunocompromised patients. Feldmann and Lott (1987) reviewed the impact of varicella in 280 children with cancer and the effectiveness of various forms of management. In their study group, passive immunization significantly reduced both the incidence and mortality of pneumonitis compared to untreated children. Even so, pneumonitis developed in 11% of children who received VZIG, requiring intensive additional antiviral chemotherapy.

It is important, particularly in a hospital environment, to be aware of the shortcomings of VZIG and to be alert to the possibility that an inoculated patient might develop varicella and become a source of infection for others.

It is also recommended that VZIG be given to susceptible pregnant women in close contact with VZV infection at any stage of the pregnancy, in the hope that it will reduce the risk of transmission of infection to the fetus and also in order to ameliorate any potentially serious VZV disease which can occur in pregnant women. Maternal varicella can still occur despite VZIG prophylaxis but a large prospective study has shown that even in such cases, the risk of fetal infection during the first 20 weeks of pregnancy, and of subsequent fetal damage, may be reduced (Enders *et al.*, 1994). Should a woman contract varicella perinatally, it is important to administer VZIG to the newborn baby (Table 7.3).

The recommended dosages for VZIG preparations available in the United Kingdom are as follows: 0–5 years, 250 mg; 6–10 years, 500 mg; 11–14 years, 750 mg; and 15 years or more, 1000 mg. A second dose can be given after three weeks if necessary.

Active Immunization

Although individual cases of varicella may be prevented or modified by VZIG or with antiviral drugs, control of varicella in the community can only be achieved by widespread vaccination. Active immunization also has the advantage in individual ‘at-risk’ patients by offering long-term protection. Varicella vaccines based on the attenuated Oka strain of VZV have been available since 1974, when it was first developed in Japan (Takahashi *et al.*, 1974). The original vaccine was derived from VZV isolated from vesicles of a three-year-old child with typical varicella and was attenuated by serial passage in guinea-pig cells and human embryo lung cells. Biologically, vOka grows less well than wild-type virus at 37 °C and better at 33 °C. The Oka vaccine strain has been shown to replicate to lower titres in models of differentiated epithelial tissue (SCID-hu epithelial implants and explant skin cultures) whereas replication in lymphocytes and neuronal tissues appears to be less impaired compared to the parental strain (Moffat *et al.*, 1995). Comparison of the sequence of the vaccine Oka strain and wild-type strains, including parental Oka, has identified approximately 20 amino acid changes unique to vOka. Eight of these are located in the IE62 protein and reduce its ability to transactivate the expression of early VZV proteins (Gomi *et al.*, 2002). Only three mutations, all in ORF62, are fixed in the vaccine strain; the rest are present as mixtures of wild-type and vaccine SNPs. As a result, the Oka vaccine contains a mixture of strains, none of which are genetically identical except at the three fixed SNPs. These in turn allow identification of the vaccine strain which accounts for 60% of the small number of rashes following immunization.

The vaccine is clinically attenuated. The infrequent occurrence of rash is thought to reduce the opportunity for cell-free virus to infect skin sensory nerve endings, which in turn reduces latent infection and zoster due to the vaccine. Where vaccine virus rashes do occur, they appear to be due to selection by the host of a subset of strains within the original vaccine mixture.

Clinical studies in Japan, the United States and Europe have shown the vaccine to be effective; 95% of healthy children seroconvert after one dose of the vaccine, while leukaemic children immunized during maintenance therapy, and healthy adults, require two doses to achieve 90% seroconversion. Clinical protection against subsequent (breakthrough) varicella appears to be about 75%, although protection against severe varicella is 96%. Vaccine failure correlates with length of time since vaccination and with younger age at vaccination. The original estimates, based on gpELISA antibody titres measured at six weeks, of 95% protection are not supported by FAMA, which shows nearer to 75% seroconversion following vaccine. Over 25% of children immunized against VZV become re-infected. Whether this is because of primary vaccine failure rather than waning immunity is still not clear. Fewer than 5% of recipients develop a mild varicella-like rash after vaccination and fever is rare. Genotyping of virus shows that vaccine-related rashes occurring within the first two weeks of immunization are more likely to be wild-type in origin, while those occurring after two weeks are more likely to be vaccine-related. No data are available for the GSK vaccine. In vaccinated leukaemic children, the incidence of adverse events in the first six weeks after immunization is higher than in healthy children, and this, coupled with a reluctance to interrupt chemotherapy for immunization, has led to other approaches to preventing serious varicella in immunosuppressed children. To this end, the vaccine has been licensed for the immunization of seronegative household contacts of children suffering with leukaemia and cancer.

Since 1995 the Oka vaccine has been given to all children in the United States at age 12–15 months. This has resulted in a fall in circulating varicella and a reduction in associated hospitalizations and mortality in both vaccinated and, by herd immunity, unvaccinated individuals (Seward *et al.*, 2002). Other countries, including Germany, Greece Australia and Canada, have since introduced childhood vaccination programmes. In the United Kingdom, considerable doubts about the economic benefits of preventing varicella in healthy children remain, especially if the costs of time lost from work by parents caring for sick children are not included in the analysis (reviewed in Breuer, 2002b). Moreover, modelling of the consequences of vaccination against VZV have shown that a reduction of circulating wild-type chickenpox would

be likely to lead to a reduced boosting of immunity in adults latently infected with VZV and an increase in the incidence of zoster due to wild-type virus. These data are supported by two studies showing that contact with children, a surrogate for contact with varicella, is inversely related to the incidence of zoster (Brisson *et al.*, 2002; Thomas *et al.*, 2002). Such data, when included in economic models, severely prejudice the likelihood of mass vaccination against varicella alone being medically or economically beneficial. As a result, some countries, including the UK, have limited vaccine programmes to vulnerable groups including seronegative health-care workers and seronegative household contacts of immunosuppressed children. Immunization of adolescents and women (post-partum) without a history of chickenpox have also been shown to be cost-effective (Pinot de Moira *et al.*, 2006).

One formulation of the vaccine (Merck) is licensed for post-exposure prophylaxis, however it is not 100% effective.

Vaccines Against Herpes Zoster

A large randomized double-blind case-controlled study of 36 000 adults over 60 years who received the Oka vaccine strain at a higher titre (18 000 pfu) than is present in the varicella vaccine (3000 pfu) showed a 51% reduction in the incidence of herpes zoster and a 66% reduction in PHN in those who received the vaccine. Overall, the burden of disease calculated from the severity of pain and its duration was reduced by 61% in vaccinees. Protection appears to persist, although follow-up has only been for four years so far. Cost-benefit analyses show economic and medical benefit from immunization of subjects aged over 60 years.

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Cytomegalovirus

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INTRODUCTION

Cytomegalovirus (CMV) infections were first described in the early years of the twentieth century, when the typical 'owl's eye' intranuclear inclusions were found by histopathologists in tissues from fetuses stillborn following cytomegalic inclusion disease. These strange inclusions were thought to result from a protozoan infection and, in 1910, one group of workers even proposed the name *Entamoeba mortinatalium* for the supposed agent. In the 1920s, the similarity of the inclusions to those produced by varicella was noted and the guinea-pig form of CMV was transmitted by salivary gland extracts passed through a Berkefeld N filter. Despite these two pieces of evidence suggesting a viral aetiology, reports were still occurring in the late 1940s attributing the disease to a strange protozoan infection. In 1956, three laboratories simultaneously isolated CMV, having successfully developed cell-culture technology, so that the true nature of the infectious agent was apparent. One of these three investigators, Weller, gave the virus its name from the effects produced in cell culture (cytomegaly), so CMV is named after its cytopathic effect (CPE).

THE VIRUS

Morphology

Electron micrographs of CMV reveal a typical herpesvirus appearance (Figure 8.1). The central DNA-containing core is surrounded by a capsid composed of 162 capsomeres, each of which is a hollow hexagon in cross-section. The capsid is in turn surrounded by a poorly-demarcated

area, the tegument, which is itself surrounded by a loosely-applied envelope.

When CMV is propagated in cell cultures, two additional morphological forms are produced from the virus-specific proteins and envelope. The first is termed a dense body and appears as a large amorphous structure without nucleocapsid or DNA. The second has been termed a noninfectious enveloped particle and consists of an empty capsid surrounded by a lipid envelope.

Nucleic Acid

CMV strain AD169 contains 235 kb of double-stranded DNA. The structure of the DNA is similar to that of herpes simplex virus (HSV) in that long and short unique sequences are bounded by terminally repetitive segments. Each long and short sequence can be orientated in one of two directions, so that four DNA isomers are produced by cells in culture. The whole genome of strain AD169 has been sequenced (Chee *et al.*, 1990). The genes are numbered according to their relative positions on one of these four isomers, termed the prototype configuration. Nonpassaged strains contain an additional 22 kb of DNA. By international agreement, the proteins they encode are designated by p (for protein), gp (glycoprotein) or pp (phosphoprotein), followed by the gene number. This formal terminology may then be followed by a trivial name, for example gpUL75 (gH) is glycoprotein H, the product of gene number 75 in the unique long region.

Productively-infected cells produce linear genomes from concatameric precursors. Cleavage is accomplished by an endonuclease (terminase) coincident with packaging. pUL89 is part of this complex, and there are thus similarities between the cleavage/packaging of herpesvirus

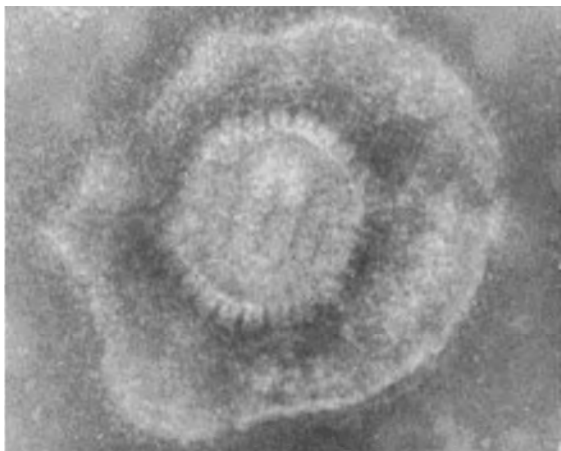


Figure 8.1 The typical electron microscope appearance of cytomegalovirus. (Photograph kindly prepared by Mr J.A. Bishop.)

DNA and that of bacteriophage T4, supported by sequence conservation of the terminase genes. The physical state of the CMV genome during latency in myeloid precursors is episomal.

Some areas of the genome are homologous with regions of human chromosomal DNA, which has practical importance for the selection of CMV DNA probes. Distinct transforming regions of DNA have been identified. One maps to a previously unrecognized open reading frame (ORF), UL111A, which is expressed in transformed cells (reviewed in Doniger *et al.*, 1999). The UL111A protein binds p53 and is spliced to two other exons within UL111A to form another protein, which binds to the cellular IL-10 receptor. It is not known whether the transformation is linked to the expression of the IL-10 homologue (Kotenko *et al.*, 2000). In addition, CMV induces specific breaks in chromosome 1 when infection occurs during the S phase of the cell cycle (reviewed by Fortunato and Spector, 2003). While such experiments are interesting, it must be emphasized that, at present, there is no confirmed evidence that CMV is naturally oncogenic.

The DNA can be digested with restriction endonucleases so that, following gel electrophoresis, oligonucleotide patterns characteristic of distinct CMV strains are produced. This technique cannot prove that two strains are identical, but if strains fail to show different patterns after digestion with at least two restriction endonucleases then it is very likely that they are identical (Huang *et al.*, 1980). Use of restriction enzyme analysis can provide useful epidemiological information, but there is no confirmed evidence to suggest that any one strain is associated with any particular type of clinical presentation (Pignatelli *et al.*, 2004).

Genetic changes also occur in the genome without acquisition of new cleavage sites for commonly used restriction enzymes. The polymerase chain reaction (PCR) followed by sequencing can be used to explore genetic variation at the fine molecular level for particular regions of interest, for example where viral variants acquire resistance to antiviral drugs.

Control of Genome Expression

Expression of the CMV genome is controlled by a cascade synthesis of proteins. The first proteins to be synthesized (α or immediate-early) are required for the transcription of the messenger ribonucleic acid (mRNA) for the second group of proteins (β or early). The early proteins allow DNA replication to proceed and this is followed by the appearance of the last proteins (γ or late). This cascade sequence is depicted in Figure 8.2, which also shows the stages at which metabolic inhibitors can be employed to manipulate the cascade. If cell cultures are prepared and infected with CMV in the presence of an inhibitor of protein synthesis then relatively large concentrations of α -mRNA build up behind the metabolic block. When this block is released by refeeding the cultures, synthesis of α -proteins will occur within minutes. To prevent α -proteins inducing β -mRNA and then β -proteins, inhibitors of transcription should be incorporated into the refeeding medium. To allow β -protein expression without inducing γ -proteins, the cultures can be refed with an inhibitor of DNA synthesis. Finally, fresh medium without added inhibitors can be used to induce the cells to produce γ -proteins. It should be emphasized that this cascade synthesis dictates that at each time after infection the appropriate proteins, together with their preceding proteins, are present in the infected cells. It is therefore not possible to produce cells containing only β -proteins or only γ -proteins by means of infection; to achieve this, individual genes must be cloned and expressed separately.

Note that some γ -genes are transcribed at early times but are only translated after DNA replication has occurred. These are sometimes termed leaky-late genes to differentiate them from true late genes, which are only transcribed after DNA replication. Some latency-associated transcripts which map in both sense and antisense orientation to the major immediate-early (MIE) region of CMV have been described (Kondo *et al.*, 1994). Some of these have unique expression at immediate-early and late times and so have been classified as λ -genes. The term virion RNAs refers to transcripts packaged within the virus which are delivered to an uninfected cell (Bresnahan and Shenk, 2000). It remains to be proven that such RNAs are specifically packaged and play the postulated role of producing viral proteins before the onset of genomic transcription.

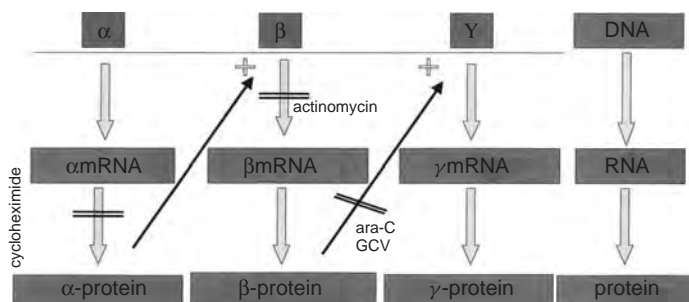


Figure 8.2 Cascade genome expression of herpesviruses. Genes labelled α (immediate-early), β (early) or γ (late) are transcribed into messenger RNA and then translated into proteins. Inhibitors of protein synthesis (cycloheximide), transcription (actinomycin-D) or DNA replication (cytosine arabinoside (ara-C) or ganciclovir (GCV)) can be used to interrupt genome expression, as discussed in the text.

The mechanism(s) which control genome expression are not fully understood. Certainly, there is no evidence for a canonical sequence upstream of β -genes which could be activated directly by α -proteins. This suggests that α -proteins mediate their effects through activation of endogenous transcriptional factors. Cellular transcription factors such as SP1 and NF- κ B have been implicated so far.

Some control of expression is also exerted at the translational level. The DNA polymerase (pUL54) has an untranslated leader region which suppresses translation. Furthermore, the presence of upstream AUG codons, which, according to the ribosome scanning hypothesis, allow production of short peptides in preference to the authentic proteins, appears to restrict early expression of some transcribed leaky-late mRNA.

Proteins

The CMV genome is sufficiently large to encode over 200 proteins of average size, and sequencing of one strain has identified 204 predicted ORFs (Chee *et al.*, 1990). A revision of the total coding capacity suggests that wild-type CMV contains 165 genes (see Dolan *et al.*, 2004 for colour gene map).

One expression unit has been studied in great detail: that encoding the MIE proteins of CMV. As shown in Figure 8.3, this genetic unit is expressed via differential splicing to produce four α -proteins of distinct M_r . The protein of M_r 86 000 (IE86) interacts with the basal transcriptional machinery, especially the TATA binding protein, to enhance formation of pre-initiation complexes. It also cooperates with the M_r 72 000 protein to synergistically increase expression of its own, and heterologous, promoters, probably through activation of endogenous transcription factors and/or by bridging between their binding sites and the TATA binding protein. The IE72

protein (together with pp71) inactivates cellular restriction factors termed Daax and promyelocytic leukaemia (PML) which would otherwise degrade incoming CMV DNA.

The M_r 55 000 protein has minor stimulatory effects, either alone or in combination with either of the other two larger proteins. The smallest protein (M_r 18 000) is expressed during replication in differentiated monocytes.

The largest of these proteins downregulates its own synthesis and so is autoregulated (Stenberg and Stinski, 1985). This downregulation is mediated by a distinct region in the carboxy terminus, which is shown stippled in Figure 8.3. This region is also present in a late protein of M_r 40 000 embedded within this region, and it is likely that activation of its promoter at late times leads to downregulation of the MIE region. IE86 also binds cellular p53, which may decrease p53-induced apoptosis, and interacts with the protein product of UL84, which is a transdominant inhibitor of IE86. Some of the latency-associated transcripts found in the bone marrow of normal donors code for proteins which are recognized by infected humans (Kondo *et al.*, 1994). Their function is obscure but, by analogy with HSV, they may play important roles in the establishment or regulation of the latent state. Expression of the MIE region was found in multiple tissues of transgenic mice containing a *lacZ* gene which correlated well with the cell types in which CMV replication is found *in vivo* (Baskar *et al.*, 1996). Thus, in summary, expression and regulation of the MIE region are remarkably complex, with the potential for exquisite control of virus replication.

Other immediate-early genes are of interest. Genes UL36–37 encode transactivators which are essential for DNA replication. The first exon of UL37 acts as a mitochondrial inhibitor of apoptosis. The UL36 protein is an inhibitor of caspase activation, preventing apoptosis driven by extrinsic death signals. The UL38 protein also

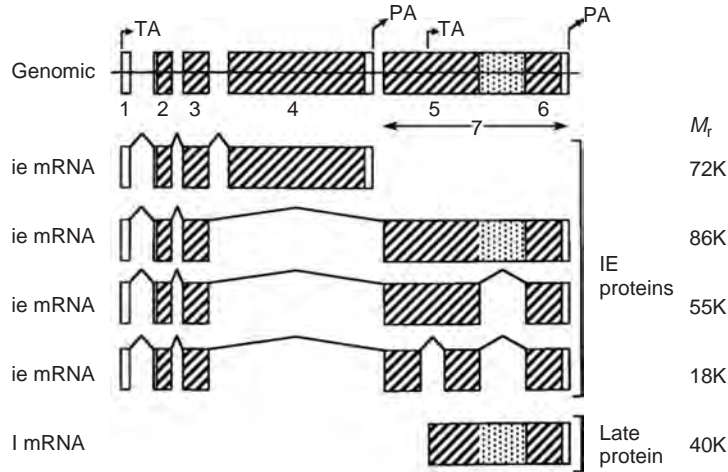


Figure 8.3 Proteins encoded within the major immediate-early region of CMV. Exons are numbered 1–7. TA, transcriptional activation; PA, polyadenylation; ie, immediate-early; I, late.

blocks apoptosis, although the mechanism remains undefined. TRS1 is required for DNA replication. pUL69 is present in the tegument of the virion, as is ppUL83. Although the latter is a late protein, in combination these two proteins appear to play a similar role to the α -transinducing factor of HSV, which is released into the cell during the process of uncoating and is then able to interact with a cellular transcription factor to upregulate the MIE promoter.

Early proteins mainly provide essential enzymic functions within the cell, for example pUL54, DNA polymerase and pUL97, a protein kinase which phosphorylates antiviral nucleosides such as ganciclovir (GCV) and aciclovir (ACV), so starting their anabolism to the functional nucleoside triphosphate inhibitors of pUL54.

Late proteins generally play a structural role in virion formation, for example surface glycoproteins are potentially important because of their interaction with the immune system. Neutralizing epitopes have been described on gB, gH, and gM/gN. gH requires gpUL115 (glycoprotein L) to facilitate surface expression. Much of the neutralizing activity of serum samples can be absorbed by recombinant gB, suggesting that this protein contains dominant neutralizing sites (Britt *et al.*, 1990). gB forms the active component of some vaccine preparations which have reached the stage of Phase II clinical testing.

Note that an alternative nomenclature system terms gB 'glycoprotein complex I' (gCI) and gH 'glycoprotein complex III' (gCIII) (Gretch *et al.*, 1988). The protein products of individual genes which combine to form these complex glycoproteins are summarized in Table 8.1.

A variety of other proteins have been identified and mapped to the genome, important examples of which will

Table 8.1 HCMV envelope glycoproteins

Complex in envelope	Constituent proteins	Mapped ORFs
gCI	gB homodimer	gpUL55
gCII	gM	gpUL100
	gN	gpUL73
gCIII	gH	gpUL75
	gL	gpUL115
	gO	gpUL74

ORF, open reading frame.

be mentioned briefly. A total of 11 proteins are required in *trans* to effect replication (see Table 8.2). Many of these perform similar functions to HSV proteins, except that CMV does not have an identified origin-binding protein and requires transactivators. pUL80A and pUL80.5 encode the protease and assembly protein, respectively. Protease, which is not packaged into the virion, is responsible for cleaving the original polyprotein at four distinct sites. The assembly protein facilitates formation of B-capsids and entry of DNA, being lost from the capsid in the process. Four genes (US27, US28, UL33 and UL78) are homologous to G-protein-coupled receptors and so may be involved in signal transduction. The US28 gene facilitates entry of HIV into CD4⁺ cells, which are otherwise not susceptible to HIV infection (Pleskoff *et al.*, 1997), and also confers chemotactic mobility on arterial smooth muscle cells, which may be relevant to the pathogenesis of atherosclerosis (Streblov *et al.*, 1999).

Note also that 22 extra genes are not present in AD169 but are found in other strains of CMV with a lower

Table 8.2 Eleven loci required for HCMV replication

DNA- <i>pol</i>	UL54
<i>pol</i> -Associated protein	UL44
ssDNA BP	UL57
Helicase-primase	UL70
	UL105
	UL101 102
Transactivators	UL36 37
	IRSI (or TRSI)
	IEI/2
	UL112 113
Binds IE86 protein	UL84

Data from Pari *et al.* (1993).

passage history (Cha *et al.*, 1996). Presumably, they were lost during the process of adapting CMV to grow in fibroblast cell lines which led to the strain termed AD169. This strain is popular with researchers because it grows rapidly to relatively high titre and releases many extracellular virions. Recent results suggest that one of the missing genes (UL138) normally maintains the state of latency, so explaining why AD169 grows relatively well.

Wild strains of CMV lack these properties and so are difficult to work with in the laboratory. As a result, one has to question whether AD169 is fully representative of CMV strains *in vivo*, and the presumed loss of these 22 genes illustrates this issue. None of the 22 genes has homology with known herpesvirus proteins. Many appear to encode glycoproteins, and their potential roles in evading host immune responses will be discussed later.

Growth *In Vitro*

The only cells which replicate CMV to high titre *in vitro* are human fibroblasts, although wild strains can also be propagated in endothelial cells, macrophages and smooth muscle cells. This finding is in complete contrast to that *in vivo*, where, at post mortem, cells infected with CMV are found in organs of epithelial origin (kidney, liver, bile ducts, salivary gland, gut epithelium, lung parenchyma, pancreas) as well as in endothelial cells. This observation again suggests that the virus propagated in the laboratory should not be assumed to be an authentic model of the wild-type virus. The genetic changes which confer tropism for endothelial cells and for polymorphonuclear leukocytes map to UL128, UL130 and UL131A. The cell surface proteins which act as receptors for CMV have not been confirmed, although several candidates have been proposed.

In fibroblast cell cultures, encapsidation occurs in the nucleus. The products of genes UL50 and UL53 combine

to digest the structural lamins which form the inner nuclear membrane (Muranyi *et al.*, 2002) and the virus envelope is then acquired by budding through this membrane. Enveloped virions are found within vesicles in the cytoplasm and these appear to fuse with cellular membranes to allow egress of the mature virus particles. Dense bodies also mature and are released from the infected cell in the same way as virions, so that they contain virus-specific glycoproteins.

At late times after infection, CMV induces the appearance in infected fibroblasts of an Fc receptor which has high affinity for human IgG but not other human immunoglobulin isotypes, and has low affinity for rabbit or mouse IgG (Keller *et al.*, 1976). One Fc receptor is encoded by gene TRL11 and its duplicate ILR11, while a second is formed by a UL119–UL118 fusion protein. The Fc receptor is found in the Golgi apparatus, which enlarges to form a perinuclear inclusion body in the concavity of the reniform nucleus. It has been suggested that HSV produces an Fc receptor so that antibody attached by its Fab portion to a virus protein can be bound by its Fc portion back on to the virion. This would have the effect of preventing immune effector mechanisms, which require an intact Fc portion after opsonization. Whether such a mechanism is operative for CMV remains to be defined. However, the production by CMV of Fc receptors *in vivo* might allow opsonized bacteria or fungi to gain access to cells which they cannot normally infect; this might explain why CMV infection is often associated with secondary bacterial and fungal infections. A similar process might operate for HIV coated in nonneutralizing antibody.

Unlike HSV, CMV does not switch off host macromolecular synthesis but actually stimulates cellular DNA, RNA and protein synthesis. The overall effect is to push the cell towards the S phase of the cell cycle without allowing cell division to occur. One cellular function which is stimulated as a result is thymidine kinase activity. It is tempting to suggest that CMV has learned to increase cellular uptake of thymidine by switching on the host enzyme responsible, whilst HSV has used a different tactic, the production of a novel thymidine kinase, to achieve the same objective. Likewise, CMV induces cellular topoisomerase II and may package this enzyme into the extracellular virions. Recent results have also shown that human complement is bound to the virion but not activated. Host-cell-complement regulatory proteins were detected in virions and might explain this phenomenon (Spiller *et al.*, 1997). Thus, like HIV, the mature virion may contain host proteins of potential importance for understanding pathogenesis, and proteomic experiments support this (Vamum *et al.*, 2004).

EPIDEMIOLOGY

CMV must be acknowledged as one of the most successful human parasites. It has learned to survive in its human host by infecting both vertically and horizontally; the virus can be transmitted by either route during primary infection, re-infection or reactivation; at all times the virus causes minimal disability, allowing infected individuals to remain active and so maintain the maximum opportunity of encountering susceptible contacts; the virus is excreted from multiple sites, so contact of varying degrees of intimacy can lead to transmission.

Infection may be acquired during delivery, following ingestion of infected maternal genital secretions, or soon afterwards by ingestion of breast milk containing CMV (Schleiss, 2006). These two means of prenatal transmission combine to infect 2–10% of infants by the age of six months in all parts of the world.

Throughout the rest of childhood, close contact is known to be required for transmission, although the precise route of infection is not known. As a result, CMV may transmit readily within family groups. The possibilities for infection must be increased where individuals are crowded together in unhygienic circumstances, and this probably explains why CMV infection is most common

in societies which are socially disadvantaged. Infection is transmitted less well in the general community, apart from child-to-child transmission, which has been documented in playgroups (Pass *et al.*, 1986). Once infected, such children can transmit CMV to their parents (Pass *et al.*, 1986) and so represent a potential threat to a future sibling should the mother be pregnant.

In populations of poor socio-economic background, the vast majority of children have experienced primary CMV infection by the onset of puberty. In countries with good social circumstances, roughly 40% of adolescents have been infected, and, as shown in Figure 8.4, seroprevalence increases by approximately 1% per year thereafter (Griffiths and Baboonian, 1984). Such primary infection can lead to vertical transmission if an individual is pregnant when she becomes infected.

The prevalence of CMV IgG antibodies in organ transplant recipients reflects their socio-economic grouping. The same applies to individuals who acquired HIV infection via blood (or blood products), heterosexually or through contaminated needles used for intravenous drug use. In contrast, HIV-positive male homosexuals have a very high prevalence of CMV IgG antibodies (typically 95%).

At whatever age primary infection occurs, the virus is not eradicated from the host but persists for the rest of the

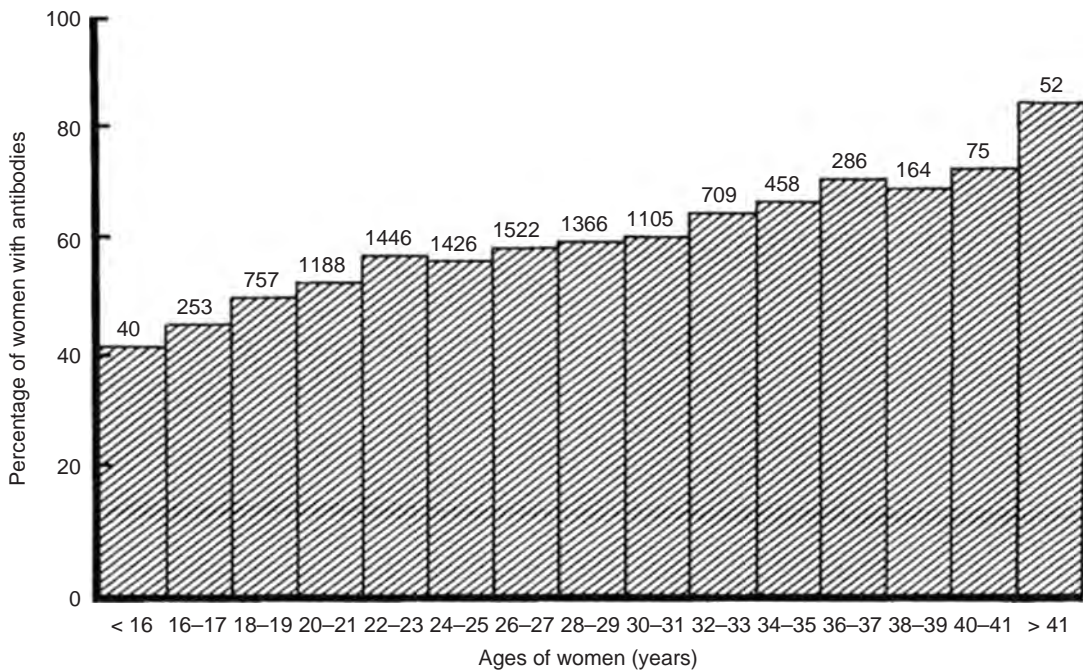


Figure 8.4 The age-specific prevalence of complement-fixing serum antibodies against cytomegalovirus. The number of women in each group is shown above each column of the histogram. (Reproduced by permission from Griffiths and Baboonian (1984).)

life of the individual. Occasionally, however, CMV reactivates from its latent state and infectious virions appear in the saliva and/or urine. These reactivations of CMV are entirely asymptomatic but form an important means by which CMV can spread horizontally. Immune hosts can also be re-infected with another or, possibly, the same strain of CMV. Epidemiologically, it is important to distinguish between re-infection and reactivation of latent infection, but in clinical practice the term 'recurrent infection' is often used to cover both possibilities. Recurrent infections can also lead to vertical transmission of CMV. This finding came as something of a surprise, since it was assumed that a woman who possessed antibody against CMV before becoming pregnant would be immune to intrauterine transmission. A recent population-based study reports that natural maternal immunity present prior to conception provides 69% protection against delivering a subsequent neonate with congenital CMV infection (Fowler *et al.*, 2003). While this protection is substantial, the high prevalence of seropositive women means that more fetuses are infected worldwide by this route than are infected as a result of primary maternal infection. Congenital CMV infection thus has its highest incidence in the poorest communities of the world, since most women in poor societies are infected before reaching childbearing age. Note, however, that primary CMV infection in the mother represents a greater risk to the fetus than recurrent maternal infection (Fowler *et al.*, 1992), so the burden of congenital disease is greatest in developed countries.

ROUTES OF INFECTION

Intrauterine Infection

As is the case with rubella, intrauterine infection is assumed to follow maternal viraemia and subsequent placental infection, although this has not been proved formally. Due to the lack of maternal illness it has not been possible to identify a series of pregnant women with primary CMV infection and show that viraemia is a risk factor for congenital infection. If viraemia is responsible for transmission then it must be determined whether cell-free virus or leukocyte-associated virus is required for placental infection. Intrauterine transmission of CMV occurs in only one third of pregnant women with primary infection but we remain ignorant of how the majority prevent the virus from infecting the fetus. It may be that the placenta acts as a form of barrier, but representation of this organ as a sieve which might or might not trap CMV must surely be simplistic. Ultrastructural studies of the placenta emphasize the importance of macrophage-mediated defence against potential virus infections. There is also the

possibility that the placental Fc receptor may be able to bind CMV coated in nonneutralizing antibody and facilitate its transfer to the fetus (Maidji *et al.*, 2006).

Perinatal Infection

Perinatal infection is acquired predominantly from one of two sites: infected maternal genital secretions or breast milk. During delivery, the fetus is surrounded by copious quantities of genital secretions, which may contain high titres of CMV as a result of recurrent maternal infection. Under these conditions, infection has been described as occurring 'during passage through the sea of cytomegalovirus'.

Breast milk, especially colostrum, has also been shown to be a good source of CMV. Although virus titres are relatively low, large quantities of milk are imbibed, so a heavy viral inoculum can be ingested. Clinical studies have demonstrated that it is not just the presence of CMV in breast milk which is required but that this milk must also be fed. Women whose only site of CMV excretion was from the breast were studied and perinatal infection occurred only when breast-feeding took place, not when such women gave formula feeds (Stagno *et al.*, 1980). Having ingested CMV, infection might be established in the neonate by infection of buccal, pharyngeal, respiratory, salivary gland or oesophageal mucosa.

Postnatal Infection

The absence of symptoms associated with postnatal CMV infection makes it impossible to implicate with certainty the routes of transmission, although evidence exists to support salivary transmission.

Saliva containing CMV has been recovered from toys at day-care centres and this would seem to be an ideal means by which the virus could be transmitted among young children unable to conform to basic standards of hygiene (Pass *et al.*, 1986). Likewise, occasional cases of CMV mononucleosis are seen in young adults and, by analogy with Epstein-Barr virus (EBV), the infection has been dubbed a 'kissing disease' (see Chapter 9).

The prevalence of CMV IgG antibodies in developed countries increases at 1% per year from puberty to middle age (see Figure 8.4). It is often stated that these infections result from sexual exposure but this remains unproven. Certainly they result from contact with an infected individual, but whether this contact takes place at the level of oral or genital mucosa is a matter of speculation. Evidence can be found to support the concept of venereal transmission, since CMV is found in semen and on the cervix. However, this is only circumstantial evidence; we do not talk of brain-to-brain transmission of poliomyelitis or urinary transmission of mumps just because these viruses

may be found at particular sites. Sexual contact is almost invariably preceded by oral-to-oral contact. Thus, even if CMV is shown to be transmitted by intimate contact, it may have resulted from salivary rather than from venereal exposure. This issue is important because we may need in the future to target CMV vaccines to particular mucosal sites in order to prevent infection, and parents may be less reluctant to vaccinate their adolescent children against a 'kissing disease' than against a perceived 'sexually transmitted' infection.

One setting in which sexual transmission of CMV does occur, however, is between male homosexuals, who have a high prevalence of CMV infection, and those who are initially seronegative run a high risk of primary infection during follow-up. At least one study has implicated rectal intercourse as an independent risk factor for CMV seroconversion, implying that the rectal mucosa provides less of a barrier to the high titres of virus found in semen than is provided by the stratified squamous epithelium of the vagina.

Blood Transfusion

In the early 1960s, when extracorporeal blood perfusion was introduced to facilitate open-heart surgery, a syndrome of leukopenia, pyrexia and atypical leukocytosis was recognized which was termed the post-perfusion syndrome. In the mid-1960s, Finnish workers showed that the syndrome was attributable to primary CMV infection acquired by fresh blood transfusion.

Although it has been established that CMV can be transmitted by blood transfusion, it is clear that this is an uncommon event, since only 1–5% of blood units taken from seropositive donors lead to infection of seronegative recipients. To date, it has not been possible to determine which donors have a high risk of transmitting the virus. It is presumed that the virus exists in the blood of healthy donors in a latent state within monocytes (Soderberg-Naucler *et al.*, 1997) and that CMV is reactivated following transfusion when these cells encounter an allogeneic stimulus (reviewed in Roback, 2002). In contrast, CMV can be grown from the peripheral blood of immunocompromised patients and pp65 antigen is found in polymorphonuclear leukocytes and macrophages. However, this is clearly a different pathogenetic situation from that found in healthy blood donors and could be the result of the phagocytic scavenging activity of these cells.

Transmission via blood products has been virtually eliminated by the routine use of filters to remove leukocytes during transfusion.

Organ Transplantation

Several studies have shown that seronegative patients undergoing renal transplantation can be divided into two

risk groups according to the serological status of the donor. Those receiving a kidney from a seronegative donor have a virtually zero risk of acquiring primary infection, whereas a seropositive kidney may transmit the virus in 60–80% of cases. Molecular typing of CMV strains excreted by multiple recipients of organs from a single donor proved that the donor organ was the source of CMV (Wertheim *et al.*, 1983). Since both organs from a single donor are usually concordant for transmission, the infectivity must be bilateral; either parenchymal cells or infiltrating leukocytes are prime suspects. Interestingly, the same techniques showed that donor virus could also infect seropositive individuals and cause CMV disease (Grundy *et al.*, 1988). Thus, recipient natural immunity acquired prior to immunosuppression cannot prevent but may alleviate CMV infection, a finding which has implications for the development and evaluation of CMV vaccines. Several studies have shown the same results following all types of solid organ transplant, so all organs from seropositive donors should be regarded as potentially infectious.

In contrast, typing of virus strains showed that the virus causing disease after bone marrow transplant is derived from the recipient and not from the donor (Winston *et al.*, 1985). Seropositive donors have been reported to adoptively transfer immunity to recipients (Grob *et al.*, 1987). This has only been reported in recipients of T-cell-depleted marrow, so it is tempting to speculate that this process removes the cells containing CMV while leaving intact immunocommitted non-T cells, which can function in the recipient.

PATHOGENESIS

Risk Factors for CMV Disease

Comparison of the results from pregnancy, from recipients of solid organ transplants, from bone marrow transplants and from patients with HIV infection reveal some interesting parallels, despite the different organs involved in CMV disease (Table 8.3). Thus, primary infection is a

Table 8.3 Risk factors for CMV disease

Factor	Pregnancy	Solid transplant	Bone marrow transplant	AIDS
Primary infection	+	+		
Viraemia	No data	+	+	+
Increased load	+	+	+	+

+, factor that has been shown to correlate with risk of CMV disease.

Table 8.4 Univariate and multivariate assessment of prognostic variables for CMV disease after renal transplant

Parameter	Univariate			Multivariate		
	OR	95% CI	<i>p</i>	OR	95% CI	<i>p</i>
Viral load (per 0.25 log)	2.79	(1.22–6.39)	0.02	2.77	(1.07–7.18)	0.04
Viraemia	23.75	(3.69–153)	0.0009	34.54	(0.75–1599)	0.07
Recipient seropositive	0.22	(0.05–0.95)	0.05	0.92	(0.002–446)	0.98

OR, odds ratio; CI, confidence interval. (Reproduced by permission from Cope *et al.* (1997b).)

major risk factor during pregnancy and for recipients of solid organ transplants but not for bone marrow transplant or AIDS patients. Viraemia has been repeatedly shown to be a risk factor following solid organ or bone marrow transplantation (Meyers *et al.*, 1990) and the same is true for AIDS patients (Bowen *et al.*, 1997). A high CMV virus load was initially shown to be important in neonates with congenital infection (Stagno *et al.*, 1975b) and more recently in renal transplant (Cope *et al.*, 1997b), liver transplant (Cope *et al.*, 1997a), bone marrow transplant (Gor *et al.*, 1998) and AIDS patients (Bowen *et al.*, 1996). Furthermore, multivariate statistical analyses show that, for renal transplant patients, once virus load in urine has been controlled for as a marker of poor prognosis, the other recognized risk factors of viraemia and donor/recipient serostatus are no longer statistically associated with CMV disease (Table 8.4). This demonstrates that high CMV load is the determinant of CMV disease and that viraemia and donor/recipient serostatus are markers of CMV disease, simply because of their statistical association with a high virus load. Similar multivariate studies in liver transplant and bone marrow patients confirm that high CMV load in blood explains the association with donor/recipient serostatus (Cope *et al.*, 1997a; Gor *et al.*, 1998). Furthermore, in all groups of transplant patients, a threshold association with CMV disease is apparent (see Figure 8.5) showing that low viral loads are tolerated by the host.

All of these results provide insight into the pathogenic stages leading to CMV disease. Figure 8.6 illustrates the concepts, using the flow of water from a tap into a bath with an open drain. This is analogous to virus-infected cells at a peripheral tissue (such as kidney or salivary gland) producing CMV virions. Their number may be controlled by local immune responses (drain), but if these are inadequate the number of virions will increase. If they overwhelm the local immune responses, virions will overflow into the systemic circulation, causing viraemia. The same process is then repeated in the target tissue (e.g. liver, retina), whose local immune responses may be able to prevent virus load reaching the critical levels required to cause disease. This explains why viraemia is a strong predictor of CMV disease, but does not guarantee that

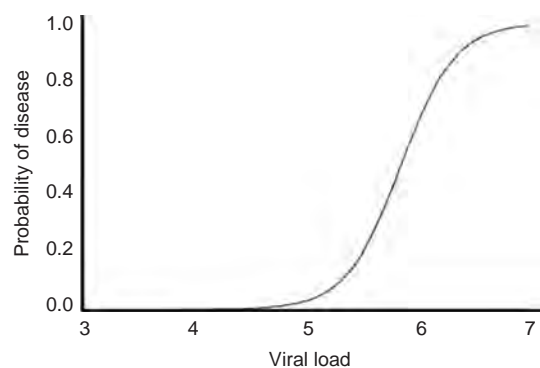


Figure 8.5 The threshold concept: non-linear relationship between increasing viral load in urine and the risk of CMV disease. (Reproduced by permission from Cope *et al.* (1997b).)

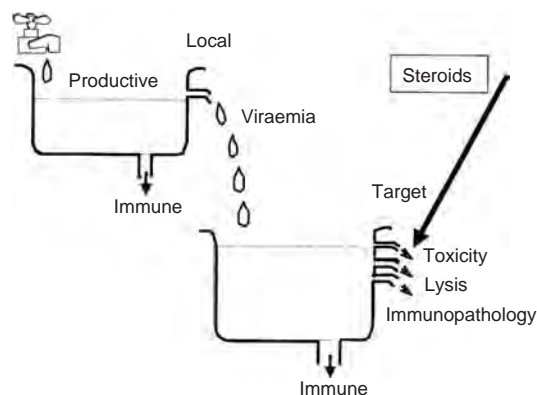


Figure 8.6 Cartoon illustrating the kinetics of CMV production leading to disease in a target organ. The tap represents production of virions by virus-infected cells. The drain represents the ability of local immune responses to control CMV accumulation.

it will occur. Finally, the model suggests that different cellular mechanisms of pathogenesis may be activated at different virus loads. Thus, immunopathology may be triggered by low virus loads, while high virus loads

may be required to damage sufficient cells by lysis to cause clinically recognized disease. Speculatively, some disease may be produced when cells are bombarded with very high virus loads without requiring replication, for example, binding of gB and gH can activate release of transcription factors (Yurochko *et al.*, 1997).

Based on the model in Figure 8.6, it has been possible to conduct clinicopathological studies to define the dynamics of CMV replication (Emery *et al.*, 1999). Remarkably, serial measures of viral load in blood show that CMV replicates with rapid dynamics, giving a doubling time (viral load on the increase) or half-life (viral load on the decrease) of approximately one day. This information has been used to predict the emergence of resistant strains of CMV during prolonged GCV treatment and explain why conventional cell culture assays frequently fail to detect this (Emery and Griffiths, 2000). The perspective of CMV replication and pathogenesis given by this application of modern molecular biology highlights the misleading impressions given about CMV when studied by fibroblast cell cultures (see Table 8.5).

Incubation Periods

Examination of three settings in which the date of infection and date of onset of virus excretion can be reliably predicted gives an estimate of four to eight weeks for the 'incubation period' of primary infection. The three informative clinical settings are where infection is acquired perinatally, where it is transmitted by organ allograft and where it is transmitted by blood transfusion.

In contrast, it is not clear whether congenital infection involves a fetal incubation period as well as a maternal one. Maternal seroconversion at different stages of pregnancy has been documented in several studies, with gestational stage recorded as the duration of pregnancy at which maternal seroconversion occurred. However, even if it is assumed that the placenta is infected following maternal viraemia at the time of seroconversion, it is not known whether the fetus can be infected immediately or whether replication in the placenta provides an

intrauterine incubation period before viral dissemination to the fetus can occur. This point is important, as the damage to the fetus should be described according to its developmental maturity when infected rather than the length of the mother's amenorrhoea; this has implications for the timing of diagnostic amniocentesis (see later).

Allograft recipients typically have recurrent CMV infections in the second month following transplantation. Studies of pregnant and nonpregnant women as well as male homosexuals have shown that up to 10% of seropositive individuals may be excreting CMV from saliva or urine, or from the cervix in females. Excretion rates are, however, very low after the age of 30 years, suggesting that a host response required for suppression may 'mature' at about this age.

Earlier reports showed that virus excretion from the cervix increased as pregnancy progressed and this was interpreted as being a response to some 'immunosuppressive' effect of pregnancy. A single later study, however, showed that CMV excretion was actually suppressed during early pregnancy, so the increase seen in virus isolation towards the end of pregnancy only brought the rate up to the level seen in nonpregnant women (Stagno *et al.*, 1975a). All studies to date have been cross-sectional rather than longitudinal, so person-to-person differences in CMV excretion could account for these results and confirmation is required before definitive conclusions can be reached.

Host Defences

The defences mounted by the host against different types of CMV infection will be outlined. In combination, these responses in people with normal immunity keep CMV suppressed into a latent state in most individuals for most of the time. Abrogation of these responses permits CMV replication, with full expression of its potential pathogenicity in some cases. Since severe CMV disease is restricted to individuals with impaired cell-mediated immunity (CMI), it can be concluded that this arm of the immune response provides most protection against

Table 8.5 Misleading impressions about CMV

Impression	Fact
<i>In vitro</i> strains represent those found <i>in vivo</i>	AD169 strain has 22 missing ORFs; Towne strain has 19
Live attenuated vaccine can be prepared in fibroblasts	No protection against CMV infection from Towne, but decreased severity of disease
<i>In vitro</i> assays correctly identify susceptibility of CMV to antivirals	Failed to detect clinically-important susceptibility to ACV
CMV is a slowly-replicating virus	CMV replicates rapidly
GCV-resistant strains occur infrequently in immunocompromised patients	Resistance is more common. Cell cultures select against detection of resistant strains

disease. Nevertheless, several lines of evidence suggest that humoral immunity may contribute towards control of CMV: (i) randomized trials of prophylactic CMV immunoglobulin report that CMV disease was reduced compared to patients receiving placebo; (ii) neonates who acquire CMV from blood products have reduced disease if they are born to seropositive women, showing that transplacental antibody from their mothers protects against disease (but not infection); (iii) results with rituximab, a humanized mouse monoclonal reactive against CD20, report severe CMV disease secondary to profound B-cell immunodeficiency.

Humoral Immunity

Antibodies of IgG class are produced promptly at the time of primary infection and persist for life. IgM-class antibodies are produced on primary but not recurrent infection of immunocompetent individuals and persist for three to four months. Some immunocompetent individuals may have IgM antibodies detectable by sensitive assays during recurrent infections. Immunocompromised patients may fail to produce IgM antibodies with primary infection and one third of them have IgM detectable with recurrent infections. With intrauterine infection, IgM antibodies are produced by the fetus, together with an IgG-class response, which only becomes detectable as passively-acquired maternal IgG antibody is catabolized.

Intrauterine CMV infection represents less of a risk to a fetus if it has been transmitted by means of recurrent maternal rather than primary maternal infection (Fowler *et al.*, 1992). Women with primary CMV infection who transmit the virus *in utero* have higher levels of total IgG but lower levels of neutralizing antibodies and lower avidity than women who do not transmit (Boppana and Britt, 1995). While all of this information is compatible with the concept that immune responsiveness can be protective, it does not prove that antibody is the beneficial component. For example, cytotoxic T cells may be protective and the ability to mount this response promptly may correlate with the ability to mount a humoral immune response. Likewise, the postulated humoral defect in women experiencing primary infection during pregnancy may be a relative failure of T helper responses rather than of B cells.

There is evidence to show that enhanced humoral immune responsiveness in the fetus correlates with poor prognosis. Cord blood levels of specific IgM, total IgM and rheumatoid factor are positively correlated with symptomatic rather than asymptomatic congenital infection. This effect has been shown to be independent of virus titre at birth and so is not simply secondary to a high virus load in symptomatic infants. Although this suggests that fetal antibody is responsible for immunopathology, it

could equally well be that there is another response of the fetus which is damaging and which indirectly correlates with the humoral immune response.

The beneficial or adverse effects of humoral immunity could be discerned better if reactivity against a particular virus-coded protein could be shown to correlate with prognosis. Many CMV proteins are recognized by the humoral immune system and work to date has not been able to identify such a pattern in congenitally- and perinatally-infected infants. The ability of serial sera to immunoprecipitate radiolabelled virus-coded proteins has simply shown that symptomatic infants are more likely to have reacted to multiple virus proteins and that their sera will precipitate more of any given virus protein than will asymptomatic infants. These results are therefore compatible with heightened immune responsiveness leading to disease production.

Cell-mediated Immunity

For studies of CMI, the lymphocyte blastogenic response to CMV antigen has mainly been used, supplemented recently with tetramer assays. Most seropositive adults have a positive test result, with a surprisingly high proportion of total CD8 cells (e.g. 1%) recognizing tetramers containing epitopes from ppUL83 (pp65). This proportion is suppressed for at least six months in transplant recipients, in direct correlation with the dose of immunosuppressive drugs received (Hassan-Walker *et al.*, 2001).

The lymphocyte blastogenic response is suppressed in congenitally- or perinatally-infected infants. This failure recovers with time and there is a direct correlation between cessation of viruria and acquisition of immune responsiveness at three to five years of age (Pass *et al.*, 1983). The defect is known not to be one of generalized T-cell immunosuppression, for three reasons. First, CMV-infected infants who also acquire HSV infections generally mount good responses against HSV but not against CMV. Second, these infants respond normally to both killed and live vaccines. Third, the suppressor and helper T-cell lymphocyte counts remain normal.

Recent work, summarized in Figure 8.7, has identified complex but plausible mechanisms which, in combination, allow the CMV-infected cell to evade lysis by T cytotoxic, macrophage and natural killer (NK) cells. Immediately after uncoating, the tegument protein ppUL83 (pp65) is available in the cell to phosphorylate the IE72 isoform of the MIE protein and so prevent its presentation as a target epitope. At immediate-early times, pUS3 retains Class I human leukocyte antigen (HLA) molecules within the endoplasmic reticulum (ER). Once the virus-infected cell has moved into the early phase of CMV gene expression, pUS2 and pUS11 act to re-export Class I HLA molecules back from the lumen of the ER into the cytoplasm, where

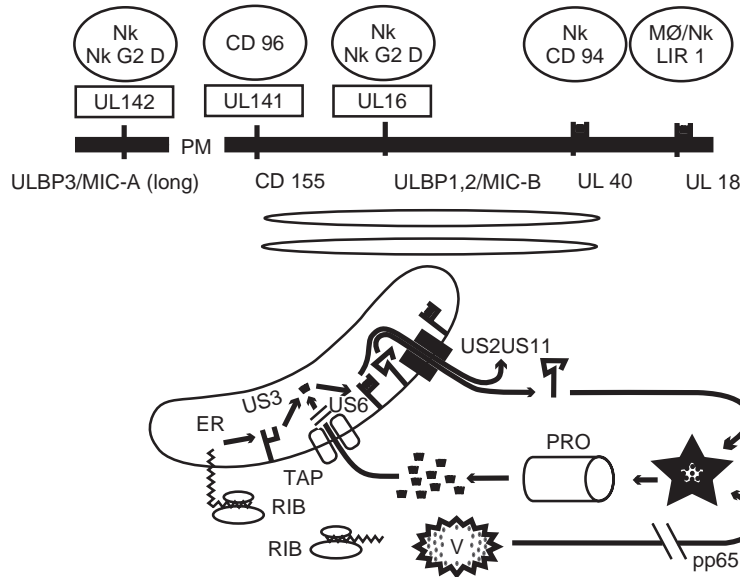


Figure 8.7 CMV gene products which interfere with the ability of cytotoxic T-cells or NK cells to recognise cells infected with this virus.

they are degraded in the proteasome. At both early and late times, pUS6 blocks the activity of the transporter associated with antigen presentation so that epitopes derived from CMV proteins cannot be presented as targets. All of these functions might, in combination, decrease surface HLA display to such an extent that the cell becomes a target for NK cells or macrophages which recognize the absence of these normally ubiquitous molecules. To prevent this, CMV has evolved a series of distinct strategies. The leader peptide of HLA-E is normally presented at the plasma membrane to indicate a healthy immunological flux of Class I molecules. Gene UL40 contains the same peptide as this leader, which is displayed to provide a negative signal to NK cells via their CD94 molecule. CMV also encodes another protein, pUL18, which is structurally strongly homologous to Class I HLA molecules and acts as a decoy to prevent macrophages attacking the CMV-infected cells. The pUL18 ligand on macrophages is leukocyte immunoglobulin-like receptor 1. Another protein, pUL16, is not membrane-bound but interferes with the ability of cellular proteins (termed UL16-binding proteins) to activate NK cells via their NKG2D receptors. Two additional CMV proteins interfere with the normal recognition of stressed cells by NK cells (Figure 8.7).

From the description above, it will be apparent that CMV has evolved a series of genes which act in a coordinated way to abrogate immune responses specific for the virus. There is much to be learned about the mechanistic aspects of how this objective is achieved. For

example, the expression of UL18 is not blocked by the action of the set of US genes which normally reduce Class I expression and, remarkably, US2 is able to block Class II expression as well as Class I. Thus, many responses may be initiated but are unable to detect their cellular targets during the initial round of replication. However, when the virus leaves the first cell to initiate infection in a second cell, it is vulnerable to display of epitopes before downregulation of HLA can be effected in that cell. Accordingly, the dominant effector CMI is directed against ppUL83 (pp65), revealed when the input virion is uncoated, thus explaining why CMI is focused on this late protein rather than those formed earlier in the viral cascade. However, it is possible that epitopes from other proteins may make contributions as well, with the MIE proteins being prime contenders.

Possible Interactions with HIV

The multiple mechanisms by which HIV may interact with herpesviruses are reviewed elsewhere (Griffiths, 1998). Many studies have shown *in vitro* that CMV infection (or transfection of particular genes) can transactivate HIV (reviewed in Ghazel and Nelson, 1993). Under some circumstances, CMV can also downregulate HIV replication, but CMV is more likely to stimulate HIV when the latter has an integrated provirus and when CMV is not actively replicating (Moreno *et al.*, 1997).

All of the postulated mechanisms of interaction would require close contact between HIV and CMV, either in

the same cell or in neighbouring cells. Studies of human autopsies have shown that CMV and HIV frequently co-infect the same organs and that individual cells can be found infected with both viruses.

Epidemiological studies report that the presence of active CMV infection or high quantities of CMV are associated with more rapid progression of HIV infection to AIDS or AIDS to death. It is therefore possible that CMV (and other herpesviruses) may act as cofactors to accelerate HIV disease. Indeed, multivariable analyses in the era of highly active antiretroviral therapy (HAART) show that the detection of CMV viraemia and a low CD4 count still correlate with death and that measurement of HIV viral load does not add further statistical predictive value (Deayton *et al.*, 2004). However, the cofactor hypothesis remains unfashionable and the reader is referred elsewhere for more details (Griffiths, 1998).

CLINICAL FEATURES

Congenital Infection

12.7% of congenitally-infected babies are born with symptoms. They are said to have ‘cytomegalic inclusion disease’ and their prognosis is poor. The remaining 87.3% appear to be normal at birth but about 13.5% develop sequelae on follow-up (Figure 8.8).

Those Symptomatic at Birth

The classic presentation is one of intrauterine growth retardation, jaundice, hepatosplenomegaly, chorioretinitis, thrombocytopenia and encephalitis, with or without microcephaly. It is often difficult, even for experienced paediatricians, to differentiate solely on clinical grounds between the several agents causing chronic intrauterine infection; laboratory tests for CMV, rubella, syphilis and toxoplasmosis are therefore invaluable. Most of the pathology outside the central nervous system (CNS) is self-limiting, although severe thrombocytopenic purpura, hepatitis, pneumonitis and myocarditis are occasionally protracted and life-threatening. The CNS involvement may present as microcephaly, hearing loss, encephalitis, seizures, apnoea or focal neurological signs. As regards congenital malformations, inguinal hernia in males, first branchial arch abnormalities, anophthalmia, diaphragmatic eventration and cerebellar hypoplasia have all been reported. However, these occur sporadically and so may be merely coincidental. There is therefore no evidence that CMV acts as a teratogen to impair normal organogenesis. Most of the clinically-apparent sequelae can be attributed to destruction of target organ cells once they have been formed.

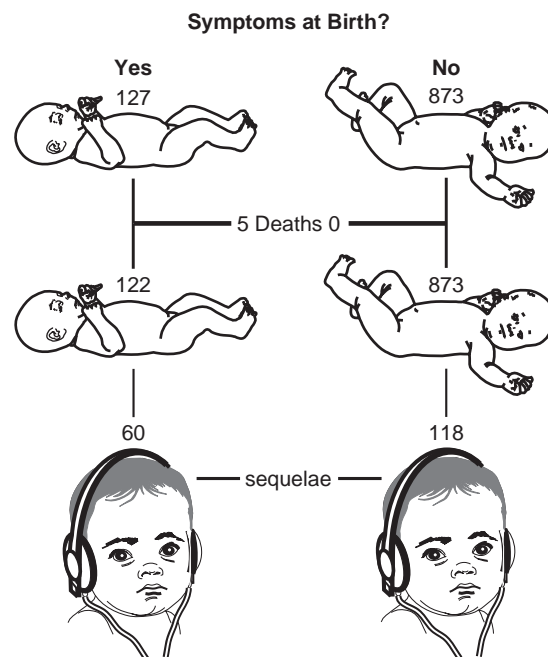


Figure 8.8 Outcomes for 1000 children born with congenital CMV infection. 12.7% are born with symptoms. They are said to have ‘cytomegalic inclusion disease’ and their prognosis is poor. The remaining 87.3% appear to be normal at birth but about 13.5% develop sequelae on follow-up.

In 0.5% of these cases the damage caused by the virus is so severe that they die during infancy. If a neonate survives, it is almost certain to have serious abnormalities for life, especially if there is microcephaly or if abnormalities are seen by computerized axial tomography (Noyola *et al.*, 2001). On follow-up, clinically-apparent extraneural damage is rare but brain damage may manifest as microcephaly, mental retardation, spastic diplegia or seizures and perceptual organ damage such as optic atrophy, blindness or deafness. Any of these abnormalities may occur alone or in combination. These defects may appear in children who do not have signs of CNS involvement at birth (including cerebrospinal fluid (CSF) examination). Milder forms of CNS damage, such as defects in perceptual skills, learning disability, minor motor incoordination or emotional lability, may be noticed as the child becomes older.

Those Asymptomatic at Birth

Long-term follow-up of such children has revealed that approximately 13.5% are likely to have hearing defects or impaired intellectual performances when compared

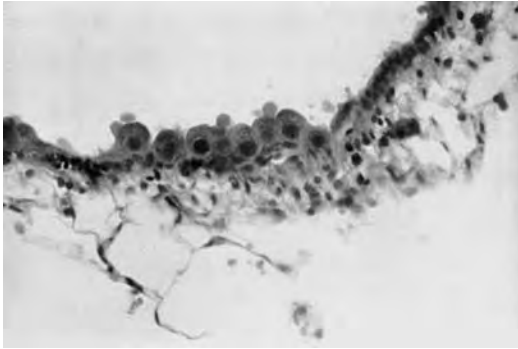


Figure 8.9 Histological preparation of an inner-ear structure from a fatal case of congenital CMV infection showing cell extension of CMV accompanied by an inflammatory response. (Courtesy of Dr S. Stagno, Alabama.)

to control children (Dollard *et al.*, 2007). The mean intelligence quotient of infected children has been reported to be significantly lower than controls. Other investigators have not noted these defects but their studies have often failed to follow the children for a sufficient length of time or have failed to use matched controls. There is now sufficient evidence to prove that the hearing loss can occur in a child born with normal hearing. In addition, a plausible pathogenesis for progressive hearing loss is apparent. Figure 8.9 shows a histological preparation of inner-ear structures from a fatal case of congenital CMV infection, demonstrating virus spread by the cell-to-cell route to produce a focus of infection surrounded by inflammation. It is tempting to suggest that this represents the infectious process in the inner ear causing progressive damage to the organ of Corti and decreased ability to perceive sound.

Perinatal Infection

Despite the continued excretion of virus for many months, the vast majority of perinatally-infected infants appear not to develop acute symptoms. The initial titres of CMV in the urine are significantly lower than those found in neonates with congenital CMV infection or disease (Stagno *et al.*, 1975b), which is compatible with the virus load hypothesis of CMV disease induction. Occasional cases of infantile pneumonitis have been attributed to perinatal CMV infection. This would appear to be an uncommon event, although CMV is a frequent pathogen in those few infants who do develop pneumonitis in the first three months of life. Disease may occur in preterm neonates (<1500 g birth weight). One study reported transmission of CMV in 17% of cases (Yeager *et al.*, 1983), while a more recent publication reported transmission in 37% (Hamprecht *et al.*, 2001). In both papers, individual cases

of neutropenia were temporally associated with CMV acquisition, sometimes accompanied by a clinical picture of sepsis with negative bacterial cultures.

Postnatal Infection

The commonest clinical outcome of primary or recurrent postnatal infection is a mild course without the production of symptoms. Most individuals identified by prospective serological studies as having seroconversions express surprise when told that they have experienced a virus infection.

Occasionally, however, primary infections are accompanied by the syndrome of infectious mononucleosis. This is similar to the syndrome produced by EBV except that lymphadenopathy is uncommon and that the Paul–Bunnell test is invariably negative. Sometimes, the hepatic component of CMV mononucleosis is prominent, so a diagnosis of viral hepatitis is considered initially. Within a few days, however, the full clinical picture becomes clear, with persisting pyrexia and atypical lymphocytosis. The post-perfusion syndrome, described earlier, is essentially CMV mononucleosis acquired by blood transfusion.

CMV is such a common virus infection that primary infection tends to occur by co-incidence with a variety of medical conditions. In addition, since CMV is an opportunist, it will tend to reactivate when a patient becomes debilitated as a result of some underlying condition. If the underlying disease is esoteric and has an unknown aetiology, a case report tends to appear describing the association. By scanning through PubMed (www.ncbi.nlm.nih.gov/pubmed), Internet surfers will be able to find literally dozens of such spurious associations, but they should be reassured: CMV is not the cause of all known diseases.

Immunocompromised Patients

Immunocompromised patients may respond to primary or recurrent CMV infections by remaining asymptomatic. More frequently, they will develop a spiking pyrexia which resolves after a few days. Some may develop viraemia with fever and leukopenia, which is sometimes termed CMV syndrome. This may progress to pneumonitis, or pneumonitis may supervene directly. In either case, once pneumonitis has become established, the prognosis is poor (mortality 80–90%). Some patients present with virus dissemination to the retina without other signs. Any part of the gut may be the site of CMV replication, which may be asymptomatic or may be associated with ulceration, which can, in extreme circumstances, cause erosion of neighbouring blood vessels, with catastrophic haemorrhage. Patients with AIDS may develop an encephalopathy of either a subacute progressive dementia or

an acute presentation with cranial nerve palsies caused by necrotizing ventriculitis. Alternatively, AIDS patients may present with a syndrome of weakness of the lower limbs, bladder paralysis and a polymorphonuclear CSF response which is termed polyradiculopathy. At post-mortem examination, AIDS patients often have CMV adenitis. Finally, CMV may induce an immunosuppressive syndrome in which the patient becomes unable to deal with superinfecting bacteria or fungi. In this instance, and in some of the other clinical presentations (pneumonia, hepatitis, gut ulceration, septicaemia), the underlying nature of the CMV infection is often not recognized by the clinical staff, who are understandably distracted by the more easily recognized superinfecting organisms.

The major clinical diseases associated with CMV are summarized in Table 8.6, according to the type of immunocompromised patient. Some complications, such as gastrointestinal involvement, are found in all patient groups. Some predominate in one group; for example, 85% of CMV disease presents as retinitis in AIDS patients compared to less than 5% in transplanted patients. While this observation remains unexplained, it is hypothesized that CMV pneumonitis in allograft patients is an immunopathological condition caused by an aggressive cell-mediated response against a lung target antigen coded by, or revealed by, CMV infection. Accordingly, AIDS patients may be relatively spared pneumonitis because, by the time they are sufficiently immunocompromised to permit CMV dissemination to the lung, they have insufficient T-cell responsiveness to mount the postulated immunopathological response. Likewise, the myelosuppressive CMV disease results when CMV replicates in stromal supporting cells,

releasing cytokines and producing a milieu unfavourable for haematopoiesis.

These observations concerning the different CMV diseases found in distinct patient groups, combined with the increasing knowledge about the importance of CMV viraemia and viral load, lead to a potential explanation for the diversity of CMV diseases. CMV viraemia may be a prerequisite for CMV disease at any target site. Increasing viral load may increase the chance that CMV can penetrate tissues to cause disease. Other organ-specific changes, distinct for each patient group, may then dictate in which organ CMV localizes; for example the marrow suppression is presumably found only after bone marrow transplant because these new cells are receptive to CMV replication.

Indirect Effects

Many studies consistently associate CMV with allograft rejection, secondary fungal or bacterial infections and accelerated atherosclerosis after heart transplant. The results (discussed later) from double-blind placebo-controlled trials of antiviral agents demonstrate that each of these is significantly reduced by preventing CMV disease, so supporting the hypothesis that CMV causes these conditions in some patients. There are no clinical features to distinguish CMV-associated graft rejection/secondary infection/atherosclerosis from the cases that are not prevented by anti-CMV drugs.

CMV is also clearly a contributor to immunosenescence. The CD8 response to CMV is prominent (discussed earlier) and may cause disease in two ways. First, it may divert immunological resources such as cytokines away from their intended function of mounting *de novo* immune responses. Second, the activated CD8 cells may trigger inflammatory diseases such as atherosclerosis. There is evidence for both of these phenomena; CMV-seropositive individuals mount an impaired response to influenza vaccine and have a significantly increased risk of death compared to age-matured CMV seronegatives in longitudinal studies (Strindhall *et al.*, 2007; Trzonkowski *et al.*, 2003).

Table 8.6 CMV diseases in the immunocompromised

Symptoms	Solid transplant	Bone marrow transplant	AIDS
Fever/hepatitis	++	+	+
Gastrointestinal	+	+	+
Retinitis	+	+	++
Pneumonitis	+	++	
Immunosuppression	+		
Myelosuppression		+	
Encephalopathy			+
Polyradiculopathy			+
Addisonian			+
Immunosuppression	+	?	?
Rejection/GvHD	+	?	
Atherosclerosis	+		
Death		+	+

GvHD, graft-versus-host disease.

DIAGNOSIS

As with all viruses, there are two potential strategies for providing a diagnosis: the detection of virus or the demonstration of a specific immune response.

Detection of Virus

Collection of Specimens

Urine can be obtained by mid-stream collection, by urine bags in neonates or by urinary catheter. Samples can be

collected at any time of day and should be sent to the laboratory without additives, since CMV is stable in urine.

Saliva should be allowed to soak onto a plain cotton-tipped swab, which is then shaken in virus transport medium and broken off. For PCR, from blood acid-citrate-dextrose or EDTA is preferred to heparin.

Tissue biopsies should be placed into plain sterile containers with no additives. Fluid and cells obtained by bronchoalveolar lavage should similarly be placed in a plain sterile container.

Amniotic fluid should be collected into a plain sterile container without any additives.

All specimens should be sent to the laboratory without delay. If delay of more than a few hours is anticipated then all samples should be sent refrigerated or on wet ice. Whole blood for PCR can be frozen at -20°C .

Selection of Sites for Examination

To diagnose congenital or perinatal infection, urine or saliva samples are usually collected. More invasive procedures, such as lumbar puncture or liver biopsy, are sometimes performed, but identification of CMV at these sites has not been shown to have any prognostic value.

If adults with mononucleosis or hepatitis are being investigated then urine and blood are the best samples. It should be possible to detect CMV from all urine samples collected within a few weeks of onset, whilst viraemia is often detected in the first few days of illness. The identification of urinary CMV excretion in a patient with such symptoms might be coincidental but the detection of viraemia strongly supports the diagnosis of CMV mononucleosis or hepatitis.

If pregnant women have symptoms, they should be investigated for viraemia. Amniotic fluid can be tested by PCR and culture but amniocentesis should not be performed before 21 weeks because fetal renal function must be established to allow CMV to appear in fetal urine and then amniotic fluid. In addition, amniocentesis performed within six weeks of primary infection may give false-negative results for CMV because of the postulated placental incubation period, which delays the transfer of CMV from the maternal to the fetal compartment. However, there is no advantage in actively screening asymptomatic women. Investigations involving the culture of urine, genital secretions, saliva and breast milk have been carried out but the results are not predictive of which women will have babies with congenital or perinatal infection. Since the 'patient' in these examples is the neonate, not the woman herself, samples should not routinely be collected from any maternal site.

Immunocompromised patients should be investigated by means of surveillance samples, taken preferably twice weekly, of blood and possibly also urine or saliva. This

must be done as routine on all patients, rather than waiting for symptoms to develop. CMV excretion from urine and saliva is very common in allograft recipients so the relative risk for future disease is typically 3. In contrast, PCR viraemia is detected less frequently in allograft patients but, when it does occur, is indicative of a relatively poor prognosis, with a relative risk of about 10. However, even the detection of viraemia does not guarantee that CMV is the cause of, say, the patient's hepatitis or pneumonitis. Ideally, samples should be collected from these target organs whenever possible.

An alternative way of detecting viraemia is through detection of antigenaemia in preparations of peripheral blood mononuclear cells as targets (van den Berg *et al.*, 1991). These are reacted with monoclonal antibodies against ppUL83 (pp65), followed by immunoperoxidase staining (see Figure 8.10). Note that the monoclonal antibody required for this assay does not recognize the immediate-early proteins as originally described. Presumably, the phagocytic activity of the leukocytes shown in Figure 8.10 has led them to ingest virus-infected material, among which ppUL83 is prominent. It is tempting to speculate that this may derive from dense bodies, since ppUL83 is a major component of these aberrant forms. In practice, antigenaemia is useful where samples can be processed by the laboratory within a few hours and where the leukocyte count is relatively normal, for example in recipients of solid organs.

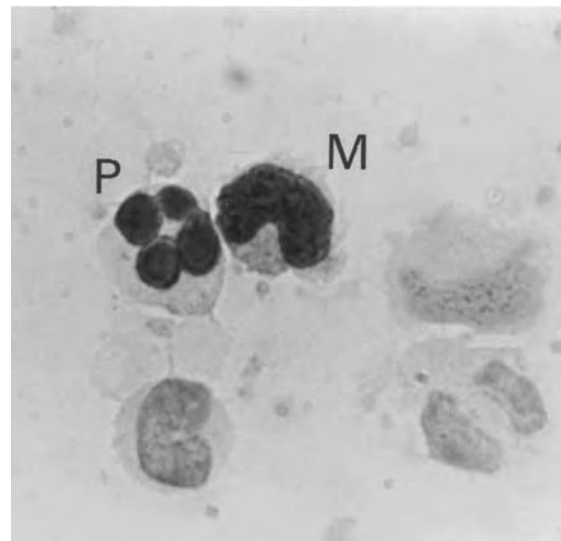


Figure 8.10 Direct detection of cytomegalovirus in peripheral blood leukocytes. M, monocyte; P, polymorphonuclear leukocyte. (Kindly provided by Professor H. Thé.)

Histopathology

CMV can be recognized in histological preparations by its characteristic 'owl's eye' inclusions. These Cowdry type-A intranuclear inclusions have a surrounding halo and margined chromatin. They can be found in kidney tubules, bile ducts, lung and liver parenchyma, gut, inner ear and salivary gland but are less prominent in brain tissue (see Figures 8.9 and 8.13 for examples).

Although histopathology provides a specific diagnosis, it is known to be insensitive. One study showed that CMV can be cultured from tissue six times more frequently than typical inclusions can be seen, while a later study showed that organs with inclusions have a median viral load two logs higher than those in which inclusions could not be seen (Mattes *et al.*, 2000).

Tissue Immunofluorescence

Some biopsy samples (e.g. liver, lung) may contain cells infected with CMV, which can be visualized by staining frozen sections with antisera to CMV. Alternatively, the tissue can be disrupted and the cells fixed to glass slides before staining. Bronchoalveolar lavage fluid contains a suspension of cells exfoliated from the respiratory tract, which can be centrifuged, washed to remove adherent mucus and then air-dried and fixed to microscope slides before staining.

Cell Cultures

For conventional cell cultures, human fibroblasts are used. Foreskins or embryo lungs may be employed as a source of fibroblasts and must be used only at low passage (<25).

To detect wild strains of CMV reliably, the cultures must be cared for obsessively. The medium must be drained and 0.2 ml of each clinical specimen inoculated and incubated at 37°C for 1 hour to permit virus adsorption. The cultures should then be refed with maintenance medium to reduce the toxic effects of the inoculum. This is especially important in the case of particulate samples, such as blood and tissue homogenates. For the detection of viraemia, buffy coat or unseparated heparinized blood can be inoculated into cell cultures. If toxicity is observed, denuded areas of the monolayer can be repaired by the addition of fresh fibroblasts.

All cultures should be observed at least twice weekly for the typical focal CPE of CMV (Figure 8.11). Occasionally, urine samples from cases of congenital infection produce widespread CPE within 24 – 28 hours, which resembles that of HSV. Usually, however, the CPE evolves only slowly, so the cultures must be maintained for a minimum of 21 days before being reported as negative. This delay in obtaining an answer

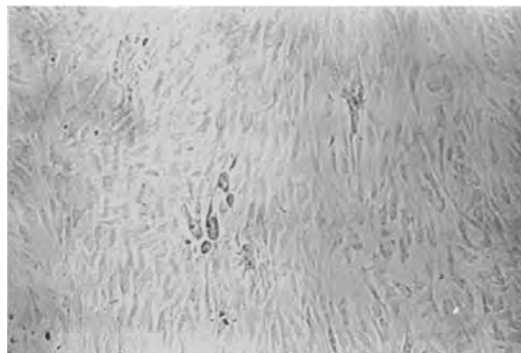


Figure 8.11 The local cytopathic effect typical of cytomegalovirus seen in monolayer cultures of human embryo lung fibroblasts. (Photograph kindly prepared by Ms A. Grzywacz.)

has stimulated research into more rapid ways of detecting CMV, which are discussed below.

Electron Microscopy

Samples of urine from infants infected congenitally or perinatally contain high titres ($10^3 - 10^6$ TCID₅₀/ml) of CMV. Using the pseudoreplica electron microscopy technique, it has been possible to demonstrate this viraemia. Several authors have reported that approximately 80% of infected infants can be detected, with the false negative results being clearly attributable to low-titre urine samples (<10³ TCID₅₀/ml). The viral specificity of the technique has been reported at 100%, simply because it would be most unusual for any other herpesvirus to be found at such high titre in urine from infants.

Electron microscopy cannot be used in immunocompromised patients for several reasons. First, the titre of CMV found in clinical samples from adults is generally lower than that found in infants. Second, all human herpesviruses frequently infect immunocompromised patients and cannot be distinguished from each other by electron microscopy. Since different antiviral therapy may be required for each herpesvirus, the results of electron microscopy would not be specific enough to influence patient management.

Detection of Early Antigen Fluorescent Foci (DEAFF)

The technique of detection of early antigen fluorescent foci (DEAFF) was developed as a means of retaining the specificity and sensitivity of cell culture without having to wait for the production of CPE as a diagnostic end point (Griffiths *et al.*, 1984).

Following inoculation on to cell cultures, CMV is absorbed into the cell within minutes. CMV rapidly starts to produce α - and β -proteins but CMV DNA synthesis is



Figure 8.12 Cytomegalovirus infection diagnosed by detection of early antigen fluorescent foci (DEAFF).

delayed and protracted until several days after infection. This explains the long delay seen in conventional cell cultures, since CMV needs to replicate, produce daughter virions and infect neighbouring cells in order to produce the CPE shown in Figure 8.11

To speed up the diagnostic process, cells are inoculated with clinical specimens and stained after only 18 hours incubation, using monoclonal antibodies directed against some of the α - and β -proteins of CMV (Figure 8.12). This technique is termed shell vial assay in the United States because of the vessels used for the sample processing (Gleaves *et al.*, 1984).

Enzyme Immunoassay (EIA)

Some workers established EIA detection systems using polyclonal hyperimmune sera or monoclonal antibodies. The results indicated that EIA could detect small amounts of CMV complement-fixing (CF) antigen or of virus when added artificially to buffer systems. However, when clinical samples containing CMV were assayed, results of low sensitivity and/or specificity were obtained.

Detection of Viral DNA

Earlier reports of the detection of CMV by dot-blot methods have been completely superseded by PCR. Different parts of the CMV genome have been chosen as targets by various authors, but there is no obvious advantage in any particular one. Ideally, one would wish to amplify a conserved region, but the degree of genetic variability found in clinical strains is only beginning to be defined. Potentially, PCR methods could be so sensitive that they detect low quantities, even latent virus, which would not necessarily be clinically relevant; accordingly, some authors have concluded that their nested PCR procedures do not provide prognostic information. We deliberately chose a non-nested procedure to avoid this problem, and found

that PCR produces good prognostic information in both transplant patients (Kidd *et al.*, 1993) and HIV-positive individuals (Bowen *et al.*, 1997). Clearly, 'PCR' is not a single procedure and each laboratory should measure the clinical significance of its results through formal assessment (Bowen *et al.*, 1997; Einsele *et al.*, 1995; Kidd *et al.*, 1993; Shinkai *et al.*, 1997). The availability of fully quantitative PCR assays for CMV, especially in a real-time format, offers another approach for further refining prognostic values, as well as for understanding pathogenesis, as discussed previously.

Amniotic fluid should be tested in multiple replicates by PCR to avoid false-negative results (Revello *et al.*, 1998).

As described later, dried blood spots can be tested by PCR to provide a retrospective diagnosis of congenital CMV infection. The testing algorithm described by Barbi *et al.* (2006) should be followed.

Characteristics of the Various Assays Described

These are summarized in Table 8.7. In the first three editions of this book, I described conventional cell culture as the gold standard against which newer assays should be compared. In the fourth edition I proposed that this process is complete for PCR, so that it can be recommended as the new gold standard for providing diagnostic information in a timely manner able to influence the management of individual patients. This conclusion is supported by a series of subsequent reports, so that any PCR assay which has had a published validation showing correlation with clinical end points can be employed (Einsele *et al.*, 1995; Kidd *et al.*, 1993; Shinkai *et al.*, 1997). Note that the samples required, the sample extraction method and the assay-specific details all differ among these validated assays, so colleagues should choose one system and follow the whole procedure exactly as described. We have always used whole blood because fractionation offers additional opportunities for sample contamination and mislabelling and so should only be performed if clear benefits can be shown. This choice is vindicated by a recent study which directly compared the utility of testing whole blood versus blood fractions (Razonable *et al.*, 2002).

Advantages and Disadvantages of Virus Detection

1. The anatomical site of the infection can be documented (e.g. lung involvement in a patient with pneumonitis).
2. The patient's immune response is not required for diagnosis, so all infected immunocompromised patients, not just those able to mount an immune response, can be identified.
3. Rapid diagnostic methods have enabled infected patients to be recruited into therapeutic trials of potential

Table 8.7 CMV detection in body fluids

Method	Sensitivity	Specificity	Reliability	Rapidity	Proven prognostic value
Conventional cell culture	++	+++	++	+	+
Detection of early antigen fluorescent foci	+	+++	++	++	+
Antigenaemia	++	+++	++	+++	+
Polymerase chain reaction	+++	+++	++	++	+

antiviral agents so that patient management may be influenced.

4. PCR methods can quantify the amount of virus in clinical samples and allow quantitative virological assessment of antiviral agents.
5. If resistant virus emerges during a course of treatment, this may be detected by the assay and so provide an opportunity for prescription of an alternative drug.
6. A potential disadvantage is that the monoclonal antibodies or DNA probes used to detect CMV may be too specific and identify only some strains of the virus, although this has not proved to be a problem to date.

Detection of Immune Response

IgG Antibody as a Marker of Past Infection

The detection of IgG antibodies against CMV is clearly indicative of infection sometime in the past. The individual is said to be seropositive and is liable to experience reactivations of their latent infection. The presence of IgG antibodies against CMV is thus a marker of potential infectivity; although a seropositive individual is 'immune' in the immunological sense, this term should not be used to imply protection from endogenous or exogenous infection.

Many assays have been described for the detection of CMV IgG antibodies. Those most frequently used

are listed in Table 8.8, together with estimates of their performance. In patients with intact immunity, the CF test is perfectly adequate, provided that an efficient antigen is employed and optimal incubation temperatures are used. Other tests (marked '+' in Table 8.8) are more sensitive in that they produce higher antibody titres, but they do not detect substantially more seropositive individuals in a population.

With immunocompromised patients, the CF test often gives false-negative results by failing to detect low levels of IgG antibody. Thus, a more sensitive technique is required, and any of those marked '++' in Table 8.8 are satisfactory, with EIA being used most frequently. If an immunofluorescence (IF) method is chosen, the IF assay-late antigen (IFALA) assay should be avoided because all human sera bind to the IgG Fc receptor induced by CMV and this perinuclear fluorescence can be difficult to distinguish from the virus-specific nuclear fluorescence found with seropositive samples. Anticomplement IF (ACIF) is the IF method preferred, since it is unaffected by IgG binding to the Fc receptor.

IgG Antibody as a Marker of Acute Infection

Rising levels of IgG antibody were employed in the past but this approach has been completely superseded by assays for detecting virus itself. However, the detection of IgG antibodies of low avidity provides evidence of

Table 8.8 Performance characteristics of several assays used to measure IgG antibodies against CMV

Method	Performance characteristics			
	Sensitivity	Specificity	Objectivity	Rapidity
Neutralization	+	++	+	
CF	+	+	+	+
IFA-LA	++			++
ACIF	++	++	+	++
Latex agglutination	++	++	+	+++
RIA	+++	++	++	++
EIA	+++	++	++	++

ACIF, anti-complement immunofluorescence; CF, complement-fixing; EIA, enzyme immunoassay; IFA-LA, immunofluorescence assay for viral late antigens; RIA, radioimmunoassay.

primary infection in the recent past, for example 16–20 weeks (Guerra *et al.*, 2000).

Detection of IgM Antibodies

Attempts to detect CMV-specific IgM antibodies have been plagued by the use of methods of poor sensitivity and specificity and by interference from rheumatoid factor. Testing for IgM antibodies may be helpful in cases of suspected CMV mononucleosis or in pregnant women. In all other cases of suspected active CMV replication, investigations using virus detection methods are recommended.

Problems Associated with Serological Diagnosis

1. Some immunocompromised patients fail to mount a typical immune response and die from disseminated CMV infection. If diagnoses are made solely by serology, these cases will not be identified.
2. The passive transfer of CMV IgG antibodies with blood products may produce 'seroconversions' if sensitive IgG assays are employed.
3. The major objection to detecting CMV infection serologically is that the diagnosis is delayed.

MANAGEMENT

Congenital Infection

The most important part of the management of these cases is to make an unequivocal diagnosis. This is usually accomplished in the case of those symptomatic at birth but is more difficult in those who are initially asymptomatic.

Babies born with symptoms are usually investigated using the appropriate tests: culture or PCR of urine or saliva for CMV. The presence of CMV in a neonate aged less than three weeks is clearly indicative of congenital infection.

Those born without symptoms are unlikely to have tests performed and typically present from the age of six months onwards with sequelae such as hearing loss or mental retardation. These cases should be tested for CMV but if the virus is present this does not guarantee that the infection was acquired congenitally rather than perinatally.

Testing for specific IgM antibodies is of no help in this situation, since they should be present following congenital infection and will presumably be produced acutely in cases of perinatal infection, although this remains to be proven rigorously.

Instead, a request should be made for retrieval of a stored dried blood spot taken in the first few days of life as part

of a screening programme for phenylketonuria, where the child was born in a country which performs such screening. Testing such dried blot spots by PCR has excellent sensitivity and specificity for detecting CMV DNA even when the card has been stored at room temperature for years (Barbi *et al.*, 2006). Provided that the sample was collected within the first three weeks of life, a positive PCR result is diagnostic of congenital CMV infection.

If CMV is found in a child who develops symptoms during infancy, it is worthwhile bleeding the mother and contacting the laboratory servicing her antenatal clinic to see if sera have been retained. Occasionally, a laboratory has kept a serum used to test for rubella status. If this serum has CMV IgG but not IgM antibodies then it is unhelpful, since the mother may have had recurrent infection during pregnancy or, if she presented later than 16 weeks, primary infection early in pregnancy. If, however, the serum has low-avidity antibodies or is IgM positive, it will confirm that she had primary infection during pregnancy, which makes it more likely that her child's CMV was acquired congenitally. Similarly, if the mother seroconverts between early pregnancy and paediatric presentation, this supports a congenital transmission. Finally, the mother may remain seronegative, in which case the child cannot have had intrauterine infection and so the diagnosis of congenital infection can be excluded.

It will be evident from this discussion that the management of these cases would be greatly facilitated if it were possible to screen all new-borns for evidence of congenital infection, in a similar fashion to the established phenylketonuria screening programme. At present, cell culture is too cumbersome and expensive a technique to be used widely but testing of dried blood spots could fulfil this role reliably if there was the political will and funding to start such screening.

Once the diagnosis of congenital infection has been established, an assessment should be made of the prognosis for the child and for a future pregnancy. An estimate of the child's prognosis can be given from the magnitude of neonatal viraemia and the presence of viraemia detected by PCR because this correlates with a poor prognosis (Bradford *et al.*, 2005).

The prognosis for a future pregnancy is clear if the diagnosis has been definitely proven and the child was symptomatic at birth; since only one example of cytomegalic inclusion disease has been reported in consecutive siblings, the risk of an identical recurrence must be very low. If, however, the child developed symptoms during infancy then the position is not so clear. No sibling cases have been reported but this might be because the correct diagnosis has not been made in the past. It remains possible, therefore, that a woman could have a future pregnancy damaged in a similar way. To be scientifically and legally

correct, the risk cannot be given as zero, but, on humanitarian grounds, the author usually emphasizes that it must be very low. Before a definitive virological diagnosis is available, the differential diagnosis often includes a recessive gene for presentations with hearing loss, which has a recurrence risk of 25%. If the parents are prepared to gamble with their genes then the virologist must not dissuade them from taking what must be a far lower risk with CMV. It is conventional to advise such women to wait one year between the estimated date of infection and trying to conceive, which approximates to two years between births, for which there is supporting evidence of low risk from a cohort study (Fowler *et al.*, 2004). However, there are no laboratory tests to determine whether it is 'safe' for an individual to proceed.

Having established the diagnosis and prognosis, treatment must be considered, including remedial therapy to compensate for hearing, speech or developmental defects. GCV has been studied in a dose-comparative trial (Whitley *et al.*, 1997), which showed that the drug was relatively well tolerated over the six weeks duration of therapy, with no excess toxicity from the higher dose of 6 mg kg⁻¹ b.i.d. intravenously. As a result, this dose was compared against no treatment in a randomized controlled trial and significantly worse hearing was reported at six months among those who were not treated (Kimberlin *et al.*, 2003). Thus, despite the toxicity of GCV, which caused neutropenia and thrombocytopenia in substantial numbers of the neonates, this drug has become the standard of care for those born with CNS symptoms. The same investigators have shown that the valganciclovir (VGCV) prodrug is bioavailable in infants and so can be used to deliver GCV orally. Further controlled trials are required to determine the optimal duration of therapy and a randomized comparison of six weeks versus six months of GCV therapy has just begun. Additional studies are required in infants born without symptoms of congenital CMV infection to determine whether the toxicity of GCV can be justified in them.

Until such trials are conducted, this author concurs with the opinions of the investigators, that GCV should not be used outside the setting of a controlled clinical trial (Kimberlin *et al.*, 2003; Whitley *et al.*, 1997). The toxicity profile of GCV includes carcinogenesis in rodents and so possible therapeutic advantages must be balanced carefully against potential adverse consequences.

Finally, the parents should be informed about the nature of the child's illness and advised on its infectivity (see below).

Perinatal Infection

These cases produce few medical management difficulties. Most remain asymptomatic and undiagnosed. Cases

of infantile pneumonitis should have samples of urine, saliva and nasopharyngeal aspirate cultured for CMV but, apart from bronchoscopy and/or lung biopsy, there is no way of proving that the pneumonitis is due to CMV in an individual case. The main medical importance of perinatal infection is the difficulty it produces for the diagnosis of congenital infection (see above) and the fear of contagion that it stimulates.

It is clear that children less than 18 months of age with perinatal infection can transmit CMV to their parents (Pass *et al.*, 1986). This presumably results from close contact with infected saliva during normal family life and so would be difficult to control completely. Control should be considered, however, if the mother is contemplating pregnancy (Adler *et al.*, 2004), although care must be taken not to induce feelings of social isolation or rejection in the older sibling. There is no evidence that these children are contagious to adults outside the family or day-care centre but it seems prudent to advise the parents that the infants should avoid intimate contact with women who may be pregnant or with immunocompromised patients. Unfortunately, this advice, when ultimately passed to school teachers, can lead to the children being treated as lepers. The teaching staff may have to be reassured strongly to ensure that the children are not treated differently from any others. It usually suffices to emphasize that 10–20% of the children in the classroom are asymptotically excreting CMV as a result of perinatal infection, so that routine hygienic precautions should be used by all staff for all children. The same advice should be given when the child has congenital CMV infection, irrespective of whether or not the virus has induced disease. It would be absurd to ostracize the occasional case of symptomatic congenital infection knowing that literally hundreds of other children of the same age are excreting similar amounts of the same virus.

Postnatal Infection

Most cases of postnatal infection are asymptomatic and so do not require management. Exceptions are CMV mononucleosis and hepatitis, which may well be treated once a safe orally bioavailable anti-CMV drug becomes available.

When postnatal infection occurs in a pregnant woman, however, the possibility of termination of pregnancy requires consideration. Recurrent maternal infections are invariably asymptomatic and there is no laboratory test currently available that can detect which immune women are transmitting the virus *in utero*. For these practical reasons, it is not possible to contemplate therapeutic intervention in these cases, even though some produce childhood damage (Fowler *et al.*, 1992), so discussion must be limited to primary maternal infections.

By analogy with rubella infection during pregnancy, it has been assumed that primary CMV infection will be most severe when it occurs during the first trimester, so therapeutic termination of such pregnancies would be justified if the diagnosis could be made sufficiently early. Studies have shown that asymptomatic primary CMV infection can be reliably diagnosed early in pregnancy by testing for IgG avidity and specific IgM antibodies (Guerra *et al.*, 2007). It has also been clearly shown that the vast majority of women in developed countries present early enough in pregnancy to allow the infection to be detected and for termination of pregnancy to be performed safely. Studies have confirmed the assumption that primary CMV infection is more severe at this stage of pregnancy—so severe that it produces a statistical excess of fetal losses. These results clearly suggest that some pregnancies involving the potentially most severely affected fetuses are terminated naturally, so that medical intervention directed towards the survivors may not be as beneficial as has been assumed.

Currently available data indicate that the risk that an individual fetus may survive to be born with cytomegalic inclusion disease following primary maternal infection before 28 weeks gestation is about 4% (Griffiths and Baboonian, 1984; Stagno *et al.*, 1986). Antenatal screening has been performed in Italy and the results of specialized reference tests show that women with positive IgM screening tests can be divided into one group with a low (1%) background risk of disease and another with a substantial (30%) risk. When given these results, most women can be supported to continue with their pregnancies (Guerra *et al.*, 2007).

When counselling women, it must be remembered that the presence of intrauterine infection does not necessarily indicate that clinically-evident disease would have presented itself in childhood, since the majority of congenitally-infected infants develop normally. There is also no evidence to suggest that a symptomatic woman is more likely to deliver an affected baby than is an asymptomatic woman; therefore, whilst the final decision rests with the parents and their obstetrician, the same virological advice should be given to all. Amniocentesis with culture and PCR of the amniotic fluid has been performed in case series. Overall, these results show that, after 21 weeks gestation, CMV can be detected in amniotic fluid in most cases. (Revello 98; Guerra *et al.*, 2000). Amniocentesis frequently gives false-negative results if performed before 21 weeks because fetal kidney function is required to excrete CMV into the amniotic fluid. Given the need to make the diagnosis rapidly at this late stage of pregnancy, PCR has a distinct advantage over culture, but the parents must be counselled about the possibility of false-negative results. In addition, quantitative PCR of amniotic fluid

shows that high viral loads correlate with a high risk of disease, but the range of values found in practice means that precise risks cannot be given to individual women.

Coupled with the results of ultrasound scanning to assess fetal growth, amniotic fluid testing for CMV aids detection of some of the potentially most severely damaged cases, but the techniques are still restricted to specialized reference laboratories. Furthermore, a randomized controlled trial of administering valaciclovir to pregnant women with PCR-positive amniotic fluid is about to start, coordinated from Paris (Ville, Y., personal communication).

In two published independent cases, female members of paediatric medical or nursing staff requested termination after discovering that they had acquired asymptomatic primary CMV infection during pregnancy. Serological tests had been performed because they were caring for infants known to have congenital CMV infection. Virus isolates were obtained in each instance from the index case, the mother and the aborted fetus. In both cases, the maternal and fetal isolates were shown to be indistinguishable from each other by restriction enzyme analysis but were quite different from their respective index case. The conclusion is clear: primary CMV infection occurs commonly during pregnancy and will be found if women of childbearing age are investigated. Typing of strains in the published cases showed that the infections were not acquired from recognized occupational exposures, a finding which has important medical implications. There is little evidence that staff exposed professionally to infectious cases have an increased risk of contracting CMV infection, but it seems prudent to advise pregnant staff to avoid such contacts if possible by practising good hygiene precautions, such as hand washing after patient contact. This cautious approach is applied to female technical staff working with CMV in the laboratory, although we have never had a case of seroconversion among our predominantly seronegative staff. It should be emphasized that the same advice is given to female staff irrespective of their serological status; clearly preconceptional humoral immunity in these women cannot be equated with a guarantee of protection for the fetus.

Immunocompromised Patients

The most important part of the management of these patients is to make the diagnosis of CMV infection rapidly. Once extensive damage to target tissues has occurred, as is shown in Figure 8.13, no antiviral therapy can reasonably be expected to have a successful outcome. To provide advance warning of CMV disease, blood should be collected twice weekly from all allograft recipients and processed by one of the rapid diagnostic methods described in the previous section.

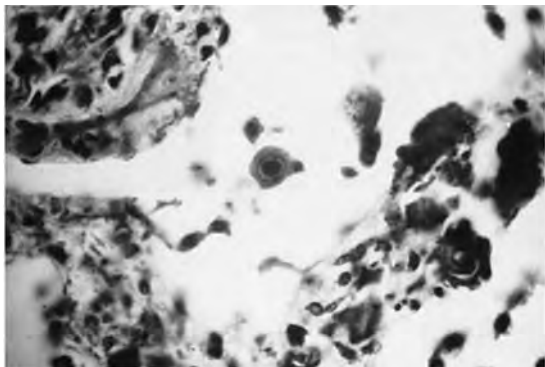


Figure 8.13 The post-mortem histological appearance of cytomegalovirus pneumonitis in a bone marrow allograft recipient. (Reproduced by permission from Griffiths (1984).)

If CMV is found in an allograft recipient then the patient's condition should be reviewed with the clinicians. Many are asymptomatic but some may subsequently become unwell and the availability of rapid virological results may allow the clinicians to reduce immunosuppressive therapy before extensive dissemination of CMV has occurred. The importance of rapid diagnosis in the clinical management cannot be overemphasized, since the differential diagnosis of some illnesses requires an increase in immunosuppressive therapy (e.g. renal allograft rejection episodes). Strategies for the use of antiviral drugs in asymptomatic patients found to have active infection with CMV will be discussed in the next section.

PREVENTION

Is Prevention Needed?

Prevention of an infection can be justified only if it leads to a reduction in ill health; in other words, the infection itself need not necessarily be the target as long as the infectious process can be modified to prevent disease. Potential strategies by which this objective may be accomplished will be detailed, but first it is necessary to consider the magnitude of the problem in the two major populations: congenital and immunocompromised patients.

Congenital Infection

While it is well recognized that babies symptomatic at birth have a poor prognosis, the full effects on those babies who appear to be normal at birth are only now becoming apparent (Fowler *et al.*, 1992). However, the figures outlined in Figure 8.8 translate into approximately 5000 children per year in the United States and 870 per

year in the United Kingdom suffering overtly as a result of congenital CMV infection. These figures confirm congenital CMV infection as the second commonest known cause of mental retardation after Down syndrome. Prevention of such a relatively common condition would be justifiable if this could be achieved safely and at low cost. A recent Institute of Medicine report estimates that \$3 billion is spent annually in the United States providing care for these unfortunate children (Stratton *et al.*, 2000).

Immunocompromised Patients

Infections form the commonest single cause of death in allograft patients. In bone marrow recipients, the single most important infection is CMV, responsible for approximately 15% of mortality before antiviral treatment became available. In recipients of solid organ transplants, several investigators report this virus to be a major cause of morbidity and mortality, with an annual cost of \$1 billion in the United States (Stratton *et al.*, 2000). In an analysis of hospital charges, CMV disease significantly increased costs, showing that if the expense of extra days spent in hospital is taken into account then prevention of CMV disease should potentially be cost-effective (Kim *et al.*, 2000). Philosophically also, it would seem sensible for health services to want to prevent CMV disease if this can be done at reasonable cost, since their investments in high technology transplantation are wasted if the procedures are effective but patients die from complications.

Prevention of Transmission

Knowledge of the potential routes of CMV transmission raises the possibility of prevention by interruption of infection. For example, seronegative renal allograft recipients could be matched to receive only kidneys from seronegative donors. Retrospective analysis by means of life-table survival curves has shown that CMV matching has a greater effect on the survival of cadaver kidney recipients than does matching the same patients for HLA Class I status. However, CMV re-infection from the donor kidney has also been shown to cause disease, albeit at a rate lower than that found after primary infection (Grundy *et al.*, 1988). If seronegative kidneys were reserved for seronegative recipients then more infected kidneys would be given to seropositive recipients and matching for other characteristics, such as HLA, might be prejudiced. Clearly, a controlled trial of the potential benefits of matching would be required before it could be justified as a clinical routine. While there is no doubt that matching could reduce morbidity and mortality due to CMV, there is equal concern that even greater morbidity and mortality due to graft rejection could result from a policy of matching for CMV. Put bluntly, patients and

their relatives would not thank us for merely substituting one specific cause for another on the death certificate.

A counsel of perfection is to give only CMV-seronegative blood or blood products to all pregnant women, all neonates and all immunocompromised patients, including those with AIDS. Alternatively, in-line filters should be used to remove leukocytes during blood transfusion, since this has been shown to reduce CMV transmission in a special-care baby unit (Gilbert *et al.*, 1989).

Transmission could theoretically be reduced in the general population by advising women of childbearing age to avoid salivary or sexual contact with seropositive consorts and children, but this clearly would be unpopular. There is no evidence that testing individuals serologically would add to the general advice given about avoiding exposure.

Pre-exposure Immunization

This strategy allows CMV exposure to occur but immunizes the recipients beforehand, in the hope of preventing disease. Several vaccine candidates have undergone Phase I clinical testing (reviewed in Schleiss and Heineman, 2005). These include recombinant soluble glycoprotein B, canarypox or alphavirus vectors expressing gB or pp65, as well as recombinant live attenuated Towne strains incorporating the novel genes from the Toledo strain. The recombinant soluble gB (with MF59 adjuvant) induced neutralizing antibodies in seronegatives and boosted the neutralizing titre in seropositives. The canarypox-gB did not induce neutralizing antibodies, although it primed volunteers to respond to subsequent challenge with the Towne strain. The canarypox-pp65 induced CMI in seronegatives. It thus appears that we have several candidate vaccines which are immunogenic and safe and so we should consider how they could be evaluated for efficacy.

In the past, vaccinologists assumed that efficacy would have to be proved by reducing the rate at which neonates

were born with symptomatic congenital CMV to mothers given vaccine or placebo many years earlier. The practicalities, logistics and expense of conducting such studies are prohibitive. However, modern virology can now be employed in distinct patient populations to determine whether a vaccine protects against primary infection and can provide immunological control against high viral loads (see Table 8.9). In the author's view, a vaccine which could protect one population of women and/or one population of transplant patients should be licensed for routine immunization (e.g. of teenagers) with post-marketing active surveillance used to document the anticipated reduction in symptomatic congenital CMV infection. As shown in Figure 8.14, herd immunity works by reducing the number of infectious individuals in a population and so should reduce both primary infections and re-infections. Since the basic reproductive number (i.e. the number of people who become infected from one infectious individual) for CMV is only 2.5, a vaccine with only 60% protective efficacy against primary CMV infection could be used to eradicate this infection from communities (Griffiths *et al.*, 2001). Certainly, there are no fears that widespread introduction of such a vaccine could affect herd immunity and so shift the incidence of primary infection into the childbearing years, because the average age at which CMV is acquired in developed countries is already greater than the average age at pregnancy (Griffiths *et al.*, 2001). Furthermore, the USA Institute of Medicine report should provide a strong financial incentive for the necessary studies to be performed, because their economic analysis shows a cost saving of \$50 000 for each quality-adjusted life year gained by a hypothetical vaccine which costs \$360 million to develop (Stratton *et al.*, 2000). At the time of writing, Dr Pass's study of gB vaccine with MF59 has shown protection against primary infection. Publication of the full report of this landmark study will undoubtedly provide support for the concept of universal immunization against CMV.

Table 8.9 Populations in whom efficacy of CMV vaccines could be evaluated using virological end points

Populations given vaccine/placebo	Outcome
Seronegative workers at day-care centres	Reduced primary CMV infection
Seronegative women with children at day-care centres	
Seronegative pregnant women immunized post-partum ^a	
Seronegative teenagers ^a	
Seronegative or seropositive patients on waiting list for receipt of solid organs ^a	Reduced CMV viraemia/need for pre-emptive therapy
Seronegative or seropositive donors of bone marrow to seropositive recipients	

^aindicates clinical trials currently underway.

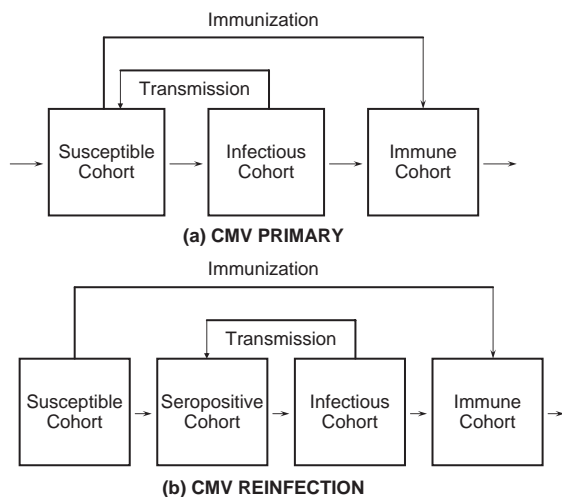


Figure 8.14 How vaccines should protect populations from (a) primary CMV infection; (b) CMV re-infection.

The live attenuated Towne strain of CMV vaccine reduced the severity of disease in seronegative recipients given seropositive kidneys (Plotkin *et al.*, 1991). Nowadays we would interpret this as evidence that the vaccine reduced the peak viral load. It could not prevent re-infection of the recipients with a different strain of CMV but no evidence was found of reactivation of Towne vaccine virus when the patients were immunosuppressed.

Homosexual men are at greater risk of acquiring CMV infection than are heterosexual men and could be considered as candidates for the assessment of the efficacy of CMV vaccines. However, most are already seropositive and so studies would have to address the question of whether vaccination could prevent recurrent infection in this group (immunotherapy). Vaccines could also be investigated for immunotherapy in patients receiving allografts. Clearly, attempting to present T-cell epitopes may be difficult because the patients would receive drugs such as cyclosporin in order to suppress these responses. However, one could aim to present B-cell epitopes with the hope of boosting the humoral immune response to keep the CMV load below that required to cause disease. In the case of bone marrow transplantation, immunization of the donor prior to marrow harvest may potentially lead to adoptive transfer of immunity to the recipient.

Passive T-cell immunotherapy has also been evaluated after bone marrow transplantation. Donor cells are expanded *in vitro* and passively administered to recipients. The clinical results show that the cells persist in recipients, and controlled trials to evaluate protective efficacy are required.

TREATMENT

The drugs GCV, foscarnet, cidofovir and fomivirsen (intravitreal only) have been licensed for serious or life-threatening CMV infections in immunocompromised patients, while valaciclovir is licensed only for prophylaxis. Note that fomivirsen and oral GCV are no longer marketed. GCV is phosphorylated by the product of the UL97 gene of CMV (Sullivan *et al.*, 1992) and then the triphosphate acts to inhibit virion DNA polymerase (UL54). Cidofovir is a phosphonate compound which is structurally analogous to a nucleoside monophosphate. It does not require activation by UL97 but is converted to the diphosphate (structurally analogous to a nucleoside triphosphate) by cellular enzymes, and then inhibits UL54. Foscarnet is a low-molecular-weight analogue of the inorganic pyrophosphate product of DNA polymerase activity and so acts at a different site and does not require prior anabolism. Foscarnet and cidofovir must be administered by intravenous infusion; GCV can now be given orally by means of the valganciclovir prodrug, which is absorbed and cleaved in gastrointestinal cells to release GCV. This prodrug offers the potential of administering GCV without the need for intravenous infusion (which is clinically important for a drug which causes neutropenia). However, clinical studies are required in each patient population to show that the drug is bioavailable. This has been done for liver transplant patients (Pescovitz *et al.*, 2000) and bone marrow transplant patients (including those with gastrointestinal graft-versus-host disease). GCV produces neutropenia, which may be dose-limiting. In animal toxicology studies, GCV has a cytostatic effect on the testis; the clinical significance of this for humans is unknown. Foscarnet is nephrotoxic, although this effect can be reduced by large volumes of normal saline. Foscarnet also affects ionized calcium levels and produces a fixed drug eruption on the skin of the genital area. Cidofovir is nephrotoxic and it is important that patients are hydrated and receive probenecid to decrease renal concentrations of drug.

Maribavir is a drug in Phase III development which directly inhibits UL97. To date, mutations in CMV UL97 selected with this drug do not give cross-resistance to GCV, but this is a subject which must be kept under review (Chou *et al.*, 2007).

Strategies for Deploying Antiviral Drugs

Based on the different stages of pathogenesis shown in Figure 8.6, four distinct treatment strategies can be envisaged for CMV (see Table 8.10). While these strategies have been evaluated formally in transplant patients, it is disappointing that they have only recently been applied to the design of studies in AIDS patients.

Table 8.10 Strategies for deploying chemotherapy against CMV

Term used	When drug given	Risk of disease	Acceptable toxicity
Prophylaxis	Before active infection	Low	None
Suppression	After peripheral detection	Medium	Low
Pre-emptive therapy	After systemic detection	High	Medium
Treatment	Once disease apparent	Established	High

Prophylaxis

This approach is to give a drug active against CMV from the time of transplant, and Table 8.11 lists the double-blind, randomized, placebo-controlled trials which have been conducted to date of such prophylaxis. GCV, the most potent drug *in vitro*, has been subjected to the rigours of several such trials but the other two licensed drugs, foscarnet and cidofovir, have not. In addition, Table 8.11 shows that interferon- α (IFN α), ACV, valaciclovir and immunoglobulin have also been studied, although many physicians do not associate these therapies with anti-CMV activity.

The results summarized in Table 8.11 show that GCV has consistently demonstrated activity against CMV infection in all patient groups. It also reduced CMV disease in most groups, with some discrepancies which require discussion. After bone marrow transplant, GCV had a strong trend towards protection, which was significant in one study (Goodrich *et al.*, 1993) but failed to reach statistical significance in a second (Winston *et al.*, 1993). After heart transplant, GCV had a significant effect in seropositive recipients (Merigan *et al.*, 1992) but not primary infections in one study, while the opposite was seen in another (Macdonald *et al.*, 1995). Note that GCV prophylaxis after bone marrow transplantation had no significant effect or even a trend in favour of survival; presumably, the drug-induced neutropenia facilitated bacterial and fungal infections, to which the patients succumbed (Goodrich *et al.*, 1993; Winston *et al.*, 1993). This illustrates the principle in Table 8.10, that prophylaxis exposes all patients to the risk of side effects and so should only be contemplated when a drug has no serious toxicity. In patients with AIDS, a trial of oral ganciclovir (oGVC), 1 g t.d.s., versus placebo was termed prophylaxis because patients did not have CMV disease at trial entry (Spector *et al.*, 1996). Note that this is not virological prophylaxis as defined in Table 8.10. Nevertheless, PCR data show that the greatest effect of GCV was seen when it was given prophylactically, because the drug had less effect in those who were already PCR-positive at trial entry.

The results in Table 8.11 also show that IFN α , ACV and valaciclovir each had anti-CMV effects *in vivo*, whereas there was no evidence of anti-CMV activity

of immunoglobulin. This suggests that, if immunoglobulin does have clinical benefit against CMV disease, this is provided by interference with secondary phenomena rather than CMV itself. Note that the anti-CMV effect of ACV is clinically important; indeed, ACV is the only antiviral drug shown to improve survival when used for prophylaxis after bone marrow transplantation, presumably because its modest antiviral activity is not offset by bone marrow toxicity, as is the case with GCV. A randomized comparison of prophylaxis with ACV or GCV after liver transplant (not included in Table 8.11) shows GCV to be superior in controlling CMV disease (Winston *et al.*, 1995), illustrating that the toxicity of GCV is less prominent if bone marrow is not the organ being transplanted.

Early Treatment of Active Infection

Where allograft recipients are monitored closely to detect active CMV infection at the earliest possibility, trials have been conducted to determine whether early intervention with antiviral drugs can provide clinical benefit. There are two main approaches: *suppression*, where the drug is given after CMV has been detected at a peripheral site, such as in urine or in saliva; and *pre-emptive therapy*, where CMV is detected systemically, either from blood or the lung sampled by bronchoalveolar lavage. The collection of the latter sample is predicated on the immunopathological nature of CMV pneumonitis, so that therapy is directed at early virus replication before disease has become established. Both approaches have produced clinical benefit (see Table 8.12). In particular, the study of GCV in bone marrow transplants by Goodrich *et al.* 1991 showed this drug was literally life-saving when used in pre-emptive mode, in contrast to its lack of effect on mortality when used prophylactically (Goodrich *et al.*, 1993; Winston *et al.*, 1993). Again, the principles summarized in Table 8.10 are prescient, showing that the toxicity of drugs for particular organs is as relevant as their antiviral potency, and that survival benefit can be achieved when the number of patients exposed to a toxic drug can be kept low by using laboratory markers to target therapy to individuals. The results in Table 8.12 also show that where GCV was compared to placebo or observation it

Table 8.11 Double-blind, randomized, placebo-controlled trials of prophylaxis for CMV infection and disease after transplantation

Patient group	Study group	Dose	Planned duration therapy (wk)	Number of patients		Markers of efficacy in whole population				Reference	
				Placebo	Drug	Reduced viraemia	Reduced excretion	Reduced disease	Increased survival		Reduced indirect
RT _x	IFN	3 × 10 ⁶ U 2/wk	6	20	21	Yes	Yes	No	No	No	Cheeseman <i>et al.</i> (1979)
RT _x	IFN	3 × 10 ⁶ U 3/wk 6 wks 3 × 10 ⁶ U 2/wk 8 wks	14	22	20	No	Yes	Yes	No	No	Hirsch <i>et al.</i> (1983)
RT _x	IFN	3 × 10 ⁶ U 3/wk 6 wks 3 × 10 ⁶ U 2/wk 8 wks	14	36	32	No	Yes	No	No	No	Lui <i>et al.</i> (1992)
RT _x	ACV	800 3200 mg/d	12	51	53	Yes	Yes	Yes	No	No	Balfour <i>et al.</i> (1989)
RT _x with rejection	Ig	100 mg kg ⁻¹	15	16	11	No	No	No	Yes	No	Metselaar <i>et al.</i> (1989)
RT _x	VACV	2 g q.d.s	13	310	306	Yes	Yes	Yes	No	Yes	Lowance <i>et al.</i> (1999)
LT _x	Ig	150 mg kg ⁻¹	16	72	69	No	No	No	No	Yes	Snydman <i>et al.</i> (1993)
LT _x	GCV	1 g t.d.s.	14	154	150	NG	NG	Yes	No	No	Gane <i>et al.</i> (1997)
LT _x	ACV	800 mg q.d.s	12	27	28	No	No	Yes	No	No	Barkholt <i>et al.</i> (1999)
HT _x	GCV	5 mg kg ⁻¹ b.d. 14 d 6 mg kg ⁻¹ o.d. 5/7 d until 28 d	4	73	76	No	Yes	Yes	No	Yes	Merigan <i>et al.</i> (1992)
HT _x	GCV	5 mg kg ⁻¹ o.d. 3/7 d until 42 d plus 14 d rejection	6(+ rejection)	28	28	NG	Yes	No	NG	No	Macdonald <i>et al.</i> (1995)
BMT _x	GCV	2.5 mg kg ⁻¹ t.d.s. days 1–7 then 6 mg kg ⁻¹ 5 d/wk after engraftment	13.5 median	45	40	No	Yes	No	No	No	Winston <i>et al.</i> (1993)
BMT _x	GCV	After engraftment 5 mg kg ⁻¹ b.d. 5 d then o.d.	10.9 median	31	33	No	Yes	Yes	No	No	Goodrich <i>et al.</i> (1993)
BMT _x	ACV	500 mg m ⁻² t.d.s. 1 m then 800 mg q.d.s. 6 m or placebo. Third arm 200 400 mg 1 m, then placebo	30	105	102 ^a	Yes	Yes	No	Yes	No	Prentice <i>et al.</i> (1994)

BMT_x, bone marrow transplant; RT_x, renal transplant; HT_x, heart transplant; NG, not given; GCV, ganciclovir; ACV, aciclovir; IFN_α, interferon- α ; Ig, immunoglobulin; VACV, valaciclovir; ^athird arm.

Table 8.12 Controlled clinical trials evaluating pre-emptive therapy

Patient group	Site sampled	Laboratory method	Patients randomized	Management allocation		Outcome compared to arm 1		Reference	
				Arm 1	Arm 2	Duration viraemia	CMV disease		Survival
BMT _x	BrW	Cytology DEAFF	40	Observation	GCV 5 mg kg ⁻¹ b.d. 14d then o.d. until 120d	NG	Reduced	Increased	Schmidt <i>et al.</i> (1991)
BMT _x	Urine Blood	DEAFF	72	Placebo	GCV 5 mg kg ⁻¹ b.d. 7d then o.d. until 100d	NG	Reduced	Increased	Goodrich <i>et al.</i> (1991)
BMT _x	Blood Urine Throat	CCC Culture (1 pos) PCR	37	Culture	PCR	NG	Reduced	Increased	Einsele <i>et al.</i> (1995)
BMT _x	Blood	Ag	226	GCV prophylaxis 5 mg kg ⁻¹ b.d. 5/7 d then 6 mg kg ⁻¹ o.d. 6/7 d until 100 d or placebo	GCV 5 mg kg ⁻¹ b.d. 15 d	NG	Same	Same	Boeckh <i>et al.</i> (1996)
BMT _x	Blood	Ag	39	GCV 5 mg kg ⁻¹ b.d. 15 d	Fos 90 mg kg ⁻¹ b.d. 15 d	Same	Same	Same	Moretti <i>et al.</i> (1998)
BMT _x	Blood	Ag or PCR	213	GCV 5 mg kg ⁻¹ b.d. 14 d	Fos 60 mg kg ⁻¹ b.d. 14 d	Same	Same	Same	Reusser <i>et al.</i> (2002)
LT _x	Blood	Ag	22	GCV 5 mg kg ⁻¹ b.d. 7 d	oGCV 2g t.d.s. 14d then 1g t.d.s. 28d	NG	Same	Same	Singh <i>et al.</i> (2000)
LT _x	Blood	Ag	60	Observation	oGCV 1g t.d.s. 14d	Reduced	Same	Same	Rayes <i>et al.</i> (2001)
LT _x	Blood	PCR	69	Placebo	oGCV 1g t.d.s. 8 wks	Reduced	Reduced	Same	Paya <i>et al.</i> (2002)
RT _x	Blood	Ag	80	Observation	oGCV 1g t.d.s. 27-70 d	NG	Reduced	Same	Sagedal <i>et al.</i> (2003)
LT _x	Blood	PCR	48	GCV 5 mg kg ⁻¹ b.d. 14d	GCV 5 mg kg ⁻¹	Same	Same	Same	Mattes <i>et al.</i> (2004)
RT _x BMT _x				b.d. 14d	o.d. 14d plus Fos 90 mg kg ⁻¹ o.d. 14d				
Neonates	Urine	Culture	100	Observation	GCV	NG	Reduced	Same	Kimberlin <i>et al.</i> (2003)
					6 mg kg ⁻¹ b.d 6 wk				

BMT_x, bone marrow (or stem cell) transplant; BrW, bronchial washings; LT_x, liver transplant; GCV, ganciclovir; oGVC, oral ganciclovir; d, days; w, weeks; NG, not given; CCC, conventional cell culture; DEAFF, detection of early antigen fluorescent foci; PCR, polymerase chain reaction; Ag, antigenaemia; Fos, foscaet.

Table 8.13 Comparison of the proposed advantages of prophylaxis and pre-emptive therapy

Proposed advantages of prophylaxis	Proposed advantages of pre-emptive therapy
Proven benefit of controlled clinical trials	Target resources on patients most at need (financial, skill)
<ul style="list-style-type: none"> • CMV disease • Indirect effects 	
Avoids complicated logistical problems	Treat when viral load lower
<ul style="list-style-type: none"> • Real-time laboratory assays • Organization of sample collection • Geographical location 	<ul style="list-style-type: none"> • Shorter treatment • Reduced recurrences? • Reduced resistance?
Protects against HSV, VZV	Allows low-level stimulation of immunity—reduces late-onset disease?
Overall, may be more cost-effective	Protects patients noncompliant with prophylaxis
<ul style="list-style-type: none"> • Cost of laboratory tests • Indirect effects of CMV • Other herpesviruses 	
	May also reduce indirect effects

usually produced less CMV disease, often accompanied by a survival benefit. In contrast, where GCV was compared to another effective management, such as foscarnet or prophylaxis with GCV, no significant benefit was seen. The results in Table 8.12 thus provide the evidence that GCV and foscarnet are each effective anti-CMV compounds, that they can be given in combination (each at half dose) to minimize toxicity but do not show synergy *in vivo*, and that pre-emptive therapy and prophylaxis are each effective for preventing CMV disease. Each of these strategies has its advantages and disadvantages (see Table 8.13) and decisions about which to choose should be made locally and supported by continuing audit of what should now be a low incidence of CMV disease in transplant patients, for example 1–2% compared to approximately 15% before treatment became available (Singh, 2006; Snyderman, 2006).

This conclusion, that prophylaxis and pre-emptive therapy are both effective ways of controlling CMV disease, is supported by the results of a recent, randomized trial which compared both strategies directly in renal allograft recipients (Khoury *et al.*, 2006). Late-onset CMV disease developed once valganciclovir prophylaxis was stopped, but such late-onset disease was not seen with pre-emptive therapy. Importantly, there were no significant differences in the incidence of CMV disease or in indirect effects such as bacterial infections or graft rejections between the two strategies.

In contrast, although pre-emptive therapy should be an effective strategy in AIDS patients, there is no evidence for this because controlled trials were not conducted

to address the possibility. However, the PCR data at trial entry from a ‘prophylaxis’ trial in AIDS patients (Feinberg *et al.*, 1998) of valaciclovir versus doses of ACV shown in two previous randomized, double-blind, placebo-controlled trials to be ineffective against CMV disease, can be interpreted to show that this drug worked best for pre-emptive therapy (Griffiths *et al.*, 1998). This interpretation was initially controversial but is now supported by natural history studies from three separate research groups, showing that PCR viraemia identifies AIDS patients at high risk of imminent CMV disease. Although HAART has revolutionized the prognosis of AIDS patients, cases of CMV disease still occur in developed countries, for two reasons. First, many individuals are unaware that they are infected with HIV and present with AIDS-defining conditions including CMV. Second, some patients have progressive HIV disease despite having tried all available antiretroviral compounds. In this latter group, a randomized placebo-controlled trial of pre-emptive therapy with valganciclovir has been conducted by the AIDS Clinical Trial Group (Protocol 5030), triggered by the detection on a single occasion of CMV DNA in plasma (Wohl *et al.*, unpublished). This is the first trial to apply the concepts of CMV pathogenesis learned from transplant patients to the parallel group of patients immunocompromised by HIV, but recruitment was difficult because of the availability of new HAART options. This underpowered study did not provide any evidence to support the use of pre-emptive therapy in AIDS patients. In addition, it is known that the institution of HAART in a patient with asymptomatic CMV

viraemia leads to prompt resolution of viraemia (Deayton *et al.*, 1999), so that HAART can be thought of as a form of pre-emptive therapy, analogous to reducing the dose of immunosuppressive drugs in a transplant patient with CMV infection.

Treatment of Established Disease

The treatment of an established disease is the most difficult strategy to pursue successfully, since the virus may trigger pathological phenomena unresponsive to antiviral drugs and because extensive tissue damage is often followed by target organ failure and secondary opportunistic agents, which present their own management problems. It is thus salutary to note that, in contrast to the proven benefits of prophylaxis and pre-emptive therapy, no double-blind randomized placebo-controlled trial of anti-CMV therapy in established CMV disease has demonstrated that treatment at this late stage can provide a clinical benefit (see Table 8.14).

One trial reported that GCV and foscarnet are equipotent for the treatment of CMV retinitis in AIDS patients, but that foscarnet is associated with a significant survival benefit (Studies of Ocular Complications of AIDS Research Group, in collaboration with the AIDS Clinical Trials Group, 1992). Although foscarnet should thus be considered the treatment of choice under these circumstances, the difficulties with intravenous administration, toxicity and cost of foscarnet usually lead clinicians to prescribe GCV instead. This illustrates that the drugs available at present for the treatment of CMV are far from ideal, so the development of safe, orally bioavailable drugs is eagerly awaited. Note also that a randomized trial in AIDS patients showed that the combination of GCV plus foscarnet was superior to either drug given alone (Studies of Ocular Complications of AIDS Research Group ACTG, 1996). In the absence of evidence for synergy between these two drugs *in vivo* against CMV (Mattes *et al.*, 2003), this outcome is best interpreted as the earliest example of combination antiretroviral therapy providing a transient clinical benefit against an opportunistic infection (because foscarnet also inhibits HIV).

The management of established CMV retinitis is complex, with multiple therapeutic options, including intravitreal injections of antiviral compounds as well as intraocular implantation of a device which slowly releases GCV into the vitreous fluid.

These therapeutic options have frequently been evaluated by recruiting patients with peripheral retinitis which is not immediately sight-threatening and randomizing them to immediate therapy versus therapy with the same drug delayed until progression of retinitis has been observed (reviewed in Thorne *et al.*, 2007).

Note that CMV lung infection in AIDS patients is not routinely treated because of the theory that they do not mount the cell-mediated response required for immunopathology, and because cohort studies in the pre-HAART era showed no excess mortality among patients with *Pneumocystis carinii* pneumonia who were co-infected with CMV. CMV pneumonitis in allograft patients is treated with immunoglobulin in addition to GCV because components of the preparation may possibly block the hypothesized immunopathological response to target antigens in the lung.

CMV strains resistant to GCV, to GCV plus foscarnet, or to GCV plus foscarnet plus cidofovir, have been described in AIDS and transplant patients (Chou *et al.*, 2005). Genetic changes in UL97 usually confer low-level resistance to GCV. Continued selective pressure may select for UL54 mutants, some of which are cross-resistant to cidofovir. Rare mutations in UL54 can confer resistance to both GCV and foscarnet (Chou *et al.*, 2005). Based on knowledge of the replication dynamics of CMV, it was predicted that CMV resistance was a greater problem than had been appreciated in the past and would be found in individuals with high-level replication receiving partially-effective antiviral prophylaxis for a long time, for example more than 100 days (Emery and Griffiths, 2000).

Clinical studies in transplant patients confirm this prediction and suggest that approximately 15–25% of patients with CMV disease in transplant (Limaye *et al.*, 2000) and AIDS patients in the pre-HAART era (Bowen *et al.*, 1996; Hu *et al.*, 2002) have ganciclovir-resistant strains.

When HAART is introduced in a patient with established CMV retinitis, an inflammatory reaction may occur, leading to vitritis, which often impairs vision more than the underlying retinitis. This pathological interaction between CMV and the immune system might be avoided if CMV retinitis were prevented by using pre-emptive therapy. Interestingly, no exacerbation or reduction of any other CMV disease has been observed with HAART, whereas an increase in CMV pneumonitis should be expected if the condition is immunopathologically mediated. In patients receiving GCV maintenance therapy for CMV retinitis, this can be stopped once the CD4 count and HIV viral load have responded to HAART for more than three months.

To summarize the main evidence base for treatment, different strategies have been described, with advantages and disadvantages for different groups of patients. For transplant patients, several strategies are available, and which is chosen by a particular unit will depend upon clinical preference and the laboratory support available (Bowen *et al.*, 1996; Emery, 2001; Hart and Paya, 2001)

Table 8.14 Randomized controlled trials of therapy for established CMV disease

Patient group affected	Drug 1	Drug 2	Planned duration of therapy (d)	No. of patients		Significant markers of efficacy reported				References	
				Drug 1	Drug 2	Reduced viraemia	Reduced excretion	Reduced disease	Reduced dissemination		Increased survival
BMT _x Upper gastrointestinal tract (GIT)	GCV 2.5 mg kg ⁻¹ t.d.s.	Placebo	14	18	19	No	Yes	No	No	No	Reed <i>et al.</i> (1990)
AIDS Retina	GCV 5 mg kg ⁻¹ b.d. 14 d then o.d.	Foscarnet 60 mg kg ⁻¹ t.d.s. 14 d then 90 mg kg ⁻¹ o.d.	14; induction then maintenance	127	107	ND	ND	No	No	Yes	Studies of Ocular Complications of AIDS Research Group Dieterich <i>et al.</i> (1993)
AIDS Lower GIT	GCV 5 mg kg ⁻¹ b.d. 14 d then o.d.	Placebo	14	32	30	No	Yes	No ^d	Yes	No	Studies of Ocular Complications of AIDS Research Group ACTG (1996)
AIDS Retina (relapsed or active retinitis despite maintenance)	GCV 5 mg kg ⁻¹ b.d. 14 d then 10 mg kg ⁻¹ o.d. or foscarnet 90 mg kg ⁻¹ b.d. 14 d then 120 mg kg ⁻¹ o.d.	GCV plus foscarnet: continue existing maintenance dose, add induction dose of second drug for 14 d. For maintenance: GCV 5 mg kg ⁻¹ o.d. foscarnet 90 mg kg ⁻¹ o.d.	Life	183	96	ND	ND	Yes	ND	No	Studies of Ocular Complications of AIDS Research Group ACTG (1996)

BMT_x, bone marrow transplant; GCV, ganciclovir; GIT, gastrointestinal tract; ND, not determined; ^dintention to treat analysis, but Yes in subsidiary analysis.

(Table 8.13). However, such patients should clearly be managed by one of the proactive strategies (prophylaxis or pre-emptive therapy), since it would be unethical to continue to allow the natural history of CMV disease in these patients to proceed unchecked in the face of overwhelming benefit demonstrated in controlled clinical trials. Similar principles can be applied to AIDS patients and congenitally-infected infants, but much less evidence is available to date.

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Epstein–Barr Virus

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INTRODUCTION

The discovery of Epstein–Barr virus (EBV) in 1964 resulted from the description by Denis Burkitt of a geographically-restricted tumour occurring in African children (Burkitt, 1958). The tumour, which characteristically arises in the jaw, is now known to be of B-lymphocyte origin and is called Burkitt's lymphoma (BL). Burkitt noticed that the geographical distribution of the tumour in Africa corresponded to that of holoendemic malaria, and was determined by the climatic conditions (high temperature and high rainfall) in which the malaria-carrying mosquito can breed. Because of this observation, Burkitt suggested that the tumour had an infectious aetiological agent for which the mosquito was the vector. This hypothesis led to electron microscopic studies on fresh tumour biopsy material, but this technique was unrewarding. Later, cell lines were grown in suspension culture from BL tumour material (Epstein and Barr, 1964) and in these cells virus particles were seen (Epstein *et al.*, 1964). Further studies showed this to be a new and distinct member of the herpesvirus group. Thus Burkitt's initial postulate of an infectious agent being involved in the aetiology of the tumour proved to be correct, and, although the mosquito does not play the role of vector for the virus, the association with hyperendemic malaria remains an important and constant finding.

Seroepidemiological studies have since shown that the virus is a ubiquitous agent; seropositivity increases with age in all communities studied, so that over 90% of adults worldwide are seropositive. When carrying out these studies, Henle and Henle (1966) made the observation that

a member of their staff seroconverted while undergoing an attack of acute infectious mononucleosis (IM). Further studies on college students in collaboration with Yale University proved that EBV is the sole aetiological agent in IM (Niederman *et al.*, 1970).

Since its discovery, EBV has been associated with a variety of other lymphoid and epithelial tumours. Early seroepidemiological and molecular studies pinpointed an association between EBV infection and anaplastic nasopharyngeal carcinoma (NPC) (Old *et al.*, 1966; Wolf *et al.*, 1973), a geographically- and genetically-restricted tumour which is very common in southern China. Other EBV-associated tumours include the lymphoproliferative lesions and lymphoma which develop in immunocompromised individuals (Crawford *et al.*, 1980), certain types of T-cell lymphoma (Jones *et al.*, 1988), a subset of Hodgkin's lymphoma (Anagnostopoulos *et al.*, 1989), as well as a minority of gastric carcinomas (Tokunaga *et al.*, 1993). The benign epithelial lesion of oral hairy leukoplakia (OHL) also contains replicating EBV (Greenspan *et al.*, 1985).

THE VIRUS

Structure

EBV is a DNA virus, which is a member of the *Herpesviridae* family, subfamily *Gammaherpesvirinae*, genus *Lymphocryptovirus*, showing a structure indistinguishable from other human herpesviruses by electron microscopy. It is a large virus with a buoyant density in

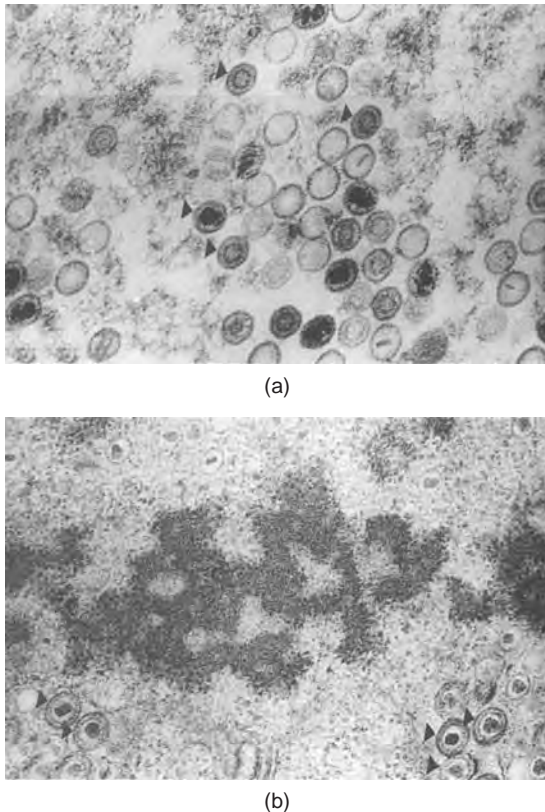


Figure 9.1 Electron micrograph of the thin section of cells from an EB virus-immortalized lymphoblastoid cell line (M-ABA). Arrows indicate (a) immature virus particles and (b) enveloped virus particles (x 39 000).

caesium chloride of 1.2–1.3 and a molecular weight of 100×10^6 Da. The central nucleic acid of the virion is surrounded by an icosahedral capsid consisting of 162 triangular capsomeres and measuring 100 nm in diameter (Figure 9.1). This, in turn, is surrounded by an outer, irregularly-shaped, lipid-containing envelope giving the mature particle a diameter of 150–200 nm. The viral envelope is derived from cellular membranes of infected cells and, like other herpesviruses, is believed to be acquired at the final envelopment sites in the *trans*-Golgi network (Mettenleiter *et al.*, 2006). The envelope is essential for infectivity, and the sensitivity of the virus to ether and other lipid solvents results from its destruction.

The Viral Genome

In the virion the EBV genome is a linear double-stranded DNA molecule 172 kb in length (De Jesus *et al.*, 2003). Structurally, the genome consists of alternating unique and internal tandem repeat regions flanked by terminal

repeat (TR) sequences (Figure 9.2). The genome sequence shows around 85 predicted open reading frames (ORFs), which are designed by a four-letter and one-number acronym; for example, BZLF1 refers to the first leftward ORF of the Bam H Z fragment of the genome (Baer *et al.*, 1984). Two strains of EBV have been defined—type 1 and type 2 (alternatively named A and B), which differ at the domains which code for the Epstein–Barr viral latent proteins. Although these types show no specific geographical restriction, in Western countries type 1 virus is more commonly isolated than type 2. *In vitro* type 1 viruses are more efficient at transforming B lymphocytes than type 2; however, neither type has a specific disease association. Variation within types occurs in the number of repeat sequences in each internal repeat, making it possible to define specific isolates by the size of their latent genes and/or their products. Analysis of these genes can therefore be used in epidemiological studies to monitor virus transmission within families or populations.

Following infection of B lymphocytes *in vitro*, EBV establishes a latent type of infection with blast transformation of the cell and a restricted (latent) viral gene expression compatible with continued cell proliferation. The viral genome does not usually integrate into cellular DNA but forms a closed circular episome by covalent linkage of the TR elements. The episome replicates to give multiple copies shortly after infection and thereafter resides in the nucleus and replicates with cellular DNA, with equal partitioning to daughter cells allowing the copy number per clone to remain constant. These episomal forms can be activated to a lytic infection with the release of multiple viral progeny and cell death.

Viral-coded Proteins and Transcripts

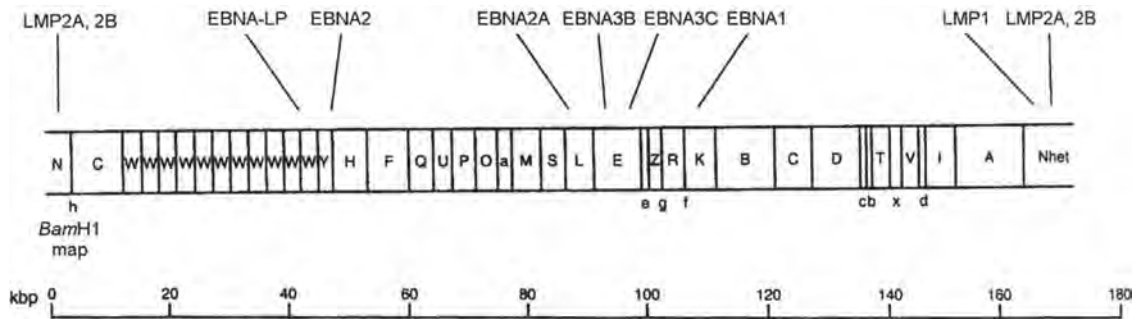
The viral genome is large enough to code for around 85 average-sized proteins, but not all of these have been identified and assigned to ORFs on the viral genome. Nine viral-coded proteins are expressed in latently-infected cells (Table 9.1; Figure 9.2) in addition to the classical herpesvirus immediate-early, early and late proteins associated with the lytic infection. The EBV genome also codes for two small RNAs (EBV-encoded RNAs, EBERs) which form abundant transcripts but are untranslated. EBERs may play a role in tumorigenesis as they confer resistance to interferon α -induced apoptosis in BL cells by inhibiting phosphorylation of RNA-activated protein kinase (Nanbo *et al.*, 2002). They also induce expression of interleukin (IL)-10 in BL cells (Kitagawa *et al.*, 2000). In addition, a complex series of highly-spliced transcripts from the Bam A region (BART transcripts) of the genome are found in latently-infected cells, but their function remains unclear. Recently, several microRNAs have been identified in the Bam A region of the genome and it is

Table 9.1 EBV-coded latent proteins and latent transcripts in different diseases

Antigens/transcripts	Expressed in						
	IM	BL	NPC	HD	PTLD	AIDS lymphoma	T/NK cell lymphoma
EB nuclear antigen (EBNA)							
1	+	+	+	+	+	+	+
2	+	-	-	-	+	+	-
3A	+	-	-	-	+	+	-
3B	+	-	-	-	+	+	-
3C	+	-	-	-	+	+	-
LP	+	-	-	-	+	+	-
Latent membrane protein (LMP)							
1	+	-	±	+	+	+	+
2A	+	-	+	+	+	+	+
2B	+	-	+	+	+	+	+
EBERs	+	+	+	+	+	+	+
BARTs	+	+	+	+	+	+	+
Latency type	3	1	2	2	3	3	2

EBNAs 3A, 3B, 3C are also termed EBNAs 3, 4 and 6, respectively. Leader protein (LP) is also termed EBNA5. LMPs 2A and B are also termed terminal protein 1 and 2.

IM, infectious mononucleosis; BL, Burkitt's lymphoma; NPC, nasopharyngeal carcinoma; HD, Hodgkin's disease; PTLD, post-transplant lymphoproliferative disease; NK, natural killer; EBERs, EBV-encoded RNAs; BARTs, Bam H1 A transcripts.

**Figure 9.2** Linear map of the EBV genome, showing the open reading frames for the major latent proteins.

postulated that these may have roles in the regulation of host and viral gene expression (Pfeffer *et al.*, 2004).

Latent Proteins

(Reviewed by Young and Murray 2003.)

EB Viral Nuclear Antigens (EBNAs)

EBNAs were first detected by anticomplementary immunofluorescence in the nuclei of latently-infected EBV transformed B cells (Reedman and Klein, 1973), and subsequently identified as six separate proteins (EBNA1, 2, 3A, 3B, 3C and leader protein (LP) (also called

EBNA1–6)) which are translated from a long polycistronic mRNA by alternative splicing (Table 9.1).

EBNA1 is coded by the Bam K ORF and characterized by a 20–45 kDa glycine–alanine (gly-ala) repeat sequence which varies in length, causing the molecular weight of the protein to vary between viral isolates (65–85 kDa). The protein binds to the viral origin of replication and to metaphase chromosomes, thereby accounting for EBV episomal maintenance within the infected cell and equal partitioning into daughter cells at cell division. EBNA1 is essential for *in vitro* transformation of B cells, and mice expressing EBNA1 as a transgene in B cells have been reported to develop lymphoma (Wilson *et al.*, 1996). It has been postulated that EBNA1 may have anti-apoptotic

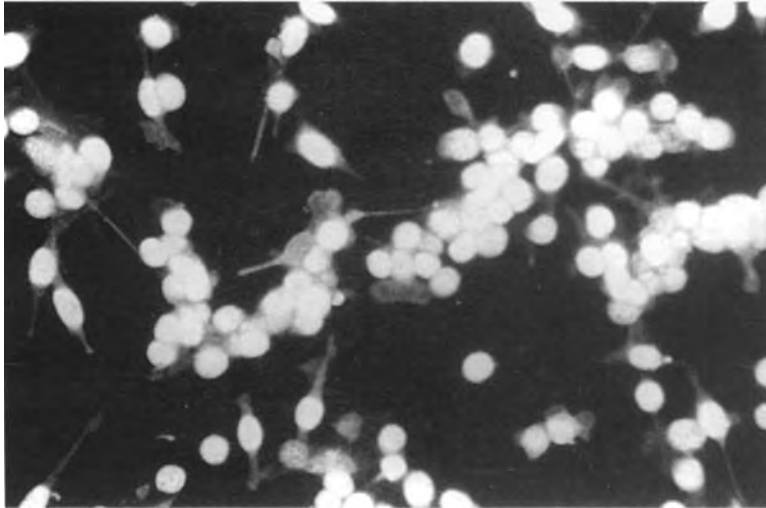


Figure 9.3 A photomicrograph of cells from an EB virus transformed adherent lymphoblastoid cell line (MABA) stained for EBNA. Bright nuclear fluorescence is seen in all cells. The cells are counterstained with Evans Blue ($\times 70$).

function in normal and tumour cells (Kennedy *et al.*, 2003).

EBNA2 is an 86-kDa protein which is coded for by the Bam WYH ORFs. Expression of *EBNA2* is essential for B-cell transformation and the protein plays a pivotal role in this event by transactivating all the other latent viral genes. In addition, *EBNA2* activates cellular genes including the B-cell activation and proliferation antigens CD21 and CD23, and the proto-oncogenes *c-myc* and *c-fgr*. *EBNA2* also upregulates BLR2/*EB11*, a G-protein-coupled receptor gene that plays a role in lymphocyte trafficking. *EBNA2* does not directly bind DNA and provides transcriptional regulation by interacting with cellular DNA-binding proteins such as retinoblastoma protein- κ and PU.1 (Johannsen *et al.*, 1995).

EBNA3A, *EBNA3B* and *EBNA3C* are a family of related proteins coded for by the BERF ORF, with molecular weights of 140–180 kDa. All three proteins inhibit transcriptional activation of *EBNA2*-responsive genes, thereby counterbalancing the action of *EBNA2*. However, only *EBNA3A* and *EBNA3C* are essential for transformation of B cells.

EBNA-LP is coded for by the BWRF1 ORF, which also forms the leader sequence of the *EBNA* RNAs. Because of multiple repeat regions in the DNA, *EBNA-LP* varies in size from 20 to 130 kDa. The protein is not absolutely essential for transformation but enhances the *in vitro* growth of infected B cells by complementing the growth-promoting effects of *EBNA2*.

Latent Membrane Proteins (LMPs)

LMP1 is coded for by the BNLFI gene, and its expression is induced by *EBNA2*. BNLFI is the most abundantly transcribed region of the genome in latently-infected cells. The protein is mainly located in the plasma membrane of infected cells, where it associates with the cytoskeleton. Structurally, *LMP1* has six membrane-spanning domains, with both the amino and carboxy termini in the cytoplasm. *LMP1* is essential for the transformation and continued proliferation of B cells and induces a tumorigenic phenotype on transfection into rodent fibroblasts (Wang *et al.*, 1985). When transfected in B cells, *LMP1* upregulates expression of the cell-adhesion molecules, CD23 and CD40, inducing B-cell activation and DNA synthesis (Wang *et al.*, 1990). These changes mimic those seen following CD40-mediated B-cell activation, many of which, in both cases, are mediated by tumour necrosis factor receptor-associated factor (TRAF)-signalling molecules and the transcription factor NF κ B. The amino terminus is essential for these effects, and a truncated form of *LMP1* lacking this region, which is expressed in lytic infection and located in the viral envelope, lacks these properties. When transfected into squamous epithelial cell lines, *LMP1* induces the membrane receptor molecules CD40 and the epidermal growth factor receptor and inhibits terminal differentiation processes.

Sequence analysis of the *LMP1* gene in EBV isolates from different tumours and geographical locations has

identified a common 30-bp deletion in the C terminus as well as several point mutations when compared to the prototype genome. However, these have not been associated with increased oncogenicity.

LMP2A and LMP2B (previously called terminal proteins 1 and 2) are formed by alternative splicing from an ORF which spans the TR sequences, and thus the intact gene is only formed and transcribed after the genome has circularized. Both are membrane proteins with 12-membrane spanning segments, but whereas LMP2A has an amino terminus of 119 amino acids, this is truncated in LMP2B. The amino terminus of LMP2A contains eight tyrosine residues, two of which form immunoreceptor-tyrosine-based activation motifs (ITAMs). These ITAMs interact with protein tyrosine kinases, leading to their degradation. This leads to blockade of B-cell-receptor (BCR) signal transduction and inhibits activation of lytic viral replication cascades. LMP2A thereby promotes cell survival and maintenance of viral latency (Miller *et al.*, 1995). LMP2B on the other hand is believed to exhibit a negative regulatory effect on LMP2A (Rechsteiner *et al.*, 2007).

Lytic Cycle Proteins

EBV lytic genes show extensive homology to those of other herpesviruses, and their expression similarly follows an orderly cascade, with the expression of each set being activated by the previous and inhibited by the following set. They are divided into immediate-early, early and late, according to whether they are transcribed before (immediate-early and early) or after (late) viral DNA synthesis.

Immediate-early genes. EBV possesses two genes that can be classified as immediate-early genes, BZLF1 (Z protein) and BRLF1 (R protein), which are activated in a latently-infected cell following BCR stimulation through cross-linking of surface immunoglobulin (Ig). Z and R proteins in turn transactivate the early genes, thereby leading to the switch from latent to lytic infection in B cells. R protein also activates viral DNA polymerase. Z protein can activate cell-cycle genes such as c-fos and human immunosuppressive cytokines such as IL-10 and tumour growth factor- β .

Early genes. First identified by the staining pattern of sera-containing antibodies to EBV when applied to the Raji cell line which lacks expression of the late genes, the early gene products (early antigens, EAs) were characterized as diffuse (D) (nuclear and cytoplasmic staining) and restricted (R) (nuclear staining) (Henle *et al.*, 1971). It is now known that there are around 30 early proteins,

most of which have enzyme functions required for viral DNA replication, including viral DNA polymerase and viral thymidine kinase. Some early gene products are involved in regulation of RNA transport and stability. BHRF1, an early lytic gene, is a homologue of the cellular anti-apoptotic gene, BCL-2, and the protein has a role in inhibition of apoptosis in EBV-infected cells.

Late genes encode viral structural proteins such as viral capsid antigen complex (VCA), tegument proteins and membrane proteins. These genes are transcribed and translated following lytic replication, thus allowing the linear viral genome to be packaged. The major capsid protein is coded for by the BcLF1 ORF. VCA can be detected by indirect immunofluorescence in around 10% of cells in a permissive cell line such as P3HR1 using an EBV-positive human serum. BCRF1, an EBV-late gene, codes for a homologue of human IL-10 which is important in immune evasion.

EBV Glycoproteins

The EBV-coded glycoproteins are involved in viral infectivity and spread. Ten have been identified, most of which are inserted into membranes in an infected cell, and several of these become components of the viral envelope (membrane antigens, MAs).

The major envelope glycoprotein gp340/220 is coded for by the BLLF1 ORF. The protein mediates virus attachment to the B-cell surface by binding to the EBV receptor CR2 (also called CD21). Antibodies to gp340/220 prevent infection by blocking attachment, and the protein has therefore been developed as a vaccine candidate.

gp85 is coded for by the BXLF2 ORF. It has homology to herpes simplex virus (HSV) glycoprotein gH and induces fusion between viral and cellular membranes. gp85 requires another glycoprotein, gp25 (homologous to HSV gL), for its transport to the cell surface. Here it forms a trimolecular complex with gp42 and gp25 which is also present in the viral envelope. The complex mediates B-cell infection by inducing fusion between viral and cellular membranes (gp85 and gp25) and viral penetration by binding to HLA class II molecules in the B-cell surface (gp42). gp42 is not required for infection of epithelial cells which are HLA class II negative (Borza and Hutt-Fletcher, 2002).

gp110 is coded for by the BALF4 ORF and has homology with the HSV glycoprotein gB. It is localized in nuclear and cytoplasmic membranes of infected cells but is not detected in the viral envelope.

Host Range and Growth *In Vitro*

The host range of EBV is limited to man, its natural host, and some subhuman primates, including the gibbon, owl monkey, squirrel monkey and some species of tamarin, all of which can be infected experimentally.

In humans only B lymphocytes are regularly infected by the virus and this restriction of infection is probably accounted for by their expression of CR2 (the receptor for the C3d component of complement, also called CD21), the cell-surface receptor used by EBV. A similar molecule found on a subpopulation of squamous epithelial cells may result in infection of these cells in the oropharyngeal cavity, although the extent and role of this infection in normal individuals is a controversial issue. Activated T cells and dendritic cells also express CR2 and may on rare occasions support EBV infection. A recent study suggests that monocytes/macrophages may become infected by EBV from B lymphocytes, migrate into the epithelium and spread virus to oral epithelial cells (Tugizov *et al.*, 2007).

EBV-induced B Cell Transformation and Immortalization

When EBV is used to infect B lymphocytes *in vitro*, cell activation occurs within 24 hours. Initially, small resting B cells undergo blastogenesis with an increase in HLA-DR expression, nuclear size and cytoplasmic volume, and the expression of B-cell-activation antigens on the cell surface. In particular, CD23 expression is reported to be essential for this so-called cellular transformation. DNA synthesis is initiated, followed by cell division, and around this time Ig can be detected in the cytoplasm of many infected B cells and is subsequently secreted into the culture medium; IgM always predominates. This latter finding indicates that EBV is a potent polyclonal activator of Ig production by B cells. These early changes seen in B cells after infection with EBV are similar to those seen after stimulation with other B-cell activators such as CD40 ligand. However, the polyclonal activation of B cells by EBV leads to transformation, which is a permanent rather than a transient event. Thus, once DNA synthesis is initiated, the cells will continue to proliferate in culture as EBV-positive B lymphoblastoid cell line (LCL). This phenomenon is also known as 'immortalization'.

EBNAs 2 and LP are the first viral antigens to be detected in infected B cells, followed by the expression of all the other EBNAs. LMPs 1, 2A, 2B are expressed last, after the genome circularizes. This pattern of viral gene expression (EBNAs 1, 2, 3A, 3B, 3C, LP; LMPs 1, 2A and 2B) is seen in virtually all cells in an LCL and is termed full latent gene expression or latency type

3 (Table 9.1). Other forms of EBV latency include: EBNA1 only (latency 1), seen in BL cells; and EBNA1, LMP1, LMP2A (latency 2), seen in NPC and Hodgkin's disease (HD). EBERs and BART transcripts are present in all forms of latency (Table 9.1). Recently, a state referred to as latency 0 has been detected in peripheral memory B cells from seropositive individuals where only EBER transcripts are present without expression of latent proteins (Hochberg *et al.*, 2004).

Only a minority (<1–10%) of cells in an LCL at any one time enter a productive phase resulting in viral progeny and cell death. LCL derived from different sources show varying degrees of permissiveness for viral replication. Tamarin-derived cell lines are the most permissive with around 10% of lytically-infected cells, and the tamarin B cell line B95–8, which was originally transformed with IM-derived EBV, is used in most laboratories to obtain infectious virus for experimental purposes (Miller *et al.*, 1972). Virus production into the culture supernatant medium can be induced by the addition of tetradecanoylphorbol-13-acetate (TPA) and sodium butyrate (Luka *et al.*, 1979; zur-Hausen *et al.*, 1978, 1979). Another cell line which can be induced to produce high levels of EBV by the addition of antibodies to surface Ig is BL-derived Akata (Takada, 1984).

Handling of EBV in the Laboratory

EBV is used as a tool for transforming B lymphocytes *in vitro*, and EBV-positive cell lines, most of which produce small quantities of infectious virus, are grown in many laboratories for research purposes. The virus is of low infectivity, and no authenticated cases of primary infection contracted in the laboratory have been reported. EBV is classified as a hazard group 2 pathogen and therefore EBV-positive cell lines should be handled according to routine microbiological laboratory practice for this group.

EPIDEMIOLOGY

In seroepidemiological studies the presence or absence of IgG antibodies to VCA is generally used to determine past infection, since these antibodies arise early in primary infection and thereafter persist for life. UK results show that seropositivity increases with age, reaching a level of around 90% in adults. Two peaks of seroconversion occur, one between the ages of 1 and 6 years and the other at 14–20 years (Figure 9.4), with infection occurring later in high compared to low socio-economic groups. These findings are very similar to those from other countries in the Western world. In developing countries

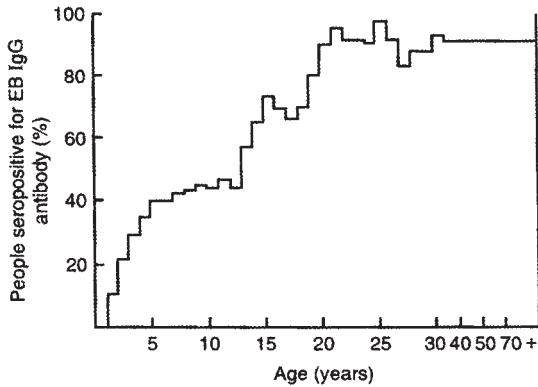


Figure 9.4 Histogram analysis of IgG anti-VCA antibodies in sera from 1469 individuals. The percentage of seropositivity increases with age to a plateau of 92%.

seroconversion occurs early in life, with more than 90% of children over the age of two years being seropositive. Most seroconversions occur subclinically but if primary infection is delayed until adolescence or early adult life acute IM may result.

EBV Infection in the Normal Seropositive Individual

In most individuals primary infection occurs subclinically during childhood, and thereafter a lifelong carrier state exists in which a balance is maintained between the level of virus infection and the cellular and humoral immune mechanisms which keep the infection controlled. Continued low-grade virus shedding in the oropharynx can be found in most seropositive individuals and can be detected by the presence of viral DNA from saliva and throat washings. Furthermore, EBV DNA is present in around 1 in 10^6 circulating long-lived memory B cells, which evade immune surveillance mechanisms by expressing no or a very restricted number of latent genes. However, details of the exact gene expression in these cells have not been elucidated. Periodic reactivation of this latent B-cell infection into lytic replication in lymphoepithelial sites such as the tonsil, perhaps in association with B-cell activation/maturation processes, allows new virus production and replenishment of latently-infected cells, and egress from the body.

EBV-specific, HLA class I-restricted, CD4- and CD8-positive cytotoxic T cells (CTLs) are present in the circulation of all normal seropositive individuals and many latent and lytic T-cell epitopes have been identified. Thus a lifelong balance between virus infection and immune mechanisms is established which successfully controls the infection at subclinical levels in the healthy

host. However, if this balance is altered by intercurrent disease or iatrogenic means which cause a decrease in the specific immune response, EBV-associated disease may occur.

EBV-ASSOCIATED DISEASES

EBV is associated with several disease states, in some of which it is the direct aetiological agent whilst in others it acts as an essential cofactor in a complex series of events which lead to the disease. The EBV-associated tumours are shown in Table 9.2. IM is the result of primary infection, whereas BL and NPC occur in seropositive individuals as a result of a series of alterations in a cell type infected by EBV. OHL and lymphoproliferative lesions occur in seropositive individuals in whom immunosuppression has allowed the cell populations naturally harbouring the virus to expand.

EBV is also associated with a variety of other tumours, including subsets of T-cell lymphomas (Jones *et al.*, 1988), Hodgkin's lymphoma (Anagnostopoulos *et al.*, 1989) and carcinoma of the stomach (Imai *et al.*, 1994). Here the tumour cells harbour clonal viral DNA and express viral antigens, suggesting an aetiological association, whereas links with salivary-gland tumours (Raab-Traub *et al.*, 1991), thymomas (Leyvraz *et al.*, 1985) and leiomyosarcomas in immunodeficient children (Lee *et al.*, 1995) are more tenuous.

Infectious Mononucleosis

IM or glandular fever is an acute, self-limiting lymphoproliferative disease resulting from primary infection with EBV. It classically occurs in adolescents and young adults (aged 15–25 years) in Western societies where a susceptible seronegative population is present in this age group. A recent study among UK university students showed that 25% were EBV seronegative at the time of entering university, that 50% of these seroconverted while at university and that clinical disease occurred in 25% of these individuals while undergoing a primary infection. The remainder (in common with most individuals before adolescence) seroconverted without overt clinical illness (Crawford *et al.*, 2006). The disease is more common in upper socio-economic classes and in the Western world because these individuals have been relatively protected from infection during childhood. The incidence of the disease varies with differing demography and lifestyles, but since in both developing and industrialized countries the majority of those reaching the susceptible age for IM are already seropositive, classical outbreaks of the disease are uncommon.

Table 9.2 EBV-associated tumours.

Tumour	At-risk population	EBV association
Lymphoid origin		
B lymphoproliferative disease (BLPD)	Post-transplant—PTLD ^a	90%
	HIV ^b infection	
	Primary central nervous system lymphoma	<100%
	Peripheral lymphoma	50%
Burkitt's lymphoma (BL)	African children—endemic BL	97–100%
	HIV infection—sporadic BL	25%
Hodgkin's disease (HD)	Children—developing countries	Overall 65%, mixed cellularity type 80%, childhood 80%
	Young adults	
	High SE ^c groups	
	History of IM	
T/NK cell lymphoma	Chronic active EBV HIV infection	10–100%, depending on histological type
Primary effusion lymphoma	HIV infection	70–80%. 100% contain HHV8 ^d DNA
Non-lymphoid origin		
Nasopharyngeal carcinoma	S. Chinese and Inuit races—high incidence	Undifferentiated 100%, nonkeratinized
	Malaysia, Indonesians	100%, keratinized 30–100%
	Filipinos, Vietnamese—intermediate incidence	
Gastric carcinoma	Not identified	Adenocarcinoma 5–15%
Leiomyosarcoma	HIV infection, immunodeficiency—mainly children	Not known

^aPTLD, post-transplant lymphoproliferative disease.

^bHIV, human immunodeficiency virus.

^cSE, socio-economic.

^dHHV8, human herpesvirus 8.

Other tumours with EBV associations: salivary gland tumours, breast carcinoma, thymoma.

Transmission

Detection of infectious EBV in multiple samples of saliva and throat washings indicates that oral excretion of the virus occurs for a prolonged period after IM and then either continuously or intermittently in most seropositive individuals. It is therefore assumed that primary infection occurs by the oral route, by close contact with a virus-excreting individual. Childhood infection probably occurs through salivary contact with family members or other children, whereas the peak of seroconversion in late adolescence, which coincides with the age at which new social contacts are often made, is likely to occur during kissing. However, EBV has also been rescued from the uterine cervix of a few IM patients and normal seropositive women (Sixbey *et al.*, 1986), and from semen from healthy males (Israele *et al.*, 1991), suggesting that sexual intercourse is a possible route of spread. This is backed up by recent data from a large study in university students showing a strong correlation between previous sexual intercourse and EBV seropositivity and IM (Crawford *et al.*, 2002), however these data do not exclude intimate kissing

associated with sexual intercourse as a means of transmission. EBV can also be acquired by transfusion of fresh blood or organ transplantation from a seropositive to a seronegative individual.

Pathogenesis

EBV usually enters the body through the mouth, and a productive infection occurs in the oropharynx, from which site infectious virus particles are shed into the oral cavity and can be recovered from saliva and throat washings. Currently there is much debate regarding the cell type first infected by EBV. Extensive examination of IM tonsils reveals infected B cells expressing lytic antigens, but no infection of the overlying epithelium (Anagnostopoulos *et al.*, 1995). Thus it is possible that EBV first infects B cells in tonsillar crypts where the surface epithelium is incomplete and that the virus could access lymphocytes directly. This initial infection of B cells would result in full latent gene expression (latency 3), cell activation and proliferation, thus amplifying the number of infected cells at the site of entry. These B lymphoblasts can be found in

peripheral blood early in IM, and are thereby disseminated throughout the body. Their presence in the circulation stimulates a massive T-cell response, which is characteristic of IM. The symptoms of IM are not caused directly by virus-infected B cells but are immunopathological in nature, resulting from massive cytokine production from CD8 T cells, and to a lesser extent CD4 T cells and natural killer (NK) cells. Together these cells are thought to control the infection by eliminating infected B lymphoblasts; however, some escape and establish lifelong persistence.

Humoral Immunity

The antibody responses to EBV-associated antigens during primary infection display a characteristic pattern (Figure 9.5). Classically, by the time of onset of clinical symptoms, IgM, and IgG antibodies to VCA are present in the serum, as are IgG antibodies to components of the

early-antigen and membrane-antigen complexes. Antibodies to the viral glycoproteins are neutralizing and probably agglutinate virus particles, thus preventing further infection and spread of the virus. IgM and IgA antibodies to VCA and IgG anti-EA antibodies rise to a peak during the acute disease and decline to low or undetectable levels during convalescence. IgG antibodies to EBNA1 are not usually detectable in the serum until the convalescent period. Heterophile antibodies regularly appear in the serum early in IM, but their relationship to the virus and their role, if any, in controlling infection remain unclear. A variety of autoantibodies may be found in IM, which include cold agglutinins, rheumatoid factors, anti-nuclear antibodies and antibodies to platelets and to smooth muscle. These antibodies, which may account for the raised total serum IgM level found in IM, are thought to be the result of the polyclonal activation of B cells caused by EBV infection. They are usually transient and harmless.

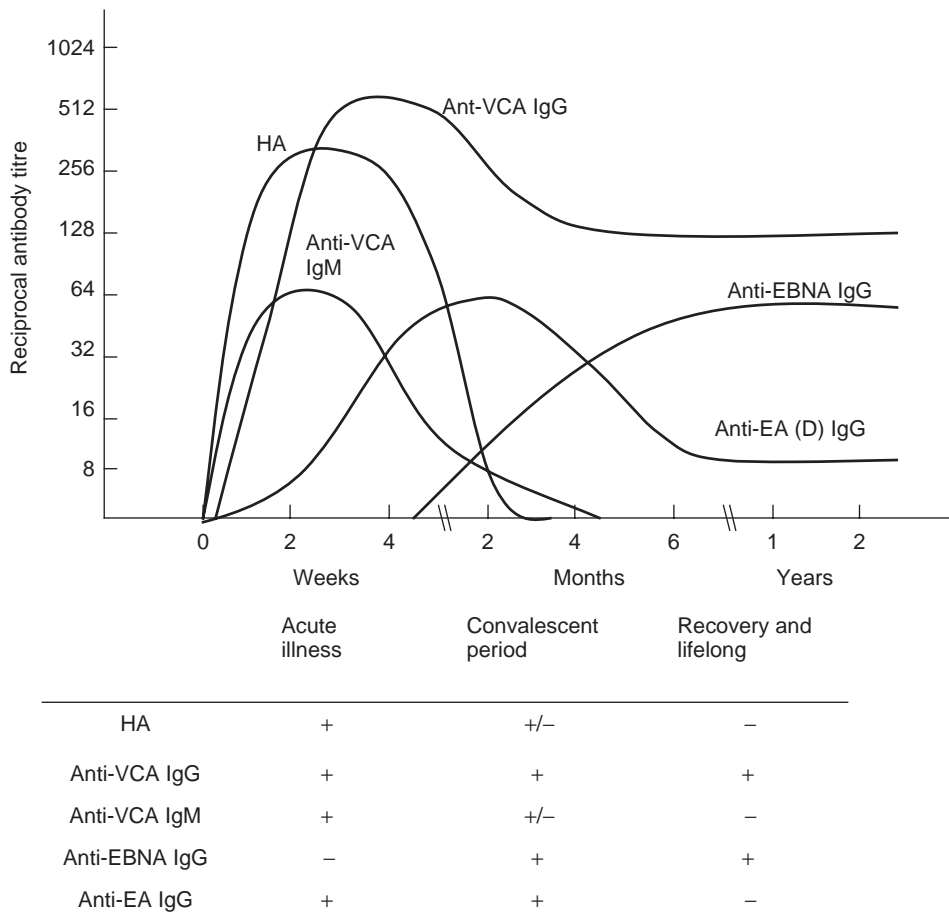


Figure 9.5 Basic pattern of serum antibodies to EBV antigens during and after primary infection. HA, heterophile antibody; VCA, viral capsid antigen; EA (D), diffuse form of early antigen; EBNA, EB nuclear antigen.

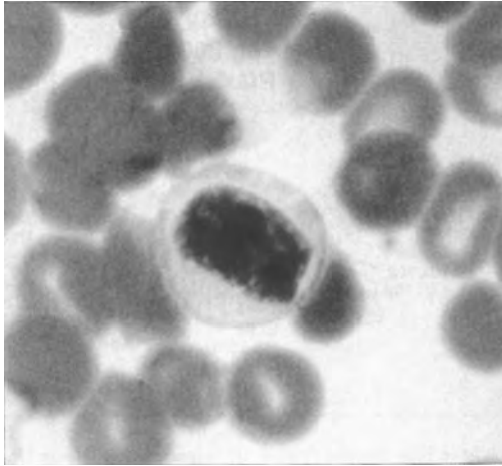


Figure 9.6 Photomicrograph of a May–Grunwald–Giemsa-stained peripheral blood film from acute infectious mononucleosis. An atypical mononuclear cell is illustrated (x 1000).

Cellular Immunity

One of the most distinctive features of IM is the presence, at the time of onset of clinical symptoms, of a lymphocytosis, and ‘atypical’ mononuclear cells in the peripheral blood (Figure 9.3). The rise of lymphocyte count, which may be very marked (up to $15 \times 10^9 \text{ l}^{-1}$), is due to a vast increase in absolute numbers of T lymphocytes. These are highly-activated, CD8-positive, HLA class I-restricted and CTL-specific for lytic (and to a lesser extent latent) antigens. During the acute phase of the disease up to 40% of the total peripheral CD8+ T-cell numbers may be directed against a single EBV epitope (Callan *et al.*, 1996). The lymphocytosis is accompanied by depression in most T-lymphocyte functions, including those measured by delayed-type hypersensitivity testing and *in vitro* mitogen stimulation.

Histological Findings

Generally, tissue from IM patients is not available for study, but occasionally liver, bone marrow or lymph node biopsies are performed before the diagnosis is made. Also, the histological findings on tissue from surgically-removed tonsils and ruptured spleens, and post-mortem specimens have been described. In all cases the tissues are infiltrated with mononuclear cells which are immunoblastic in appearance. These mainly consist of CD8+ T cells with a minority population of EBER-positive B cells. In the lymph node the pattern is generally described as ‘reactive’, with the infiltrate found in widely-dilated sinuses and intersinusoidal cords

extending into the interfollicular compartment, often obscuring the follicular centres. Virus-infected B cells are localized in the paracortical region, where occasional Hodgkin or Reed–Sternberg-like cells may be present. In the liver the portal areas are infiltrated and in the spleen the white pulp may be obscured by the extensive infiltrate which extends throughout the parenchyma. Small aggregates of monocytoïd cells and immunoblasts can be seen in the bone marrow. The histological findings are not diagnostic of IM and may be difficult to distinguish from other causes of immunoblastic proliferations, Hodgkin’s disease and non-Hodgkin’s lymphomas (NHLs).

Clinical Features

Incubation Period As the source of infection is generally not determined, the incubation period of IM is difficult to calculate. However, a period of 30–50 days is usual before symptoms occur.

Symptoms Characteristically, IM begins abruptly with a sore throat and swelling of the neck, accompanied by non-specific symptoms such as malaise, fever, sweating, chills, headaches, stiff neck, anorexia and vague abdominal discomfort. A prodromal period characterized by lassitude and slight fever is described by some patients. The sore throat, which occurs in 80–90% of patients, is usually mild and clears after 7–14 days. It may, however, be severe enough to cause extreme pain and difficulty with swallowing, and occasionally gross tonsillar enlargement may lead to pharyngeal obstruction. In 25% of cases secondary infection of the pharynx occurs, commonly with a β -haemolytic streptococcus. Less commonly, patients present with jaundice, cough, myalgia or symptoms of one of the neurological complications of IM (see below).

Physical Signs Lymphadenopathy is present in the majority of cases at some time during the acute illness. The cervical nodes are most obviously involved, but generalized lymphadenopathy may occur, with the nodes often remaining palpable for several weeks. Glands are discrete and not severely tender. The additional presence of rubbery small glands in the axilla and groin indicates IM rather than a throat infection with involvement of cervical glands. Clinically-detectable splenic enlargement occurs in 50–60% of patients and is usually accompanied by tenderness, however ultrasound scanning detects splenomegaly in almost all cases. Mild hepatomegaly occurs in 15–25% of patients, and jaundice is clinically apparent in 5–10% of patients. Fevers of 38–40°C are a regular feature during the first 1–2 weeks of IM, with the highest temperature often occurring at midday and being followed by drenching sweats.

Pharyngitis and palatal petechiae occur during the first week of the illness. These may be accompanied by a grey-white membrane, which, when associated with pharyngeal oedema and tonsillar enlargement, can result in obstruction to the pharynx or trachea. Periorbital oedema is common early in this disease.

Two types of skin rash can occur: a faint morbilliform eruption which lasts 24–48 hours, or a maculopapular rash which occurs in almost all patients receiving ampicillin. The cause of the latter is unknown, but its presence is regarded by some as diagnostic of IM.

Other associated clinical conditions include encephalitis, meningitis, delirium, coma, psychosis, transverse myelitis, polyneuritis, mononeuritis, pericarditis, myocarditis, interstitial pneumonia and pleural effusions. None of these are common.

IM in Children and the Elderly When IM occurs outside the classic age range of 15–25 years, it tends to present a less typical clinical picture. In children the disease is usually mild and does not require medical attention. Sore throat and cervical lymph-node enlargement are usually present but not invariably so. Occasionally children exhibit classic IM even as early as the age of two years, and in these cases the heterophile antibody (HA) test is often negative.

The clinical onset of IM in the elderly is often insidious and occasionally bizarre. The disease can be severe, with hepatic, neurological and renal involvement.

IM in Pregnancy IM during pregnancy is uncommon, but where it has occurred there has rarely been deleterious effect on the fetus, and termination of pregnancy is not indicated.

IM in the Immunosuppressed When primary EBV infection occurs in immunocompromised patients, particularly after organ transplantation, it is often asymptomatic due to the inability to mount a T-cell immune response. However, it may result in atypical disease with gastrointestinal symptoms and/or signs of renal-graft rejection and failure. Antibodies to EBV antigens may be slow to develop, with neither IgM antibodies to VCA nor the HA test being invariably positive. Some of these primary infections progress to lymphoproliferative disease and lymphoma (discussed later).

Course and Convalescence

The illness usually resolves in one to two weeks but may last for several weeks, often with continued exhaustion on the slightest exertion and complaints of an inability to concentrate for several weeks after apparent recovery. Occasional patients, particularly those over 25 years of age, may experience intermittent fatigue over the following

two years. Other patients suffer ‘relapses’ during the six months to one year following IM, with return of fever, sore throat and lymphadenopathy accompanied by a positive HA test. The exact nature of these relapses is unclear since the serological markers of primary infection may remain positive for up to a year even in subclinical cases of seroconversion.

Complications

In the vast majority of cases IM is a benign and self-limiting disease from which complete recovery is the rule. However, certain morbid complications have been described in the literature, which account for around 30 deaths per year in the United States. The main causes of death are neurological complications, splenic rupture, hepatic failure and secondary infection. Approximately half the deaths are associated with X-linked lymphoproliferative syndrome (X-LPS; see below).

Neurological Complications These include meningitis, encephalitis and the Guillain–Barre syndrome. Each of these conditions may precede, accompany or postdate IM by several weeks. Recovery is usual.

Hepatic Complications Although most IM patients have biochemical evidence of hepatocellular damage giving abnormal liver-function tests, overt jaundice is uncommon (5–10%) and complete recovery is the rule. However, more severe cases have been reported, and these include massive hepatic necrosis resulting in death if untreated by liver transplant.

Splenic Rupture This is a well-known but rare complication of IM, which may occur spontaneously or after mild trauma. The rupture gives rise to severe abdominal and shoulder pain and requires immediate surgical intervention.

Pharyngeal and Tracheal Obstruction These may occur due to massive enlargement and oedema of the tonsils, adenoids, uvula and epiglottis, giving rise to an inability to swallow or to stridor with eventual cyanosis. A short course of corticosteroids usually gives a dramatic improvement, but intravenous hydration and feeding or tracheotomy may be necessary as emergency measures.

Immunological Complications These include haemolytic and aplastic anaemia, thrombocytopenia, hypogammaglobulinaemia, agranulocytosis and haemophagocytosis. These disorders may result from excess autoantibody production, such as to the blood group antigens, due to B cell stimulation, or to abnormal suppression of haemopoiesis by T cells. Several of these conditions may be found in patients with X-LPS (see below).

Chronic Active EBV (CAEBV) (Reviewed by Kimura, 2006.)

Chronic active EBV (CAEBV) infection, where symptoms of IM persist for more than six months, occurs rarely and may result in death from hepatic failure, lymphoma, interstitial pneumonitis, sepsis or haemophagocytic syndrome. In these cases there is a persistence of the acute IM-like serological profile, usually with grossly elevated titres of IgG antibody to VCA and EA-D, absence of IgG antibody to EBNA1, a positive monospot test and often detectable IgM antibodies to VCA. An atypical lymphocytosis is also present. The viral load in saliva and peripheral blood is very high and in some cases EBNA-positive cells can readily be detected in the circulation. There is now growing evidence that there are clonal expansions of EBV-infected T or NK cells which infiltrate into the organs and play a central role in pathogenesis of CAEBV. These are likely to be due to inadequate immunological control of viral replication.

CAEBV has been treated with aciclovir and a variety of immunomodulating agents such as steroids, interferons, IL2, cyclosporine A and etoposide, but the results are disappointing. More recently, stem cell transplantation with cord blood and adoptive immunotherapy with autologous EBV-specific CTLs have been used with some success (Nakagawa *et al.*, 2007; Savoldo *et al.*, 2002).

CAEBV can be distinguished from chronic fatigue syndrome (CFS) on clinical grounds and by an EB viral antibody screen. Around 10% of CFS patients have mildly elevated VCA and/or EA antibody titres, but no gross abnormalities can be found. These changes do not denote a specific aetiological association between EBV and CFS.

Laboratory Findings

The classic finding in IM, after which the disease is named, is the 'atypical' mononuclear cells in the peripheral blood, which were first described by Downey and McKinlay (1923) (Figure 9.6). Morphologically, 'atypical' cells are large activated lymphocytes (10–20 µm in diameter), which, when stained with May–Grunwald–Giemsa stain, show abundant pale blue, vacuolated cytoplasm and an elongated or indented nucleus with coarse nuclear chromatin. These are mainly EBV-specific CD8+ CTLs and account for the leucocytosis regularly seen in the first two weeks of IM. A few atypical mononuclear cells occur in the peripheral blood in other acute virus infections, including cytomegalovirus, hepatitis B, influenza B and rubella, but they are most prominent in IM. Activated CD4+ cells and NK cells are also increased in numbers in acute IM. B lymphocytes are usually present in normal or slightly raised numbers and around 1 in 10⁴–10⁵ are infected with EBV and express all the latent viral proteins.

Diagnosis

The diagnosis of IM may be suspected on clinical grounds. However, infections with other agents such as cytomegalovirus, toxoplasmosis and human immunodeficiency virus (HIV) seroconversion should also be considered in the differential diagnosis. The diagnosis of IM is substantiated by the haematological findings of 'atypical' lymphocytes and is confirmed by the serological demonstration of antibodies to EBV-related antigens. A pre-illness specimen is rarely available to prove absence of antibody before the illness, and IgG antibodies to VCA are almost invariably present in the first serum sample received, having often already reached their peak. However, high levels of IgG anti-VCA antibodies are not of diagnostic significance since variable levels are reached during the illness, and higher levels may occur in other conditions. The presence of IgM antibodies to VCA, with or without IgG antibodies to EA (D) and an absence of IgG anti-EBNA1, are diagnostic. Commercial enzyme-linked immunosorbent assay (ELISA) tests are reliable and are widely used. False-positive results in the IgM test may result from cross-linking between specific EB viral IgG and anti-IgM conjugate by rheumatoid factor, and therefore if this factor is present in the serum it should be absorbed out before testing. There is also a simple slide-based test for detecting HA, called the monospot test (see below).

Following primary EBV infection, IgG VCA persists for life whereas IgM VCA can be detected for three to six months after infection. IgG EBNA becomes detectable six to eight weeks after the infection and persists for life (Figure 9.5).

The Monospot Test The presence of IgM HA in IM serum causes agglutination of red blood cells from species other than humans. Quick and reliable slide tests are available, which involve absorption of the serum with guinea-pig kidney (GPK) emulsion to remove nonspecific agglutinating Forssman antibodies, or with ox cell stroma (OCS) to remove HA, followed by the addition of horse red cells. Drops of serum are placed on two squares on a slide; GPK is stirred into one drop and OCS into the other. Horse red cells are then added to each square and stirred into the absorbed serum. Agglutination in the GPK square and not in the OCS square is indicative of IM.

The monospot test is positive in around 85% of IM cases (as confirmed by positive anti-VCA IgM) and can persist for up to six months after recovery. Negative results are more common in sera from children under 14 years with IM than in those from older children or adults. This may be because HA arise due to the polyclonal stimulation of memory B cells, so if the priming exposure to the unknown 'heterophile' antigen has not yet occurred,

no specific memory B cells will be present and no secondary rise can be induced. False-positive HA tests have been extensively recorded, particularly in association with pregnancy and autoimmune disease.

Treatment

This is largely supportive. High-dose aciclovir reduces virus production in the throat but does not shorten the duration of the illness, probably because the symptoms are immunopathological in nature and not caused directly by virus infection of B cells. The sore throat may be extremely painful and regular analgesics are then essential. Corticosteroids curtail the severity and duration of the symptoms, but are best reserved for severe cases of pharyngeal or tracheal obstruction, and neurological and haematological complications.

Burkitt's Lymphoma

BL is a tumour which occurs endemically in equatorial Africa and Papua New Guinea and sporadically worldwide. The African (endemic) form of the tumour is geographically restricted to those areas in which hyperendemic falciparum malaria occurs (Figure 9.7). These are

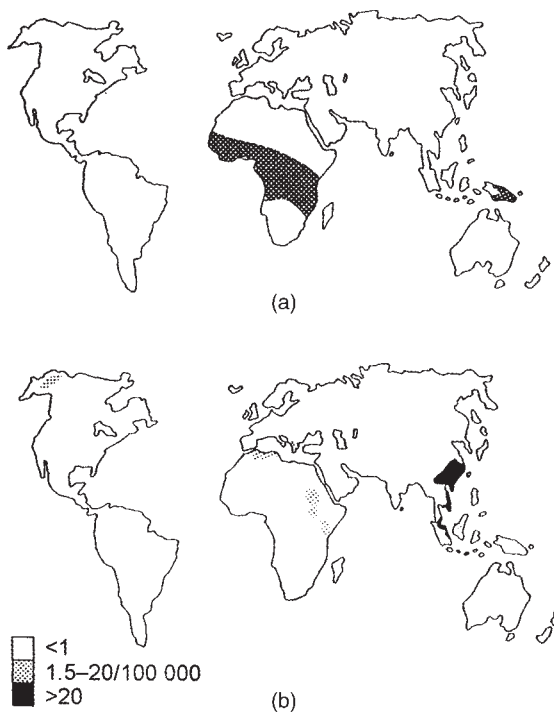


Figure 9.7 Maps showing the worldwide distribution of (a) Burkitt's lymphoma and (b) nasopharyngeal carcinoma.

the low-lying areas of equatorial Africa and Papua New Guinea with a rainfall of over 60 cm per year and a minimum temperature of 16°C.

The endemic and sporadic forms of BL are both monoclonal tumours of B lymphocytes which have indistinguishable histological appearances. However, whereas almost 100% of cases of African BL are associated with EBV, only 12–25% of the sporadic cases in developed countries are EBV-related. An intermediate incidence (25–80%) of EBV-associated BL is reported in countries in South America, such as Argentina and Brazil (Rao *et al.*, 2000).

Seroepidemiology

In the geographical areas where BL is endemic almost all children over the age of two years have been infected by EBV and have IgG antibodies to VCA. However, in BL the pattern of antibodies to EBV antigens is altered when compared to normal matched controls. Sera from BL patients have IgG antibody titres to VCA with a geometric mean 8–10 times greater than matched control sera. IgG anti-EA (R) and anti-MA antibodies are also raised, and the levels of these serum antibodies vary with clinical events. Thus, after treatment, a drop in anti-EA (R) indicates a good prognosis, whereas a rise in anti-EA (R) and a fall in anti-MA may precede a recurrence of clinical disease.

Pathogenesis

Association with EBV Although it is very difficult to obtain absolute proof that a virus is involved in the aetiology of a human tumour, there is now much evidence which points to the widely-accepted view that EBV is involved in the aetiology of BL. In addition to the seroepidemiological evidence outlined above, multiple copies of the viral genome can be detected in the tumour cells of around 97% of African BL biopsy samples. The viral genome is clonal in the monoclonal BL B-cell population, indicating that the infection event occurred before proliferation of the malignant cell. However, the viral gene expression is restricted to the latent antigen EBNA1, which is essential for maintenance of the viral genome in the cell and EBERS and BARTs (latency 1) (Table 9.1). No direct oncogenic role for these genes has been identified, although mice transgenic for EBNA1 develop lymphoma (Wilson *et al.*, 1996), and EBV has been suggested to increase cell survival by inhibiting apoptosis (Komano *et al.*, 1999) and inducing expression of the inhibitory cytokine IL-10 (Kitagawa *et al.*, 2000).

BL-derived Cell Lines Lymphoid cell lines that are grown directly from BL biopsy material show

the characteristic features of BL cells, with cellular markers consistent with a germinal-centre B-cell phenotype (CD10, CD77) and viral protein expression to restricted EBNA1 (latency 1). These differ from their *in vitro*, EBV-transformed counterpart in several important respects, which indicates their increased malignant potential. Thus, BL-derived cell lines grow as colonies in soft agar and subcutaneously in nude mice whereas *in vitro*-transformed LCLs do not. BL cell lines are monoclonal, with all cells bearing surface Ig of one heavy-chain isotype (usually M) and one light-chain type, whereas *in vitro* transformed cell lines are polyclonal in origin. There are also differences in growth characteristics, cellular gene expression and cytological appearances, reflecting their differing stages of B-cell maturation (germinal-centre cell versus activated lymphoblast). However, with prolonged culture, the viral and cellular gene expression in BL cell lines may drift to resemble LCLs. Finally, BL cell lines consistently carry specific chromosomal translocations which are not present in *in vitro*-grown LCL derived from normal individuals (see below).

Chromosomal Abnormalities It has been recognized for many years that fresh BL tumour cells and the derived cell lines (whether EBV-associated or not) show a reciprocal chromosomal translocation between the long arm of one chromosome 8 and chromosomes 14, 2 or 22. Each of these translocations results in the *c-myc* oncogene on chromosome 8 coming under the regulatory control of either the Ig heavy-chain genes on chromosome 14 or, more rarely, the κ or λ light-chain genes on chromosomes 2 and 22, respectively. The positions of the chromosomal break points suggest that these translocations are the results of mistakes occurring during Ig gene rearrangements, Ig class switching or somatic hypermutation in a germinal-centre cell. Furthermore, the translocated allele of *c-myc* frequently contains mutations. *c-myc* is a normal cellular gene which codes for a nuclear protein involved in the control of cell activation and proliferation. The BL translocations deregulate the gene, giving constitutive expression. In addition to this characteristic chromosomal translocation, BL cells often contain other genetic abnormalities, including a mutated p53 gene in about 30% of biopsy samples.

Cofactors in the Pathogenesis of BL Since EBV is a ubiquitous agent, whereas EBV-associated BL occurs almost exclusively in those geographical areas of the world where malaria is hyperendemic, it is probable that malaria infection acts as one factor in the multifactorial aetiology of this disease. The association between BL and malaria is further substantiated by the findings that where

malaria eradication has been successfully accomplished the incidence of BL has dropped dramatically, and that the incidence of BL in children with the sickle-cell trait, which confers partial protection from malaria, is low.

Malaria infection acts as a chronic stimulator of germinal-centre B cells and also as an immunosuppressant, causing a decreased EBV-specific CTL activity and increased numbers of EBV-infected B cells in the peripheral blood. It is postulated, therefore, that the combined lymphoid stimulation and immunosuppressive effects of malaria cause an increase in B-cell turnover and a decrease in the elimination of EBV-positive B cells. However, at present it is unclear whether EBV acts to increase the population of cells susceptible to a chromosomal translocation, or to enhance the survival and proliferation of a cell population bearing the translocation. Whichever scenario is correct, it is assumed that the latent EBV growth-promoting genes (latency 3) must be expressed at this early stage of lymphomagenesis, but that a switch to the nonimmunogenic EBNA1-only phenotype (latency1) occurs once their function is replaced by deregulated *c-myc*.

Clinical Features

African BL is a tumour which occurs in children aged 3–15 years, with a peak age incidence of 6–7 years. In those areas of Africa and Papua New Guinea where BL is endemic it occurs with an incidence of 15 per 100 000 children aged 5–10 years, and is the commonest malignancy in this age range. It is more common in boys than girls and arises extranodally, typically in the area of the jaw, giving a characteristic presentation (Figure 9.8). The tumour is usually found to be multifocal at presentation, the other sites commonly involved being the postorbital region, gastrointestinal tract, thyroid, liver, kidney, skeleton, testicles and ovaries, and the breast in adolescent girls. BL is a highly malignant tumour, with death supervening within a few months of clinical onset in untreated cases.

Diagnosis

The diagnosis of BL in an endemic area is very often clear from the clinical features described above; however, histological evidence should be sought (Figure 9.9). The tumour shows a characteristic histological picture of a poorly-differentiated lymphocytic lymphoma, with variable numbers of infiltrating histiocytes giving the classic 'starry sky' appearance. BL is a monoclonal tumour of B-cell origin, expressing the markers of a germinal-centre B cell: CD10, CD77 and, in over 90% of cases, IgM.



Figure 9.8 Male child with a Burkitt's lymphoma of the jaw, (a) before treatment and (b) after treatment with cyclophosphamide.

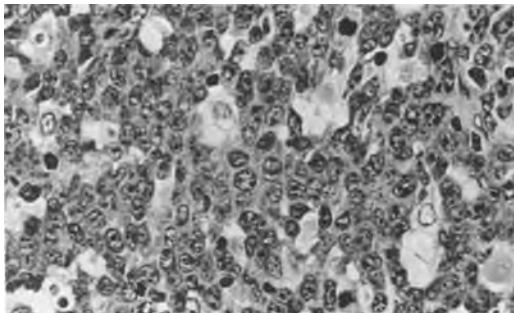


Figure 9.9 Histological section of Burkitt's lymphoma, showing a uniform population of nucleolated lymphoid cells and scattered vacuolated macrophages (H&E x 800). (Courtesy of Professor P. Isaacson, University College Hospital.)

Treatment

BL is very sensitive to chemotherapy, one dose of cyclophosphamide often being enough to cause complete regression of the tumour mass. Relapses do occur, however, and are progressively less responsive to therapy. For this reason a full course of treatment should be given initially, in which case the prognosis is good.

Prevention

The prevention of BL may theoretically be achieved by the eradication of malaria, which has been achieved in

the past in small areas, such as some of the islands of Papua New Guinea, with a coincident fall in BL in these regions. Alternatively, prevention of BL development by a vaccine which prevents EBV infection has been suggested (see later).

Nasopharyngeal Carcinoma

NPC is a malignant tumour of the squamous epithelium of the nasopharynx which is highly prevalent in southern China, where it is the commonest tumour in men and the second most common tumor in women. In most other areas of the world the tumour is rare, but pockets of high incidence occur in North and Central Africa, Malaysia, Indonesia, Vietnam, the Philippines and in the Inuit races of Alaska, Greenland and Iceland (Figure 9.7). The most undifferentiated form of the tumour, which is the most common, shows a 100% association with EBV regardless of the geographical location, whereas the association with the rarer, more differentiated forms is less consistent.

Seroepidemiology

The association between NPC and EBV was first demonstrated serologically (Old *et al.*, 1966) and later studies showed that sera from 100% of cases of undifferentiated NPC have high-titre antibodies to VCA. As in BL, the antibodies are present at a 10-times higher geometric mean titre than in matched controls and show a unique reaction

pattern. Thus the anti-EA (D) component is the most frequently seen and is present at a higher titre than anti-EA (R). IgG and IgA anti-EA (D) antibody titres rise as the disease progresses, fall in remissions, and may be undetectable in long-term survivors. Similarly, IgA antibodies to VCA are present in NPC sera and correlate with disease progression. These IgA antibodies are also found uniquely in the saliva of NPC patients.

Pathogenesis

Association with EBV The seroepidemiological data referred to above, and the finding of multiple clonal copies of the EBV genome in the malignant epithelial cells of 100% of undifferentiated NPC biopsy specimens, strongly suggest that the virus is involved in tumour aetiology. All the malignant epithelial cells express the EBV-coded antigen EBNA1, and LMP1 and 2 are expressed in around 50% of tumours (latency 2). Although the exact mechanisms involved in squamous epithelial cell transformation by EBV have not been resolved, the fact that LMP1 is a viral oncogene which can drive epithelial cell proliferation *in vitro* (Dawson *et al.*, 1990) and induce severe epithelial hyperplasia in transgenic mice (Wilson *et al.*, 1990) provides compelling evidence for an oncogenic role for EBV in NPC. However, a recent study using a quantitative real-time reverse transcriptase polymerase chain reaction (PCR) method detected significant levels of EBNA1, LMP2A and BART transcripts but failed to detect LMP1 transcripts in 12 NPC biopsies (Bell *et al.*, 2006). LMP2A has been shown to cause epithelial cell proliferation by stabilising β -catenin through PI3K and Akt signal transduction pathways (Morrison *et al.*, 2003).

Cofactors in the Pathogenesis of NPC NPC is a genetically-restricted tumour, being most common in southern Chinese and Inuits. It has been noted that the first-generation immigrants from southern China to the USA retain the high frequency of the disease, although later generations show a declining incidence which may be due to intermarriage with non-Chinese races. These data suggest a genetic factor in the aetiology of the disease, and this is backed up by family clustering and the finding of an association with certain HLA haplotypes (A2-B5In2, BW17-AW19, BW17-A blank). Polymorphism in the HLA-A locus has also been associated with the development of NPC (Lu *et al.*, 2005). Environmental factors may play a role in the aetiology of NPC, in particular dietary components such as salted fish which contain carcinogenic nitrosamines, and traditional herbal medicines containing phorbol esters, which are taken as snuff.

Clinical Features

NPC occurs at a rate of 98 per 100 000 of the population in southern China and is more common in men than in women. The age of onset of NPC varies with geographical location and histological type, the undifferentiated type being more common in high-risk areas in young patients, whereas the more differentiated types occur in older patients and constitute the bulk of sporadic cases. The tumour most commonly arises on the posterior wall of the nasopharynx in the fossa of Rosenmüller, where it often remains silent, and rapidly metastasizes to the draining lymph nodes. Thus the most frequent presenting symptom of NPC is bilateral enlargement of lymph glands in the neck, which are firm, nontender and fixed. The upper cervical chain of glands is most often involved in the initial spread. At this stage the primary tumour may be very small and difficult to locate. Less frequently, the presenting symptoms are associated with invasion by the primary tumour and include nasal obstruction, postnasal discharge, epistaxis, partial deafness and cranial nerve palsies. If untreated, the disease is rapidly fatal, with death being most often due to laryngeal and pharyngeal obstruction.

Diagnosis

The diagnosis of NPC is made on biopsy material from the primary tumour or an enlarged cervical lymph node. The cells are squamous epithelial in origin and three histological types are described in the World Health Organization classification: (i) *WHO1*, a well-differentiated squamous cell carcinoma with intercellular bridges and keratinization; (ii) *WHO2*, a nonkeratinizing carcinoma; and (iii) *WHO3*, an undifferentiated carcinoma in which a heavy lymphocytic infiltration is often present, which may be so extensive as to lead to the mistaken diagnosis of lymphoma (Figure 9.10). However, the lymphocytes, which are mainly T cells, are nonmalignant. The term 'lymphoepithelioma' has been used to describe this third type, which occurs most commonly in the high-risk areas.

Serum antibody titres to EBV antigens can be used to confirm the diagnosis of NPC and to monitor the progress of the disease. Large-scale screening programmes in China have identified individuals with persistent IgA antibodies to VCA in serum and/or saliva, and follow-up of these cases has successfully detected 'precancerous' and early lesions.

Treatment

NPC is difficult to treat surgically because of the characteristic feature of early metastasis to regional lymph

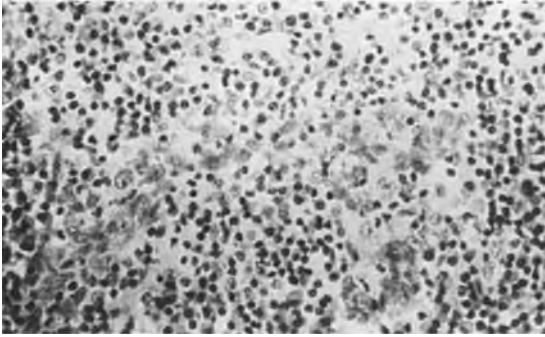


Figure 9.10 Histological section of undifferentiated nasopharyngeal carcinoma, showing scattered malignant epithelial cells and a heavy infiltrate of small lymphocytes (H&E x 400).

nodes. The tumour is resistant to chemotherapy, and therefore radiotherapy to the primary tumour and cervical lymph nodes is the treatment of choice. The overall prognosis is poor; however, five-year survival rate for early-stage disease is around 60%.

Prevention

Although the precise role of EBV in the aetiology of NPC remains to be elucidated, it has been argued that prevention of primary infection by EBV using vaccination may be enough to break the chain of events which culminates in tumour formation. Vaccine preparations are discussed later.

Hodgkin's Disease

An association between EBV and HD was long suspected because IM in the previous five years is a risk factor for HD, and HD patients have high antibody titres to EBV lytic-cycle antigens months or years before development of the disease. This association was firmly established when EBV DNA and expression of viral-coded proteins were demonstrated in malignant Hodgkin Reed–Sternberg (HR-S) cells in a proportion of cases. Multiple copies of clonal, circular EBV DNA can be detected in 40–60% of HD biopsies by Southern blotting, with confirmation of the HR-S cell location by detection of EBERS by *in situ* hybridisation (Anagnostopoulos *et al.*, 1989). These cells also express EBNA1 and high levels of LMP1 and 2A, in the absence of other latent or lytic proteins (latency 2). The mixed cellularity type of HD is most often associated with EBV.

The age distribution of HD is similar to that of primary EBV infection, showing a bimodal distribution with one peak in childhood and another in adulthood. Furthermore,

the age of the early peak varies with geographical location, being later in Western societies (15–35 years) than in developing countries (5–10 years). This similarity initially suggested that HD might represent an atypical outcome to primary EBV infection; however, although most childhood HD in developing countries is EBV-associated, in Western societies non-EBV-associated HD predominates in the young, with EBV association increasing with age (Jarrett, 2002).

The pathogenesis of EBV-associated HD is still unclear, although the presence of clonal viral DNA in HR-S cells indicates infection at an early stage. In addition, expression of LMP1 which induces cell activation and proliferation while inhibiting apoptosis, and LMP2A which enhances cell survival, is consistent with an oncogenic role for the virus. HR-S cells have been definitively identified as germinal-centre B cells with functional Ig-gene rearrangements but defective Ig-gene transcription (Marafioti *et al.*, 2000), and it is therefore possible that EBV infection of these atypical cells allows the survival of a cell which would otherwise undergo apoptosis in the germinal centre.

An association between EBV-positive HD and genetic microsatellite markers (D6S510 and D6S265) in the HLA-class-1 locus has recently been identified (Diepstra *et al.*, 2005). Similarly, others have found that these HLA-class-1 alleles are significantly more frequent in patients with IM when compared with EBV-seropositive individuals (McAulay *et al.*, 2007). Since IM is a well-documented risk factor for developing EBV-positive HD, the increased frequency of certain HLA-class-1 alleles in both diseases suggests that differences in the efficiency of EBV-peptide presentation to T cells can influence the effectiveness of the immune response and predispose to EBV-related diseases.

EBV INFECTION IN THE IMMUNOCOMPROMIZED HOST

EBV establishes a lifelong persistent infection in over 90% of the world's population with latency in B lymphocytes and virus production into saliva (see above). This persistent infection is controlled mainly by EBV-specific CTLs, and it is not surprising therefore that when cell-mediated immunity is decreased there is often increased EBV production in saliva, high antibody titres to lytic-cycle antigens (VCA and EA) and an increased viral load in peripheral blood lymphocytes. This pattern is sometimes called a 'reactivated infection', although generally no clinical symptoms ensue. In a few patients, however, EBV-associated lymphoproliferative lesions and lymphoma develop.

X-Linked Lymphoproliferative Syndrome (X-LPS)

(Reviewed by Gaspar *et al.* 2002.)

This rare syndrome (first called Duncan's syndrome) was recognized in 1974 by Purtilo *et al.*, who described a family in which six male kindred died of acute IM and/or malignant lymphoma (Purtilo *et al.*, 1974). Since then many such families have been reported, with affected members having an apparent inability to mount an effective immune response to primary EBV infection. X-LPS accounts for about half of the fatal IM cases reported, the other half being sporadic, with an equal sex distribution.

Clinically, the affected males are generally healthy until primary EBV infection occurs. The course of the disease is then fulminating and rapidly fatal in the majority of cases, with death commonly resulting from hepatic necrosis. A minority of patients progress to a chronic phase, often culminating in a fatal B-cell lymphoma. These tumours are mostly extranodal, commonly occurring in the central nervous system or gastrointestinal tract.

Histological studies on fatal cases show infiltration of tissues throughout the body with EBV-positive lymphoblastoid and plasmacytoid cells and activated T cells. These infiltrates are a particularly prominent feature in the liver, where dysregulated cytokine release from T cells leads to hepatic necrosis. Haemophagocytosis is seen in the tissues in almost all cases.

There is no elective treatment for this disease, although etoposide, a cytotoxic drug used in the treatment of malignancies such as small-cell carcinomas of lung, Hodgkin's lymphomas, retinoblastomas and testicular tumours, may have some effect. Recently success has been reported with bone marrow transplantation. Many abnormal immunological findings have been reported in X-LPS, although none is consistently found. These become more marked following EBV infection when combined T, B and NK cell abnormalities are seen.

The defective gene in X-LPS was identified in 1998 (Coffey *et al.*, 1998; Nichols *et al.*, 1998; Sayos *et al.*, 1998) and the development of a diagnostic test rapidly followed. This identified a spectrum of clinical manifestations associated with the syndrome, including dysgammaglobulinaemia (incorporating some cases of common variable immunodeficiency), aplastic anaemia, lymphoid vasculitis with aneurysm formation and, rarely, non-EBV-associated lymphoma.

The X-LPS gene codes for a small *src*-homology 2 (SH2) domain containing cytoplasmic protein, alternatively called SH2D1A or SAP (signalling lymphocytic activation molecule (SLAM) associated protein), which is expressed in T and NK cells. SAP appears to play a modulating role in T-cell activation and is required for NK-cell cytotoxicity; it is likely that the loss of these functions explains the X-LPS phenotype. However, this

does not explain the link between EBV and X-LPS, since SAP expression is not specific to primary EBV infection. In this regard, the finding of rare X-LPS lymphoma in EBV-negative individuals indicates that the syndrome is not restricted to primary EBV infection, and it is now postulated that X-LPS is a more generalized immunodeficiency which can be triggered by a variety of virus infections. However, the exact nature of the immunological abnormality is still unclear.

Post-transplant Lymphoproliferative Disease (PTLD)

(Reviewed by Burns and Crawford 2004.)

Iatrogenic immunosuppression following stem cell or solid organ transplantation results in an increased incidence of virus-associated tumours. B-cell lymphomas are the commonest tumour in paediatric transplant recipients and the second-commonest tumours in the adult transplant population (following skin tumours), and have a high morbidity and mortality.

Histology

These tumours are mainly diffuse large-cell lymphomas with the term 'post transplant lymphoproliferative disease' (PTLD) being used to reflect the morphological diversities. Histologically, PTLT is classified into three major categories: (i) *Hyperplastic PTLT*, which includes plasmacytic hyperplasia, IM-like lesions and other atypical lymphoid hyperplasias. These lesions are of early-onset, and characteristically noninvasive. They are typically polyclonal in composition, containing a mixture of B and T cells. (ii) *Polymorphic PTLT*, which characteristically invade and disrupt underlying tissue architecture. The majority of cells are B cells and most tumours are monoclonal. (iii) *Lymphomatous or Monomorphic PTLT*, which include monoclonal B cell tumours that resemble conventional NHL, with rare cases of Burkitt's and Burkitt's-like lymphomas.

Risk Factors and Association with EBV

PTLT develops in up to 10% of transplant recipients. Risk factors include high levels of immunosuppressive drugs (often in association with rejection episodes), EBV seronegativity before transplant and primary EBV infection following transplant (Haque *et al.*, 1997). The latter is more common in children, who therefore have a higher incidence of PTLT than adults. Over 90% of PTLTs are EBV-positive and the tumour cells generally express all the latent viral genes (latency 3) (Table 9.1). However, more restricted patterns of gene expression are detected in some lesions. Early lesions are often polyclonal-B-cell proliferations, but progression to a monoclonal lymphoma

regularly occurs. Although most PTLD tumours are of B-cell origin, rare T-cell and NK-cell tumours have also been described.

Pathogenesis

The pathogenesis of PTLD appears to be straightforward: immunosuppressive therapy following transplantation inhibits EBV-specific CTLs, leading to uncontrolled proliferation of EBV-infected B cells. However, the lesions are often single and monoclonal, suggesting that, in addition to EBV, cellular factors are required for tumour outgrowth. The finding of nonfunctional Ig genes in a proportion of these tumours (Timms *et al.*, 2003) suggests that EBV infection may have rescued an abnormal cell from apoptosis within the germinal centre. In a recent large multicentre retrospective study, the use of polyclonal Ig (which is likely to contain anti-VCA, EBNA and LMP antibodies) for cytomegalovirus prophylaxis during the first four months following cadaveric renal transplantation was shown to prevent PTLD development in the first post-transplant year, whereas antivirals (aciclovir or ganciclovir) had no protective effect (Opelz *et al.*, 2007).

Clinical Features

Clinically, PTLD may present as an IM-like syndrome, commonly in children undergoing primary infection (in which the virus may be acquired from the transplanted organ), and often within the first year of transplant. Alternatively, discrete tumours, frequently in the lymph nodes, gastrointestinal tract, brain or transplanted organ, are seen in seropositive recipients, often several years after transplant.

Diagnosis

Definitive diagnosis of EBV-associated PTLD is made on tumour biopsy materials by *in situ* hybridisation for EBERS and by immunostaining for EBNA and LMP antigens. Because of the variable clinical presentation, diagnosis is often difficult and measurement of the viral load in peripheral blood has been used as an indicator of risk. However, although high viral load is found in the majority of PTLD patients, levels are very variable in asymptomatic recipients, with some reaching those seen in PTLD. Therefore, single estimations are not helpful and regular monitoring of viral load in blood is required. In patients with rising high viral load, pre-emptive reduction of immunosuppression has been reported to reduce the incidence of PTLD when compared with historic control (Lee *et al.*, 2005).

Treatment

Reduction of immunosuppressive therapy is now the first line of treatment for PTLD, and the antiviral drugs aciclovir and ganciclovir are sometimes added to this regimen, although their role in tumour regression is unproven. Complete tumour regression may be achieved by this treatment alone, particularly in early lesions, but relapses and recurrences are common, and despite additional chemotherapy and/or radiotherapy the overall mortality is over 50%. Death is usually due to tumour progression, graft failure and/or opportunistic infections. Recent trials of immunotherapy for PTLD, including the humanized antibody to the B-cell surface antigen CD20, rituximab (Choquet *et al.*, 2006) and autologous (Rooney *et al.*, 1998) or allogeneic (Haque *et al.*, 2002) *in vitro*-grown EBV-specific CTLs, have reported some success, although randomized controlled trials have not been performed. In a recent multicentre Phase II clinical trial using partially HLA-matched allogeneic CTL, tumour response was seen in 64% of PTLD patients at five weeks and 52% of PTLD patients at six months (Haque *et al.*, 2007).

Acquired Immunodeficiency Syndrome (AIDS)

The incidence of NHL in individuals with untreated HIV is increased some 60-fold over the general population, and its development is an AIDS-defining illness. However, with the use of antiretroviral therapy, the incidence of NHL is falling (Kirk *et al.*, 2001). Three principal types of HIV-associated NHL have been described: (i) Primary central nervous system lymphoma (PCNSL) is a rare tumour in the general population which has a thousand-times increased incidence in HIV-infected individuals. It occurs at a late stage of AIDS and is invariably EBV-associated. Definitive ante-mortem diagnosis may be difficult, but EBV DNA can be found in cerebrospinal fluid in most cases and detection is considered to be diagnostic. Brain biopsy can be stained for EBV antigens (EBNA2, LMP1) and EBERS. (ii) BL develops at a relatively early stage of AIDS and all AIDS BL show the typical *c-myc* translocation. Around 25% are EBV-associated, showing the cellular and viral gene expression described for African BL (see above). (iii) Peripheral NHL occurs at a late stage of the disease and often presents at extranodal sites. Around 50% of cases are EBV-associated.

Other types of lymphoma seen in HIV-infected individuals include HD, which shows a modestly raised incidence and a strong EBV association, and the human herpesvirus 8-associated primary infection lymphoma, of which 70–80% of cases show dual infection with EBV. All lymphomas associated with HIV infection respond poorly to treatment, due to the underlying disease.

OHL was first described in HIV-seropositive individuals, forming multiple characteristic corrugated white lesions on the lateral margin of the tongue. DNA hybridization and immunocytochemical staining revealed replicating EBV in these lesions (Greenspan *et al.*, 1985). OHL has now been recognized in other groups of immunocompromized patients. The lesion is painless and apparently harmless, and it can be successfully treated if required with continuous aciclovir therapy.

VACCINE DEVELOPMENT

Over the last two decades work has been undertaken to develop a vaccine which would prevent primary infection by EBV. The antigen chosen for vaccine development is the MA antigen gp340/220 (see above), since it is this antigen to which neutralizing antibodies are mainly directed. It was argued that this would prevent the development of BL and NPC by breaking a link in the chain of events which leads to the evolution of these diseases (Epstein, 1984). Such a vaccine preparation would have to be given very early in life to prevent natural infection in the BL- and NPC-susceptible populations. An effective vaccine preparation could also be useful in seronegative organ transplant recipients, and those at risk of developing severe IM, such as male offspring of X-LPS carriers.

The recently-published results of two randomized controlled studies using recombinant, subunit, gp350 EBV vaccine preparations in 148 healthy adult volunteers showed that the vaccines had a good safety profile and were well tolerated. They were also immunogenic, inducing gp350-specific antibody responses including neutralization antibodies. A total of 117 EBV-seronegative subjects were monitored over a period of seven months and there was only one possible case of IM. Seventeen individuals who were still seronegative at the end of the clinical trial were followed up for three years and no cases of IM were reported, although ten patients developed antibodies to EBV nonvaccine antigen without any disease. This observation suggests that the vaccine is efficacious in the prevention of symptomatic disease rather than in the induction of sterile immunity and prevention of infection (Moutschen *et al.*, 2007).

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Roseoloviruses: Human Herpesviruses 6A, 6B and 7

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INTRODUCTION

Human herpesvirus (HHV)-6 and HHV-7, first discovered in 1986 and 1990 respectively, are genetically related to human cytomegalovirus (CMV) and as such are grouped with the latter in the *Betaherpesvirinae* subfamily. Two variants of HHV-6 have been defined (termed HHV-6A and HHV-6B). These have not been formally designated as individual virus species despite the fact that they meet the criteria of the International Committee on Taxonomy of Viruses (ICTVs) for such classification. There are readily discerned differences between the two variants found throughout their genomes, and recombinants between the two viruses have never been identified. They also differ in their biological, pathological and epidemiological properties. Where possible, the two variants of HHV-6 (A and B) are discussed here separately, and the more general term HHV-6 is reserved for studies where either the distinction was not made or there is no clear point to be elucidated.

In common with the other HHVs, HHV-6 and HHV-7 establish lifelong infection following initial exposure. Both HHV-6 and HHV-7 are highly prevalent worldwide and infection usually occurs in early childhood. Within this age group, HHV-6B is a causative agent of febrile illness including exanthem subitum (ES) and convulsions, and the same applies to HHV-7.

The more extensively studied HHVs (herpes simplex types 1 and 2, CMV, varicella-zoster virus and Epstein-Barr virus (EBV)) exhibit enhanced pathogenicity and fre-

quency of reactivation in the immunocompromised. There is now considerable evidence that active infections with both HHV-6 and HHV-7 are common in transplant recipients and have the potential to cause disease, for example HHV-6 encephalitis. For CMV, both the indirect and direct pathological effects of the virus are generally appreciated, and it may be that the other betaherpesviruses, namely HHV-6 and HHV-7, likewise have indirect effects, perhaps even more significant than their ability to cause direct-end organ disease.

A unique feature of HHV-6 amongst the HHVs is the persistence of viral genomic sequences in a chromosomally-integrated state in a minority of infected persons, with the capacity in these circumstances for vertical transmission. This feature highlights a most fascinating biological aspect of the virus which has unwittingly confused the interpretation of earlier studies examining disease associations and the pathogenicity of HHV-6. Importantly, it continues to confound laboratory diagnosis of active HHV-6 infection, potentially resulting in the unnecessary use of antiviral intervention.

BIOLOGY OF THE VIRUSES

Virus Structure and Replication

In common with all herpesviruses, HHV-6 and HHV-7 have a double-stranded DNA genome surrounded by an

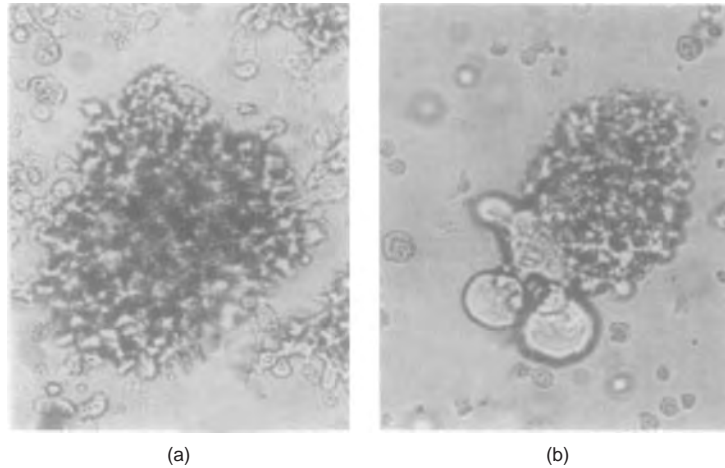


Figure 10.1 HHV-6 cytopathic effect (CPE). Peripheral blood lymphocytes showing the characteristic CPE induced by HHV-6 infection. (a) Mock-infected cells; (b) HHV-6 infected ballooning cells within aggregates of cells. (Source: Di Luca *et al.*, 1990, with permission.)

icosahedral capsid that is separated by amorphous material, the tegument, from an outer envelope containing viral glycoproteins. HHV-6 and HHV-7 are characterized by growth in T lymphocytes (although, as described below, they can infect other cell types). Replication is slow *in vitro*, with the production of swollen (cytomegalic) cells (Figure 10.1), and results in cell death. Gene regulation for HHV-6 and HHV-7 appears to follow the same cascade as in other herpesviruses, with immediate-early (IE), early and late genes, although the temporal classification of the majority of HHV-6 and most HHV-7 genes has yet to be determined (for a recent account of the HHV-6 replication cycle, see review by De Bolle *et al.*, 2005).

Viral Genomics

Conserved genes (core genes) among the herpesviruses can be organized into seven gene blocks, the arrangement of which is subfamily-specific, and there is an additional block found only in the betaherpesviruses. The closer genetic relationship between HHV-6 and HHV-7 compared to CMV underlies their separate classification in the *Roseolovirus* genus. Table 10.1 gives a selected list of HHV-6 and HHV-7 genes and gene products—see two reviews (Black and Pellett, 1999; De Bolle *et al.*, 2005) for fuller descriptions of the genomic properties of HHV-6 and HHV-7.

HHV-6A and HHV-6B

The complete nucleotide sequences of HHV-6 variant A (U1102 strain) and variant B (strains Z29 and HST) are known. The genomes are 160–162 kb in size, including a unique (U) region of 143–144 kb bound by terminal direct

repeats (DRs) of 8–9 kb. Open reading frames (ORFs) encoded in the DR are prefixed ‘DR’, and those in the unique region are termed U1–100 from the left to the right hand of the sequence. HHV-6B strain Z29 is predicted to contain 119 ORFs, 9 of which are absent in HHV-6A (strain U1102), and splicing is predicted to result in 97 U genes (88 of which have counterparts in HHV-6A).

Comparative genomic analysis reveals that the HHV-6A and HHV-6B genomes are co-linear, with an overall nucleotide sequence identity of 90%. The differences are such that clinical isolates can be classified as variant A or B on the basis of characteristic restriction enzyme profiles. Regions of significant variation in the genome include DR and a 24 kb segment located to the right of U86 (the IE-1 region) where there are differences between splicing patterns and temporal transcription regulation in the two variants. An exception is U94, a gene with homology to the adeno-associated virus type 2 (AAV-2) *rep* gene, in which variants A and B differ by only 2.4% at the amino acid level. At present, the full implications of genomic and proteomic differences between variants are unknown, although it is clear that such differences may have profound biological effects.

Variation Within HHV-6A and HHV-6B There is little evidence for variation, although two distinct subgroups within HHV-6B (strains Z29 and HST) have been suggested by analysis of the IE-A regions of the viral genome. However, examination of the U94 gene from 13 HHV-6B isolates from the United States, Africa and Japan showed 100% amino acid identity (Rapp *et al.*, 2000), suggesting an intolerance of variation in this particular gene because

Table 10.1 Selected HHV-6 and HHV-7 genes and gene products

Gene/gene product	HHV-6A	HHV-6B	HHV-7
Nucleic acid metabolism			
U94/rep protein ^a	+	+	–
U86 and U90/immediate-early proteins (IE-A)	+	+	+
U69/protein kinase confers ganciclovir sensitivity ^b	+	+	+
Structural proteins			
U57/major capsid protein	+	+	+
U11/tegument phosphoprotein	+ ^c (p100) ^d	+ ^c (101K) ^d	+ (pp89) ^d
U14/tegument phosphoprotein	+	+	+(pp85) ^d
U39/glycoprotein B (gB)—virus attachment protein	+	+	+
U48/glycoprotein H (gH)—membrane fusion protein	+	+	+
U82/glycoprotein L (gL)—membrane fusion protein	+	+	+
U100/glycoprotein Q (gQ)—membrane fusion protein	+ ^e	+ ^e	+
Immunomodulatory molecules			
U12/chemokine receptor	+	+	+
U51/chemokine receptor	+	+	+
U83/potential viral chemokine	+	+	–

^aHomologue of adeno-associated virus type 2 (AAV-2) *rep* gene.

^bHomologue of CMV UL97.

^cOnly 80% amino acid identity between the variants.

^dMajor antigenic target for human antibody response.

^eOnly 79% amino acid identity between the variants.

it has an essential function (see ‘Viral Latency’ and ‘Chromosomal Integration of HHV-6’).

HHV-7

Two laboratory isolates of HHV-7 (strains JI and RK) have been fully sequenced. The genome is 153 kb in size and is arranged in the same way as HHV-6, with a unique region of 133 kb flanked at either end by a 10 kb DR region. Over 90% of the ORFs of HHV-7 are similar in amino acid sequence to those of HHV-6A and HHV-6B. There are only a small number of potential ORFs in HHV-6 that do not have a counterpart in HHV-7, and vice versa.

Intrastrain Variation of HHV-7 Comparison of the two fully-sequenced HHV-7 isolates (RK and JI) shows little variation overall, although it is more apparent in the repeat regions close to either end of the genome. However, analysis of three HHV-7 genes encoding the tegument phosphoprotein, glycoprotein B (gB) and major capsid protein in viral nucleic acid from 297 people derived from populations in Africa, Asia, Europe and America have suggested the presence of distinct variants (Franti *et al.*, 2001).

Cell Tropism

HHV-6A and HHV-6B

Both variants are tropic for T lymphocytes *in vitro* and can infect CD4⁺ T cells, but CD8⁺ T cells can only be

infected by HHV-6A. Typically, variant A viruses replicate in the lymphoblastoid T-cell line HSB-2 whereas variant B viruses grow in the less differentiated MOLT-3 T-cell line. HHV-6 also has the ability to infect a wide variety of other cell types. These include monocytes, epithelial cells, endothelial cells, fibroblasts and a range of neural cell types. Notably, HHV-6A has a greater capacity than HHV-6B for replication in cultured cells of neuronal origin, for example astrocytes. CD46 (also known as membrane cofactor protein, which functions as a regulator of the complement cascade) is a major cellular receptor for both HHV-6A and HHV-6B (Santoro *et al.*, 1999). The fact that CD46 is expressed on all nucleated cells is in keeping with the broad cellular tropism of HHV-6 *in vitro*, but activated peripheral blood CD4⁺ T cells and monocytes appear to be the site of fully permissive replication in HHV-6B primary infection *in vivo*, with monocytes mainly responsible for HHV-6 viraemia (Kondo *et al.*, 2002; Takahashi *et al.*, 1989).

Interaction with CD46 has been reported to involve the participation of the viral glycoproteins gH, gL and gQ (Mori *et al.*, 2003) and seems to be critical for HHV-6 fusion. In the case of gQ, there is only 79% amino acid identity between variants, which may influence their ability to target different cells (See ‘Tissue Tropism’). In this context it is interesting to note that a variety of cultured cells are susceptible to syncytium induction by HHV-6A but not HHV-6B—a fact with obvious implications for pathogenicity.

HHV-7

Tropism appears to be more restricted for this virus, which utilizes CD4 as a cellular receptor to infect T cells and replicates efficiently in the T-lymphoblastoid cell-line Sup-T1. Alternative receptors such as heparin sulphate, which binds to the HHV-7 protein gQ, are likely to be utilized for other cells (Skrincosky *et al.*, 2000).

Viral Latency

HHV-6 and HHV-7 remain in the host throughout life following primary infection. This persistence probably includes both a latent state (see below), with virions only produced during episodes of reactivation, and chronic replication, with continuous or frequent but intermittent production of infectious virus. Salivary glands are a candidate site for chronic replication of HHV-6B and HHV-7 (see 'Transmission').

Sites of Latency

HHV-6 and HHV-7 can be detected in the peripheral blood mononuclear cells (PBMCs) of healthy individuals by any sensitive polymerase chain reaction (PCR) when sufficient quantities of DNA are tested, with HHV-6B found more frequently than HHV-6A. The actual sites of latency have yet to be established, although candidates for HHV-6 include monocytes and early bone marrow progenitor cells. In the latter case, haematopoietic differentiation can lead to HHV-6 reactivation (Andre-Garnier *et al.*, 2004); thus there are obvious similarities to CMV, where latent virus is well established to reactivate in this way. For HHV-7, CD4⁺ T cells have been proposed as a site for latency (Miyake *et al.*, 2006).

Latency-associated HHV-6 Gene Expression

One group has reported the detection of U94 mRNA in the peripheral blood of healthy individuals, suggesting latency-associated gene expression (Rotola *et al.*, 1998). In addition, HHV-6 latency-associated transcripts that share the protein coding region of the IE1 and -2 transactivators and are maximally expressed in an intermediate stage between latency and reactivation have been identified (Kondo *et al.*, 2003); again, and as noted above, there are obvious parallels with CMV latency, where IE1 protein expression has been proposed as a control point for virus reactivation.

Reactivation from Latency

HHV-6 can be reactivated *in vitro* from latency by superinfection with HHV-7 (Katsafanas *et al.*, 1996). Razonable *et al.* (2002) investigated betaherpesvirus reactivation

in critically-ill immunocompetent hosts. Reactivation of HHV-6A, detected by PCR analysis of PBMC, was significantly increased in such patients compared to controls, suggesting that stress-related mechanisms may favour the reactivation of this variant. Further stimuli for HHV-6 or HHV-7 reactivation *in vivo* are uncharacterized, but are likely to include immunosuppression (see below).

Chromosomal Integration of HHV-6A and HHV-6B

An alternative form of persistence, characterized by very high HHV-6 DNA loads in blood (>6 log₁₀ HHV-6 DNA copies/ml), is due to integration of viral sequences into host leucocyte chromosomes; this situation occurs in a small but significant subset of the human population (see 'Epidemiology and Pathogenesis'). Such integration is unique amongst the HHVs in that it occurs naturally *in vivo*. Instances of EBV chromosomal integration have also been reported—but only very rarely—in Burkitt lymphoma cell lines after repeated passage *in vitro*, rather than as part of the normal virus biology.

The phenomenon of HHV-6 chromosomal integration was first described in 1993 by Luppi *et al.* (1993), who reported three individuals with HHV-6 DNA levels so high in PBMC that they could be detected even by Southern blotting rather than the very much more sensitive technique of PCR. Subsequently fluorescent *in situ* hybridization (FISH) using HHV-6-specific probes identified integrated viral sequences on chromosome 17 in all three cases (Torelli *et al.*, 1995). Further studies suggested that the sites of integration were close to, or in, the telomeres of the short arm of chromosome 17 (Morris *et al.*, 1999) (see example in Figure 10.2).

Sites of Chromosomal Integration

Since the earliest report from Italy (Luppi *et al.*, 1993), FISH-confirmed cases of integrated HHV-6 have also been identified in Japan and the United Kingdom. Both HHV-6A and HHV-6B sequences can be integrated but the sites of integration are not random, being within or close to telomeres, although on a seemingly random range of chromosomes and on either the short or the long arms (Table 10.2). Each individual with integration has viral sequences from either HHV-6A or HHV-6B on only one copy of the particular chromosome involved. Although only 23 individuals have now been reported worldwide, already 7 different chromosomal sites have been identified. Further epidemiological study is warranted to investigate the possibility of additional sites and of geographical or racial variation in them.

Copies of Viral DNA/Cell

In cases of viral chromosomal integration there is at least one copy of HHV-6 DNA/leucocyte (best estimate, about

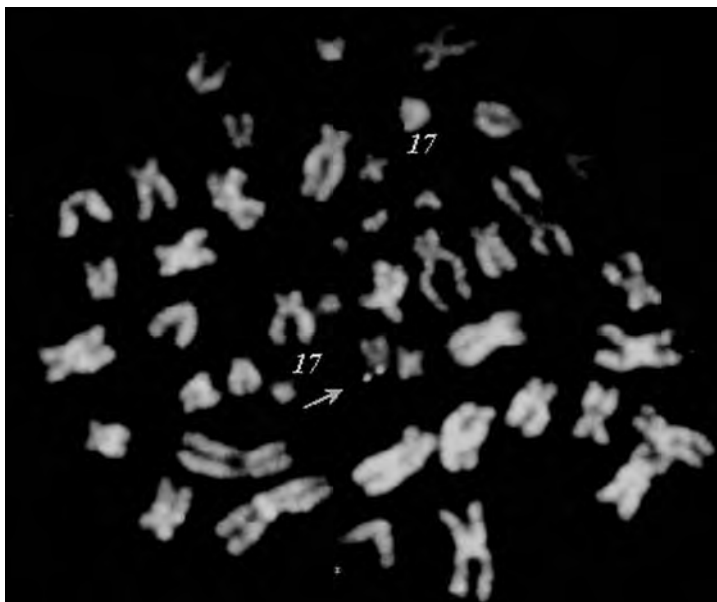


Figure 10.2 HHV-6A integration at the telomeric end of the short arm of chromosome 17 as mapped by fluorescent *in situ* hybridisation (FISH). Example of a dividing cell showing an HHV-6 FISH signal at 17p13.3 from one homologue of the chromosome only. The arrow points to two bright dots at the lower tip of chromosome 17 representing the location of the integrated viral sequence on the sister chromatids. Adapted from Clark *et al.*, (2006). Please refer to this publication to see the original colour illustration showing the FISH signal as green.

four) (Nacheva *et al.*, 2008; Ward *et al.*, 2006). Where FISH was undertaken to identify the site of chromosomal integration, 100% of the metaphase spreads gave an HHV-6 signal from the involved chromosome. Notably there is also at least one copy of HHV-6 DNA/hair follicle cell (Ward *et al.*, 2006), and chromosomally-integrated HHV-6 has been found by FISH in both the skin fibroblasts and peripheral blood cells of a Japanese individual (Daibata *et al.*, 2000). Thus, it seems likely that viral DNA is present in every nucleated cell in the body; that is, present in the germ line (see ‘Vertical Transmission’).

Mechanism of Chromosomal Integration

Exactly how HHV-6A and HHV-6B sequences integrate into the ends of chromosomes is not understood, although the presence of telomeric-like repeats (similar to those found in vertebrate telomeres) at either end of the viral genome in the DR regions may plausibly mediate such targeted integration. Interestingly, another herpesvirus, Marek’s disease virus of chickens, has analogous telomeric repeat sequences within its genome, and has been shown to be integrated into avian chromosomes in newly-harvested lymphoma cells at several different telomeric sites. HHV-7 also contains telomeric-like repeat sequences within its DR regions, but notably lacks

an HHV-6 U94 homologue, as do all the other HHVs. There is no evidence to date that HHV-7 integrates into chromosomes. Finally, it is interesting to note that, in addition to a putative role in virus latency (see above), the HHV-6 U94 gene may be involved in chromosomal integration, since it is a homologue of the AAV *rep* gene, which is implicated in the integration of the AAV genome at specific chromosomal sites.

Chromosomal Integration and the Possibility of Reactivation

It seems that almost the full length of the HHV-6 genome, if not all of it, is integrated (Clark *et al.*, 2006a; Daibata *et al.*, 1999; Torelli *et al.*, 1995) but the very few studies conducted to date have not shown that integrated virus is capable of full replication *in vivo* or *in vitro*, although in one case there is evidence for transcription of a range of viral genes in PBMC (Clark *et al.*, 2006b). However, individuals with HHV-6 chromosomal integration have strikingly high levels of HHV-6 DNA in blood, despite the apparent absence of full virus replication. Such high levels of viral DNA are found not only in whole blood but also in serum. The explanation for the serum DNA lies in the fact that human chromosomal DNA is inevitably found in serum, originating

Table 10.2 The HHV-6 chromosomal-integration sites that have been identified so far, together with variant and country of origin.

Chromosomal integration site	No. of individuals identified and HHV-6 variant		
	Europe		Japan
	United Kingdom	Italy	
1q44	—	—	3B ^a , 3NT ^{b,c}
9q34	2B ^d	—	—
10q26	1A ^d	—	—
11p15.5	1B ^e	—	—
17p13.3	1A ^e , 2B ^d	3B ^f	—
19q11	1B ^d , 1B ^d	—	—
22q13	—	—	4B ^g , 2NT ^b

^aDaibata *et al.* (1998).

^bDaibata *et al.* (1999)—one person had HHV-6 integrated on not only chromosome 1 but also chromosome 22 (see Family 1, Figure 10.4).

^cWatanabe *et al.* (2008).

^dNacheva *et al.* (2008).

^eClark *et al.* (2006a).

^fLuppi *et al.* (1993).

^gTanaka-Taya *et al.* (2004).
NT, not tested.

from cells damaged before or during separation from clotted blood. Thus, where HHV-6 is integrated in a chromosome, viral DNA will be released into serum. This has important implications for epidemiological studies and for molecular diagnosis of active HHV-6 infections (see 'Epidemiology and Pathogenesis' and 'Laboratory Diagnosis').

EPIDEMIOLOGY AND PATHOGENESIS

Incidence and Prevalence

HHV-6A and HHV-6B

As regards the distribution of the two HHV-6 variants in the population, the antibody responses is unhelpful as variant-specific assays are not available (see 'Laboratory Diagnosis') and can only provide the combined seroprevalence of both variants. Virus detection is therefore required but distinction must be made between viral chromosomal integration and the very much more usual acquisition of active infection by the classical process of primary infection.

Chromosomal Integration This has been estimated in populations as between 1 and 3% in England and Wales

(Leong *et al.*, 2007; Ward *et al.*, 2005b) and as 0.2% in Japan (Tanaka-Taya *et al.*, 2004). About a fifth to a third of individuals with this phenomenon have HHV-6A integrated rather than HHV-6B (Tanaka-Taya *et al.*, 2004; Ward *et al.*, 2005b, 2007b).

HHV-6A and Primary Infection In contrast to HHV-6B, natural primary infection with this variant has never been identified. Variant A was initially isolated in cultured lymphocytes from immunocompromised adults but has since been detected in African (Kasolo *et al.*, 1997) and American (Hall *et al.*, 1998) subjects, although it was not determined whether these were actually cases of integration, as was found for British (Ward *et al.*, 2007b) and Japanese (Tanaka-Taya *et al.*, 2004) children with this variant. Thus the true prevalence of HHV-6A due to primary infection remains to be determined.

HHV-6B and Primary Infection This has occurred in almost all children worldwide by the time they are two years old, the peak age of acquisition being between 9 and 21 months (Figure 10.3).

HHV-7

Infection with HHV-7, like HHV-6B, is almost universal and likewise occurs early, but usually somewhat later than the latter, being gradually acquired over the first three or four years of life (Figure 10.3).

Tissue Distribution

HHV-6A and HHV-6B

The distribution of HHV-6A in the body differs from that of HHV-6B. Variant B is commonly found in saliva and blood whereas variant A has only rarely been found at these sites (Hall *et al.*, 1998; Ward *et al.*, 2005b). HHV-6B is also the predominant variant found in brain tissue (Challoner *et al.*, 1995; Tuke *et al.*, 2004). On the other hand, HHV-6A occurs relatively more frequently than variant B in lungs (in this case often accompanied by variant B) (Cone *et al.*, 1996), cerebrospinal fluid (CSF) (Hall *et al.*, 1998) and skin (Di Luca *et al.*, 1996), suggesting different natural histories for the two variants. Since the above studies were based on detection of HHV-6 DNA by PCR rather than by isolation of replicating virus, further work is required to elucidate the possible confounding contribution of chromosomal integration.

HHV-7

Although *in vitro* the host range is limited to T lymphocytes, *in vivo* HHV-7 is commonly found in blood and saliva but also in lungs, skin and mammary glands (Kempf

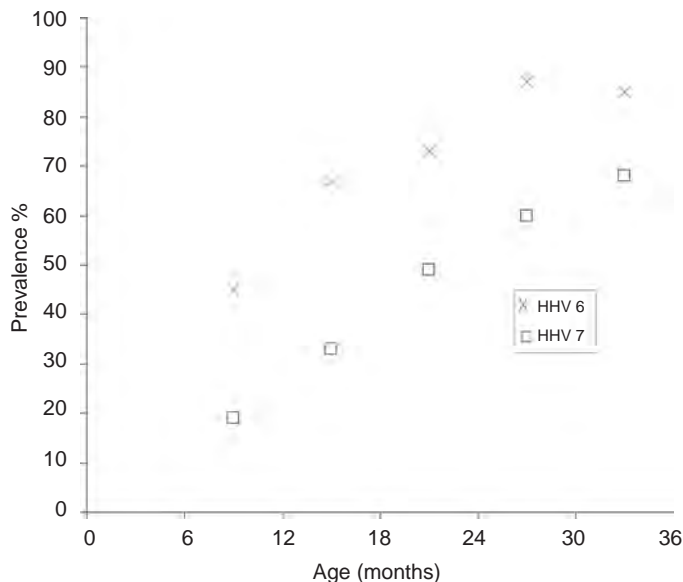


Figure 10.3 Prevalence of HHV-6/HHV-7 infections in British children in relation to age. Prevalence = number of cases with past infection/number with past infection plus uninfected $\times 100$. (Source: Ward, 2005, with permission.)

et al., 1998). In contrast, the virus has rarely been found in brain tissue.

Transmission

Horizontal Transmission of HHV-6B and HHV-7

Both viruses are frequently detectable in the saliva of seropositive individuals, and often at high level in the case of HHV-7, suggesting that *in vivo* salivary glands are a major site of viral replication and that saliva is therefore the source for transmission of both viruses. Molecular characterization of HHV-6 strains detected within a limited number of families suggests that the virus may be transmitted primarily from mother to child. Recently, it has been reported that being exposed to parental saliva and having older siblings were independently associated with HHV-6B infection in early childhood (Rhoads *et al.*, 2007). 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 even through three generations in the same household (Takahashi *et al.*, 1997). Considering that HHV-6B and HHV-7 are both frequently shed in saliva, it is not obvious as to why infection with the latter virus is more delayed in childhood. One possibility is that the restricted cellular tropism of HHV-7 hinders its ability to replicate at the portal of infection, a restriction that does not affect the more widely tropic HHV-6. On the other hand the fact that titres of maternally-acquired neutralizing antibodies

to HHV-7 are higher than those to HHV-6 may account for the later acquisition of HHV-7.

Vertical Transmission of HHV-6A and HHV-6B

A large study by Hall *et al.* (2004) in Rochester, USA investigated the occurrence of congenital HHV-6 infection. Using nested PCR, HHV-6 DNA was detected in 57 of 5638 (1%) cord blood samples, and one third of the positives were variant A, as opposed to the control postnatal infections, all of which were variant B. The high proportion of variant A in cord blood is striking given its apparently low prevalence overall and it seems highly likely that a main route for HHV-6A infection is vertical. The question of vertical transmission of chromosomally-integrated HHV-6 was not investigated in this study, although the proportion of individuals estimated to exhibit such a phenomenon is similar to the actual incidence found (see 'Incidence and Prevalence'). In fact there is considerable evidence that chromosomally-integrated HHV-6A and HHV-6B can be inherited vertically. This has been reported in six different families in Japan (Daibata *et al.*, 1998, 1999; Tanaka-Taya *et al.*, 2004); in one from both parents and in another through two generations (Figure 10.4). In addition, two cases of transmission from mother to child have been reported in the United Kingdom (Ward *et al.*, 2005b).

Finally, a very recent report from Rochester (Caserta *et al.*, 2007) suggests the possibility of two routes of HHV-6 congenital infection, one being transmission of

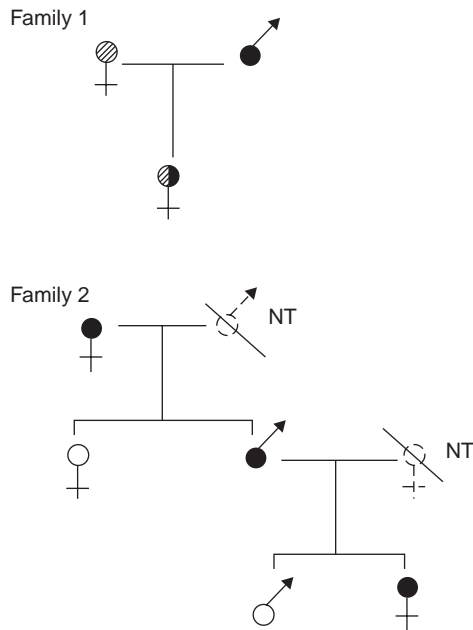


Figure 10.4 Vertical transmission of chromosomally-integrated HHV-6 from both parents in one family and through two generations in another. (Adapted from Daibata *et al.*, 1999 and Daibata *et al.*, 1998, respectively.)

Key: Chromosomal integration site 1q44 (◐) and 22q13 (◑); female; ♂, male; NT, not tested.

integrated HHV-6 through the germ line and the other a conventional infectious process whereby virus has crossed the placenta.

Vertical Transmission of HHV-7

In contrast to HHV-6 there is no evidence for vertical transmission of HHV-7. In the same large study described above, Hall *et al.* (2004) investigated the occurrence of congenital HHV-7 infections, but even using nested PCR, HHV-7 DNA was not detected in 2129 cord bloods.

Perinatal Transmission of HHV-6 and -7

Although there is no general agreement as to whether perinatal transmission occurs, HHV-6 (predominantly variant B but also variant A) and HHV-7 DNA have been detected in 7.5 and 3% respectively of cervical swabs from pregnant women (Caserta *et al.*, 2007), pointing to this as a potential route. HHV-7 DNA, 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).

Nosocomial/iatrogenic Transmission

Primary HHV-6 Infection Transmitted from a Solid Organ Donor This was first reported in 1989 after primary HHV-6 infection in a patient following liver transplantation from a seropositive donor (Ward *et al.*, 1989). More recent evidence supports transmission of HHV-6 infection to infants through liver transplantation from their HHV-6 seropositive mothers (Yoshikawa *et al.*, 2001). Similarly, since HHV-6 has been identified in bone marrow progenitor cells, it is likely that HHV-6 can be transmitted by donor marrow. Indeed, a case of HHV-6B transmission has been well documented in an infant who received a bone marrow transplant from his non-identical twin brother who had ES at the time of marrow donation (Lau *et al.*, 1998).

Chromosomally-integrated HHV-6 Transmitted from a Haematopoietic Stem Cell Donor This was reported for the first time by Clark *et al.* (2006a). In such cases the resultant extremely high HHV-6 DNA load in the blood of the engrafted recipient must always be distinguished from active infection (see also comments in ‘Laboratory Diagnosis’).

Immune Responses

Although fixed infected tissue culture cells and cell lysates have been the most common source of antigens in serological assays for HHV-6 and HHV-7, there is limited characterization of specific antigenic targets or of those antibodies that may be protective (for review see Black and Pellett (1999)). Maternal antibodies appear to be relatively effective in limiting primary infection in the first six months of life and the incidence of seroconversions increases after this time as passively-acquired maternal antibodies wane. For HHV-6, the tegument proteins—p100 for HHV-6A and 101K for HHV-6B—encoded by U11 are a major target of the antibody response. However, there is 80% amino acid identity between HHV-6A and HHV-6B in this ORF and substantial antibody cross-reactivity exists. Antibody responses to HHV-7 are directed predominantly against pp85 and pp89 (encoded by U14 and U11 respectively). There is a low level of cross-reactivity between the antibody responses to HHV-6 and HHV-7 in indirect immunofluorescence tests (see ‘Laboratory Diagnosis’). Neutralizing antibodies have been described for a number of HHV-6 glycoproteins including gB, the gH/gL complex and gQ. For HHV-7, neutralizing antibodies to the HHV-7 gQ homologue have been reported (Skrincosky *et al.*, 2000).

There is only a very limited characterization of the cellular immune responses to HHV-6 and HHV-7 but, as for antibodies, there seems to be some level of

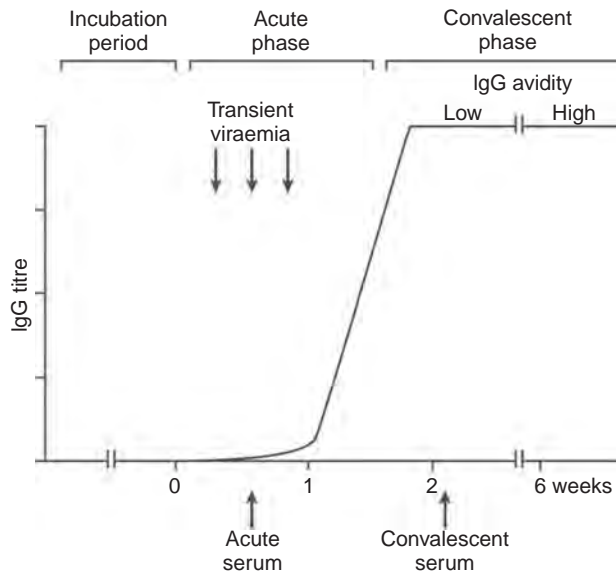


Figure 10.5 Virological features of primary HHV-6 and -7 infections. For avidity see P. 233. (Source: Ward, 2005, with permission.)

cross-reactivity. In the case of HHV-6, virus reactivation is common after immunosuppression accompanying organ transplant, indicating the importance of T lymphocytes in controlling virus infection.

Primary Infection

HHV-6B IgM antibodies can be detected five days after the onset of infection and IgG antibodies by day 7, the latter increasing for up to three weeks and persisting thereafter. There is a transient viraemia that subsides with the appearance of neutralizing antibodies (Figure 10.5).

HHV-7 Although less well studied, the immune response after primary infection is similar to that of HHV-6; some cases are accompanied by a rise in HHV-6 antibodies (see 'Laboratory Diagnosis').

DISEASE ASSOCIATIONS

HHV-6 in particular has been alleged to cause many different diseases, although so far—apart from the well-established case of ES—without compelling evidence for an aetiological role. In fact, the association of HHV-6A, HHV-6B and HHV-7 with disease is fraught with difficulty since, as with all the herpesviruses, both primary infection and reactivation/re-infection may be asymptomatic, and virus replication may continue intermittently thereafter. In addition, HHV-6B and

HHV-7 are universal in the human population from an early age. Finally, in the case of HHV-6 A and HHV-6B, matters are further complicated by the phenomenon of viral chromosomal integration.

Chromosomally-integrated HHV-6 and Disease

Two decades ago a small group of patients with predominantly malignant lymphoid disorders were identified with an unusually high level of HHV-6 DNA in tissue biopsies (Jarrett *et al.*, 1988), suggesting a role for HHV-6 in neoplasia. In 1993 Luppi *et al.* (1993) reported similar high levels in three patients, one with Hodgkin's disease, one with a non-Hodgkin's lymphoma and one with multiple sclerosis (MS), and showed that this resulted from HHV-6 chromosomal integration. Since then viral chromosomal integration has been identified in patients with a variety of dissimilar clinical presentations, including acute lymphatic leukaemia (Daibata *et al.*, 1998), Burkitt lymphoma (Daibata *et al.*, 1999), aplastic anaemia (Hubacek *et al.*, 2007b), anticonvulsant hypersensitivity syndrome (also known as drug-induced hypersensitivity syndrome (DIHS), see below) (Watanabe *et al.*, 2008), various disparate neurological syndromes (Ward *et al.*, 2007b), and even one case of measles (Tanaka-Taya *et al.*, 2004). Thus, it seems that the phenomenon of viral chromosomal integration was come upon by chance rather than because it caused any of the above diseases. Indeed, HHV-6 chromosomal integration has also been found in several apparently healthy individuals, some of whom were blood

donors (Leong *et al.*, 2007) or stem cell donors (Clark *et al.*, 2006a; Kamble *et al.*, 2007).

There are nowhere near enough data available to unravel the possible long-term clinical consequences of HHV-6 integration at various telomeric chromosomal sites. The telomeric region of the chromosome is notoriously unstable and prone to homologous recombination in a mechanism involving the telomere repeats, and it is not known what effect HHV-6 integration may have on this propensity. Large-scale prospective epidemiological surveys are required to determine whether viral chromosomal integration has a role to play in any disease.

Although no clinical consequences have yet been linked to HHV-6 integration it is worth emphasizing that the phenomenon has important implications in clinical practice. Since every cell in the body of an affected person carries the viral genome, high levels of the viral DNA will be found in body fluids such as blood. Unless recognized as the consequence of chromosomal integration, such high viral DNA levels may be interpreted as 'chronic active' HHV-6 infection causing disease. This misunderstanding can lead to inappropriate use of antiviral drugs, such as ganciclovir or foscarnet, which can result in unnecessary toxicity but may well have no effect on the HHV-6 DNA load (Figure 10.6a).

Congenital HHV-6 Infection and Disease

In three surveys of congenital HHV-6 infection as defined by the detection of HHV-6 DNA in cord blood (Adams *et al.*, 1999; Dahl *et al.*, 1999; Hall *et al.*, 2004) no morbidity was found. Although the congenitally-infected children were asymptomatic at birth, more subtle long-term effects could not be excluded, nor could the likelihood that, as with CMV infection, only a low proportion of infected children might be adversely affected. None of these surveys took account of the possibility of germ-line transmission due to chromosomal integration. Prospective studies are needed that differentiate between a classical congenital infection and germ-line inheritance as there may be very different outcomes depending on the mode of transmission. The exact contribution of HHV-6A and HHV-6B to these two possibilities also remains to be determined.

HHV-6B and HHV-7 Primary Infection and Disease in Young Children

Exanthem Subitum

Primary infection with HHV-6B has been conclusively proven to cause ES ('roseola infantum' or sixth

disease) (Yamanishi *et al.*, 1988), a classic febrile rash illness of early childhood which is typically accompanied by a transient viraemia, reduced numbers of circulating leucocytes (both lymphocytes and neutrophils) and sometimes thrombocytopenia and hepatitis. Primary HHV-7 infection also causes ES in a minority of children (Tanaka *et al.*, 1994).

This childhood illness classically presents in an infant with three to five days of high fever with no localizing signs and hence is a worry to both parents and physicians. Defervescence follows, accompanied by the acute onset of a rose-pink, nonpruritic, macular rash, predominantly seen on the neck and trunk. Because of the abrupt onset of the rash, the disease was named exanthem subitum ('*subitum*' means 'sudden' in Latin). The clinical presentation of the disease is not always so characteristic and the rash is commonly misdiagnosed as measles, rubella or an allergic reaction to antibiotics. Finally, it should be noted that both HHV-6B and HHV-7 primary infections may cause a febrile illness without a rash or may be clinically silent.

Convulsions and Encephalitis/Encephalopathy

Although usually mild, it was known long before the aetiology of ES was established that convulsions, and rarely encephalopathy, can accompany this febrile illness. Since the discovery of HHV-6 and HHV-7 and the laboratory diagnosis of primary infection became available, primary HHV-6B infection has been confirmed as the cause of about a third of febrile convulsions (Hall *et al.*, 1994). HHV-7 also causes febrile convulsions, although these are less well documented (Caserta *et al.*, 1998).

As regards more severe neurological manifestations, the convulsions associated with primary HHV-6B infection are often recurrent or otherwise atypical (Hall *et al.*, 1994). Other severe central nervous system (CNS) complications of primary HHV-6B infection (such as meningoencephalitis), although not usually accompanied by pleocytosis in the CSF, have been suggested in isolated case reports. In the case of HHV-7, a primary infection associated with encephalitis/encephalopathy has been described (van den Berg *et al.*, 1999) in which the child recovered. These hints that primary HHV-6B and possibly HHV-7 infections may make a significant contribution to neurological morbidity have now been confirmed by a recent prospective British Isles-wide survey of young children with suspected encephalitis. Seventeen percent of serious neurological diseases in children up to two years old were found to be due to primary infection with HHV-6B or HHV-7; this is just the age when routine childhood

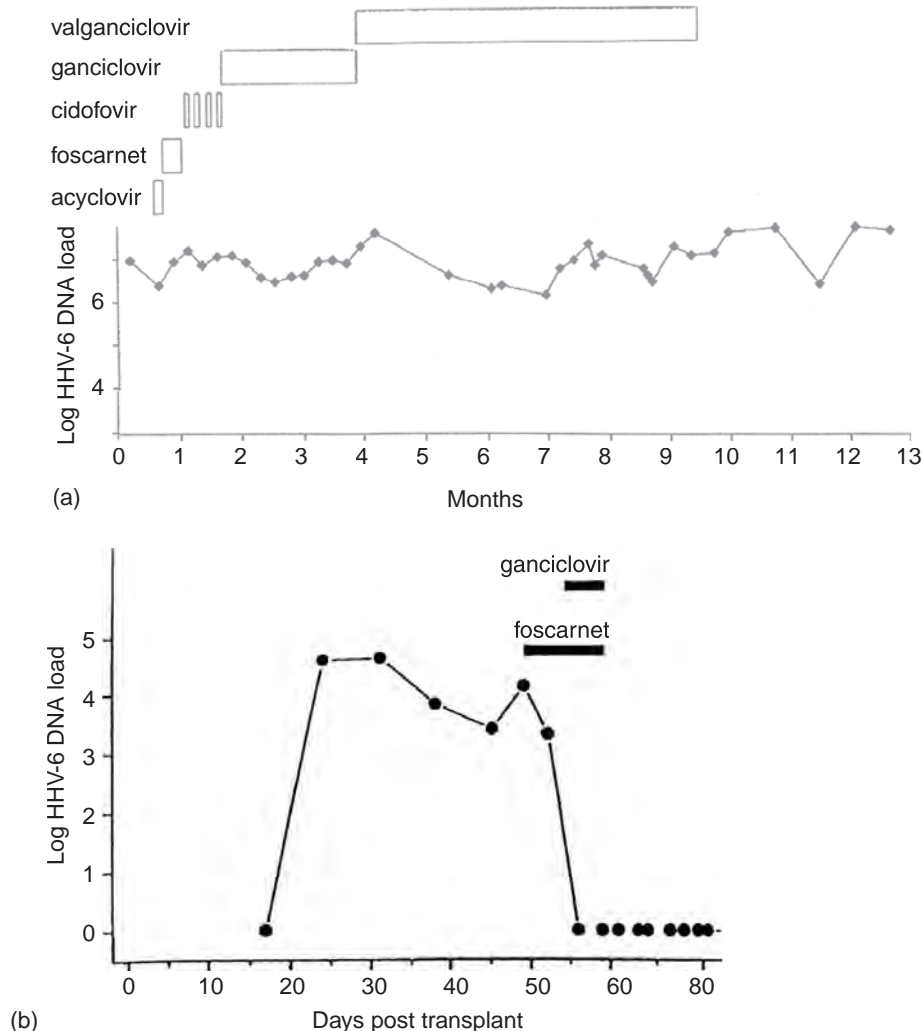


Figure 10.6 Comparison of the effects of antiviral drugs on (a) HHV-6 chromosomal integration and (b) active HHV-6 infection. (a) Failure of multiple antivirals to affect high HHV-6 DNAemia resulting from viral chromosomal integration in a case of severe aplastic anaemia. (Adapted from Hubacek *et al.*, 2007b.) (b) Virological response to antiviral therapy in a case of HHV-6-related graft failure following allogeneic bone marrow transplantation. (Adapted from Johnston *et al.*, 1999.)

vaccines such as measles, mumps, rubella (MMR) are administered and thus neurological complications wrongly attributed to the vaccine may in fact be due to coincidental primary HHV-6B or HHV-7 infections. Many of the cases of primary infection in the survey were in status epilepticus with fever (Ward *et al.*, 2005a) but fitted the criteria for complex febrile convulsions rather than encephalitis (Ward *et al.*, 2007a). In this connection, a recent preliminary study has identified HHV-6B in the mesial temporal lobe of patients with epilepsy originating

from this part of the brain (Fotheringham *et al.*, 2007) and such disease has been associated with complex febrile convulsions.

Summary of Clinical Manifestations of Primary Infection

In the case of HHV-6B, most children have at least some symptoms (only about 5% are asymptomatic), ranging from fever, occasionally accompanied by the rash of

ES (Zerr *et al.*, 2005), to febrile convulsions to status epilepticus with fever (Ward *et al.*, 2005a). The severity of disease depends on the population under study. It is mildest in the community (Zerr *et al.*, 2005), more serious in children requiring medical attention but not necessarily admitted to hospital (Hall *et al.*, 1994), and most severe in children admitted to hospital (Ward *et al.*, 2005a). Primary infection with HHV-7 has been much less extensively investigated but the pattern of disease is similar.

HHV-6B and HHV-7 Primary Infection in Older Children and Adults

With virtually all children acquiring HHV-6 infection by the age of two years and HHV-7 usually slightly later primary infections are bound to be unusual. But, by analogy with the other HHVs, such delayed primary infection might be expected to cause more severe disease than in early childhood. The evidence is scanty but in adults primary HHV-6B infection has been associated with heterophile antibody-negative mononucleosis (Akashi *et al.*, 1993) and primary HHV-7 infection with encephalitis and flaccid paralysis (Ward *et al.*, 2002).

HHV-6 and HHV-7 Reactivation and Disease in the Immunocompetent

Studies on this topic are more difficult to carry out than those on primary infection and depend on the detection of increased viral replication by one means or another (see 'Laboratory Diagnosis' for a discussion of the problems and pitfalls). In addition, the distinction between re-infection and reactivation has rarely, if ever, been attempted, and in what follows the blanket term 'reactivation' is used to cover both possibilities. Thus, HHV-6 reactivation has been described apparently without effect on morbidity or mortality in 3.3% of children (Caserta *et al.*, 2004), commonly in pregnancy (Dahl *et al.*, 1999), during infection with other viruses such as measles and dengue, and in critically-ill but otherwise immunocompetent patients (Razonable *et al.*, 2002).

HHV-6 Reactivation and Disease

Drug-induced Hypersensitivity Syndrome (DIHS)

This is also known as drug hypersensitivity syndrome (DHS) or drug rash with eosinophilia and systemic symptoms (DRESS) and is characterized by a severe multi-organ hypersensitivity reaction that usually appears three to six weeks after exposure to certain drugs, including anticonvulsants. The exact pathogenesis of this

syndrome is unknown although it is likely that a genetic predisposition is involved. Recently HHV-6 reactivation has been associated with DIHS because of very large increases in HHV-6 IgG titres three to four weeks after onset. However, it remains to be seen whether HHV-6 reactivation plays a causal role in this syndrome by contributing to either its initiation or its exacerbation, rather than merely being a consequence of pre-existing immune activation (Wong and Shear, 2004).

Multiple Sclerosis (MS) In 1995 Challoner and colleagues (1995) proposed an association of HHV-6 with MS. Their findings provoked substantial controversy and the generation of conflicting data from many later studies (for review see Clark (2004)). With hindsight it seems that the ready identification of HHV-6 DNA by representational difference analysis in an MS brain sample may have been due to viral chromosomal integration with its consequent high HHV-6 DNA load (Clark *et al.*, 2006a). Any link of either HHV-6 variant to MS remains unclear.

Chronic Fatigue Syndrome/Myalgic Encephalopathy

There is no convincing scientific evidence for an aetiological role of HHV-6 in this syndrome but the lingering belief in this unproven association leads to frequent unnecessary requests for HHV-6 diagnosis.

Encephalitis As regards neurological disease, since HHV-6B commonly persists in brain, viral reactivation from this site might be expected occasionally to cause encephalitis. HHV-6 was the only agent detected by PCR in CSF in a small proportion of cases of older children and adults with encephalitis (McCullers *et al.*, 1995), leading to the conclusion that the virus was replicating in the nervous system. Since then there have been about a dozen case reports suggesting HHV-6A or HHV-6B as the cause of encephalitis in adults. Moreover, several large surveys of the prevalence of HHV-6 DNA in the CSF of patients in America, Europe and the Middle East all gave a result of 1–2%. Although unrecognized in any of the above studies, their finding of HHV-6 DNA in about 1–2% of samples is of exactly the same order as that shown for the prevalence of HHV-6 chromosomal integration in UK blood donors. Indeed, a recent study (Ward *et al.*, 2007b) has demonstrated that chromosomal integration rather than active virus replication is the most likely cause of HHV-6A or HHV-6B DNA in the CSF of the immunocompetent—the higher the number of leukocytes in that fluid the greater the

amount of chromosomally-integrated HHV-6 that will be detected.

HHV-7 Reactivation and Disease

Very little is known about HHV-7 reactivation in the normal host although there is no evidence of this in critically-ill patients (Razonable *et al.*, 2002). HHV-7 DNA is rarely, if ever, found in the CSF of older children and adults and therefore no link between virus reactivation and encephalitis has been suggested. Likewise, a postulated association of HHV-7 with 'pityriasis rosea' remains unproven (Chuh *et al.*, 2004).

HHV-6 and HHV-7 and Immunomodulation

Both HHV-6 and HHV-7 infect CD4⁺ T lymphocytes, the cornerstone of the adaptive immune response, and it seems likely that in common with the other herpesviruses HHV-6 and HHV-7 have genes that function to facilitate the avoidance of immune surveillance during primary infection, latency and reactivation. It has therefore been suggested that infection with either of these two viruses (but primarily HHV-6; see Table 10.3) interferes with the functioning of the host immune response, and therefore contributes to overall mortality by enhancing the pathogenic effects of agents such as human immunodeficiency virus (HIV) and CMV, or exacerbates graft-versus-host disease (Ljungman and Singh, 2006; Lusso, 2006). Further studies are required to establish the clinical impact, if any, of immunomodulation due to HHV-6 and HHV-7.

HHV-6 and Disease in the Immunosuppressed after Organ Transplant

Since HHV-6 seroprevalence is almost universal in older children and adults, it is not surprising that HHV-6 reactivation is common after solid organ transplant and haematopoietic stem cell transplant (HSCT). Such reactivations occur up to half of all patients within two to four weeks of transplant. In addition, as discussed above, HHV-6 can sometimes be transmitted with the donor allograft. Unfortunately, very few studies of HHV-6 infection after transplant distinguish between the two variants, though in those that do it is clear that HHV-6B is identified much more frequently than variant A. Finally, associations between HHV-6 and disease are often based on case series, in which the described cases are frequently complex, involving several different possible pathological mechanisms, which makes conclusions about aetiology very difficult.

HSCT Recipients

In this context various nonspecific clinical manifestations have been attributed to HHV-6, including fever, skin rash and hepatitis, but the best documented are bone marrow suppression and encephalitis (Ljungman and Singh, 2006; Zerr, 2006).

Bone Marrow Suppression HHV-6 can infect haematological progenitor cells *in vitro* and reduce colony formation. *In vivo* HHV-6 infection has been associated with delayed engraftment after HSCT, especially of monocytes and platelets.

Table 10.3 Principal mechanisms of immunomodulation by HHV-6

Mechanisms	Viral variant
Lytic infection of CD4 ⁺ T cells	A, B
Lytic infection of cytotoxic effector cells (CD8 ⁺ T cells, $\gamma\delta$ T cells and natural killer cells)	A
Phenotypic and functional impairment of antigen-presenting cells (macrophages, dendritic cells)	A, B
Suppression of IL-12 secretion by macrophages and dendritic cells	A, B
Suppression of IL-2 secretion	A
Induction of inflammatory and immunosuppressive cytokines and chemokines (e.g. IFN- α , IL-1 β , IL-10, IL-15, TNF- α , RANTES)	A, B
Expression of viral chemokines and chemokine receptors	A, B
Downmodulation of the CD3/T-cell receptor complex	A, B
<i>De novo</i> induction of CD4	A
Downmodulation of CD46	A > B
Induction of T-regulatory type 1 cells	A, B
Synergy with HIV-1	A > B

IL, interleukin; IFN, interferon; TNF, tumour necrosis factor.
From Lusso (2006), with permission.

Encephalitis HHV-6 encephalitis is emerging as a significant but relatively rare clinical entity after HSCT, with a mortality of about 40%. In the first case of HHV-6 encephalitis described there was evidence of HHV-6B infection, namely viral protein in astrocytes and neurons, in the diseased area of the brain including the hippocampus, and the pathology was characterized by necrosis and demyelination (Drobyski *et al.*, 1994). Since then there have been many publications describing HHV-6 encephalitis in over 40 patients, mostly as single case reports, and in over three quarters of these a mismatched related or unrelated HSCT was involved (only one patient had received an autograft rather than an allograft). Where the virus had been typed it was predominantly variant B. Of these case reports about a third had a limbic encephalitis, for which the term post-transplant limbic encephalitis (PALE) has recently been suggested (Seeley *et al.*, 2007), whereas the remainder had a more diffuse or multifocal encephalitis. In addition, longitudinal observational studies have established a correlation between HHV-6 reactivation in peripheral blood and CNS disease.

Patients with HHV-6 encephalitis characteristically present early after transplant (median 24 days) with depressed consciousness, convulsions, confusion and disorientation, often with short-term memory loss. Few patients have focal findings on neurological examination. Apart from HHV-6 DNA in CSF, elevated CSF protein levels are present in about two thirds of patients, and about half have CSF pleocytosis. Computed tomography (CT) of the brain, especially when conducted early in the course of illness, is often normal. Acute abnormalities are seen more often in magnetic resonance imaging (MRI) of the brain; these include multiple non-enhancing, low-attenuation lesions in the grey matter, sometimes involving the temporal lobes (especially the medial temporal). Electroencephalography (EEG) studies are usually diffusely abnormal but sometimes involve the temporal region (Zerr, 2006). However, before the diagnosis of HHV-6 encephalitis is finally accepted, other infectious agents should be excluded by culture, microscopy or nucleic acid test. Malignant disease (based on CT or MRI, and microscopy and immune staining of CSF) and HHV-6 chromosomal integration must likewise be excluded.

Solid Organ Transplant Recipients

As for HSCT, there is some evidence for bone marrow suppression and encephalitis due to HHV-6 infection after solid organ transplant, although this occurs less frequently than after HSCT. Patients receiving a liver graft seem to be the most prone to developing symptoms due to HHV-6. However, a potential role for indirect effects of HHV-6

(see 'HHV-6 and HHV-7 and Immunomodulation') on disease in transplant recipients has been suggested by a number of prospective studies and these may be more significant than direct effects in terms of medical impact (Clark and Griffiths, 2003).

HHV-7 and Disease in the Immunosuppressed after Organ Transplant

In contrast to HHV-6, HHV-7 reactivation after HSCT is less common (Boutolleau *et al.*, 2003) and there is little evidence for an association with direct end organ disease, although a handful of cases with CNS symptoms have been ascribed to the virus (Ljungman *et al.*, 2008). However, as for HHV-6, findings from prospective studies suggest that there may be important indirect effects of HHV-7, such as an increased risk of CMV disease in renal transplant recipients.

LABORATORY DIAGNOSIS

Antibody Detection

IgG and IgM

The first tests for HHV-6 IgG were developed by Yamanishi and colleagues using indirect immunofluorescence (Yamanishi *et al.*, 1988). Many other assays have subsequently been introduced including those for neutralizing antibody, enzyme immunoassay (EIA) and immunoblot. However, systematic comparisons of these assays have rarely been carried out and more are urgently needed; furthermore, unfortunately none of them can distinguish between the antibody response to HHV-6A and B. In the case of HHV-7, much less developmental work has been done and indirect immunofluorescence is the most commonly used test.

Immunofluorescence tests are disadvantageous in that they are labour-intensive, subjective and limited by non-specific fluorescence. In addition, IgM is not always detected with primary infection because of low sensitivity, although this can be improved somewhat by separation of IgM from IgG, for example, by using ion-exchange chromatography. There is also cross-reactivity at the IgM level between HHV-6 and -7, and probably with CMV and EBV. Nevertheless, the indirect immunofluorescence tests for HHV-6 and -7 antibody remain the gold standard and are the most commonly used (kits are commercially available). Such tests allow evaluation of antibody development and titres in sequential serum samples, and when modified to detect IgG antibody avidity can distinguish primary from long-standing infection (see below).

IgG and Antigenic Cross-reaction

As discussed earlier there is antibody cross-reactivity between HHV-6 and HHV-7 IgG. However, in indirect immunofluorescence tests this is limited and titres >32 can be accepted as true positives. As regards other tests, immunoblot assays can eliminate cross-reaction by using the immunodominant antigen specific to each virus, namely for HHV-6 101k (or p100) and for HHV-7 pp89, but the reagents are not readily available. Likewise, some of these problems might be solved by the use of EIAs based on recombinant antigens specific to HHV-6 and -7 but much further developmental work is required. Finally, the neutralizing epitopes for HHV-6 and HHV-7 appear to be distinct and immunological cross-reactivity between neutralizing antibodies for the two viruses has not been reported (Yoshida *et al.*, 2002) but such tests are very labour-intensive.

It should be noted that rises in pre-existing IgG antibody to HHV-6 have been reported following primary CMV and EBV infections. These may be due to polyclonal stimulation, antigenic cross-reaction eliciting a secondary HHV-6 response, or reactivation.

Antibody Avidity Tests

In primary infection the first IgG antibodies produced are of low avidity but with time the immune response matures as specific B-cell clones undergo somatic mutation and IgG antibody of higher avidity is produced. *In vitro* tests for antibody avidity rely on the fact that a protein-denaturing agent disrupts the antigen-antibody reaction preferentially, affecting low- but not high-avidity antibody. Such tests have been used for the diagnosis of many different human virus infections. HHV-6 and HHV-7 IgG immunofluorescence tests are easily adapted for the assessment of antibody avidity by the use of a urea wash after the primary incubation with antibody; if antibody avidity is low this confirms recent primary infection but if the avidity is high primary infection must have occurred at least six weeks previously (Figure 10.5).

Virus Detection

Unlike antibody tests, these methods have the ability to discriminate between HHV-6A and HHV-6B.

Virus Isolation

HHV-6A and HHV-6B, and HHV-7 may be grown by co-culture of a sample of the individual in question's PBMC with phytohaemagglutinin-stimulated cord blood lymphocytes. The cytopathic effects (CPEs) for all three viruses are very similar (see Figure 10.1 for HHV-6 CPE) but they may be individually identified by the use of tests

for antigen or nucleic acid (see below). Unfortunately, although culture is the gold standard for diagnosis of active infections this is too labour-intensive for routine diagnostic use and cord blood cells are not generally available.

Antigen Detection

Although specific monoclonal antibodies are available (some commercially) (Wang and Pellett, 2007) and an HHV-6 antigenaemia test has been developed, there is very little experience with it as its use has not been widespread. Similarly, the use of monoclonal antibodies directed against virus structural proteins for the immunohistochemical diagnosis of tissue infections has only been used in research studies.

Detection of Viral Nucleic Acid

Both qualitative and quantitative PCR methods have been published but the optimal genomic target is not known. Multiplex PCR is recommended to distinguish HHV-6A, HHV-6B and HHV-7. As regards the detection of infection, real-time quantitative PCR is the method of choice, although no quantitative threshold has been formally established to differentiate latent HHV-6 and HHV-7 infection from active viral replication. The lack of a standardized upper limit is also a concern in the case of HHV-6, where abnormally high levels in whole blood and serum due to chromosomal integration will be misdiagnosed as active infection responsible for disease. Reverse transcription PCR directed against a gene activated in the lytic replication cycle of HHV-6 and HHV-7 is a potential alternative, although in the one case reported so far, low levels of HHV-6 transcripts from IE, early and late genes were detected in the PBMC of a person with chromosomally-integrated HHV-6 (Clark *et al.*, 2006b).

Diagnosis of HHV-6 Chromosomal Integration

This phenomenon should always be suspected if there is an abnormally high HHV-6 DNA load in blood. A way to confirm viral chromosomal integration is to test hair follicles for the presence of HHV-6 DNA but this is impractical for routine diagnosis. Instead, viral DNA load should be measured in both whole blood and serum, preferably alongside quantitation of cellular DNA, for example β -globin DNA, so as to calculate the number of HHV-6 copies per cell. If there is chromosomal integration of HHV-6 then there should be at least one copy of HHV-6 DNA per leucocyte, and similarly for serum at least one copy per lysed leucocyte.

Diagnosis of Primary Infection in Young Children

In all that follows, it should be remembered that maternally-acquired antibody may confuse the interpretation of results in children less than six months old.

Using Paired Sera

This is the traditional method, requiring an acute serum preferably taken within the first week of illness, and a convalescent serum taken 10–14 days later (Figure 10.5). Testing the two sera together will readily demonstrate IgG seroconversion and thus primary HHV-6 infection in an HHV-7 seronegative individual (Table 10.4, example 2) and vice versa (Table 10.4, example 4). However, difficulty commonly arises in primary HHV-7 infection in the presence of previous HHV-6 infection since rising titres of antibodies to both viruses may occur, presumably due either to antigenic cross-reaction eliciting a secondary HHV-6 response or to concomitant HHV-6 reactivation. Antibody avidity tests prove very useful in this situation, distinguishing the low-avidity IgG response in HHV-7 primary infection from the high-avidity HHV-6 response which may accompany it (Table 10.4, example 5). Coinciding HHV-6 and HHV-7 primary infections may also be differentiated in this way (Table 10.4, example 1).

However, differentiating primary HHV-6A from HHV-6B infection requires the detection and characterization of virus or viral nucleic acid.

Using Paired Saliva Samples

This innovative method using the detection of viral DNA in saliva samples was pioneered by Zerr *et al.* (2005). It is useful for field studies but the time interval between last negative test and first positive test should not be more than 14 days; the problem in using more extended intervals is that in the first few years after primary infection viral DNA will inevitably be found in saliva.

Using a Single Acute Serum

Viral DNA in serum in the absence of IgG is usually taken to reflect the transient viraemia that occurs in the acute phase of HHV-6 and HHV-7 primary infections (see Table 10.4, examples 1 and 2, and 3 and 4 respectively). However, in the case of HHV-6 the possibility of chromosomal integration must be borne in mind and excluded. Importantly, it is not always possible to detect viral DNA in serum or plasma in the acute phase of primary infection (see Table 10.4, example 1 primary HHV-7 infection) (see also Ward *et al.*, 2007b).

Table 10.4 Examples of HHV-6 and -7 antibody responses and detection of DNA in primary infections in young children (Source: Ward, 2005).

Example no. and type of infection	Day of illness	HHV-6 IgG		HHV-6 DNA ^a	HHV-7 IgG		HHV-7 DNA ^b
		Titre	Avidity		Titre	Avidity	
(1) Dual primary HHV-6B and -7 infections	1	nt	nt	+	< 16	nt	–
	2	< 16	nt	nt	nt	nt	nt
	17	2048	Low	nt	256	Low	nt
(2) Primary HHV-6B infection without HHV-7 infection	0	< 16	nt	+	< 16	nt	–
	4	< 16	nt	+	< 16	nt	–
	10	2048	Low	–	nt	nt	–
(3) Primary HHV-6B infection with past HHV-7 infection	55	512	High	nt	< 16	nt	nt
	3	32	nt	+	128	High	–
	14	128	Low	–	128	High	–
(4) Primary HHV-7 infection without HHV-6 infection	4	< 16	nt	–	< 16	nt	+
	11	16	nt	–	512	Low	–
	41	< 16	nt	nt	512	Low	nt
(5) Primary HHV-7 infection with past HHV-6 infection	168	16	nt	–	512	High	–
	3	512	High	–	64	Low	+
	15	8192	High	nt	8192	Low	nt
	96	1024	High	–	4096	Low	–
	119	512	High	–	256	High	nt

^aHHV-6B detected in plasma or serum.

^bDetected in plasma or serum.

nt, not tested.

See Ward *et al.* (2001) for details of methods. Low-avidity antibody results are highlighted in bold.

Using a Single Convalescent Serum

The high prevalence of both HHV-6 and HHV-7 makes it difficult to interpret single positive IgG results in convalescent sera, although low avidity antibody suggests infection within the previous six weeks.

Diagnosis of Convulsions with Fever and Encephalitis

If HHV-6 or HHV-7 is suspected as the cause of fever and convulsions in a young child, serological tests for primary infection are the key to diagnosis as viral nucleic acid is not always detected in CSF (Caserta *et al.*, 1998; Hall *et al.*, 1994). On the other hand it is not enough for the diagnosis of HHV-6 encephalitis to detect viral DNA in CSF. The amount of viral DNA must be quantitated and other possible causes, including HHV-6 chromosomal integration, must be excluded before any conclusions are drawn. Although, as discussed above, HHV-6 encephalitis is accepted as an emerging disease after organ transplant, formal proof of suspected HHV-6 encephalitis under any other circumstances requires evidence in CSF of active virus infection and cell destruction in the brain.

Diagnosis of Primary Infection or Reactivation in Older Children/Adults and the Immunosuppressed

It is of note that diagnosis of primary infections in older children/adults and those who have received chemotherapy definitely requires the use of avidity tests as antibody titres may have decreased to the point of seronegativity and a secondary antibody response may therefore appear to be that of a primary. The diagnosis of primary infection in the severely immunocompromised, for example HSCT patients, who are unable to make an antibody response, is especially difficult and requires rigorous exclusion of pre-existing infection by molecular means. IgM tests are unhelpful in this group since as many as 5% of adults may be positive at any given time, suggesting either re-infection/reactivation or nonspecific reactions.

As regards reactivation, antibody tests (even when including those for avidity) may be unhelpful, especially for HHV-6, since as noted previously pre-existing antibody may rise in primary CMV and EBV infections. In addition, as discussed earlier, the detection of HHV-6 DNA in CSF in those already HHV-6 seropositive may well be due to HHV-6 chromosomal integration rather than virus reactivation.

Diagnosis of Disease after Organ Transplant

As discussed above antibody tests have little to offer in the immunocompromised. Diagnosis therefore relies on the careful interpretation of the results of virus detection

in the context of clinical features and exclusion of other possible causes.

Pitfalls due to HHV-6 Chromosomal Integration

In the past, detection of HHV-6 DNA in the blood of transplant patients was interpreted as evidence of virus reactivation, with very high DNA levels taken to indicate high levels of active virus replication. Unfortunately such an interpretation should not be made in an individual with HHV-6 chromosomal integration and this phenomenon must be excluded before assuming active HHV-6 infection. For example, after HSCT from a donor with chromosomal integration of HHV-6 there will be asymptomatic elevation of HHV-6 DNA load in the recipient after transplant, rising to characteristically and strikingly high levels that correlate with leucocyte engraftment (Clark *et al.*, 2006a). On the other hand, if the stem cell recipient rather than the donor has viral chromosomal integration the converse will occur, although a persistent low-level HHV-6 DNA will remain in blood, presumably due to release of chromosomal DNA from the recipient's non-haematopoietic cells (Hubacek *et al.*, 2007a). A comparison of the different possible findings regarding HHV-6 after HSCT is given in Table 10.5. If HHV-6 chromosomal integration is suspected, it can most easily be established by checking donor and recipient blood prior to transplant for high levels of HHV-6 DNA using quantitative PCR.

HHV-6 Induced Bone Marrow Suppression or Encephalitis

The diagnosis of virus-induced bone marrow suppression must be based on delayed engraftment, together with HHV-6 DNA in blood or ideally virus isolation in culture. Similarly the diagnosis of encephalitis must be based on features consistent with HHV-6 disease, namely CNS symptoms and signs, together with an abnormal MRI, or diffuse EEG changes, together with detection of HHV-6 DNA in CSF. In both cases other possible causes of disease and HHV-6 chromosomal integration must be excluded.

Other HHV-6 or HHV-7 Disease

Symptoms and signs from the organ in question, together with HHV-6 or HHV-7 detection in blood, are not enough for the diagnosis of disease. Tests on tissue are required to establish evidence of viral replication and consequent pathology. Possible techniques include *in situ* hybridization and immunohistochemistry. PCR for viral DNA is not recommended as its detection may merely reflect the presence of virus in blood rather than tissue

Table 10.5 Comparison of findings after HSCT in various forms of HHV-6 infection

Clinical/laboratory observations	HHV-6 status		
	Horizontal acquisition	Donor chromosomal integration	Recipient chromosomal integration
≥ 1 HHV-6 copy/leucocyte	No	Yes ^a	No ^b
≥ 1 HHV-6 copy/hair follicle	No	No	Yes ^c
HHV-6 variant/prevalence	B/>95%	A or B/about 1%	A or B/about 1%
HHV-6 DNA in blood	No or intermittent	+ + + +	+/-
Disease due to HHV-6	Encephalitis, delayed engraftment	No?	No?
HHV-6 response to antiviral drugs	Yes, decrease in HHV-6 DNA level	No	No

^aHHV-6 found persistently at extremely high level (chromosomal integration) in haematopoietic tissue, for example blood, bone marrow and spleen.

^bChromosomally-integrated HHV-6 'not' found in the haematopoietic tissue.

^cHHV-6 found persistently at extremely high level (chromosomal integration) in cells and tissues of the body that are not involved in haematopoiesis.

cells, and hence the specificity and the positive predictive value are too low.

ANTIVIRAL THERAPY

To date there have been no controlled trials of antiviral drugs against either HHV-6 or HHV-7 (Clark and Griffiths, 2003). The drugs that might be effective, that is ganciclovir, foscarnet and cidofovir, all have serious side effects, although there are case reports in the literature of antiviral efficacy, as evidenced by reduction of HHV-6 DNA load or simply a clinical response to treatment. Therefore no specific recommendations can be made and it should be emphasized that use of antiviral drugs should not be contemplated for the immunocompetent patient, except perhaps in life-threatening situations. In particular, it should be remembered that there is good evidence that antiviral therapy will not reduce the HHV-6 DNA load in chromosomal integration, as was shown in a recent case report (Hubacek *et al.*, 2007). Figure 10.6 compares the lack of an antiviral response in chromosomal integration (Figure 10.6a) with the good response in a case of HHV-6-induced bone marrow suppression in which there was not only a rapid 10 000-fold reduction in amount of HHV-6 DNA (Figure 10.6b) but also a corresponding increase in white cell count (see Johnston *et al.*, 1999).

Organ Transplant Recipients

Given the low risk of HHV-6 disease and the toxicity of the available drugs, antiviral prophylaxis cannot be recommended (Ljungman *et al.*, 2008). In addition, it should be remembered that since ganciclovir and foscarnet

are commonly used in the HSCT setting for pre-emptive therapy against CMV, this may facilitate the emergence of drug-resistant HHV-6.

As regards HHV-6 encephalitis, most of the patients reported with HHV-6 encephalitis after HSCT received either foscarnet or ganciclovir (Ljungman and Singh, 2006; Zerr, 2006). However, although one series demonstrated a virological response to these drugs, there remained a high morbidity and mortality. Despite the lack of controlled trials and based solely on expert opinion, the International Herpes Management Forum recommends foscarnet or ganciclovir, either alone or in combination, for treatment of HHV-6 CNS disease (Dewhurst, 2004).

Future studies in organ transplant patients (in whom the relevant drugs are in any case used for control of CMV infection) are needed to determine the concurrent impact of antiviral therapy on HHV-6 and HHV-7 infections and hence the possible effectiveness of treatment.

CONCLUDING REMARKS

HHV-6 reactivation has been claimed as a cause of many different diseases, although for most there is no compelling evidence for an aetiological role. In some cases HHV-6 may play an immunomodulatory role, but alternatively it may merely be a bystander reactivated by inflammation or immune reactivation. In other cases association with active infection and disease may have been wrongly suggested by the phenomenon of HHV-6 chromosomal integration.

The scale of the contribution of HHV-6 and especially HHV-7 primary infections to serious neurological disease

in young children has not been appreciated in the past but such cases must now be fully investigated for these two viruses. This is most important where the illness is temporally related to vaccinations, which might otherwise be wrongly blamed for the condition.

It is clear that a great deal more is known about the behaviour and role of HHV-6 than of HHV-7 and it is therefore necessary for future studies to include the latter virus. Although much has been accomplished in the last decade, further improvements in the diagnosis of primary HHV-6 and -7 are required, in particular convenient rapid tests for use on acute specimens. It is anticipated that this will be achieved at least in part by the development of standardized quantitative real-time PCR and the use of antibody tests based on recombinant proteins.

The recently-recognized phenomena of chromosomal integration and germ-line inheritance of HHV-6 must always be borne in mind, and clearly require further study. It is also important that the role of HHV-6 variants A and B be distinguished in various situations by use of the appropriate tests, and that that of HHV-6A, in particular, be more fully explored.

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Kaposi's Sarcoma-associated Herpesvirus (Human Herpesvirus 8)

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INTRODUCTION

The eighth human herpesvirus is associated with three forms of human neoplasia. It was first discovered in Kaposi's sarcoma (KS), with which it is strongly associated, hence the designation Kaposi's sarcoma-associated herpesvirus (KSHV) given to it by its discoverers (Chang *et al.*, 1994). It is also consistently found in a very rare form of B-cell lymphoma, body cavity-associated lymphoma (BCBL) or primary effusion lymphoma (PEL) (Cesarman *et al.*, 1995a). Finally, it is found in the plasma cell variant of multicentric Castleman's disease (MCD) (Soulier *et al.*, 1995), particularly in human immunodeficiency virus (HIV)-infected individuals, and may also play a role in occasional cases of bone marrow failure in immunosuppressed individuals (Luppi *et al.*, 2000b). To reflect its association with conditions other than KS, and to keep within the nomenclature adopted for other human herpesviruses, the term human herpesvirus 8 (HHV-8) is preferred by some authors.

The discovery of KSHV was initiated by careful epidemiological studies which had, for several decades, suggested the involvement of a transmissible agent in the pathogenesis of KS. In spite of these leads, attempts by several groups to identify such an organism by conventional culture or morphological approaches had been unsuccessful. The development of molecular techniques combining polymerase chain reaction (PCR) amplification with subtractive hybridization (representational difference analysis, RDA) allowed Chang *et al.* (1994) to search for unknown DNA sequences present in KS, but not a control tissue. They initially identified two small DNA fragments

with homologies to two oncogenic gammaherpesviruses, Epstein-Barr virus (EBV) and herpesvirus saimiri (HVS) of squirrel monkeys. These were later shown to be part of a complete viral genome (Russo *et al.*, 1996) present in the endothelial tumour (spindle) cells of KS lesions (Boshoff *et al.*, 1995). After the discovery of KSHV in PELs (Cesarman *et al.*, 1995a) several groups succeeded in establishing persistently-infected cell lines from such lymphomas, and subsequently in visualizing KSHV virions after chemical induction of the lytic replication cycle (Renne *et al.*, 1996). The viral particles show the characteristic morphological appearance of *Herpesviridae* (see Chapter 6). An example of a KSHV virion, produced by the PEL-derived KS-1 cell line after induction of the lytic cycle by treatment with phorbol esters, is shown in Figure 11.1.

ORIGIN AND EVOLUTION OF KSHV

Phylogenetic analysis of its genomic sequence (Russo *et al.*, 1996) places KSHV with the gamma-2 subgroup of herpesviruses, the rhadinoviruses. Following the discovery of KSHV, attempts to understand its origin have resulted in the isolation or identification of closely-related rhadinoviruses in many primates (reviewed in Greensill and Schulz, 2000). Thus, as shown in Figure 11.2, fragments of viral DNA polymerase genes with close similarity to KSHV have been found in chimpanzees (PtRV-1 or PanRHV1), gorillas (GoRHV-1), mandrills (MnRHV1), African green monkeys (ChRV1) and macaques (RFHVMm, RFHVMn). In addition, a second

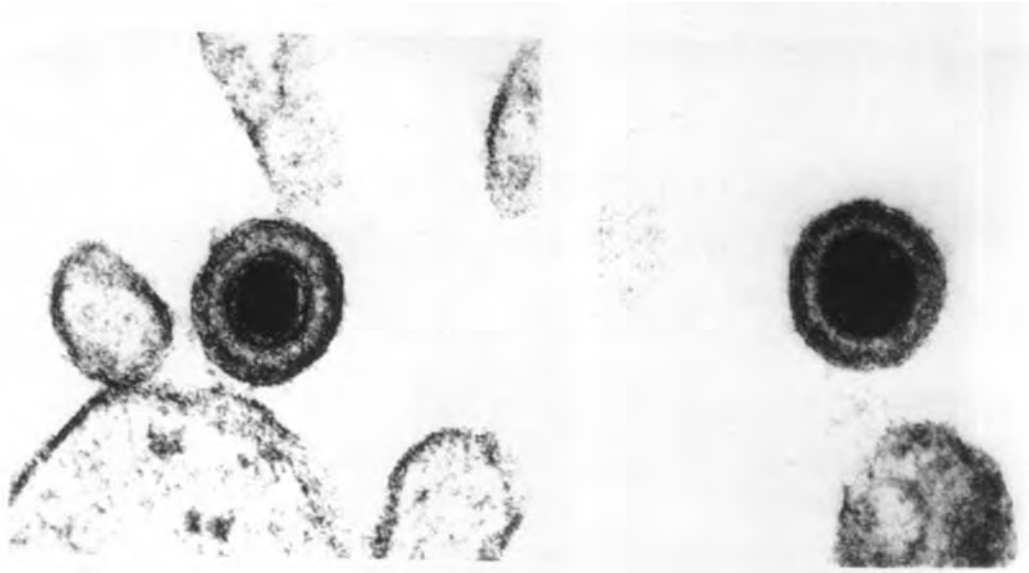


Figure 11.1 Electron micrograph of KSHV in lytically-induced KS-1 cells. The KSHV virion has the characteristic features of a herpesvirus. (Courtesy of D. Ablashi, Advanced Biotechnologies.)

group of gamma-2 *Herpesviral* DNA polymerase fragments has been identified in various primate species, which appear to be slightly more distantly related to KSHV. The fact, that for many nonhuman primate species a member of each group has now been found suggests the existence of two distinctly evolved lineages of gamma-2 herpesviruses (rhadinoviruses), termed RV-1 and RV-2. The similarity of the different identified viral sequences mirrors that of their host species and would thus be in keeping with the accepted view of herpesvirus–host co-evolution (McGeoch *et al.*, 2000).

The study of genomic variation of KSHV isolates has also revealed the existence of particular KSHV variants in certain geographic regions, suggesting that KSHV has co-evolved with human populations. This is best, but not exclusively, illustrated by the pattern of sequence variability found in the K1 gene at the left end of the KSHV genome (see Figures 11.3 and 11.4). K1 encodes a type I transmembrane protein with an immunoglobulin superfamily-like extracellular domain containing two highly variable regions, VR1 and VR2, which allow classification of different viral isolates (Zong *et al.*, 1999). As illustrated in Figure 11.3, K1 variants can be grouped into four major clades, A–E. These five clades are all thought to be derived from one common ancestor, termed ‘P’ (‘prototype’). Clade B is found exclusively in Africa and individuals descended from an African ancestor, while clade D is confined to old Asian populations, such as the Ainu, an old population on Hokkaido in the north

of Japan (Meng *et al.*, 2001; Zong *et al.*, 1999). Clade E, which is closely related to clade D, was found in Amerindian populations of Brazil and Ecuador (Biggar *et al.*, 2000), an observation that would be compatible with the well-established migration of the original Amerindian people from Asia to the Americas. In contrast, KSHV clades A and C are now found in many parts of the world, most likely reflecting the mixing of populations over the last three millennia (Zong *et al.*, 1999). However, geographic or ethnic associations can occasionally still be seen within individual clades, for example K1 sequences found in Ashkenazi and Sephardi Jews in Israel belonging to particular A and C subtypes (Davidovici *et al.*, 2001).

In addition to this pattern of evolution within human populations, recombination between different KSHV genomes and, in at least two cases, with related gamma-2 herpesviruses, appears to be another important factor in the evolution of KSHV. Evidence for multiple recombination events among P-type sequences has been found in many KSHV sequences (Zong *et al.*, 2002). Further, remnants of two so-far unidentified but more distantly related gamma-2 herpesviruses have been found in currently circulating KSHV variants. One, the ‘M’ type, has recombined into P-type variants at the right end of the viral genome. Both the fact that this M type has been seen and the observation of two separate lineages of the M type indicate that this recombination event took place possibly in the order of 100 000 years ago (Zong *et al.*, 2002). The

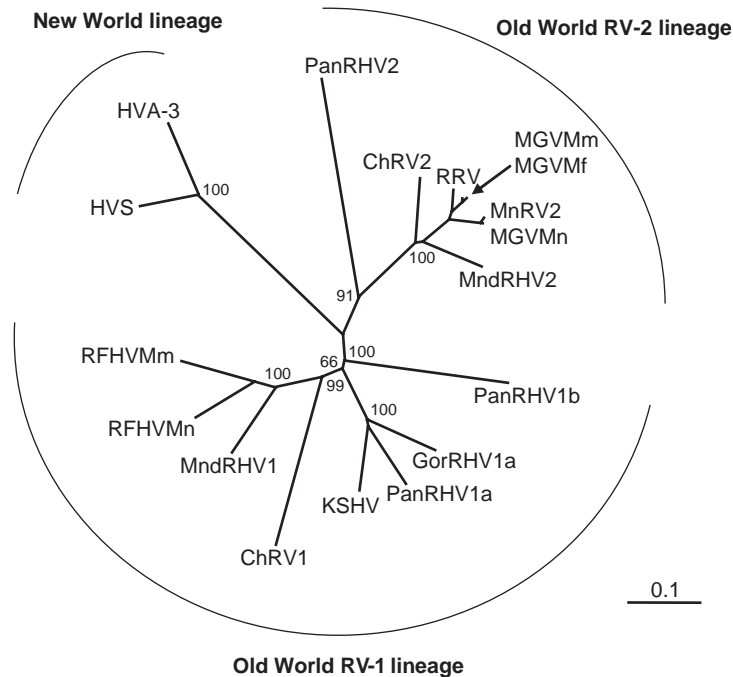


Figure 11.2 Phylogenetic relationships among primate gamma-2 herpesviruses. The phylogenetic tree shown here was created by the neighbour-joining method of partial viral DNA polymerase sequences of the gamma-2 herpesviruses shown. Numbers at branchpoints denote bootstrap values to indicate the reliability of this analysis (values over 75% are taken to indicate a robust assignment to a branch). The figure shows that KSHV belongs to the RV1 subgroup of gamma-2 herpesviruses, with closely related viruses being found in many Old World primate species. In addition, a second group, RV2, comprises slightly more distantly related gamma-2 herpesviruses in many primate species (see text). The New World primate gamma-2 herpesviruses form a third branch (see text).

other type ('Q' type) also originated from a recombination event with yet another gamma-2 herpesvirus at the right end of the KSHV genome, but has so far only been seen in South Africa (Zong *et al.*, 2002).

WORLDWIDE DISTRIBUTION OF KSHV IN THE GENERAL POPULATION

In contrast with other human herpesviruses, the seroprevalence of KSHV varies widely in different geographic regions. Many seroprevalence studies to detect KSHV-specific antibodies have now been conducted using a variety of different immunologic techniques (see 'Diagnostic Assays'; for references see Schulz, 1999). As outlined below, none of these assays is 100% sensitive or specific. This means that exact seroprevalence rates, particularly in low-prevalence areas, have been difficult to determine. However, these assays have proved good enough to work out the distribution of KSHV in different countries and regions, and to define modes and risk

factors of transmission in endemic countries as well as in population groups at high risk for sexually-transmitted diseases (STDs) (see Schulz, 1999 for detailed references).

Europe

The currently available seroepidemiology data indicate that KSHV is relatively rare in most northern, western and central European countries, with less than 5% of blood donors having antibodies to either KSHV latency-associated nuclear antigen (LANA), the orf65-encoded SCIP protein or the K8.1-encoded virion membrane glycoprotein (different antigens were used in the individual studies and the results are therefore difficult to compare) (Simpson *et al.*, 1996). In comparison, infection with KSHV appears to be more widespread in the Mediterranean basin, namely some parts of Italy, Greece and, to a lesser extent, Spain (Gao *et al.*, 1996b; Simpson *et al.*, 1996; Whitby *et al.*, 1998). In Italy, infection with KSHV appears to be more common among blood donors from regions previously reported to have a

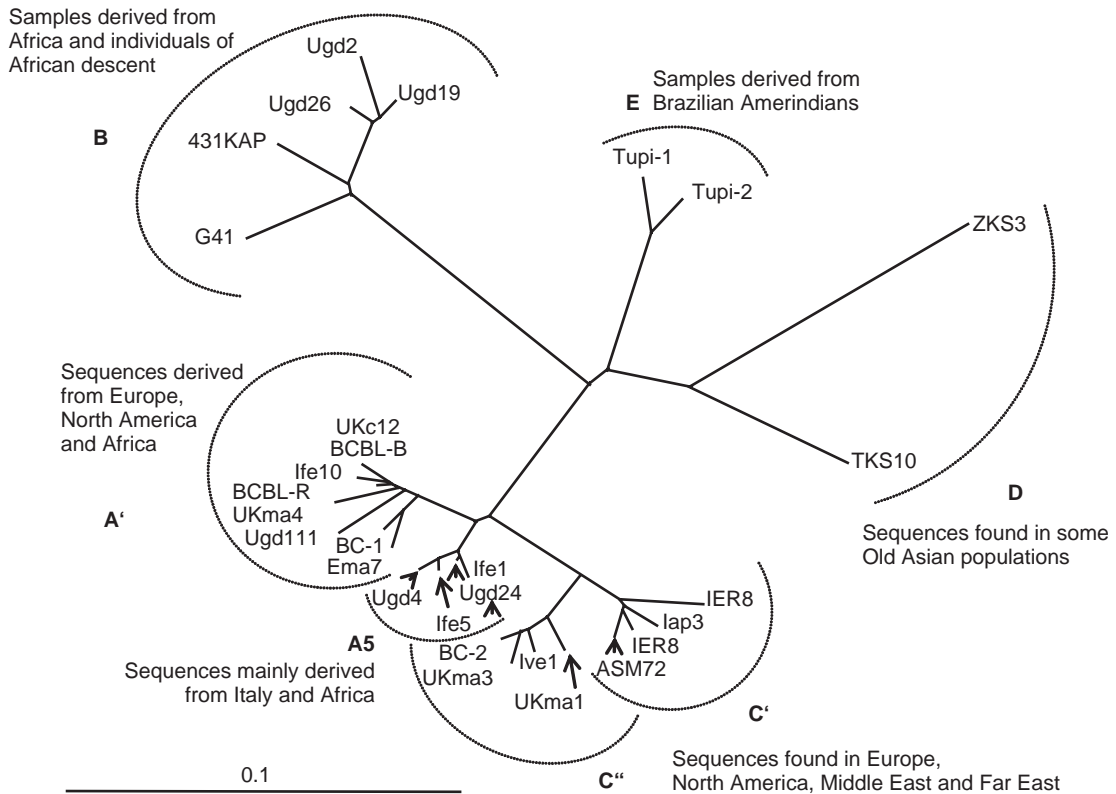


Figure 11.3 Evolution of the K1 gene of KSHV. K1, located at the 'left' end of the KSHV genome (see Figure 11.4), exists in five major variants. Some of these are associated with particular geographic regions or populations, indicating a possible co-evolution of K1 with human populations (see text) in isolates from different parts of the world.

higher incidence of classic KS, such as Sicily or Sardinia, than in the centre or in prealpine regions (Whitby *et al.*, 1998).

North America

In the United States, reported prevalence rates in blood donors vary between 0 and up to 25% in different assays (Gao *et al.*, 1996b; Kedes *et al.*, 1996; Simpson *et al.*, 1996). Data from the National Health and Nutrition Examination Survey III, however, post the seroprevalence in the general population to 1.6%, thus in the same range as many non-endemic European countries (Engels *et al.*, 2007). There may also exist remarkable regional and socio-economical variations in United States seroprevalence, since a higher prevalence rate is reported in particular parts of New York and individuals of African-American or Mexican descent (Engels *et al.*, 2007). These variations in seroprevalence may therefore reflect increased transmission in particular communities or risk groups (see below).

South America

In South America, the prevalence of antibodies to LANA among blood donors and healthy or young adults was in the range of 3–7% (Mohanna *et al.*, 2005). Like in the United States, higher prevalence rates (up to 16%) are found in socio-economically weaker populations of Belem, North Brazil (Freitas *et al.*, 2002). In contrast to the low prevalence of the non-native population, KSHV was found to be highly prevalent (LANA antibody prevalence rates 4–63%) in different Amerindian tribes and language groups in Brazil and Ecuador that had only little contact to the outside world (Biggar *et al.*, 2000). As mentioned above, these individuals are infected with a KSHV variant that is related to those found in old Asian populations.

Asia

In the Asian countries, like India, Sri Lanka, Thailand and Japan, KSHV prevalence was found to be low, ranging between 0 and 4% (Ablashi *et al.*, 1999; Fujii

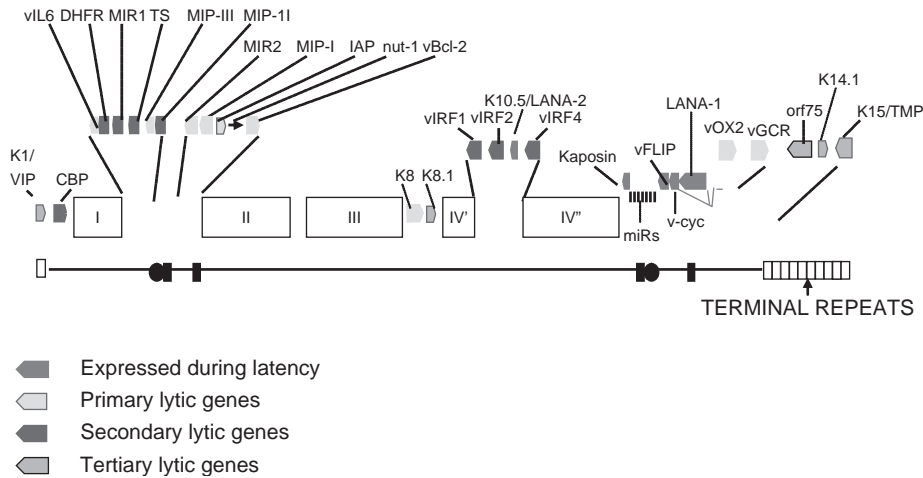


Figure 11.4 Genome diagram of KSHV. Open boxes with Roman numerals denote groups of structural or metabolic genes which are conserved among γ herpesviruses and many other herpesviruses. The solid line represents the long unique (coding) region; open rectangles, internal or terminal repeat regions; solid circles, putative origins of lytic replication (ori-(L), ori-(R)). The position and transcriptional orientation of viral genes discussed in the text is indicated by pointed boxes.

et al., 1999; Satoh *et al.*, 2001). For South East Asia the data are still inconclusive, spanning a seroprevalence of <5% in Eastern Melanesia and Malaysia to >20% in Melanesian residents of remote villages in Papua New Guinea (Ablashi *et al.*, 1999; Satoh *et al.*, 2006).

Middle East

Only a few data are available for the Middle East, where KSHV infection seems to be found only infrequently (Almuneef *et al.*, 2001). Intermediate KSHV prevalence rates have been found in Israel.

Africa

In contrast to the low or intermediate seroprevalence in the rest of the world, all studies reported so far concur that antibodies to KSHV are highly endemic in most African countries, particularly in sub-Saharan Africa. Antibodies to LANA, orf65/SCIP, K8.1 glycoprotein or undefined productive-cycle antigens are found in approximately 40–60% of adults and adolescents in most parts of sub-Saharan Africa investigated so far, for example Uganda, Tanzania, the Gambia, Cameroon, South Africa, Eritrea, Malawi, Kenya and Egypt (Gao *et al.*, 1996b; Simpson *et al.*, 1996; for detailed references see Schulz, 1999). Occasional studies have even reported higher antibody prevalence rates by immunofluorescence assays (IFAs) for lytic KSHV antigens. These high seroprevalence rates, obtained even with assays which detect antibodies to defined antigens, suggest that

KSHV is widespread in Africa, and does not only occur in those regions (east and central Africa) where KS was known to exist before the arrival of HIV-1 (endemic KS) (see below). This could suggest an involvement of additional cofactors in the pathogenesis of endemic KS.

General Observations

A consistently observed feature of all these studies investigating the distribution of KSHV in the general population is the increase of KSHV prevalence with age (for references see Schulz, 1999). This is likely to reflect ongoing transmission throughout adult life, but may also be due to increased viral replication after reactivation of a latent KSHV infection in elderly individuals.

Thus, these studies conducted in different parts of the world indicate that, in spite of being an 'old' human virus that has co-evolved with human populations, KSHV has apparently become rare in many geographic regions, although in some of these it may have been retained in defined populations. It has, however, remained common in Africa.

TRANSMISSION

Transmission in Childhood

In endemic countries or population groups (Africa, remote African or Amerindian populations in South America;

see above) the rapid increase of KSHV infection in children aged 1–12 years indicates that KSHV can be efficiently transmitted in childhood (Andreoni *et al.*, 2002; Plancoulaine *et al.*, 2000). In these situations young children are more likely to be infected if their mothers or siblings are infected, suggesting mother-to-child, or sibling-to-sibling transmission as a possible source of infection (Dedicoat *et al.*, 2004; Plancoulaine *et al.*, 2000). However, molecular analysis of KSHV strains found in Malawian families and Ugandan mother–child pairs have suggested that both intrafamilial and extrafamilial transmission of KSHV can occur (Mbulaiteye *et al.*, 2006). In keeping with this, an epidemiological study among Egyptian children found that contact with at least two other children in the community was a risk factor for KSHV infection (Andreoni *et al.*, 2002), indicating that—as is the case for cytomegalovirus—close or intimate contact with persons who are excreting the virus is necessary to acquire KSHV infection. There is increasing evidence that saliva is the main source of virus, as high viral loads are found in this specimen (Andreoni *et al.*, 2002; Dedicoat *et al.*, 2004). *In utero* or perinatal KSHV transmission has been described (Brayfield *et al.*, 2003) but is thought to be infrequent. Although low levels of KSHV DNA can occasionally be found in breastmilk, breastfeeding is thought to play only a minor role in mother-to-child transmission, in view of the steep rise in seroprevalence curves in children greater than one to two years (Dedicoat *et al.*, 2004). Using segregation analysis, one study (Plancoulaine *et al.*, 2003) predicted the existence of a recessive gene which increases susceptibility to KSHV infection in childhood.

Transmission in Adult Life

The gradual increase in seroprevalence during adult life, noted in many studies, would be compatible with the notion of continuing acquisition of KSHV infections. While in endemic countries or populations the majority of infections appear to occur before puberty, transmission during adulthood accounts for most infections in non-endemic countries. Sexual transmission is now believed to be the most common route of KSHV transmission in adults, even though viral loads found in vaginal, seminal and prostatic secretions are much lower than in saliva (de Sanjose *et al.*, 2002; Pauk *et al.*, 2000; further references in Schulz, 1999).

Homosexual Transmission

Evidence for sexual transmission came largely from studies of homosexuals in industrialized countries, who, in accordance with the high incidence of acquired immune deficiency syndrome (AIDS) KS in HIV-1-infected homo/bisexual men, are more frequently infected with KSHV than the general population of their country (Gao

et al., 1996a; Kedes *et al.*, 1996; O'Brien *et al.*, 1999; Pauk *et al.*, 2000; Rezza *et al.*, 2000; Simpson *et al.*, 1996). Numerous studies conducted on this topic agree that KSHV infection among gay men is linked to behavioural patterns that predispose to infection with a sexually-transmitted agent. Thus, KSHV infection is consistently seen to increase with the number of sexual partners as well as with a history of sexually-transmitted disease (Dukers *et al.*, 2000; Martro *et al.*, 2007; Melbye *et al.*, 1998; O'Brien *et al.*, 1999). Epidemiological studies investigating the impact of detailed sexual behavioural variables on KSHV transmission have, however, arrived at different conclusions. While several studies reported anal intercourse, either passive or active, as a risk factor (Melbye *et al.*, 1998; O'Brien *et al.*, 1999), others have identified oral–penile contact (Dukers *et al.*, 2000) or deep kissing (Pauk *et al.*, 2000) as predisposing behavioural variables. The use of amyl nitrate ('poppers') has also emerged as a risk factor in some (Pauk *et al.*, 2000) but not other (Melbye *et al.*, 1998) studies.

Interestingly, in some MSM cohort studies, a decline of KSHV prevalence or incidence has been noted during the last two decades (Melbye *et al.*, 1998; Renwick *et al.*, 1998), but in other cohorts the rate of KSHV infection has remained stable (Osmond *et al.*, 2002; Rezza *et al.*, 2000). KSHV appears to have spread in this group as a separate epidemic, independently of HIV-1 (Dukers *et al.*, 2000; Melbye *et al.*, 1998; O'Brien *et al.*, 1999). Attempts have been made to back-calculate the beginning of this epidemic, but these are associated with some uncertainty and the answers may well differ for homosexual communities in different countries; thus in Denmark KSHV may have been introduced into Danish homosexual men in the late 1970s through contact with US partners (Melbye *et al.*, 1998), whereas in Holland the beginning of this epidemic is inferred to have occurred much earlier (Dukers *et al.*, 2000), and KSHV was already highly prevalent in California in 1978–1979 (Osmond *et al.*, 2002).

Heterosexual Contacts

Among heterosexual adults, transmission during sexual contacts is not so obvious. On the one hand, there was no association between KSHV infection in husbands and wives in a population-based study among the Noir Marron of French Guyana (Plancoulaine *et al.*, 2000) or in the general population of the United States (Engels *et al.*, 2007). On the other hand, an elevated KSHV seroprevalence was found to be associated with an increased number of sexual partners and with a history of STDs in women with risk-taking sexual behaviour or prostitutes and men reporting contacts with prostitutes (de Sanjose *et al.*, 2002; Perna *et al.*, 2000). In particular, a history of genital warts seems to be a risk factor for KSHV infection.

Thus, given the well-documented transmission of KSHV during sexual contact among gay men, sexual transmission among heterosexual individuals would appear likely. The reasons why this is seen in some but not other studies are currently not clear.

Other Forms of Transmission

Another controversial issue is the importance of blood-borne transmission, as KSHV DNA can be detected in PBMCs, as well as in plasma or serum, of about 5–10% of infected individuals (Whitby *et al.*, 1995). Multiple parental routes of transmission have been discussed in recent years.

IVdrug Use

While early studies (Simpson *et al.*, 1996) and a large prospective cohort study of more than 1000 drug users in Amsterdam (Renwick *et al.*, 2002) found no evidence for an increased seroprevalence of KSHV among intravenous drug users, evidence for blood-borne transmission of KSHV among this cohort came from four US studies (Atkinson *et al.*, 2003; Cannon *et al.*, 2001; Goedert *et al.*, 2002; Greenblatt *et al.*, 2001), revealing a longer injection-drug use and concomitant HCV infection (as surrogate marker for IVdrug use) as risk factors for KSHV seropositivity. An association of increased KSHV seroprevalence with high-risk (sexual) behaviour for HIV infection supports the idea that KSHV may be transmitted via behaviour accompanying the use of drugs rather than through the drug use itself.

Blood Transfusions

Concern about an iatrogenic transmission via blood transfusion was expressed as early as 1997 (Blackbourn *et al.*, 1997), when viral DNA was found in blood (Whitby *et al.*, 1995), even blood from blood donors (Blackbourn *et al.*, 1997; Hladik *et al.*, 2006). Increasing evidence for KSHV transmission by this route came when Hladik *et al.* (2006) reported a significantly higher risk of seroconversion among Ugandan recipients of HHV-8-seropositive blood than among recipients of seronegative blood, with an excess risk of 2.8%. The occurrence of seroconversion between three and ten weeks after transfusion, as well as a decrease in the transmission rate when the storage of the blood products exceeded four days, further hinted at a viral transmission. However, transmissibility may be limited by the cell-associated nature of the virus and by the low frequency of circulating virus in the blood of asymptomatic seropositive individuals, suggesting that blood transfusion is associated with only a small risk of KSHV transmission (Dollard *et al.*, 2005; Mbulaiteye *et al.*, 2003).

Organ Transplantation

Among transplant patients, KSHV transmission from donor to recipient has clearly been documented in individual cases (Barozzi *et al.*, 2003; Luppi *et al.*, 2000a; Regamey *et al.*, 1998). KSHV-infected endothelial precursor cells may be transmitted from organ donor to recipient during transplantation and may develop into (donor-cell derived) KS (Barozzi *et al.*, 2003). Nevertheless, although transmission of KSHV through organ or bone marrow transplantation may occur, it appears that in endemic countries the majority of individuals who have developed post-transplant KS were already infected with KSHV at the time of transplantation, rather than being infected through the transplanted organ (Cattani *et al.*, 2001; Farge *et al.*, 1999; Parravicini *et al.*, 1997b). To what extent this also applies in non-endemic countries and whether there is therefore a need, in non-endemic countries, to screen organ or bone marrow donors, or blood to be transfused to immunosuppressed individuals, is still under investigation.

CLINICAL MANIFESTATIONS

Kaposi's Sarcoma

The 'classic' form of KS was first described in 1872 by the Austro-Hungarian dermatologist Moritz Kaposi and is characterized as a skin disease affecting elderly (>50 years) men of Mediterranean, Eastern European or Jewish heritage. It appears as indolent, firm, blue-reddish-brown patches/plaques and later on as nodules on lower extremities that may ulcerate and bleed. Internal organ involvement, mainly the gastrointestinal system and lungs, is only seen rarely today (although several of the clinical cases in Kaposi's early description did show visceral involvement). The 'endemic' form of KS was noted in East Africa before the spread of HIV. Visceral involvement is more frequent than in the classic form. 'AIDS-associated' KS is clinically more aggressive and is characterized by widespread cutaneous and visceral involvement and associated complications such as bleeding. While it has become less frequent in HIV-infected gay men in Western countries, it now represents the commonest tumour in sub-Saharan Africa (Dedicoat and Newton, 2003). Finally, 'iatrogenic' KS in immunosuppressed individuals, mainly after organ transplantation, is more frequent in transplant recipients from KSHV-endemic countries.

Epidemiological Findings

Epidemiological studies allow the conclusion that KS only develops in KSHV-infected individuals but that this remains a rare event unless there is additional immune

suppression, as a result of either organ transplantation or HIV infection. The presence of antibodies to KSHV in non-endemic areas is strongly associated with having KS, or being at increased risk for KS, and, among HIV-1-infected individuals, is strongly predictive of the subsequent appearance of KS lesions (Gao *et al.*, 1996b; Kedes *et al.*, 1996; Renwick *et al.*, 1998; Simpson *et al.*, 1996). However, while classic KS remains a rare tumour in immunocompetent KSHV-infected individuals—the incidence rate range from 1 to 3 per 100 000 inhabitants, even in regions of Italy where KSHV seroprevalence rates are in the range of 20–30% (Calabro *et al.*, 1998)—it is much more common in immunosuppressed individuals. Among transplant recipients, KSHV infection increased the risk of post-transplant KS by a factor of 40–80 in different studies (Cattani *et al.*, 2000; Farge *et al.*, 1999; Parravicini *et al.*, 1997b). Among HIV-infected homosexual men, approximately 40–50% of KSHV-infected individuals developed KS within 5–10 years after primary infection with KSHV before the introduction of highly active retroviral therapy (HAART) (O'Brien *et al.*, 1999; Renwick *et al.*, 1998). Individuals who are already HIV-infected when they contract KSHV progress more rapidly towards disease than those who acquire HIV after KSHV (Renwick *et al.*, 1998). In the Gambia, West Africa, AIDS KS is markedly more common among HIV-1-infected than HIV-2-infected individuals, although the KSHV prevalence appears to be comparable in these two groups (Ariyoshi *et al.*, 1998). In Africa, endemic KS is mainly found in east and central Africa, in spite of a high KSHV prevalence in most of sub-Saharan Africa (Dedicoat and Newton, 2003). This observation strongly suggests the existence of other environmental cofactors that promote the development of KS in KSHV-infected individuals.

Pathology and Molecular Biology Findings

KSHV is present in the neoplastic component of KS lesions, the endothelial cell-derived spindle cell (Boshoff *et al.*, 1995; Rainbow *et al.*, 1997; further references in Schulz, 2001). Latent viral genes (orfK12/kaposin; orfK13/vFLIP (viral FLICE (Fas-associated death-domainlike IL-1b-converting enzyme)-inhibitory proteins); orf72/v-cyclin; orf73/LANA) are expressed in these cells (Parravicini *et al.*, 2000; Rainbow *et al.*, 1997; further references in Schulz, 2001) (see Figure 11.4). KS biopsies contain mainly circular episomal viral DNA, which is consistent with the latent infection (Judde *et al.*, 2000). However, some spindle cells in KS lesions can undergo lytic replication of KSHV (Katano *et al.*, 2000). Given the functional properties of some KSHV lytic-cycle genes, for example induction of vascular

endothelial growth factor (VEGF), angiogenic properties, and so on (see 'Pathogenesis', below), it is thought that some viral proteins expressed during the lytic cycle could contribute to the development of KS.

Primary Effusion Lymphoma (PEL)

PEL, also called BCBL, is a rare lymphoma in AIDS patients, characterized by its presentation as a malignant effusion in the peritoneal, pleural or pericardial space, most often in the absence of an obvious tumour mass. The lymphoma cells have pleomorphic or anaplastic features combining morphological aspects of large-cell immunoblastic and anaplastic large-cell lymphomas (references in Schulz, 2001). They are usually monoclonal and of B-cell origin, as shown by a rearranged immunoglobulin locus and monotypic immunoglobulin light-chain pattern, but express only a few of the usual markers of B-cell differentiation. The majority of PEL cases described so far have been from AIDS patients, but there are also several reports of PEL in HIV-uninfected individuals (Cesarman *et al.*, 1996). The presence of KSHV is now often considered an essential criterion for the diagnosis of PEL. However, rare cases of KSHV-negative effusion lymphomas of the B-cell lineage, with an indeterminate surface marker phenotype and pleomorphic appearance, some of them CD30-positive, have been described (references in Schulz, 2001). In most PEL cases the lymphoma cells are co-infected with EBV (Cesarman *et al.*, 1995a, 1995b; further detailed references in Schulz, 2001). However, several examples of PEL which contain only KSHV have now been reported and stable cell lines have been derived from some of these. Such 'KSHV-only' cell lines can induce lymphomas with malignant effusions in immunodeficient (SCID, BNX) mice, suggesting that—at least in this experimental model—the presence of EBV is not required for the induction of lymphoma (detailed references in Schulz, 2001).

During latency, EBV and KSHV genomes persist as covalently-closed episomal circles of defined length, whereas lytic replication is associated with the presence of linear concatameric genomes of varying lengths. As judged by this criterion, different PEL cases have been shown to harbour either latent or lytically-replicating KSHV genomes (Judde *et al.*, 2000). Where latent (i.e. circular episomal) KSHV genomes were found, these were present as either a single episome or as multiple episomes with a varying number of terminal repeat units, indicating, respectively, a monoclonal or an oligoclonal population (Judde *et al.*, 2000). Experiments on several PEL cell lines, as well as immunohistochemistry, immunofluorescence and *in situ* hybridization studies on pathology specimens, indicate that four viral genes, LANA, v-cyc, vFLIP and kaposin/K12, may

be expressed in a substantial proportion of lymphoma cells (Katano *et al.*, 2000; Parravicini *et al.*, 2000; Rainbow *et al.*, 1997; further references in Schulz, 1999, 2001). Where pathology samples have been studied by immunohistochemistry or immunofluorescence, LANA has been found to be expressed in the vast majority, while vIL-6 protein expression is confined to a small proportion (2–5%) of tumour cells (Katano *et al.*, 2000; Parravicini *et al.*, 2000). Other lytic proteins, for example K8 (a homologue of EBV Zta), K8.1 (a viral membrane glycoprotein), K9, K10 and K11 (proteins with sequence homology to interferon regulatory factors (IRFs)), orf59/PF-8 (a processivity factor) and orf65 (a minor capsid protein) have been detected in very few cells (<1%) or not at all (Katano *et al.*, 2000; Parravicini *et al.*, 2000). This expression pattern is consistent with the notion that KSHV persists in most PEL tumour cells in a latent form, but that a small population of cells can switch into lytic viral replication. That vIL-6 is expressed in more lymphoma cells (2–5% by immunohistochemistry/immunofluorescence; significantly more by *in situ* hybridization) than other lytic viral proteins (0–<1%) could be due to the fact that it is among the first 'early' genes to be expressed after induction of the lytic cycle in PEL cell lines *in vitro* (Sun *et al.*, 1999). Its increased detection in uncultured PEL cells may therefore reflect higher protein levels in cells during the early stages of the lytic cycle. Alternatively, it has been suggested that there may be a particular latency program for KSHV in PEL cells and that vIL-6 expression can occur independently from the activation of the lytic cascade, perhaps indicating a tissue-specific regulation of viral gene expression (Parravicini *et al.*, 2000). Another example for preferential expression of a KSHV gene in B cells is the recently-described homologue of IRFs, vIRF 10.5/LANA-2 (Rivas *et al.*, 2001).

Multicentric Castleman's Disease (MCD)

Castleman's disease is a localized lymphoproliferative condition, often found in the mediastinum or in mesenteric or peripheral lymph nodes and characterized histologically by expanded germinal centres with B-cell proliferation and vascular proliferation. Two histological types have been recognized; one is characterized by a pronounced plasma-cell proliferation and persistence of the nodal architecture ('plasma-cell variant') and the other by abnormal germinal centres and abundant hyalinized vessels ('hyaline vascular variant'). Variants combining the features of these two types also occur. Involvement of multiple lymph node sites occurs in MCD. MCD in AIDS patients is often associated with KS and usually belongs to the plasma-cell variant. KSHV can nearly

always be detected by PCR in the lymph nodes or spleen affected by MCD in AIDS patients, but is much less common in MCD outside HIV infection (Soulier *et al.*, 1995). By immunohistochemistry or *in situ* hybridization, KSHV is found in the B cells surrounding the follicular centres of MCD. As assessed by immunohistochemistry, KSHV-infected B cells express LANA, a hallmark of all KSHV-infected cells; 10–50% of follicular-mantle cells have been found to express LANA in different studies. About 5–25% of LANA-expressing follicular-mantle B cells in MCD tissue also express vIL-6 and vIRF-1/K9 (Katano *et al.*, 2000; Parravicini *et al.*, 2000; further references in Schulz, 2001) (Figure 11.5). Another viral vIRF homologue, vIRF-3/K10.5/LANA-2, is also expressed in a significant number of KSHV-infected B cells (Rivas *et al.*, 2001), and a third vIRF homologue, encoded by orfK10, in about 5% of all mantle zone cells (Katano *et al.*, 2000). In addition, a small proportion of mantle-zone cells also express other viral proteins that are associated with the early stages of lytic viral replication, such as PF-8 (encoded by orf59) and the K8 protein, a homologue of EBV BZLF-1, an activator of the lytic replication cycle (Katano *et al.*, 2000; Parravicini *et al.*, 2000). Compared to PEL and KS tissue, KSHV thus appears to adopt a less restrictive pattern of gene expression in MCD tissue and this suggests that MCD may represent the result of active lytic viral replication in lymphoid tissue. In keeping with this notion, the intensity of clinical symptoms in MCD patients such as fever, or of laboratory parameters reflecting disease activity such as C-reactive protein, have been shown to correlate with the KSHV viral load in peripheral blood (Grandadam *et al.*, 1997; Oksenhendler *et al.*, 2000). Fluctuations of KSHV viral load in HIV-infected

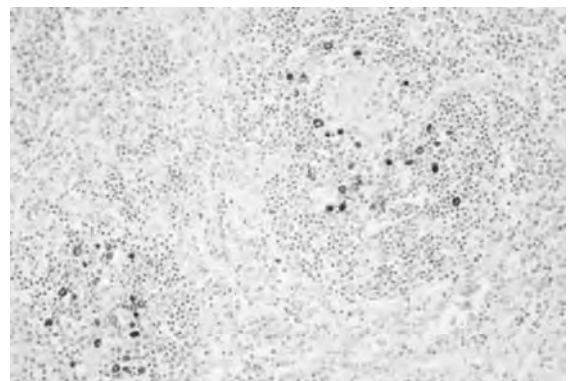


Figure 11.5 Expression of vIL6 in B cells of MCD. vIL6 is expressed in a small number of KSHV-infected B cells, but may affect others through paracrine action (see text). (Photograph kindly provided by Drs Y. Chang and P. Moore.)

patients with MCD also mirror fluctuations in the plasma levels of human IL-6 and IL-10 and in plasma HIV RNA copy numbers, indicating that active HIV infection can increase the severity of MCD by increasing KSHV viral load (Oksenhendler *et al.*, 2000). MCD can be a manifestation of a primary infection with KSHV in an HIV-infected individual or in transplant recipients (Oksenhendler *et al.*, 1998; Parravicini *et al.*, 1997a). This is in keeping with the idea that MCD may reflect active viral lytic replication in lymphoid tissue. It also highlights that co-infection with HIV-1, which can have a marked effect on KSHV replication (Goudsmit *et al.*, 2000; Oksenhendler *et al.*, 2000), may however not be required and that immunosuppression is sufficient to allow lytic replication in lymphoid tissue to an extent that produces clinically-visible MCD lesions. Clinically-visible MCD is, however, a rare outcome of primary KSHV infection, despite about 10% of HIV-1-infected individuals that seroconvert to KSHV having detectable KSHV viraemia in their peripheral blood (Goudsmit *et al.*, 2000). Given the functional properties of vIL-6 (see below), it is likely that this viral protein plays a role in the pathogenesis of MCD. The plasmacytic phenotype of B cells in KSHV-associated cases of MCD may be the result of the activity of vIL-6 in this condition. Some authors prefer the term 'plasmablast' for these cells, to describe their large size and large vesicular nucleus with one or two prominent nucleoli (Dupin *et al.*, 2000). Interestingly, these KSHV-infected plasma cells have expressed IgM with λ -immunoglobulin light chains in all cases examined so far, indicating light-chain restriction and the presence of a monotypic cell population (Dupin *et al.*, 2000). In some cases, these KSHV-infected plasmablasts are found in small clusters surrounding or replacing follicles, suggesting the emergence of microscopic plasmablastic lymphomas in MCD lesions (Dupin *et al.*, 2000).

Other Disease Associations

KSHV has been detected in occasional cases of HIV-negative angioimmunoblastic lymphadenopathy and germinal-centre hyperplasia (detailed references in Schulz, 2001). Histologically, there may be prominent plasma-cell proliferation and angiogenic changes in these cases, suggesting that viral proteins like vIL-6 and viral macrophage inflammatory protein (vMIP)-I/II may have induced these transient changes. In addition, a small number of case reports indicate that primary infection with KSHV, or KSHV reactivation, in transplant recipients may be associated with a transient thrombocytopenia, leukopenia and/or anaemia (Luppi *et al.*, 2000b), or a febrile maculopapular skin rash in immunocompetent children (Andreoni *et al.*, 2002; further references in Schulz, 2001).

PATHOGENESIS

This section focuses on the biochemical and cell-biology features of individual KSHV genes. Understanding their contribution to pathogenesis is currently still hampered by the lack of a good animal model. However, a broad understanding of the functional roles of individual KSHV proteins has been developed. Below, these are discussed in groups defined by their main functions, and these groups are illustrated in Figures 11.4, 11.6 and 11.7.

The KSHV Genome

KSHV contains a long unique region (LUR) of 140.5 kb, with a GC content of 53.5%, flanked by 801 bp terminal tandem repeats (TRs) rich in GC (84.5%) (Neipel *et al.*, 1997; Russo *et al.*, 1996) (see Figure 11.4). Within the LUR there are at least 90 putative open reading frames (ORFs). These ORFs are named and numbered following the nomenclature initially adopted for the prototypic rhadinovirus, HVS. Genes unique to KSHV are designated by the letter K, followed by a number.

Culture Systems

PEL-derived cell lines may be latently infected with KSHV only, or may be co-infected with EBV if the original tumour also contained EBV (Renne *et al.*, 1996; further references in Schulz, 2001). In some cell lines (e.g. BCP-1, BCBL-1, KS-1) most cells only express latent KSHV genes (see below) but some will spontaneously switch into lytic replication, whereas in others, for example the KSHV/EBV dually-infected cell line HBL-6 (or BC-1), KSHV is much more strictly latent. The lytic replication programme can be switched on in all these cell lines by treatment with either phorbol esters (Renne *et al.*, 1996) or sodium butyrate, but some cell lines are better virus producers (e.g. KS-1, BCBL-1, JSC-1, Cro-AP3) than others. KSHV virus preparations obtained in this manner can be used to infect a number of permanent cell lines or primary endothelial cell cultures (Ciuffo *et al.*, 2001; Flore *et al.*, 1998; Moses *et al.*, 1999; Renne *et al.*, 1998). In primary endothelial cell cultures most cells are infected in a latent form, with minimal viral gene expression, expression of the viral 'LANA' (see below) in the majority and evidence of lytic-cycle or structural viral proteins in only a few cells (Ciuffo *et al.*, 2001; Moses *et al.*, 1999). Stable persistence of KSHV in endothelial cells *in vitro* may depend on the culture system used: in primary endothelial cell cultures and telomerase-immortalized endothelial cells KSHV appears not to be retained in infected cultures upon subsequent passage, whereas stable persistence has been found in an HPV-immortalized endothelial cell line (Moses *et al.*, 1999). Infection of endothelial

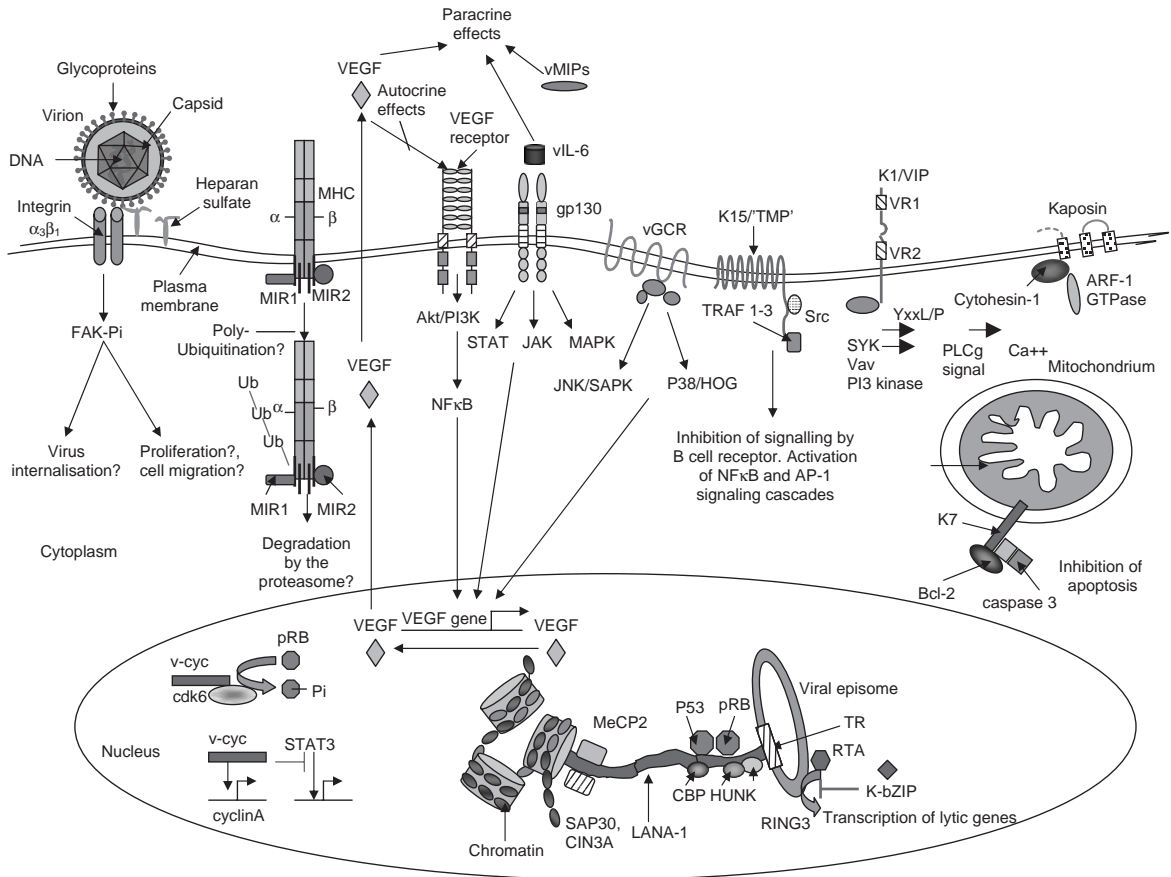


Figure 11.6 Overview of some KSHV-encoded proteins that may contribute to pathogenesis. See text for detailed explanation of the pathways and receptors engaged by KSHV proteins.

cells results in morphological changes (spindle-cell formation, piling up of infected cells) that vary with the cell culture system used (Ciuffo *et al.*, 2001; Moses *et al.*, 1999). It is also possible to isolate replicating KSHV on 293 cells from saliva (Vieira *et al.*, 1997), but this is very inefficient and requires detection of replicating KSHV by PCR. The inefficient persistence of KSHV in endothelial or epithelial cells is reminiscent of the rapid loss of KSHV from primary cultures of KS biopsies (Lebbe *et al.*, 1997) and contrasts with its stable long-term persistence in PEL-derived cell lines. It is therefore possible that cell-lineage-specific factors affect the long-term latent persistence of KSHV *in vitro* as well as *in vivo*.

Cell Entry

Cell-free infection *in vitro* is inefficient and only low titres are achieved with virus produced from PEL cell

lines (Flore *et al.*, 1998; Moses *et al.*, 1999; Renne *et al.*, 1998). These virus stocks appear to have a particle : infectivity ratio in the range of 10^3 – 10^4 , and there is some evidence that a significant proportion of virions in these stocks are defective (Nealon *et al.*, 2001). Four structural glycoproteins, gB, gD, gH and the orfK8.1-encoded glycoprotein, have been documented in KSHV virions. These glycoproteins are candidates for promoting initial contact with KSHV receptor(s) and initiating cell entry. KSHV interacts with ubiquitous host-cell-surface heparan sulphate and α₃β₁ integrin molecules through gpK8.1 and gB (orf8) (Akula *et al.*, 2002; Birkmann *et al.*, 2001) (see Figure 11.6). The gB glycoprotein contains an RGD motif, known to be important for interaction with cell-surface integrins. KSHV and its counterpart in macaques, RFHV (see 'Origin and Evolution of KSHV' and Figure 11.2), are the only herpesviruses known to date to have a gB protein containing an RGD motif. It has been shown that this motif plays a central role in KSHV entry at a

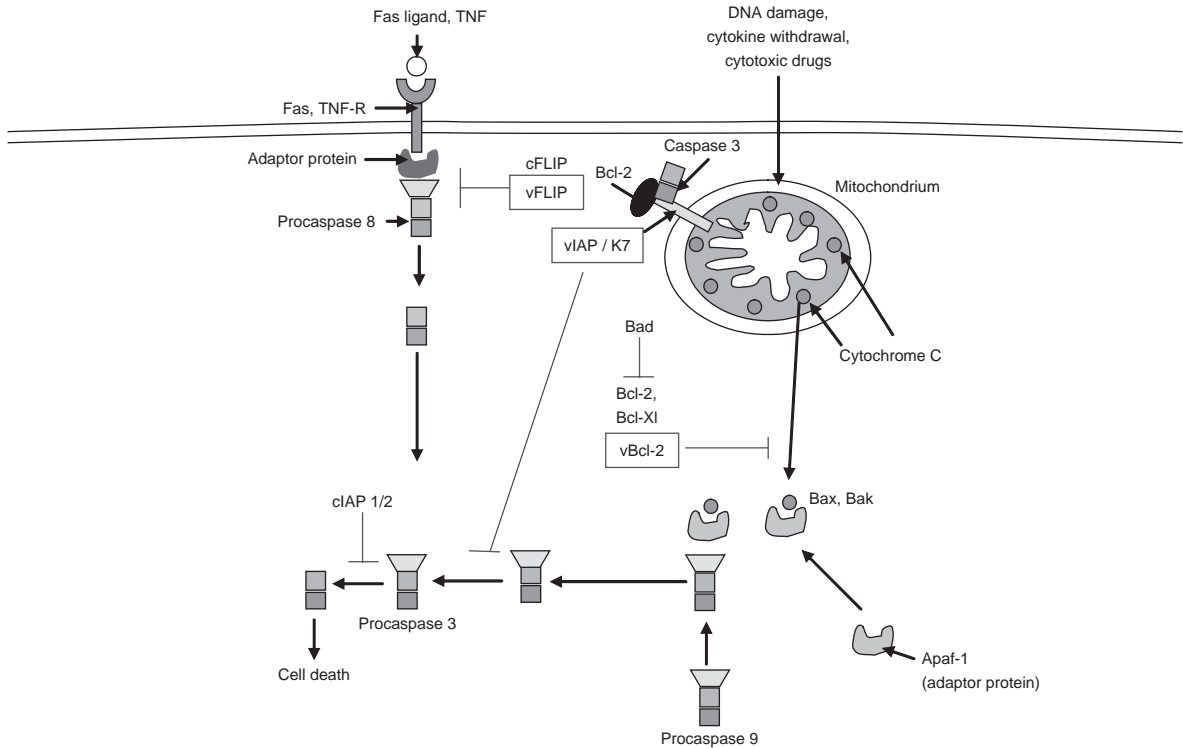


Figure 11.7 KSHV-encoded proteins involved in apoptosis. Two apoptosis pathways exist in human cells and are regulated by a number of cellular and viral components. The extrinsic pathway is initiated by Fas-L or tumour necrosis factor (TNF) and assembly of the procaspase-8 complex is inhibited by FLIP (FLICE inhibitory protein; see text). A KSHV-encoded FLIP homologue, vFLIP, acts in a similar manner. The intrinsic pathway is triggered by DNA damage, cytokine withdrawal and cytotoxic drugs, and is regulated by the Bcl/Bad/Bax group of proteins. A viral bcl-2 homologue, vbcl-2, acts at this stage. Finally, vIAP acts in the intrinsic pathway and on procaspase 3, as discussed in the text.

post-adhesion step, suggesting the presence of more than one receptor for KSHV entry (Akula *et al.*, 2002). In addition, xCT, the 12-transmembrane segment light chain of the cystine/glutamate exchange transporter system, has recently been identified as a receptor mediating membrane fusion and entry of KSHV (Kaleeba and Berger, 2006).

Entry experiments using the human B-cell-line BJAB indicate that KSHV is endocytosed. KSHV infection induced the integrin-mediated activation of focal adhesion kinase (FAK), implicating a role for integrin and the associated signalling pathways in the entry of the virus, which results in morphological changes and cytoskeletal rearrangements (Naranatt *et al.*, 2003). Also, a role for phosphatidyl-inositol (PI) 3-kinase in the early stages of the entry process, and for PKC- α , MEK and Erk at later stages, has been proposed (Naranatt *et al.*, 2003). It has been suggested that the interaction of KSHV with

cell-surface molecules, such as integrins, might generate a cellular state more prone to infection.

Latent Replication

KSHV persists in a latent form in the majority of infected cells. During latency, the KSHV genome replicates and persists as extrachromosomal episomal DNA circles in the absence of virion production. Persistence is very efficient in rapidly-dividing B-cell lines obtained from PEL patients (PEL cell lines). Cell cultures established from KS tumours rapidly lose the KSHV genome after a few passages (Lebbe *et al.*, 1997). Primary endothelial cells, as well as endothelial and epithelial cell lines, infected in culture also lose the virus after several passages. The reason for the efficient persistence of KSHV in PEL cell lines is currently unknown. Latency is characterized by the presence of episomal viral DNA (Judde *et al.*, 2000) (see above) and a restricted

pattern of viral gene expression (see Figure 11.4). Only five viral genes are currently known to be expressed during latency: the latency-associated nuclear antigen-1 (LANA-1/orf73); the viral homologue of a D-type cyclin (v-cyc/orf72); a homologue of the cellular inhibitor of the FLICE apoptosis complex (vFLIP/orfK13); and a group of short membrane-associated proteins, kaposin A, B, C (orfK12) (see Figure 11.4 and above). In addition, a cluster of virally-encoded miRNAs, located in the vicinity of orfK12 (see Figure 11.4), is generated by processing a latent transcript (Cai *et al.*, 2005; Pfeffer *et al.*, 2005; Samols *et al.*, 2005). One of these, miRK12-11, may be a functional homologue of a cellular miRNA, miR-155, and appears to modulate the expression of several cellular genes known to be involved in cell-growth regulation (Gottwein *et al.*, 2007; Skalsky *et al.*, 2007). A considerable proportion of persistently-infected B cells and B lymphoma cells in PEL and MCD, but not in KS endothelial cells, express K10.5/LANA-2, one of the KSHV vIRF homologues (Rivas *et al.*, 2001) (see above). Among these five proteins, only LANA-1 has been detected in virtually all KSHV-infected cells by IF or IHC (Katano *et al.*, 2000; Rainbow *et al.*, 1997). LANA-1 is required for the persistence of the KSHV episomal genome (Ballestas *et al.*, 1999; Garber *et al.*, 2002; reviewed in Viejo-Borbolla *et al.*, 2003). LANA-1 binds to mitotic chromosomes through its amino-terminal region and to two short motifs of 16 nucleotides (LANA-binding sites (LBS)-1 and -2) in the terminal repeat subunit (TR) of the KSHV genome via its carboxy-terminal region (Garber *et al.*, 2002) (see Figures 11.4 and 11.6). In this way, LANA-1 tethers viral episomes to mitotic chromosomes, and it is assumed that this ensures the distribution of KSHV genomes to daughter cells upon mitosis, at least in PEL cells. LANA-1 also mediates the replication of TR-containing plasmid DNA in transfected cells, thus illustrating its role in the replication of latent episomes (Garber *et al.*, 2002; further references in Viejo-Borbolla *et al.*, 2003). LANA-1 has also been shown to bind to p53 and retinoblastoma protein (pRb) (see Figure 11.6) and, in this way, to inhibit the activation of p53-dependent promoters and induce the activation of E2F-dependent genes (Friborg *et al.*, 1999; Radkov *et al.*, 2000). Moreover, LANA-1 can transform cells in cotransfection assays with a constitutively-active Ha-ras (Radkov *et al.*, 2000). Furthermore, LANA-1 acts as a transcriptional activator and/or repressor, and associates with the mSin3 repressor complex as well as cellular factors involved in the methylation of DNA (DNMT3a) and in interaction with methylated DNA (MeCP2) (Krithivas *et al.*, 2002; Shamay *et al.*, 2006). Further cellular interaction partners of LANA-1 include brd2 (RING3), brd3 and brd4 (HUNK), members of

the BET protein family involved in transcriptional regulation (Ottinger *et al.*, 2006; Platt *et al.*, 1999) (see Figure 11.6).

Activation of the Viral Lytic Replication Cycle

Despite KSHV remaining latent in most of the infected cells (see above), a small population of virus-infected cells undergoes lytic (productive) viral replication, which allows the production and release of viral particles. This process, required for virus spread, normally results in cell death. Nevertheless, it is thought that periodic or continuous infrequent activation of the lytic cycle contributes to disease progression. This hypothesis is supported by two clinical observations: drugs that inhibit lytic KSHV replication prevent the development of clinical KS (see below); and an increase in KSHV replication is associated with the appearance of MCD lesions in immunocompromized patients (see above). Moreover, several observations (Bais *et al.*, 1998; Flore *et al.*, 1998; Montaner *et al.*, 2003; further detailed references in Schulz, 2001; Viejo-Borbolla *et al.*, 2003) indicate that lytic genes with autocrine and paracrine effects might play an important role in KSHV-related malignancies. The switch between latent and lytic cycle is mediated by transcriptional activators of early and late lytic genes. The viral immediate-early transactivator RTA, encoded by orf50, is the key element in the activation cascade. Ectopic expression of RTA in PEL cells triggers the lytic cycle, leading to the production of infectious virus (Sun *et al.*, 1998; further references in Viejo-Borbolla *et al.*, 2003).

RTA acts through binding to specific DNA sequences (Chang *et al.*, 2002) and by interacting with cellular transcription factors (Wang *et al.*, 2003). RTA can activate its own promoter and cellular genes. The product of KSHV orfK8, K-bZIP (Gruffat *et al.*, 1999), represses or enhances RTA-mediated gene activation (Izumiya *et al.*, 2003; Wang *et al.*, 2003). K-bZIP also binds to p53, thereby repressing p53-mediated apoptosis, promotes both CCAAT/enhancer binding protein alpha (C/EBPa) and p21CIP-1 expression and, through interaction with C/EBPa, is able to promote p21CIP-1-mediated inhibition of entry into S phase (Park *et al.*, 2000; Wu *et al.*, 2002). Together, these features of K-bZIP probably contribute to the creation of a suitable environment for lytic viral replication by antagonizing apoptosis and preventing competition with host-cell DNA synthesis for limited resources. C/EBPa, RTA and K-bZIP form a complex that associates with and activates the K-bZIP promoter (Wang *et al.*, 2003). Therefore, C/EBPa and K-bZIP can activate each other.

Regulation of the Cell Cycle and DNA-Damage Response

Infection of primary endothelial cells with KSHV results in the induction of antiproliferative checkpoints and DNA-damage response pathways; these effects are also seen in early KS lesions (Koopal *et al.*, 2007). The viral D-type cyclin homologue, v-cyc, plays an important role in this process. On its own, v-cyc mediates phosphorylation, and thereby inactivation, of pRb through association with CDK6, and promotes the progression of resting cells into the S phase of the cell cycle (Chang *et al.*, 1996; Swanton *et al.*, 1997) (see Figure 11.6). In contrast to cellular D-type cyclins, v-cyc targets a broader range of cellular proteins for phosphorylation, including histone H1, the cdk inhibitor p27kip and bcl-2 (detailed references in Verschuren *et al.*, 2004). Also, unlike cellular D-type cyclin/CDK6 complexes, the CDK6/v-cyc complex required for phosphorylation of pRb is resistant to the cellular CDK inhibitors p16, p21 and p27 and to p16INK4a, conferring resistance to the antiproliferative action of these inhibitors (Platt *et al.*, 2002; Swanton *et al.*, 1997). However, in spite of these biochemical properties, v-cyc induces a growth arrest and cytokinesis defects in primary cells by activating DNA-damage response pathways (Koopal *et al.*, 2007; Verschuren *et al.*, 2002). This v-cyc-mediated growth arrest is dependent on a functional p53 (Koopal *et al.*, 2007; Verschuren *et al.*, 2002).

LANA-1 is also able to promote the entry into S phase of transfected cells by interacting with, and sequestering, GSK-3 β , a kinase involved in phosphorylation and consequent degradation of β -catenin by the proteasome (Fujimuro *et al.*, 2003). Association of GSK-3 β with LANA-1 leads to its transfer to the nucleus, the stabilization of β -catenin and increased β -catenin levels in infected cells, which allows activation of promoters containing Lef/Tcf binding sites and entry into S phase (Fujimuro *et al.*, 2003).

Angiogenesis and B-cell Proliferation: vIL-6, vMIP-I-III, vGCR

KSHV-associated diseases express high levels of VEGF and its receptor, kinase insert domain-containing receptor (KDR), which induces angiogenesis (for a review see Hayward, 2003). In KS lesions, VEGF and other angiogenic factors stimulate the inflammatory and neovascular responses determining proliferation of spindle cells, the predominant cell type within these lesions (Bais *et al.*, 1998; detailed references in Hayward, 2003).

There is considerable evidence of a role for vIL-6 in the proliferation of infected B cells (reviewed in Schulz, 2001). Like its cellular counterpart, hIL-6—known to be important in B-cell proliferation—vIL-6 is able to support

the growth of IL-6-dependent B cells *in vitro*. However, there are differences between the two cytokines. Human IL-6 needs both subunits of the cell-surface IL-6 receptor, IL-6Ra and gp130, to fulfil its stimulatory role, whereas vIL-6 only requires the latter (see Figures 11.4 and 11.6). IL-6Ra and gp130 differ in expression patterns, with gp130 being more widely expressed. This may allow vIL-6 to stimulate a broader spectrum of cells. In line with this prediction, vIL-6 has been shown to induce neurite outgrowth in the rat pheochromocytoma cell line PC12 and to promote colony formation of human CD34⁺ bone marrow progenitor cells. *In vivo*, vIL-6-transfected fibroblasts inoculated into nude mice induced hepatosplenomegaly, lymphadenopathy and polyclonal hypergammaglobulinaemia, accompanied by increased haematopoiesis of the myeloid, erythroid and megakaryocytic lineages and plasmacytosis in spleen and lymph nodes. Tumours developing in these animals were more extensively vascularized than those in control animals and expressed high levels of VEGF, which correlated with the amount of vIL-6 in these tumours. VEGF is also expressed in PEL-derived cell lines, and a neutralizing antibody to VEGF blocked the formation of effusion lymphoma and bloody ascites in mice inoculated with PEL cell lines. VEGF can also be detected in the malignant effusions of PEL patients. VEGF-induced stimulation of vascular permeability may therefore be critical to the formation of the malignant ascites characteristic for this AIDS lymphoma. Finally, vIL-6 can also activate STAT 3, Janus tyrosine kinase (JAK1) and the mitogen-activated protein kinase (MAPK) pathway (detailed references for the effects of vIL-6 can be found in Schulz, 2001).

A viral homologue of a G-protein-coupled receptor (vGCR) is encoded by KSHV orf74. KSHV vGCR is homologous to the human IL-8 receptor. In contrast to the latter, KSHV vGCR shows ligand-independent, constitutive activity due to the presence of a point mutation in a sequence motif (DRY) that is highly conserved among GCRs (Arvanitakis *et al.*, 1997; Burger *et al.*, 1999). vGCR transforms murine cells and induces VEGF-dependent angiogenesis and KS-like lesions in transgenic mice, animals inoculated with transfected cells and transgenic animals following endothelial cell-specific infection by a retroviral vector expressing vGCR (Bais *et al.*, 1998; Montaner *et al.*, 2003; Yang *et al.*, 2000). Survival of vGCR-immortalized human umbilical endothelial cells (HUVECs) was dependent on autocrine signalling through KDR, a protein present in KS lesions, leading to the activation of the phosphatidylinositol 3'-kinase Akt/PI3K pathway and consequent NF- κ B transcription factors (Bais *et al.*, 2003; Montaner *et al.*, 2001). The transcription of angiogenesis-regulating genes, cytokines and pro-inflammatory genes, including

angiopoietin 2, was also found to be modulated upon vGCR expression. vGCR activates the MAP kinases p38 and ERK-2, augments transcription of several KSHV lytic genes (e.g. orf57) and increases production of vIL-6 and VEGF (Cannon *et al.*, 2003). Interestingly, despite its tumorigenic and angiogenic functions, vGCR is only expressed in approximately 10% of the KS cells (Chiou *et al.*, 2002). Similarly, infection of transgenic mice with a retroviral vector allowing endothelial cell-specific expression of vGCR resulted in the development of KS-like lesions, but expression of vGCR was confined to a small proportion of tumour cells. Expression of vGCR appeared to lead to the recruitment of other endothelial cells, suggesting that vGCR acts through paracrine mechanisms, probably by secreting angiogenic factors (Montaner *et al.*, 2003). The role of vGCR in disease progression in haematopoietic cells was analysed by using PEL cell lines expressing vGCR under the control of an inducible promoter (Cannon *et al.*, 2003). In an animal model of KS, involving murine bone marrow endothelial cells transfected with a complete KSHV genome and transplanted into mice, down-modulation of vGCR by siRNA reduced the formation of endothelial cell tumours (Mutlu *et al.*, 2007). All these observations point to vGCR as a major player in the angiogenesis and thereby pathogenesis of KSHV.

Three chemokine homologues, vMIPs I–III, encoded by KSHV orfK6, orf4 and orf4.1, respectively, have been proposed to be important in promoting leukocyte chemotaxis, eosinophil migration and angiogenesis. They are members of the MIP family, hence their name. Among them, vMIP-I has been reported to induce the expression of VEGF in PEL cell lines, in a similar way to vIL-6 (Liu *et al.*, 2001) (see above). Other viruses, such as mouse and human cytomegaloviruses, also encode functional chemokines (Alcami, 2003). The vMIPs are also required for evading the immune response and inhibiting apoptosis (see below).

Inhibition of Apoptosis

Programmed cellular death, or apoptosis, is a complex process involving several cellular proteins. Viral infection triggers pathways resulting in apoptosis. To overcome this, viruses have developed mechanisms interfering with apoptotic pathways. This allows them to increase the survival rate of virus-infected cells, thus increasing the time available for viral replication and spread within the host and to other individuals. Since inhibition of apoptotic pathways is one of the hallmarks of tumour cells, it is possible that viral proteins interfering with apoptosis could also contribute to virus-mediated transformation. Figure 11.7 summarizes some of the ways by which KSHV inhibits programmed cellular death. Two different

apoptotic pathways in mammalian cells result in the activation of effector caspases. The extrinsic pathway requires the activation of procaspases 8 and 10 by so-called 'death receptors', which belong to the tumour necrosis factor (TNF) receptor gene superfamily, whereas in the intrinsic pathway mitochondria release caspase-activating proteins.

vFLIP, encoded by orf K13, is expressed on the same bicistronic transcript as v-cyc (Rainbow *et al.*, 1997). It can block Fas-induced apoptosis and has been postulated to act as a tumour progression factor by interfering with apoptotic signals induced by virus-specific T-killer cells (Djerbi *et al.*, 1999) (see Figure 11.7). It has also been proposed to contribute to the continuous NF- κ B activation observed in PEL cells (Liu *et al.*, 2002). Inhibition of NF- κ B and down-modulation of vFLIP by RNA interference induces apoptosis in PEL cell lines, indicating that vFLIP-induced NF- κ B activation may be important for their survival (Guasparri *et al.*, 2004). Transduction of vFLIP into primary endothelial cell cultures induces spindling, suggesting that the vFLIP-induced NF- κ B activation may also play an important role in the formation of spindle cells, the histological hallmark of KS lesions (Grossmann *et al.*, 2006).

K7 is a glycoprotein structurally related to the apoptosis regulator survivin, as revealed by computational analysis (Wang *et al.*, 2002). Human survivin, a member of the IAP family, protects cells from apoptosis by an unknown mechanism. K7 anchors to cellular membranes in the vicinity of Bcl-2 and binds to Bcl-2 (but not Bax), as shown by GST-pulldown assays, via its putative BH2 domain, and to caspase-3 via its BIR domain (Wang *et al.*, 2002) (see Figure 11.7). Thus, K7 seems to be an adaptor molecule bringing together Bcl-2 and effector caspases, allowing the inhibition of the latter by Bcl-2 (Wang *et al.*, 2002).

Like all other gammaherpesviruses for which genomic sequences are available, KSHV expresses a viral homologue of human Bcl-2 (Cheng *et al.*, 1997). The vBcl-2 mRNA is detected in PEL cell lines (Sarid *et al.*, 1997) and protein expression could be shown for late stages of KS lesions. vBcl-2 forms heterodimers with hBcl-2 (Sarid *et al.*, 1997) and it is believed that it may inhibit apoptosis in KSHV-infected cells (Cheng *et al.*, 1997; Sarid *et al.*, 1997). While human Bcl-2 forms heterodimers with members of the Bcl-2 family with pro-apoptotic roles (such as Bax and Bak), the viral homologue does not seem to do so (Cheng *et al.*, 1997). As mentioned above, the CDK6–v-cyc complex phosphorylates and inactivates human Bcl-2, which results in apoptosis. In contrast, vBcl-2 is not phosphorylated by the CDK6–v-cyc complex, allowing KSHV to overcome the v-cyc-induced apoptosis (Ojala *et al.*, 2000). These differences seem to be due to variations in the protein structure between the two proteins.

IRFs are a family of interferon-responsive transcription factors that regulate expression of genes involved in pathogen response, cell proliferation and immune modulation through binding to interferon-stimulated response elements (ISREs) in the promoters of interferon-responsive genes. Among the members of the IRF family, IRF-3 and IRF-7 seem to be the key regulators for the induction of type I interferons (IFNs), the primary response against viral infection (for a review, see Stark *et al.*, 1998). KSHV encodes four IRF homologues, named vIRFs (Moore *et al.*, 1996; Russo *et al.*, 1996). vIRF-1, encoded by orfK9, is a multifunctional protein that inhibits signalling (Gao *et al.*, 1997; Li *et al.*, 2000; Moore *et al.*, 1996). Expression of vIRF-1 inhibits IFN-signal transduction in reporter assays, downregulates expression of the cell-cycle inhibitor p21WAF-1/CIP-1 and transforms NIH 3T3 cells (Gao *et al.*, 1997). vIRF-1 is weakly expressed in KSHV-infected B cells, while it is absent from spindle KS cells (Gao *et al.*, 1997).

KSHV orfK10 encodes vIRF-2 (Burysek and Pitha, 2001), another protein that also inhibits IFN-mediated cell death. It is conceivable that, by blocking interferon-induced signal transduction and/or apoptosis, vIRF-1 and vIRF-2 allow KSHV-infected cells to escape the effect of virus-specific T cells (see below).

KSHV orfK10.5 is a latent gene whose product is another KSHV IRF homologue (vIRF-3), also referred to as LANA-2 in view of its latent expression in KSHV-infected B cells (Rivas *et al.*, 2001). vIRF-3/LANA-2 inhibits the activation of p53-dependent promoters and thereby restrains p53-mediated apoptosis (Rivas *et al.*, 2001). It also interferes with the activation of caspase 3 induced by interferon-activated protein kinase (PKR) and can therefore be considered part of the KSHV defense mechanism against the interferon system (Esteban *et al.*, 2003). Furthermore, vIRF-3/LANA-2 has been reported to inhibit the transcriptional activity of FOXO3a and to block the G2/M arrest induced by 14-3-3 protein overexpression (Munoz-Fontela *et al.*, 2007).

KSHV Proteins with Transforming and Intracellular Signalling Activity

Kaposin

A group of transcripts originating in the K12/kaposin locus has been reported to encode several proteins (Sadler *et al.*, 1999). Among these, kaposin A, a type II transmembrane protein, is able to transform rodent fibroblasts to tumourigenicity (Kliche *et al.*, 2001; Muralidhar *et al.*, 1998). Kaposin A induces lymphocyte aggregation and adhesion, probably through direct interaction with cytohesin-1 (Kliche *et al.*, 2001), a guanine nucleotide exchange factor for ARF GTPases and regulator

of integrin-mediated cell adhesion (Figure 11.6). Kaposin B binds to the cellular kinase MK2 and enhances the stabilization of certain cytokine mRNAs that are normally regulated by the p38/MK2 pathway (McCormick and Ganem, 2005).

ORFsK1, K9, 74 and K15

The products of three early and late genes, orfK1, orfK9 and orf74 (VIP, vIRF-1 and vGCR, respectively), have been shown to have transforming properties and to induce intracellular signalling pathways. K1/VIP and vGCR cause tumours in transgenic mice and activate several intracellular signal transduction pathways, including stress- and mitogen-induced kinases (Lee *et al.*, 1998) (for references for vGCR, see above). K1/VIP transforms rodent fibroblasts and is able to immortalize T lymphocytes when replacing the saimiri transforming protein (STP) in the HVS genome (Lee *et al.*, 1998).

In line with their classification as early or late lytic genes, neither K1/VIP nor K9/vIRF-1 have so far been found to be expressed in the majority of tumour cells of KS, MCD or PEL, although low-level expression remains a possibility. The expression of vGCR in a small percentage of tumour cells in PEL, MCD and KS lesions has been observed by immunohistochemistry (Chiou *et al.*, 2002), in accordance with a proposed role for this membrane-signalling protein in paracrine effects (Bais *et al.*, 1998; Montaner *et al.*, 2003) (see above).

A family of alternatively-spliced transcripts is transcribed late in the lytic replication cycle from eight exons located between orf75 and the TR. Proteins (terminal membrane proteins (TMPs)) derived from these transcripts are predicted to contain up to 12 transmembrane domains and a common cytoplasmic domain containing a putative TRAF as well as SH2 and SH3 (Src homology domain) binding sites (Glenn *et al.*, 1999; Poole *et al.*, 1999) (Figure 11.6). The largest K15-derived protein activates the NF- κ B, MEK/Erk and JNK pathways and induces the production of inflammatory cytokines (Brinkmann *et al.*, 2007). Intracellular localization studies have shown that TMP resides in the endoplasmic reticulum (ER) and mitochondria, where it interacts with HS1-associated protein X-1 (HAX-1), an inhibitor of Bax-induced apoptosis (Sharp *et al.*, 2002).

Escape from the Immune System

The interaction between viruses and their hosts during evolution is thought to have conditioned the host immune system and has resulted in the development of viral strategies to evade the immune system. Many viruses encode proteins that target essential pathways of the immune system. KSHV has also acquired several mechanisms to protect infected cells from an attack by the immune system.

The K3 and K5 proteins, unique to KSHV, are involved in protecting virus-infected cells against NK cells or cytotoxic T lymphocytes (Coscoy and Ganem, 2000). These membrane proteins, also termed modulator of immune recognition (MIR) 1 and 2, respectively, have been shown to downregulate major histocompatibility complex (MHC) class I molecules (Coscoy and Ganem, 2000). MIR1 targets HLA-A, B and C, whereas MIR2 primarily targets HLA-A, ICAM-1 (intercellular adhesion molecule 1; CD 54) and B7.2 (CD 86); both also target the gamma interferon receptor 1 (Li *et al.*, 2007). MIR1 and MIR2 increase the rate of endocytosis and degradation of their target proteins by acting as E3 ubiquitin ligases and mediating their ubiquitination (Lorenzo *et al.*, 2002; Means *et al.*, 2002) (Figure 11.6). Downregulation of MHC-I molecules by MIR1 involves the formation of lysine-63-linked polyubiquitin chains on MHC-I, followed by endocytosis and lysosomal degradation (Duncan *et al.*, 2006). Both MIR1 and MIR2 contain an amino terminal RING finger motif required for their function as E3 ligases. This domain and the carboxy-terminal region are responsible for MIR-mediated endocytosis, whereas the transmembrane region is required for target specificity. Reports studying the expression of these proteins have shown that both MIR1 and MIR2 are expressed as early and late lytic genes following reactivation in PEL cell lines, whereas K3 transcripts were not detected in KS lesions (Rimessi *et al.*, 2001; Sun *et al.*, 1999).

Like many other viruses, KSHV has also developed defense mechanisms against components of the interferon system. The initial immune response against viral infection is regulated by IRFs through binding to ISREs in the promoters of interferon-responsive genes. The KSHV vIRFs are involved in counteracting these effects. The mechanism by which vIRFs inhibit IFN-signal transduction seems to involve direct binding to IRFs p300/CREB binding protein (CBP) and other transcription factors impeding the formation of the transcriptional active complexes (Li *et al.*, 2000; Lin *et al.*, 2001).

vIRF-1 is able to bind to the transcriptional coactivator CBP and inhibit its transcriptional activity, and it also interacts with the histone acetyl transferase p300, impeding its activity and modifying chromatin structure and blocking IRF3 recruitment of p300/CBP (Li *et al.*, 2000; Lin *et al.*, 2001) (see 'Inhibition of Apostosis', above).

Similarly, vIRF-2 can also bind to IRFs and CBP/p300 and thereby inhibit virus-mediated induction of IFN type I gene transcription (Burysek and Pitha, 2001). Another mechanism of action of vIRF-2 involves the interaction with the IFN-induced, double-stranded, RNA-activated serine-threonine kinase (PKR) (Burysek and Pitha, 2001), a main component of the host antiviral defence (for a review on PKR, see Levy and Garcia-Sastre, 2001).

Such interaction blocks autophosphorylation of PKR and subsequent phosphorylation of PKR targets (Burysek and Pitha, 2001). The role of vIRF-3/LANA-2 in the PKR pathway has been discussed above. The KSHV orf45 protein blocks phosphorylation and nuclear accumulation of IRF-7, and thereby virus-mediated induction of type I IFN-activated genes (Zhu *et al.*, 2002).

The three viral β chemokines, vMIPs I–III (see 'Angiogenesis and B-cell Proliferation: vIL-6, vMIP-I-III, vGCR', above) may also play a role in modulating the immune response. Other herpesviruses and poxviruses encode homologues of chemokines (see Alcami, 2003 and references cited therein). Little is known regarding the role of vMIP-III. vMIP-II binds to several chemokine receptors, either as an agonist or as an antagonist (Boshoff *et al.*, 1997; Kledal *et al.*, 1997), whereas vMIP-I is more selective, binding exclusively to and acting as an agonist of CCR8 (Endres *et al.*, 1999). The leukocyte infiltrate within KS lesions is composed mainly of mononuclear phagocytes and T cells, with the CD4⁺ and CD8⁺ cells having a marked type II cytokine profile. This is probably due to the fact that both vMIP-I and -II act as chemoattractants for monocytes and Th2 cells and not Th1, NK or dendritic cells (Weber *et al.*, 2001). This may allow the virus to skew the immune response from a type I, antiviral, response pattern towards a type II pattern (Weber *et al.*, 2001).

The product of orf4, named KCP for KSHV complement control protein, has kinetics characteristic of a lytic protein which specifically increases the decay of classical C3-convertase (Spiller *et al.*, 2003). It is conceivable that KCP could enhance virus pathogenesis through evading complement attack, opsonization and anaphylaxis (Spiller *et al.*, 2003).

DIAGNOSTIC ASSAYS

Serological Assays

Several serological assays of varying sensitivity and specificity, such as immunofluorescence, enzyme-linked immunosorbent assays and immunoblotting, have been developed. Among the first KSHV antibody assays, latently-infected PEL cell lines, such as BCP-1 or BCBL-1, were used as antigens for IFA by incubating these cells, dried on to microscope slides, with patient sera and detecting specific antibody bindings with fluorescein-conjugated secondary antibodies (Gao *et al.*, 1996b; Kedes *et al.*, 1996) (see Figure 11.8). Positive cells show a typical speckled nuclear pattern, which represents the main latent nuclear antigen of KSHV, the 225–234 kDa LANA-1 protein encoded by orf73 (Rainbow *et al.*, 1997). The sensitivity of this assay

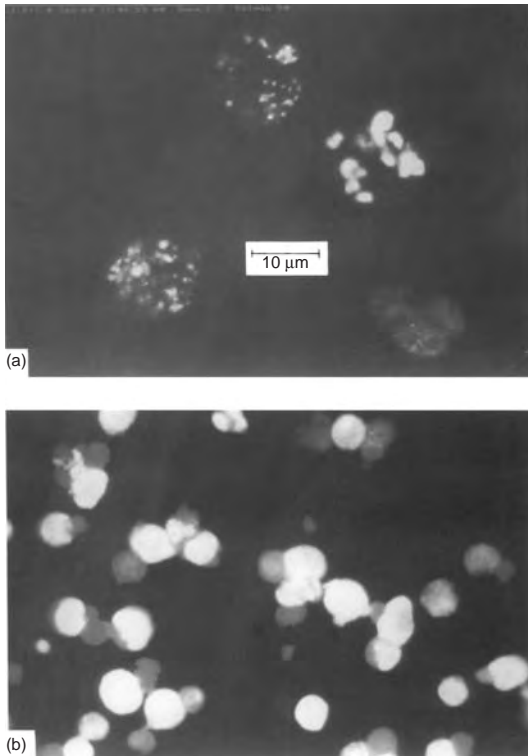


Figure 11.8 Immunofluorescence pattern for (a) latent and (b) lytic KSHV antigens in PEL cells. (a) shows the characteristic speckled nuclear pattern for the ‘latency-associated nuclear antigen’, LANA (Kedes *et al.*, 1996; Gao *et al.*, 1996b), which is encoded by orf73 (Rainbow *et al.*, 1997). (b) shows the diffuse nuclear and cytoplasmic fluorescence seen on TPA- or sodium-butyrate-induced PEL cells with patient sera.

lies in the order of 80% or higher when suitable PEL cell lines (e.g. BCP-1) and sera from KS patients are used as standards. A 3% reactivity rate in this assay among healthy individuals from low-prevalence countries indicates a high specificity. In addition, an IFA performed on PEL cell lines, in which the lytic cycle can be efficiently induced by treatment with phorbol esters or sodium butyrate (e.g. BCBL-1; KS-1), detects antibodies to several viral structural proteins, in particular the orf K8.1-encoded virion membrane glycoprotein (Rezza *et al.*, 1998) (see Figure 11.8). While thought to be more sensitive than the IFA for LANA-1 antibodies, the ‘lytic’ IFA suffers from a lower specificity in many laboratories. However, with some protocols good specificities for this assay have been reported (Rezza *et al.*, 1998), although the issue of inter-laboratory comparability still remains problematic.

Enzyme-linked immunosorbent assays (ELISAs) are based on either whole-virus lysates, recombinant viral proteins—in particular LANA-1, K8.1 glycoprotein and orf65/vSCIP—or short chemically-synthesized peptides derived from a combination of these three proteins (Lam *et al.*, 2002; Raab *et al.*, 1998; Simpson *et al.*, 1996; see Schulz, 1998 for further references). Sensitivity and specificity of these ELISAs are in the range of 81–94% and 70–80%, respectively. Specificity of these assays can be increased by verifying a positive result in a more specific immunoblot. ELISAs based on the highly immunogenic K8.1 glycoprotein, which has no homologue in other herpesviruses, seem to perform with a comparatively high specificity.

Combinations of different antigens or assays increase sensitivity and thus make it possible to detect even lower antibody titres, as in the early stages of asymptomatic infection or in the late stages of AIDS when KSHV-specific antibodies can vanish.

These assays have proved useful in epidemiology studies of KSHV. However, serological diagnosis of a KSHV infection in an individual, particularly from a low-risk population, may still be associated with a degree of uncertainty if antibodies to only one KSHV antigen or reactivity in only one assay are found. Simultaneous reactivity in several assays affords much higher certainty.

Molecular Biology Assays

By qualitative PCR, KSHV is easily detectable in fresh or frozen biopsies of KS, PEL or MCD, although the amount of KSHV DNA can be quite variable, particularly in KS biopsies. Detection in paraffin-embedded specimens generally may require the use of nested PCR, as does detection in peripheral blood, saliva or semen. Detection of KSHV DNA in the peripheral blood of HIV-infected individuals has long been known to predict the onset of KS (Whitby *et al.*, 1995). Several quantitative PCR protocols, including competitive and real-time PCR assays (de Sanjose *et al.*, 2002) have been developed. In several, but not all, studies, peripheral blood viral load has been found to predict clinical progression. MCD in particular seems to be associated with a high KSHV viral load (Boivin *et al.*, 2002; Oksenhendler *et al.*, 2000). In AIDS patients, KSHV viral load is higher in patients with active KS than in patients with KS in complete remission, and highest among patients with progressing KS (Laney *et al.*, 2007; Marcelin *et al.*, 2004). However, in HIV-unrelated KS patients KSHV viral load does not correlate significantly with clinical stage (Guttman-Yassky *et al.*, 2007; Marcelin *et al.*, 2004), suggesting KSHV viral load might only reflect the intensity of immunosuppression rather than the extension of KS in a patient. Nevertheless, as KSHV viral load not only increases with progressing disease but

also decreases and/or becomes negative after complete or partial clinical response (Laney *et al.*, 2007; Lebbe *et al.*, 1998), quantifiable PCR methods can serve as a diagnostic tool to monitor immunocompromised patients at high risk of KSHV-related diseases.

ANTIVIRAL THERAPY

HAART

Since the introduction of HAART in 1996 the incidence of AIDS KS in Western countries has markedly reduced. This may be the consequence of an enhanced KSHV-specific immune response following restoration of patients' immune systems by effective suppression of HIV, but a direct effect of HIV proteinase inhibitors, for example indinavir, on KS has also been postulated (Sgadari *et al.*, 2003).

Anti-herpesviral Drugs

Nevertheless, virus-specific therapy of KSHV-associated diseases still remains a problem, since the classical anti-herpesviral drugs known so far are only effective in combating lytic and not latent infection. As discussed above, most tumour cells in KS, PEL and MCD are latently infected with KSHV; of these, MCD is characterized by the highest percentage of cells expressing lytic KSHV proteins. In keeping with this observation, ganciclovir treatment may lead to substantial clinical improvement in MCD cases (Casper *et al.*, 2004). *In vitro* assays have established that the replicative cycle of KSHV in PEL cell lines can be inhibited with cidofovir, ganciclovir and foscarnet, but not with aciclovir (Kedes and Ganem, 1997; Medveczky *et al.*, 1997). However, clinical studies with these antiviral drugs have produced controversial results. The use of intravenous ganciclovir, foscarnet or cidofovir therapy to treat cytomegalovirus disease did not affect the KSHV DNA load in PBMCs, but was associated with disease progression (Boivin *et al.*, 1999; Little *et al.*, 2003). On the other hand, some retrospective studies and anecdotal reports do support the notion that these drugs are active against KSHV *in vivo*. Thus, both foscarnet and ganciclovir, but not aciclovir, had some activity in preventing the occurrence of KS (Mocroft *et al.*, 1996). It therefore appears that ganciclovir and foscarnet can inhibit the replication and dissemination of KSHV prior to the emergence of KS, but that they are of limited benefit in treating established disease.

Experimental Approaches

The growing knowledge on KSHV pathogenesis now offers multiple starting points for the development of new

therapeutic strategies, some of them already being tested in phase I and phase II clinical trials. In addition, it may explain the modes of action of long-established therapeutic regimens. Thus there are hints that the silencing of the still-functional p53 tumour suppressor gene by viral oncogenes like LANA can be overcome by the application of p53-activating drugs such as the liposomal anthracyclines. These DNA-damaging, hence p53-activating, chemotherapeutic drugs are known to induce KS regression (Petre *et al.*, 2007; Sarek *et al.*, 2007). Similarly, nutlin 3a, an inhibitor of the interaction between p53 and its ubiquitin E3 ligase, mdm-2, has been shown to restore p53 function and p53-mediated apoptosis in a PEL-tumour model (Sarek *et al.*, 2007). Another promising strategy is the inhibition of the VEGF-induced Akt/mTOR pathway, which emerged as a major driving force in KS development, by for example rapamycin, an immunosuppressive drug used to prevent organ rejection after transplantation. By switching a cyclosporine-based regimen to rapamycin, which unlike cyclosporine inhibits mTOR, all 15 renal allograft recipients with cutaneous KS showed complete regression (Stallone *et al.*, 2005). Nevertheless, failures to rapamycin have also been described (Lebbe *et al.*, 2006). Other angiogenesis-inhibiting drugs like COL-3 (inhibits MMP-2 and -9) or interleukin-12 (Dezube *et al.*, 2006; Little *et al.*, 2006) are currently being evaluated.

Other antiviral agents might also be effective against KSHV infection. Glycyrrhonic acid, a component of liquorice, decreases the expression of LANA and increases that of v-cyclin, which results in apoptosis of latently KSHV-infected cells. Although the required concentration of glycyrrhonic acid is far too toxic, better components may be developed by pharmacological engineering, expanding anti-herpesviral treatment options to the reservoir of latently-infected cell (Curreli *et al.*, 2005). KSHV encodes a dihydrofolate reductase (DHFR) (orf2). Although this early lytic enzyme has been shown to be non-essential for viral replication in cultured PEL cells and its activity was not detected in PBMCs, DHFR inhibitors like methotrexate (MTX) or trimethoprim might represent starting points for the design of new specific anti-KSHV agents (Cinquina *et al.*, 2000). MTX has already been shown to completely prevent TPA-induced lytic viral DNA replication and to strongly decrease viral lytic transcript levels *in vitro* (Curreli *et al.*, 2002). Whether this is suitable for a therapeutic approach has to be further examined.

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Hepatitis Viruses

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INTRODUCTION

Hepatitis, characterized by necrosis and inflammation in the liver, may result from infection with a variety of viruses and is the main clinical outcome of infection with viruses from several different families. The predominantly hepatotropic viruses, which vary in prevalence throughout the world, may be considered together, although the biology of these viruses and the spectrum of disease they cause differ considerably (see Table 12.1 for an overview).

The clinical spectrum of acute disease ranges from an asymptomatic or mild anicteric illness to acute disease with jaundice to severe prolonged jaundice or fulminant hepatitis (acute liver failure). Where the infection persists, chronic hepatitis may ensue and, here, the outcome is also variable. Inapparent or subclinical and anicteric infections are common. Hepatitis A virus (HAV) and hepatitis E virus (HEV) do not persist in the liver and there is no evidence of direct progression to chronic liver damage. However, hepatitis B virus (HBV), with or without its satellite, hepatitis D virus (HDV or the delta agent) and hepatitis C virus (HCV), may be associated with persistent infection, a prolonged carrier state and progression to chronic liver disease, which may be severe. In addition, there is substantial evidence of an aetiological association between infection with chronic hepatitis B or C and hepatocellular carcinoma.

Hepatitis A, B and D can be differentiated by sensitive laboratory tests for specific antigens and antibodies, and the respective viruses have been characterized. Enzyme immunoassays (EIAs) and recombinant immunoblot assays (RIBAs) have been developed for the detection of antibodies to HCV, and viraemia may be confirmed by testing for the viral genome, for example by using the

polymerase chain reaction (PCR). Assays for the nucleocapsid antigen of this virus currently are under evaluation. EIAs have been developed for the detection of antibodies to HEV and the genome may be detected using PCR.

Pathology

Acute Hepatitis

The pathological features that are constant in all types of acute viral hepatitis consist of parenchymal cell necrosis and histiocytic periportal inflammation. The reticulin framework of the liver is usually well preserved, except in some cases of massive and submassive necrosis. The liver cells show necrotic changes that vary in form and intensity. The necrotic areas are usually multifocal, but necrosis tends to be frequently zonal, with the most severe changes occurring in the centrilobular areas. Individual hepatocytes often are swollen and may show ballooning, but they can also shrink, giving rise to acidophilic bodies.

Dead or dying rounded liver cells are extruded into the perisinusoidal space. There are variations in the size and staining quality of the nuclei. Fatty changes in the liver are usually not marked, but some steatosis can be observed in chronic HCV infection. A monocellular infiltration, which is particularly marked in the portal zones, is the characteristic mesenchymal reaction. This is also accompanied by some proliferation of bile ductules.

Kupffer cells and endothelial cells proliferate and the Kupffer cells often contain excess lipofuscin pigment. In the icteric phase of typical acute hepatitis, the walls of the hepatic vein tributaries may be thickened and frequently are infiltrated, with proliferation of the lining cells in the terminal hepatic veins. Cholestasis may occur in the early stages of viral hepatitis, and plugs of bile thrombi may

Table 12.1 The principal agents of viral hepatitis

	HAV	HEV	HBV	HDV	HCV
Classification	<i>Picornaviridae</i> <i>Hepatovirus</i>	Calicivirus-like <i>Hepevirus</i>	<i>Hepadnaviridae</i>	Unclassified (viroid-like)	<i>Flaviviridae</i> <i>Hepacivirus</i>
Size (nm)	27	27–38	42	36–40	36
Genome	7.5 kb linear	7.5 kb linear	3.2 kb circular	1.7 kb circular	9.4 kb linear
Structural proteins	ssRNA VP1–VP4	ssRNA ORF2 product	dsDNA ^a HBcAg HBsAg	ssRNA HDAg (HBsAg)	ssRNA E1 (gp35) E2 (gp70)
Transmission	Enteric	Enteric	Parenteral	Parenteral	Parenteral
Persistent infection	No	No	Yes	Yes	Yes
Vaccine	Yes	Under trial	Yes	No ^b	No

^aThe HBV genome contains a single-stranded region.

^bHepatitis B vaccine protects against HDV–HBV co-infection.

be found in the bile canaliculi; this is a more common feature in hepatitis E.

Spotty or focal necrosis with the associated mesenchymal reaction may also be found in anicteric hepatitis, but on the whole the lesions tend to be less severe than in the icteric type of illness. At the other extreme, there is rapid massive necrosis of the liver cells in fulminant hepatitis.

Repair of the liver lobules occurs by regeneration of hepatocytes; frequent mitoses, polyploidy, atypical cells and binucleated cells are found. There is gradual disappearance of the mononuclear cells from the portal tracts, but elongated histiocytes and fibroblasts may remain. The outcome of acute viral hepatitis may be complete resolution or fatal massive necrosis.

Chronic Hepatitis

The pathological features of chronic hepatitis B depend upon the stage of the disease, the host immune response and the degree of virus replication. In chronic hepatitis B with mild activity, only rare piecemeal necrosis is seen. Characteristic hepatocytes with eosinophilic 'ground-glass' cells are relatively common in anti-HBe-positive patients with low levels of virus replication. Lobular hepatitis is more common in patients with active virus replication and raised serum aminotransferases. CD8-positive cells predominate in areas of piecemeal necrosis. Hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg) can be detected by immunoperoxidase staining in routinely-fixed liver biopsy sections. Patients with high levels of viraemia may have minimal hepatitis.

The pathological features of HCV infection are quite characteristic, albeit not pathognomonic. The presence of HCV RNA in serum tends to correlate with some degree of hepatitis, and disappearance of HCV RNA, for example following successful interferon alpha (IFN- α) treatment, is

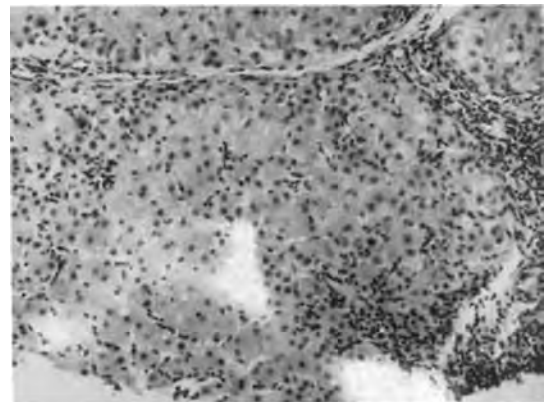


Figure 12.1 Section of a liver biopsy from a patient with chronic hepatitis C seen at low magnification. There is a moderate lymphocytic infiltrate in the portal tracts, with piecemeal necrosis. A scattered mild parenchymal inflammatory infiltrate is also present. Stained with haematoxylin and eosin. (Courtesy of Dr A.P. Dhillon, Department of Histopathology, Royal Free Hospital and UCL Medical School.)

followed by histological improvement. Typically, patients with chronic hepatitis C have mild portal tract inflammation with lymphoid aggregates or follicles and mild periportal piecemeal necrosis (Figure 12.1). Parenchymal steatosis, apoptosis and mild lobular inflammation are present and portal fibrosis or portal-central fibrosis may be present in later stages of disease. Bridging necrosis is not common. Rarely, granulomas can be observed. Although many of the lymphoid follicles are associated with bile ducts, ductopaenia is not observed. Advanced disease, with cirrhosis or hepatocellular carcinoma, is not generally associated with distinguishing features.

HCV antigens have been detected in scattered groups of cells, with granular cytoplasmic staining. The periportal lymphocytes around lymphoid follicles are mixed, but

contain relatively large numbers of CD4 lymphocytes. A characteristic histological pattern of mild chronic hepatitis with portal lymphoid follicles and varying degrees of lobular activity is found in many patients with persistent hepatitis C infection.

Biochemical Tests of Liver Function

The serum levels of aspartate and alanine aminotransferase (ALT) are elevated in acute hepatitis, as are levels of other enzymes released by the damaged liver cells. Usually, the levels of ALT are higher than those of aspartate aminotransferase (AST), a difference particularly marked in hepatitis C. However, with progression of the disease to cirrhosis, the AST/ALT ratio may be reversed. Elevation of these enzymes may be the only abnormality to be found in individuals with asymptomatic and anicteric infections who are tested because of known exposure. Bilirubin is found in the urine and conjugated and total serum bilirubin levels are raised in most symptomatic infections. The leukocyte count is usually normal but some atypical lymphocytes are frequently found.

A progressive decline in serum albumin concentrations and prolongation of the prothrombin time are characteristically observed after decompensated cirrhosis has developed. A subset of patients with chronic hepatitis C infection may test positive for autoantibodies, including LKM1 antibody.

Clinical Manifestations

Differences between the clinical syndromes of acute hepatitis A, acute hepatitis B and other forms of viral hepatitis become apparent on analysis of large numbers of well-documented cases, but these differences are not sufficiently reliable for the diagnosis of individual patients with icteric disease. Epidemiologic risk factors, for example travel, injections or sexual risk, can indicate a possible aetiology. Fever and headache are more frequent during the prodrome of acute hepatitis A. The late incubation period–early clinical phase is frequently heralded by a variety of nonspecific symptoms such as fatigue, anorexia, malaise and myalgias. A few days later, anorexia, nausea, vomiting and right-upper-quadrant abdominal pain can appear, followed by passage of dark urine and clay-coloured stools and the development of jaundice of the sclera and skin. With the appearance of jaundice there is usually a rapid subjective improvement in symptoms. The jaundice usually deepens during the first few days and persists for one or two weeks. The faeces then darken and the jaundice diminishes, at first rapidly and then more slowly, over an additional period of two weeks or so. The liver may be palpable in acute severe hepatitis, but only a minority of patients have palpable splenomegaly. Convalescence

may be prolonged, although complete recovery in adults usually takes place within a few months. In children, the prodromal features may be mild or even absent, although anorexia, when present, tends to be severe. The icteric or post-icteric phase in children is short. The prodromal phase of hepatitis B and C is often prolonged and more insidious. Low-grade fever, arthralgias and skin rashes, particularly in hepatitis B, are not uncommon. The clinical features of the icteric phase are similar in all types of acute viral hepatitis. The mortality rate of acute hepatitis is low, approximately 0.1–3 deaths per 1000 cases.

Fulminant hepatitis can occur following acute hepatitis A–E; it is more common in hepatitis B. Hepatocellular failure develops rapidly; the patient may be deeply jaundiced or encephalopathy may occur before conspicuous jaundice is evident. Widespread haemorrhage occurs. The prothrombin time is prolonged; an altered prothrombin time is a more reliable indicator of prognosis, and of the need for liver transplantation, than the serum bilirubin or serum aminotransferases.

Fulminant hepatitis is unusual following hepatitis C infection, but has been reported, particularly following chemotherapy or withdrawal of chemotherapy. High mortality rates for hepatitis occurring during pregnancy have been reported from India, the Middle East and north Africa, associated particularly with HEV infection.

HEPATITIS A

Hepatitis A is endemic in all parts of the world, but the precise incidence is difficult to estimate because of the high proportion of asymptomatic and anicteric infections, differences in surveillance and differing patterns of disease. The degree of under-reporting is known to be very high. Serological surveys have shown that infection with HAV is almost universal and, in developing countries, 80–90% of children have serological markers of past infection by the age of five. In industrialized countries (particularly in northern Europe, North America and Australia) improvements in sanitation have decreased the incidence of hepatitis A. The prevalence of antibodies in young adults in such countries is 5–20%.

Incubation Period

The incubation period of hepatitis A is between three and five weeks, with a mean of 28 days. Subclinical and anicteric infections are common, particularly in children, and, although the disease has, in general, a low mortality, adult patients may be incapacitated for many weeks. There is no evidence of persistence of the infection in the liver and progression to chronic liver damage does not occur.

The virus replicates *in vivo* in the liver but it seems likely that the initial site of virus replication may be in the gut. This is not proven and the mechanism by which the virus reaches the liver is unknown, although a transient viraemia has been postulated.

Mode of Spread

HAV is spread by the faeco-oral route, most commonly by person-to-person contact, and infection is particularly common in conditions of poor sanitation and overcrowding. Common source outbreaks result most frequently from faecal contamination of drinking water and food, but water-borne transmission is not a major factor in industrialized communities. On the other hand, many food-borne outbreaks have been reported in developed countries. This can be attributed to the shedding of large amounts of virus in the faeces (Figure 12.2) during the incubation period of the illness in infected food handlers, and the source of outbreaks often can be traced to uncooked food or food which has been handled after cooking. The consumption of raw or inadequately-cooked shellfish cultivated in polluted water is associated with a high risk of HAV infection. For example, raw clams were implicated as the source of an epidemic with approximately 300 000 cases in Shanghai in 1988. However, although hepatitis A is common in developed countries, the infection occurs mainly in small clusters and often with only a few identified cases. Sporadic cases result from person-to-person contact. Hepatitis A is highly endemic in many tropical and subtropical areas, with the occasional occurrence of large epidemics. The infection is acquired frequently by travellers from areas where it is of low prevalence to areas where hepatitis A is hyperendemic.

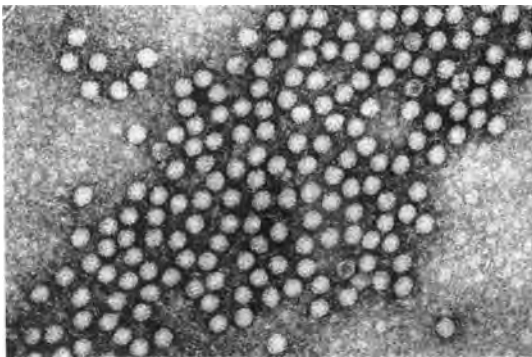


Figure 12.2 Electron micrograph showing HAV in faecal extracts obtained during the early acute phase of illness. The particles measure about 27 nm in diameter ($\times 170\,000$). (From a series by Anthea Thornton and A.J. Zuckerman.)

Age Incidence

All age groups are susceptible to hepatitis A and disease severity increases with age. As noted above, most individuals in highly-endemic areas are infected before five years of age but in many countries in northern Europe and in North America most clinical cases occur in adults. In countries where there has been improvement in socio-economic conditions and sanitation, such as southern Europe and China, there has been an increase in the mean age of infection. In many developed countries, the prevalence of antibodies to hepatitis A has fallen to 5–10% of young adults and there is thus a large susceptible population. The diminishing incidence of hepatitis A is now matched by an increasing incidence of clinically-apparent disease. This shift in age incidence is similar to that which occurred with poliomyelitis during and after the Second World War, reflecting improvements in socio-economic and hygienic conditions and a consequent shift in herd immunity.

The Biology of Hepatitis A Virus

The hepatitis A virion is a non-enveloped particle measuring 25–28 nm in diameter (Figure 12.3) and containing a linear genome of positive-sense, single-stranded RNA, approximately 7500 nucleotides in length and coding for three major structural polypeptides with molecular weights of 33 000, 29 000 and 27 000 (VP1, VP2 and VP3). A fourth polypeptide (VP4) of only 17 amino acids has been predicted from the nucleotide sequence of the virus but has not been detected experimentally. X-ray crystallographic studies have not yet been reported but the

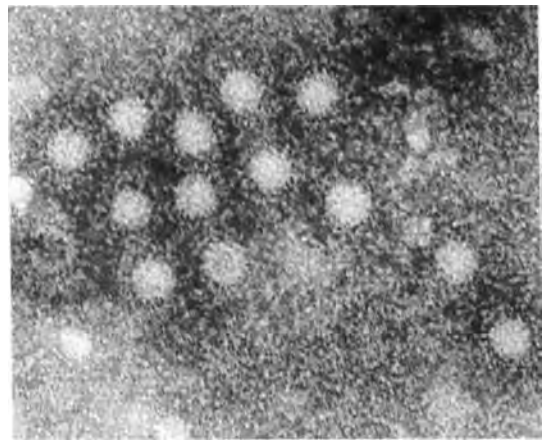


Figure 12.3 Electron micrograph showing a large aggregate of HAV particles heavily coated with antibody, giving the appearance of a 'halo'. 'Full' and 'empty' particles are shown ($\times 340\,000$). (From a series by Anthea Thornton and A.J. Zuckerman.)

basic structure of the capsid may be predicted from such studies of other picornaviruses; the antigenic structure has been defined further by analysis of neutralization-escape mutants selected by monoclonal antibodies. These studies suggest that the immunodominant neutralization site is a conformational epitope comprising residues of VP1 and VP3. It is believed that secondary or higher orders of protein structure may play essential roles in this antigenic site, because it has not been possible to detect this predominant antigen in virus preparations disrupted with detergent or following expression of recombinant protein (although expression of the entire HAV polyprotein may enable assembly of antigenic virus-like particles in cell culture).

HAV is exceptionally stable; it is ether-resistant, stable at pH 3.0 and relatively resistant to inactivation by heat and to degradation by environmental conditions. HAV retains its physical integrity and biological activity at 60 °C for 10 hours but is inactivated after 5 minutes at 100 °C. The virus may also be inactivated by ultraviolet irradiation and by treatment with a 1 : 4000 concentration of formaldehyde solution at 37 °C for 72 hours. There is also evidence that HAV is inactivated by chlorine at a concentration of 1 mg l⁻¹ for 30 minutes.

Genetic Organization

The organization of the genome of HAV is similar to other picornaviruses and it was classified originally as *Enterovirus* type 72. However, there are substantial differences between HAV and the *Enteroviruses* and other genera of the *Picornavirus* family and it has been reclassified in its own genus of the *Picornaviridae*, *Hepatovirus*.

Cloning and sequencing data indicate that the genome of HAV consists of 7478 nucleotides with a 5' noncoding region of 733 nucleotides and a shorter noncoding region and poly(A) tract at the 3' terminus. A small protein (VPg) is bound covalently at the 5' terminus. A single open reading frame (ORF) extends from nucleotide 710–750 to about 60 nucleotides in advance of the 3' terminal poly(A) tract. Comparison of the predicted amino acid sequence of the polyprotein with other *Picornaviruses* suggests that the 5' region of the ORF codes for the three

major structural proteins of the virus along with the fourth, small VP4. The 3' region encodes a protease, the polymerase and other functions involved in genome replication. Dipeptide cleavage sites which have been identified in a number of *Picornavirus* polyproteins are not conserved in HAV, but attempts have been made to predict the cleavage pattern and post-translational processing of the polyprotein (Figure 12.4). The cellular receptor for HAV has been identified as TIM1, a member of the T-cell immunoglobulin mucin family.

Cell Culture

The successful propagation of HAV in primary monolayer and explant cell cultures, and in continuous cell lines of primate origin, was a major advance and opened the way to the preparation of hepatitis A vaccines. The viral capsid antigens are detectable by immunofluorescence and radioimmunoassay, and the viral RNA by an indirect, quantitative autoradiographic plaque assay and by complementary DNA–RNA hybridization and reverse transcriptase polymerase chain reaction (RT-PCR). HAV does not induce cytopathic changes in culture but tends to establish persistent infections and remain largely cell associated. However, primary isolation of wild-type virus is difficult and several weeks elapse before antigen is detectable; thus, virus isolation is not a practical diagnostic technique in routine laboratories.

Adaptation to growth in cultured cells occurs with repeated passage, with more rapid production of intracellular antigen and higher final yields. Virus adapted to growth in cell culture may become attenuated and the nucleotide sequences of wild-type and attenuated strains have been compared. There is evidence that changes in the 5' noncoding region and the region encoding non-structural (2B/2C) polypeptides may be associated with attenuation and adaptation to cell culture.

Only one serotype of hepatitis A has been identified in human volunteers infected experimentally, in patients from different outbreaks of hepatitis A and in naturally- and experimentally-infected chimpanzees and other primates. This also has been confirmed by cross-neutralization tests and by the protective efficacy

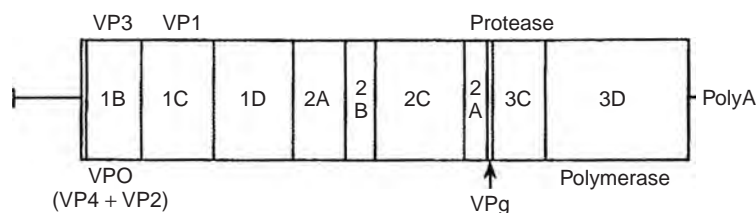


Figure 12.4 Organization of the HAV genome. See text for details.

of pooled human immunoglobulin obtained from different geographical regions. However, strain-specific differences exist, at least at the level of genomic nucleotide sequences from different isolates of HAV. Phylogenetic analysis of the nucleotide sequences of various HAV strains reveals that these can be classified into six genotypes; genotypes I, II and III are of human origin and genotypes IV, V and VI were isolated from Old World monkeys. All strains have a highly-conserved single immunodominant epitope which generates cross-reactive, neutralizing antibodies.

Pathogenesis

The mechanisms underlying liver injury in hepatitis A are not understood. The initial noncytopathic phase, during which virus replicates and is released, is followed by decreased virus multiplication and inflammatory cell infiltration, suggesting that immune mechanisms are involved in pathogenesis. Experimental evidence suggests that HLA-restricted, virus-specific T cells play a significant role in HAV-related hepatocellular injury. T-cell clones have been derived from patients with acute hepatitis A and analysed for their phenotype. CD8⁺ clones isolated during the acute phase of the disease predominate over CD4⁺ clones; these CD8⁺ clones have cytotoxic activity and show specific cytotoxicity against autologous fibroblasts infected with HAV. These data support the hypothesis that liver-cell injury in acute HAV infection is mediated by HAV-specific CD8⁺ T lymphocytes and is not entirely due to an intrinsic cytopathic effect of the virus itself. The molecular targets of these cells are unknown. Serum-neutralizing antibodies protect against HAV infection.

Laboratory Diagnosis

Specific diagnosis of hepatitis A can be established by demonstrating the virus in faeces by EIA and radioimmunoassay, or by immune electron microscopy (IEM) and RT-PCR. Isolation of the virus in cell cultures is not appropriate for routine diagnosis.

As stated above, the capsid antigen is highly conserved and there is only a single serotype of HAV. Specific serological tests for hepatitis A antigen (HAAg) and antibodies include radioimmunoassay and EIAs. Hepatitis A antibody (anti-HAV) is always demonstrable by such assays during the early phase of the illness, and titres increase rapidly (Figure 12.5). Because antibody develops very early in the course of the infection, serological diagnosis of recent infection can be established by titrations of serial samples of serum or, more conveniently, by the demonstration of hepatitis A antibody of the immunoglobulin M (IgM) class, which is the simplest and most economical method of establishing the diagnosis. Hepatitis A IgM is detectable in serum for 45–60 days after the onset of symptoms. Liver biopsy is not usually required in acute hepatitis A. Titres of anti-HAV IgG rise with convalescence and the antibody usually persists for many years. Recovery from infection is associated with lifelong immunity.

Clinical Course

Clinical disease with jaundice is uncommon in infants and young children and the infection may pass unnoticed in this group. The high seroprevalence of anti-HAV antibodies in developing countries (more than 70% of adults) is largely due to a high rate of asymptomatic

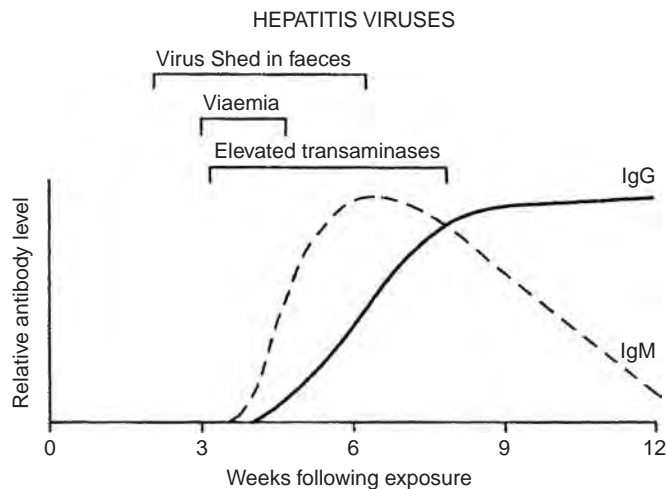


Figure 12.5 Serological profile during uncomplicated hepatitis A.

infection in childhood. Infection in adulthood results in acute icteric hepatitis in more than 70% of cases. The case fatality rate is 0.3–1.8%; the risk of serious complication increases significantly with age, and severe hepatitis is correspondingly more common in older persons. Recurrent hepatitis has been observed in patients (and experimentally-inoculated Saimiri monkeys with acute disease) and may be associated with shedding of virus in stools. The relapses are generally benign, with eventual complete resolution. The absence of chronic infection indicates the effectiveness of the host immune response to HAV. In genetically-susceptible individuals, with a defect in suppressor-inducer T lymphocytes controlling immune responses to the asialoglycoprotein receptor, it is possible that hepatitis A may trigger an autoimmune chronic hepatitis. Such individuals may develop a persistent response to the asialoglycoprotein receptor and, rarely, autoimmune hepatitis after subclinical hepatitis A. Approximately 5% of patients with acute hepatitis A will develop cholestatic hepatitis, characterized by pruritis and steatorrhea.

Prevention and Control of Hepatitis A

Control of infection is difficult. Strict isolation of cases is not a useful control measure because faecal shedding of the virus is at its highest during the late incubation period and prodromal phase of the illness. Spread of hepatitis A is reduced by simple hygienic measures and the sanitary disposal of excreta.

Normal human immunoglobulin (NHIG), containing at least 100 IU/ml of anti-HAV, will prevent or attenuate a clinical illness if given intramuscularly before exposure to the virus or early during the incubation period. The dosage should be at least 2 IU of anti-HAV/kg body weight, but in special cases such as pregnancy or in patients with liver disease the dosage may be doubled. Immunoglobulin does not always prevent infection or excretion of HAV, and inapparent or subclinical hepatitis may develop. The efficacy of passive immunization is based on the presence of hepatitis A antibody in the immunoglobulin, but the minimum titre of antibody required for protection has not been established. Immunoglobulin is used most commonly for close personal contacts of patients with hepatitis A and for those exposed to contaminated food. Immunoglobulin has also been used effectively for controlling outbreaks in institutions such as homes for the mentally handicapped and in nursery schools.

Prior to the availability of a vaccine, pre-exposure prophylaxis with immunoglobulin was recommended for persons without hepatitis A antibody visiting highly-endemic areas. After a period of six months the administration of immunoglobulin for travellers had to be repeated, unless it could be demonstrated that the recipient had developed

his own hepatitis A antibodies. Because the shift in herd immunity led to reduced titres of anti-HAV in normal immunoglobulin in developed countries and a highly immunogenic vaccine became available, a single dose of vaccine may be offered to travellers, with the option of a booster dose 6–12 months later. NHIG (given at a contralateral site) is a valuable addition to the vaccine for individuals who may have been exposed to infection.

Hepatitis A Vaccines

There has been considerable interest in the development of both killed and attenuated hepatitis A vaccines, and inactivated vaccines are now licensed in many countries. The virus grows poorly in cell culture but yields have been improved by adaptation and are sufficient to permit gradient purification. This virus is inactivated with formaldehyde; the antigen is adsorbed to aluminium hydroxide and given intramuscularly. These preparations are safe and immunogenic in man and have been shown to induce a protective immune response. Safety and immunogenicity studies and efficacy trials have been undertaken in volunteers with formaldehyde-inactivated, Al(OH)₃-adsorbed vaccines (Havrix, GlaxoSmithKline (GSK), or Vaqta, Merck Research Laboratories). The anti-HAV response includes neutralizing antibodies. The currently-licensed vaccines appear to be well tolerated and immunogenic at doses of 720–1440 arbitrary ELISA units (GSK) or 125–50 U (Merck). In the case of the GSK vaccine licensed in the United Kingdom, a single dose of 720 ELISA units of hepatitis A viral protein is sufficient for protection of adults of 16 years and over, with the option of a booster dose 6–12 months later. A juvenile formulation with 320 ELISA units per dose is available. Active immunization induces higher levels of both total and neutralizing antibodies than NHIG.

Inactivated vaccines of this type are useful for the protection of travellers from prosperous countries but are likely to be too costly for use in the developing world, where exposure usually occurs early in life. In developed countries the vaccine should be given to travellers to countries where HAV is endemic, as well as to armed forces personnel, diplomats, staff of children's day-care centres and institutions for intellectually-handicapped individuals, male homosexuals, intravenous drug abusers, haemophiliacs and sewage workers. The addition of human immunoglobulin used to be recommended when travel was to occur within four weeks of receipt of vaccine, because of concern about the time taken to develop neutralizing antibodies. However, a single dose of vaccine (1440 ELISA units) is effective. Vaccine should be offered even when given up to the day of travel; ideally travellers should be given prophylaxis at least four weeks before, but they should not be denied prophylaxis on the strength

of the available evidence. After vaccination, anti-HAV antibodies may be detectable rapidly, although the minimum protective antibody level is uncertain.

Data in chimpanzees suggest protection against infection if vaccine is administered shortly after exposure. The curtailment of Alaskan and Italian outbreaks, achieved by immunization with a single dose without concurrent administration of immune globulin, provides supportive evidence for at least some efficacy of one dose for post-exposure prophylaxis (McMahon *et al.*, 1996; Sagliocca *et al.*, 1999). Serological screening for susceptibility may be indicated in appropriately-selected patients. The cost-benefit ratio of HAV vaccination is probably most beneficial when vaccine is given to frequent travellers to endemic areas. Where practical, testing for antibodies to hepatitis A prior to immunization may be indicated in those aged 50 years or over, those born in areas of high endemicity and those with a history of jaundice. HAV vaccine also is recommended for individuals with significant chronic liver disease due to hepatitis B or C and who may have an increased risk of fulminant hepatic failure following HAV infection. A combined hepatitis A and B vaccine is available and licensed for use in adults and children at risk of both infections, and a combined hepatitis A and typhoid vaccine is available and appropriate for use in travellers to areas where typhoid is endemic. Other combined inactivated polyvalent vaccines are under development (Zuckerman, 2006).

Attenuated strains of HAV have been developed and potentially may be useful as vaccines. This approach is attractive because live vaccines may be cheaper to produce (the attenuated virus grows more efficiently in cell culture), can be given orally and may induce a mucosal antibody response. As for vaccine strains of polioviruses, attenuation may be associated with mutations in the 5' noncoding region of the genome which affect secondary structure. There is also evidence that mutations in the region of the genome encoding the nonstructural polypeptides (Figure 12.4, region 2B/2C) may be important for adaptation to cell culture and attenuation. However, the markers of attenuation of HAV are not as well defined as those of the *Polioviruses* and reversion to virulence may be a problem also. There is also concern that 'over-attenuated' viruses may not be sufficiently immunogenic. However, despite these caveats, live attenuated hepatitis A vaccines (H2 and L-A-1 strains) are licensed and used effectively in China.

HEPATITIS E

Epidemic hepatitis, which resembled but was serologically distinct from hepatitis A, was reported initially in

the Indian subcontinent and later in central and South East Asia, the Middle East, north Africa and Central America (reviewed by Panda *et al.*, 2007). The disease is also a common form of acute sporadic hepatitis in these areas and other developing countries. Sporadic cases have been observed in developed countries among migrant labourers and travellers returning from such areas. In contrast to prior epidemics of enterically-transmitted non-A, non-B hepatitis (ET-NANBH), HEV was found to be a common cause of acute hepatitis in a paediatric population in Egypt. Seroprevalence studies in Hong Kong suggest that hepatitis E accounts for a third of non-A, non-B, non-C hepatitis, and that co-infection of hepatitis A and E can occur. More recently, indigenous cases of hepatitis E have been reported in the United States and several European countries, including the United Kingdom. Rarely, HEV may be transmitted by blood transfusion in countries where the disease is endemic.

The average incubation period is slightly longer than for hepatitis A, with a mean of six weeks. The infection is acute and self-limiting. Clinical disease occurs predominantly in young adults, and high mortality rates (up to 20%) have been reported in the third trimester of pregnancy. The infection is spread by the ingestion of contaminated water and probably by food, but secondary clinical cases seem to be uncommon.

Virus-like particles have been detected in the stools of infected individuals by IEM using convalescent serum (Figure 12.6). However, such studies have often proved inconclusive and a large proportion of the excreted virus may be degraded during passage through the gut. The particles are slightly larger than those of hepatitis A, with a mean diameter of 32–34 nm. Cross-reaction studies between sera and virus in stools associated with a variety of epidemics in several different countries suggest a single viral serotype.

Biology of HEV

HEV was first transmitted to cynomolgous macaques, and subsequently to a number of other species of monkeys and chimpanzees. The gallbladder bile of infected monkeys was found to be a rich source of virus, enabling the molecular cloning of DNA complementary to the HEV (RNA) genome and elucidation of the entire 7.5 kb sequence (Tam *et al.*, 1991). The organization of the genome is distinct from the picornaviruses; the non-structural polypeptides are encoded in the 5' region and the structural polypeptides at the 3' end. Although HEV resembles the *Caliciviruses* in the size and organization of its genome, as well as the size and morphology of the virion, the HEV genome differs in having a capped 5' end (rather than a genome-linked protein) and in the organization of the ORFs. Therefore, HEV has been classified

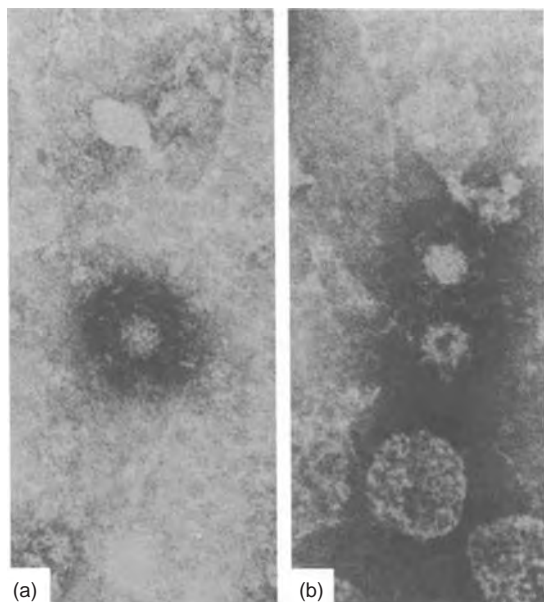


Figure 12.6 IEM of HEV from a faecal extract. (a) Particle coated with predominately IgG. (b) Particles coated with predominately IgM. (Source: Purcell and Ticehurst, 1988, with permission.)

in its own genus, *Hepevirus*, but has not been formally assigned to a virus family.

Organization of the HEV Genome

The HEV genome is a polyadenylated, positive-sense RNA of around 7500 nt and contains three ORFs (Figure 12.7). The first, of approximately 5 kb, begins 28 nt from the 5' end of the genome and encodes motifs associated with NTP-binding, helicase and RNA-dependent RNA polymerase activities. A second ORF of around 2 kb begins 37 nt downstream of the first, terminates 68 nt from the poly(A) tail and is believed

to encode the structural polypeptides. The third ORF is very short (369 nt) and overlaps the other two. The viral proteins may be subject to proteolytic processing and other post-translational modifications.

Sequencing of the HEV genome has enabled the development of a number of specific diagnostic tests. For example, HEV RNA was detected using RT-PCR in stool samples obtained during an epidemic in Kanpur (north India) and may also be detected in infected liver. EIAs, which detect IgG and IgM anti-HEV, have been developed and are commercially available.

Pathogenesis

Macaque monkeys develop changes in acute viral hepatitis associated with a rise in liver enzymes, the presence of HEV-specific viral particles in the stool and histological changes in the liver from 21 to 45 days after HEV inoculation. Subclinical hepatitis E also may occur in experimental primates, and subclinical infections of humans may provide a reservoir for transmission. Ultrastructural changes in the livers of these experimental monkeys include infiltration of lymphocytes and polymorphonuclear leucocytes around the necrotic area, swelling of mitochondria, dilation of smooth endoplasmic reticulum (ER) and the presence of 27–34 nm virus particles during the acute phase of the disease. It is not known whether these changes reflect cytopathic liver injury or are immune-mediated.

Diagnosis

With the availability of recombinant antigens and synthetic peptides, serological assays were developed to test for antibody to hepatitis E virus (anti-HEV). IgM is detected infrequently at initial presentation and disappears by three months after jaundice. IgG titres initially can be quite high, but tend to wane over time. The diagnosis can be confirmed by RT-PCR on faecal material from acutely-infected patients, and the serum may also be positive with a transient

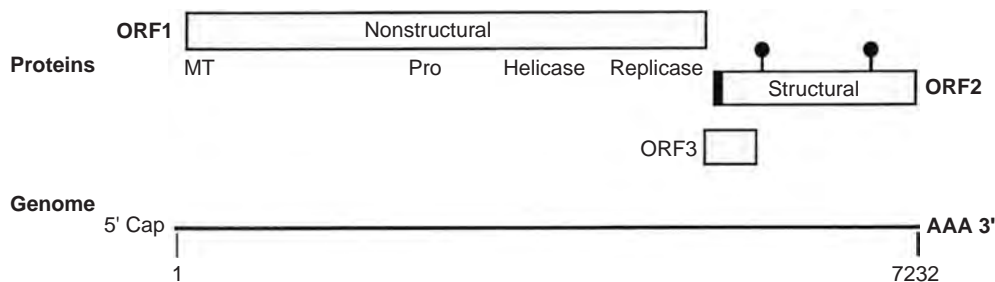


Figure 12.7 Organization of the hepatitis E virus genome. Motifs within the ORF1 protein include a methyl transferase (MT), protease (Pro), helicase and RNA-dependent RNA polymerase (replicase). The shaded area at the amino terminus of the ORF2 protein indicates a signal sequence, and 'lollipop' indicate potential glycosylation sites.

viraemia. Attack rates have been higher in males than females and for adults rather than children. Evidence of secondary intra-familial spread is uncommon.

Clinical Features

In general, the disease is self-limited, with no evidence of chronic infection. Cholestatic features are common and may be prolonged. Liver biopsies obtained during the acute illness show portal inflammation and cytoplasmic cholestasis. Stool specimens may reveal 27–32 nm virus-like particles when tested by IEM but RT-PCR is a more reliable assay for the virus. An observational study appears to confirm a high rate of severe or fulminant hepatitis in pregnant women, with a mortality rate of 20% during the third trimester. Babies born to women with acute disease are at risk of vertical transmission and may be at risk of perinatal morbidity, and infant mortality of up to 30% has been observed. Liver transplantation is indicated for fulminant hepatitis E if survival otherwise seems unlikely.

Immunization

Although individuals who recover from hepatitis E mount an antibody response, including to putative capsid antigens, it is not clear whether these antibodies protect against subsequent infection or, if so, how long that immunity lasts. Adult populations in endemic areas are susceptible to hepatitis E, with high attack rates in epidemics. The degree and longevity of protective immunity of macaque monkeys following recovery from experimental infection or immunization with recombinant DNA or antigen preparations is controversial. Immunization with a bacterially-expressed fusion protein, derived from the capsid region of the Burmese strain, protected cynomolgus macaques from challenge with the homologous strain of virus. However, monkeys challenged with the Mexican strain excreted virus in their faeces, although there was no biochemical evidence (i.e. raised serum aminotransferases) of liver disease and in another study an immunogen derived from the capsid region of the Pakistan strain was found to protect against infection with the Mexican strain. Aside from the duration of a protective antibody response, other caveats are that animals are frequently challenged intravenously with HEV, rather than via the natural (oral) route, and that macaques do not suffer overt symptoms of hepatitis during infection; protection often is defined as the absence of elevated aminotransferases despite evidence of virus replication (excretion in the faeces). Nonetheless, a baculovirus-expressed HEV vaccine (spanning aa 112–607 of the ORF 2 protein of the Pakistan strain) is undergoing a phase II/III clinical trial in Nepal, and the initial data show that the immune response protects against infection (Shrestha *et al.*, 2007).

Sporadic Hepatitis E in Industrialized Countries Is Predominantly Zoonotic in Origin

The genomic sequences of HEV initially isolated from several Asian countries (Pakistan, China, India) were found to be similar to the Burmese prototype (now recognized as genotype 1) and less closely related to the strain isolated from Mexico (genotype 2). Genotype 1 viruses are associated with epidemic and sporadic hepatitis E throughout Asia and Africa, and genotype 2 viruses have been described in Nigeria. A third genotype of HEV was discovered in several herds of swine in the United States (Meng *et al.*, 1997) and has been implicated as the cause of rare cases of indigenous hepatitis E in individuals in North and South America, Europe and Japan. Genotype 4 HEV, which has been described in China (Wang *et al.*, 2000), Taiwan, Japan, Vietnam and India, seems to be the major cause of sporadic hepatitis E in some Asian countries and has also frequently been found infecting pigs. Thus, it seems that genotype 3 and 4 HEV are responsible for sporadic cases of hepatitis E in humans that arise from zoonotic infection, with farmed pigs as a major reservoir. In contrast, genotype 1 viruses, which have been implicated in all the major Asian epidemics of hepatitis E, including in the Xinjiang Uighur autonomous region of China in 1988–1990, have not been isolated from or transmitted to pigs. Such epidemics typically result from massive faecal contamination of the water supply and the source of such contamination remains to be elucidated. In the United Kingdom, the rare, sporadic cases of hepatitis E are typically genotype 1 infections of travellers returning from endemic areas or genotype 3 infections acquired locally (Ijaz *et al.*, 2005).

HEPATITIS B

Epidemiology

The discovery in 1965 of Australia antigen (now referred to as hepatitis B surface antigen, HBsAg) and the demonstration by Blumberg and his colleagues, and others, of its association with type B hepatitis (Blumberg *et al.*, 1965) led to rapid and unabated progress in the understanding of this complex infection. Hepatitis B remains a globally-important disease and over one third of the world's population has been infected with HBV. Low- (less than 2% of the population seropositive for HBsAg), intermediate- (2–8%) and high- (more than 8%) prevalence areas are recognized (Table 12.2). Several epidemiological studies indicate that the reported rates of hepatitis B infection have declined in western and northern Europe and the United States. Infection rates in children have declined in high-prevalence areas where universal immunization of infants has been introduced.

Table 12.2 Prevalence of hepatitis B

	Northern Europe	Eastern Europe	Parts of China
	Western Europe Central Europe North America Australia	Mediterranean USSR South-west Asia Central America South America	South East Asia Sub-Saharan Africa
HBsAg	0.2–0.5%	2–7%	8–20%
Anti-HBs	4–6%	20–55%	70–95%
Neonatal infection	Rare	Frequent	Very frequent
Childhood infection	Infrequent	Frequent	Very frequent

In the past, hepatitis B was diagnosed on the basis of infection occurring approximately 60–180 days after the injection of human blood or plasma fractions or the use of inadequately-sterilized syringes and needles. The development of specific laboratory tests for hepatitis B confirmed the importance of the parenteral routes of transmission, which may result from accidental inoculation of minute amounts of blood or fluid contaminated with blood during medical, surgical and dental procedures; immunization with inadequately-sterilized syringes and needles; intravenous and percutaneous drug abuse; tattooing; body piercing; acupuncture; laboratory accidents; and accidental inoculation with razors and similar objects that have been contaminated with blood. However, several factors have altered the epidemiological concept that hepatitis B is spread exclusively by blood and blood products. These include the observations that under certain circumstances the virus is infective by mouth, that it is endemic in closed institutions and institutions for the mentally handicapped, that it is more prevalent in adults in urban communities and in poor socio-economic conditions, that there is a reservoir of over 350 million carriers of HBV in the human population, and that the carrier rate and age distribution of the surface antigen vary in different regions. There is much evidence for the transmission of hepatitis B by intimate contact and by the sexual route. Those with a large number of sexual partners, particularly male homosexuals, are at very high risk. HBsAg has been found in blood and in various body fluids such as saliva, menstrual and vaginal discharges, seminal fluid, colostrum and breast milk, and serous exudates, and these have been implicated as vehicles of transmission of infection.

Perinatal Transmission

Viraemic mothers, especially those who are seropositive for hepatitis B e antigen (HBeAg), almost invariably transmit the infection to their infants. The mechanism of perinatal infection is uncertain, but it probably occurs during or shortly after birth as a result of a leak of maternal

blood into the baby's circulation, or of its ingestion or inadvertent inoculation. Such perinatal infections lead to a high rate of chronicity, estimated at around 90%, and individuals infected at such an early age exhibit a high degree of immune tolerance and may remain viraemic for decades. Perinatal transmission is an extremely important factor in maintaining the reservoir of the infection in high-prevalence regions. There is also a substantial risk of perinatal infection if the mother had acute hepatitis B in the second or third trimester of pregnancy or within two months of delivery. Although HBV can infect the fetus *in utero*, this appears to be rare and is generally associated with ante-partum haemorrhage and tears in the placenta.

However, mother-to-infant transmission does not account for at least 50% of infections in children, and horizontal transmission—that is, from child to child—is equally important. The prevalence in children is quite low at 1 year of age but increases rapidly thereafter, and in many endemic regions reaches a peak in children 7–14 years of age. Clustering of HBV also occurs within family groups, but does not appear to be related to genetic factors and does not reflect maternal or venereal transmission. The probability of a childhood infection becoming persistent declines with age, from around 90% in neonates to below 5% in adolescence.

Chronic Hepatitis B

Chronic hepatitis B is defined as persistence of HBsAg in the circulation for more than six months. The 'carrier state' may persist for life and may be associated with liver damage, varying from mild chronic hepatitis to severe, active hepatitis, cirrhosis and primary liver cancer. Several risk factors have been identified in relation to its development. It is more frequent in males, more likely to follow infections acquired in childhood than those acquired in adult life, and more likely to occur in patients with natural or acquired immune deficiencies. Chronic hepatitis B infection occurs in 90% of neonates or young infants, but in only 1–5% of immunocompetent adults. In countries

where hepatitis B infection is common, the highest prevalence of HBsAg is found in young children, with steadily declining rates among older age groups. HBeAg has been reported to be more common in young than in adult carriers of hepatitis B, whereas the prevalence of anti-HBe seems to increase with age.

Survival of HBV is ensured by the reservoir of carriers, estimated to number over 350 million worldwide. The prevalence of carriers, particularly among blood donors, in northern Europe, North America and Australia is 0.1% or less; in central and eastern Europe it is up to 5%; in southern Europe, the countries bordering the Mediterranean, and parts of Central and South America the frequency is even higher; and in some parts of Africa, Asia and the Pacific region as much as 20% of the apparently healthy population may be HBsAg positive (Table 12.2).

Biology

In addition to the human HBV, a number of similar viruses which infect mammals and birds (but are not infectious for humans) have been described and the virus family has been named *Hepadnaviridae* (hepatotropic DNA viruses). The viruses have a similar genetic organization and mode of replication and are characterized by a high degree of host specificity and tropism for the liver. Of the mammalian viruses, the woodchuck hepatitis virus (WHV) and Beechey ground squirrel hepatitis virus (GSHV) have been well characterized, the former being of interest as a model system for primary liver cancer because there is a high probability of progression to tumour for WHV-infected woodchucks. Of the avian viruses, the Pekin duck hepatitis B virus (DHBV) has been well characterized, and this animal model has been valuable in the elucidation of the *Hepadnavirus* replication process and for testing antiviral drugs.

Structure of Hepatitis B Virus

Electron microscopy of HBV-positive serum reveals three morphologically-distinct forms of particle (Figure 12.8). The small, 22 nm spherical particles and the tubular forms of roughly the same diameter are composed of the virus-surface protein embedded in lipid and are synthesized in vast excess over the 42 nm, double-shelled virions. The latter comprise a 27 nm, electron-dense core surrounded by HBsAg that is distinct from the 22 nm spheres in that pre-S1 epitopes are present (see below). The core or nucleocapsid consists of the genome surrounded by a second protein, HBcAg. HBeAg is found in soluble form in virus-positive sera and is related to the core antigen as described below. The genome is DNA (Figure 12.9) and comprised of two strands held in a circular configuration by base-pairing at the 5' ends (cohesive

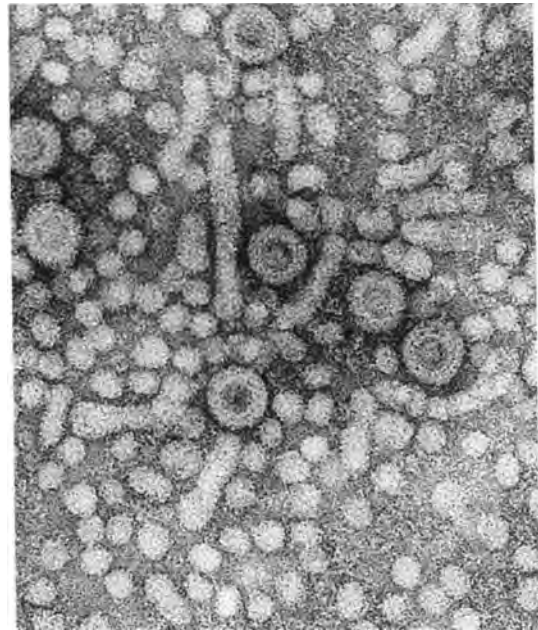


Figure 12.8 Electron micrograph of serum containing HBV after negative staining. The three morphological forms of the antigen are shown: (1) small pleomorphic spherical particles 20–22 nm in diameter (hepatitis B surface antigen); (2) tubular structures (a form of the surface antigen); (3) the 42 nm double-shelled virions, with a core surrounded by the surface antigen ($\times 300\,000$). (Source: (Zuckerman, 1975).)

end regions). One of the strands is incomplete (usually 50–80% of full length) and is associated with a polymerase which is able to fill in the single-stranded region when provided with suitable substrates.

The genomes of a variety of isolates of HBV have been cloned, and the complete nucleotide sequences determined. There is some variation in sequence (up to 12% of nucleotides) between these isolates, and up to eight genotypes (A to H) have been described on the basis of >8% nucleotide sequence divergence, and recombinant forms have also been recognized. However, the genetic organization and other essential features are conserved. The genome is around 3200 bp in length and analysis of the protein-coding potential reveals four conserved ORFs, the products of which are described below. These four ORFs are located on the same DNA strand, and the strands of the genome have accordingly been called 'plus' (incomplete strand) and 'minus' (complete strand), as shown in Figure 12.9. Other features include a motif of 11 bp, which is directly repeated near to the 5' end of each strand of genomic DNA (DR1 and DR2) and plays an essential part in the replication strategy, two transcriptional enhancer

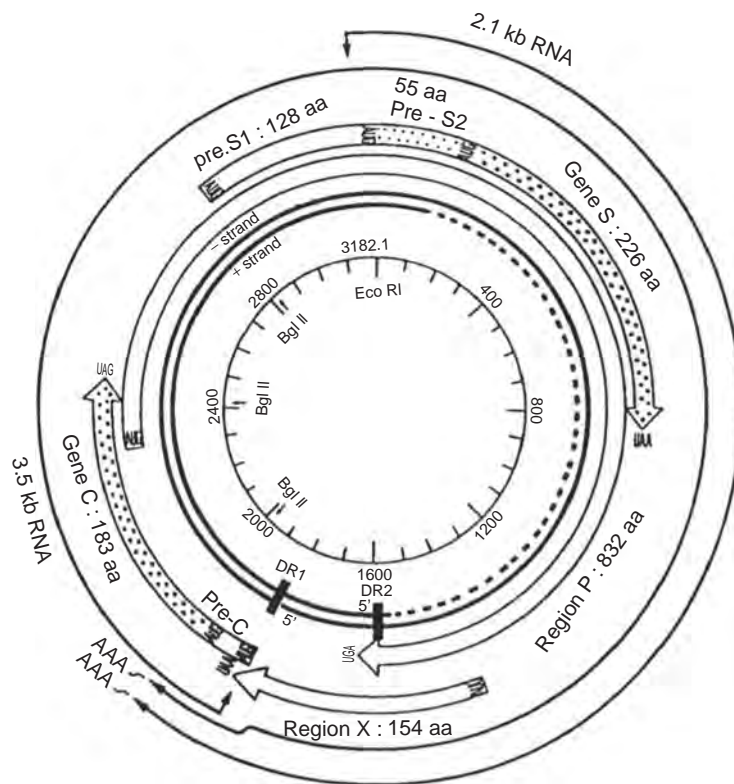


Figure 12.9 Structure and genetic organization of the HBV genome. The broad arrows surrounding the genome represent the four large ORFs of the L (–) strand transcript. The number of amino acids (aa) encoded is indicated in each case. The two thin arrows surrounding the broad arrows represent the two major HBV mRNAs. The partial restriction map and the numbering of the nucleotides indicated on the inner circle correspond to the ayw3 genome. DR1 and DR2 are the directly-repeated sequences involved in the replication of the genome. (Source: (Tiollais *et al.*, 1985).)

elements and binding sites for glucocorticoids and other cellular factors.

Hepatitis B Surface Antigen, HBsAg

The major (or small) surface protein is 226 amino acids long and found in nonglycosylated (p24) and glycosylated (gp27) forms. It is encoded in the 3' half of the surface ORF and translated from the third of three in-phase initiation codons. Larger, pre-S proteins are translated utilizing the two upstream initiation codons; translation from the second results in two intermediate-sized glycoproteins (gp33 and gp36) with a glycosylated 55 amino acid N-terminal extension, the pre-S2 domain. These middle surface proteins are minor components of virions and subviral particles. Translation of the entire ORF (pre-S1 + pre-S2 + S) gives rise to the large surface proteins (p39 and gp42) which are found predominantly in virions, but also in the 22 nm tubular forms. A domain within the pre-S1 region seems to be responsible for the attachment

of the virus to the (unidentified) receptor on the hepatocyte. Synthesis of the pre-S1 protein also may act as a signal for virion assembly in the infected cell.

The subviral particles and the virion surface are composed of HBsAg anchored in a lipid bilayer derived from the ER of the host cell. The major antigenic determinant on the particles is the common, group-specific antigen, *a*, which may form a 'double loop' structure (aa 124–137 and aa 139–147) on the surfaces of the virions and subviral particles. The formation of anti-*a* antibodies following vaccination seems to be sufficient to confer protective immunity. The major HBsAg protein also carries a pair of mutually-exclusive subdeterminants, *d* or *y* and *w* or *r*, which, in each case, correlate with variation at single amino acid positions (aa 122 and 160, respectively). Thus, four principal phenotypes of HBsAg are recognized—*adw*, *adr*, *ayw* and, more rarely, *ayr*—and these show differing geographical distributions. For example, in northern Europe, the Americas and Australia subtype *adw* predominates, whilst *ayr* occurs in a

broad zone that includes northern and western Africa, the eastern Mediterranean, eastern Europe, northern and central Asia and the Indian subcontinent. Both *adv* and *adr* are found in Malaysia, Thailand, Indonesia and Papua New Guinea, whereas subtype *adr* predominates in other parts of South East Asia, including China, Japan and the Pacific Islands. Historically, the subtypes provided useful epidemiological markers of HBV, but today phylogenetic analysis is rather more informative.

Unusual variants which lack the group-specific antigen *a* may be selected by antibody in immunized infants infected perinatally and in persistently-infected individuals following treatment with hepatitis B immune globulin or a natural antibody response. Surface variants of HBV were first described in Italy, infecting children and adults in the presence of specific hepatitis B surface antibodies (anti-HBs) several months after successful immunization with two generally-licensed hepatitis B vaccines given with and without hepatitis B immunoglobulin (HBIG). Epitope mapping of HBsAg in these patients revealed that monoclonal antibodies which normally bind to the *a* determinant failed to bind, and further work established that, in at least one case, the infection was caused by a variant with a mutation leading to a substitution of arginine for glycine at amino-acid position 145 in the immunodominant domain. This change, which seems to have been selected by antibody, has since been observed independently in the United States, Singapore, Japan and elsewhere. Other mutations that lead to altered HBsAg, which escapes neutralization by anti-HBs, have been described, but the arginine-for-glycine substitution at aa 145 seems to be the most common (reviewed by Harrison, 2006).

Hepatitis B Core Antigen and Other Viral Proteins

The core protein (p22) is the major component of the nucleocapsid and has, at its carboxyl terminus, an arginine-rich domain which presumably interacts with the viral nucleic acid. The importance of antibodies to this protein (anti-HBc) in diagnosis of infection is discussed below. The core protein is translated from the second initiation codon in the core ORF (Figure 12.9). Translation from the upstream initiation codon yields a precursor protein (p25), which is processed to yield HBeAg. The precore region, between the two initiation codons, encodes a signal sequence which directs p25 to the ER, where it is cleaved by a cellular signal peptidase. HBeAg is secreted following further proteolysis, which removes the carboxyl-terminal domain. HBeAg is also expressed on the surface of the infected hepatocyte and is a major target for the cellular immune system. HBeAg is not an essential protein of the virus but it may cross the placenta during pregnancy, inducing tolerance in

the fetus and increasing the probability that a perinatal infection will progress to chronicity. Variants of HBV with mutations in the precore region (precore mutants), and which are defective for the synthesis of HBeAg, are discussed below.

The P ORF, which overlaps the other three, encodes the viral polymerase. This enzyme has both DNA- and RNA-dependent activities and the predicted amino acid sequence has been shown to have homology with retroviral reverse transcriptases (RTs). The polymerase protein also acts as the primer for minus-strand DNA synthesis and has an 'RNase H' activity which degrades the RNA pregenome during minus-strand synthesis. The fourth ORF has been termed X because the function of its product originally was obscure. It is now known that this protein acts as a transcriptional transactivator and may enhance the expression of the other viral proteins. Experiments using the woodchuck model (see below) confirm that a functional X gene is required for the establishment of infection *in vivo*.

Replication of the Virus

The essential elements of the replication strategy of the *Hepadnavirus* genome were elucidated by Summers and Mason (1982) in an elegant series of experiments utilizing subviral cores isolated from DHBV-infected duck hepatocytes. The *Hepadnaviruses* are unique among animal DNA viruses in that they replicate through an RNA intermediate (Figure 12.10). On infection of the hepatocyte, the viral DNA is uncoated and converted to covalently-closed circular deoxyribonucleic acid (cccDNA) in the nucleus and forms a minichromosome that is the template for transcription of the viral RNAs. There are at least four viral promoters and all of the RNAs are 3'-co-terminal, being polyadenylated in response to a signal in the core ORF. The largest RNAs are greater than genome length (approximately 3500 nucleotides) and comprise the precore RNA, the messenger ribonucleic acids (mRNAs) for the synthesis of HBeAg, and the pregenomic RNA, which, in addition to being the mRNA for HBcAg and the viral polymerase, is also the template for reverse transcription. Binding of the polymerase to the packaging signal (ϵ) at the 5' end of the pregenome leads to packaging into immature viral cores in the cytoplasm (Figure 12.10, step 1). The amino terminal domain of the viral polymerase acts as the primer for minus-strand DNA synthesis and, following synthesis of a four-nucleotide nascent strand, translocates to a complementary four-base sequence (τ) near to the 3' end of the RNA template (Figure 12.10, steps 2a and 2b) This protein remains covalently attached to the 5' end of that strand in the mature virion. Minus-strand synthesis proceeds with

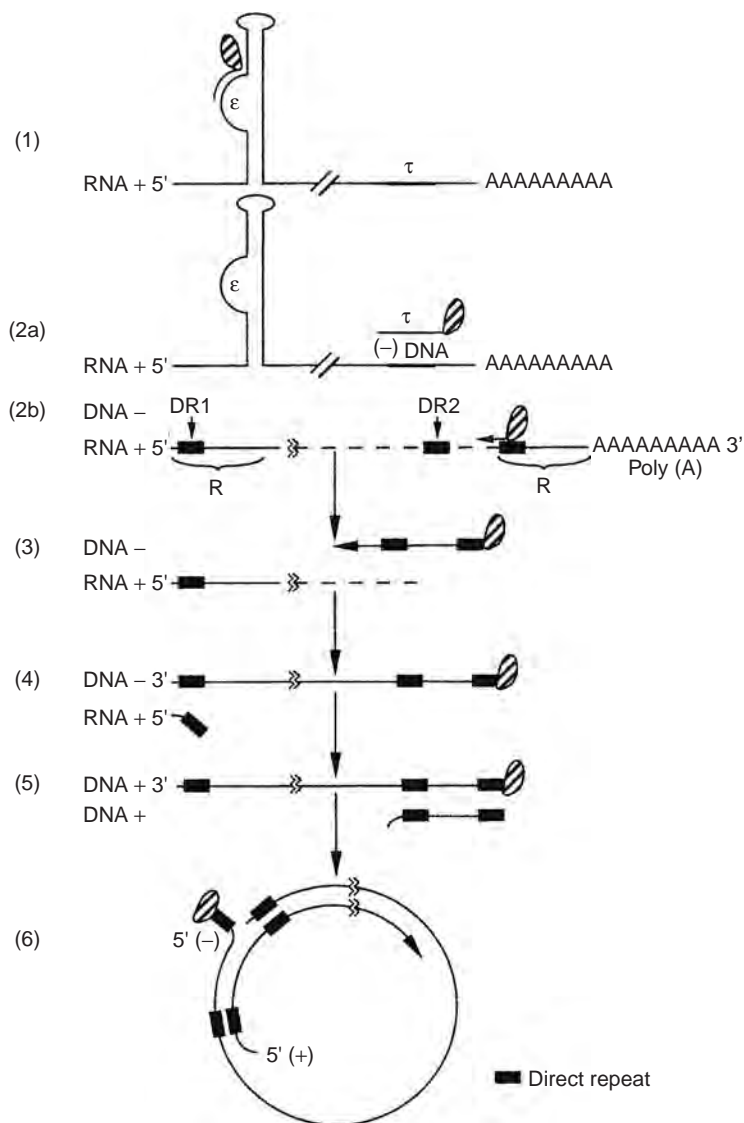


Figure 12.10 The replication strategy of the HBV genome: (1) priming of minus (-) strand synthesis by the terminal protein (cross-hatched) on the bulge of the ϵ signal near the 5' end of 3.5 kb pre-genomic RNA; (2a) translocation of the primer to a position (τ) near the 3' end of the RNA template; (2b) the template shown as a linear structure, solid boxes indicate the direct repeat (DR) sequences and R the redundancy of the RNA; (3) minus (-) strand DNA synthesis by reverse transcription with concomitant hydrolysis of the RNA template (RNase H-like activity); (4) and (5) translocation of the remaining 5' oligoribonucleotide from DR1 to DR2 and priming of plus (+) strand DNA synthesis; (6) circularization of the genome through the redundancy in the minus (-) strand and continuation of plus (+) strand synthesis.

reverse transcription of the pre-genome by the viral polymerase and with concomitant degradation of the template (RNase H-like activity; Figure 12.10, step 3). The remaining oligoribonucleotide (which was the 5' end of the pregenome) is at the position of the direct

repeat, DR1 (Figure 12.10, step 4), and is now believed to translocate to the other copy of the direct repeat, DR2, on the minus strand, and to prime synthesis of the plus strand (Figure 12.10, step 5). The minus strand has a short terminal redundancy (approximately eight

nucleotides) which permits the circularization of the genome as the plus strand is synthesized (Figure 12.10, step 6). Completion of the core presumably starves the polymerase of precursor nucleoside triphosphates, leaving the plus strand incomplete. The cores then bud through internal membranes embedded with HBsAg to form mature virus particles.

HBV Infection and Chronic Hepatitis B

Following infection, the first marker to appear in the circulation is HBsAg, which becomes detectable two to eight weeks prior to biochemical evidence of liver damage or the onset of jaundice (Figure 12.11). Next to appear are the markers of the virion, such as the virus-specific DNA polymerase activity and the viral DNA, along with the soluble antigen, HBeAg. Antibody to the core (anti-HBc) is detectable two to four weeks after the appearance of the surface antigen; this persists throughout the infection and

after recovery. In acute infections, clearance of the virus is marked by seroconversion, with the disappearance of HBeAg and the appearance of antibodies to it (anti-HBe). Later, during convalescence, HBsAg also disappears, with the production of anti-surface antibody (anti-HBs).

In 2–10% of infected adults, and a much larger percentage of children infected perinatally, the immune system fails to clear virus replication and the infection persists. Chronic hepatitis B may be divided into several phases (Figure 12.12). In the first, the immune-tolerance phase, high levels of virus replication occur and the patient is seropositive for markers of the virion and for HBeAg but lacks biochemical or histological evidence of hepatitis. During the immune-clearance phase, viraemia and HBeAg continue but with increasing inflammatory necrosis of hepatocytes, often leading to clearance of HBeAg and the development of anti-HBe. In the immune-control phase, the patient is anti-HBe-positive with low levels of HBV

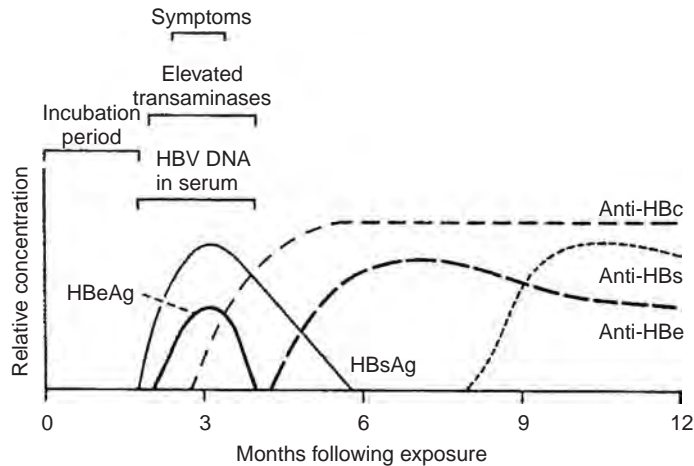


Figure 12.11 Serological profile of acute, resolving hepatitis B.

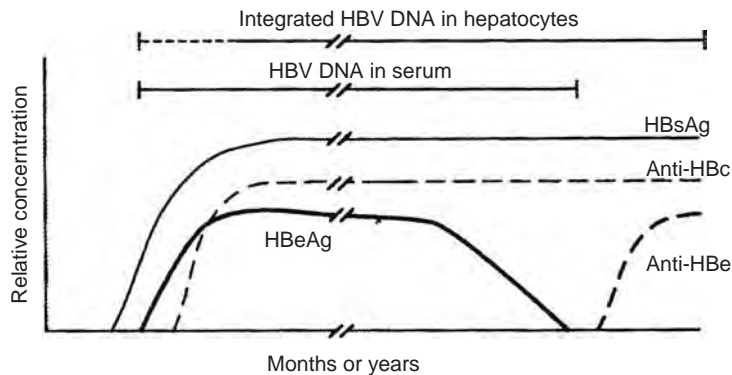


Figure 12.12 Serological profile of chronic hepatitis B with seroconversion.

replication and little inflammatory activity. However, viraemia and hepatitis may return in the absence of HBeAg, reflecting the emergence of the HBe-negative virus (pre-core or core promoter mutants).

Thus, although HBeAg correlates with the presence of the virus, in some cases when virus replication declines to very low levels, or when precore or core promoter mutants are present, seroconversion to anti-HBe may occur without cessation of virus replication. Direct tests for the virus, for example detection of viral DNA by hybridization or PCR, are more reliable for establishing infectivity. During the first phase of chronicity, replicative forms of the HBV genome may be detected in the liver by southern hybridization of DNA extracted from biopsy material (Figure 12.13, lane A7). This replicative phase may persist for years, and even lifelong in individuals who were infected perinatally and whose immune systems are tolerant of the virus. More usually, levels of virus replication decline gradually until this is eliminated with seroconversion to anti-HBe. Rarely, there will also be seroconversion to anti-HBs, but in many cases HBsAg will persist in the absence of detectable levels of virus

replication. Examination of liver biopsies from patients in this later phase of chronicity often reveals that HBV DNA is now integrated chromosomally in the hepatocytes (Figure 12.13, lane B), and HBsAg seems to be produced following transcription of this integrated DNA. In fact, integration of virus DNA into the hepatocyte chromosomes seems to take place throughout the period of virus replication, and expansion of clones of such cells may be a stage in progression to neoplasia (see below). Integration of the viral genome is not required for replication and seems to be a 'dead-end' for the virus. Although replicative and integrated HBV DNA may sometimes be observed in an individual biopsy specimen (Figure 12.13, lane C) it is not clear that both may be present in an individual hepatocyte.

Occurrence of HBV in Extra-hepatic Tissues

As stated above, *Hepadnaviruses* are essentially hepatotropic and it is not clear that they can replicate in other tissues. Sensitive hybridization techniques have, however, enabled the detection of viral DNA at other sites, particularly in peripheral leucocytes, the bone marrow and

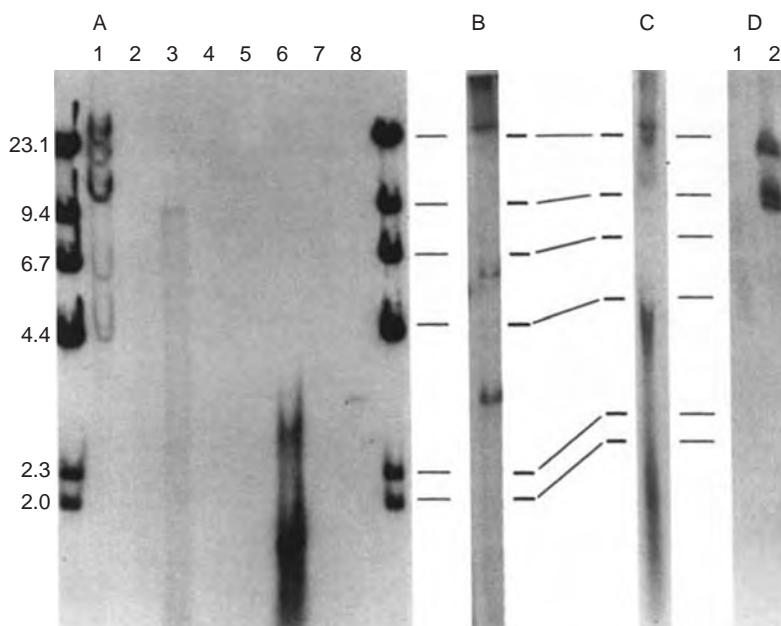


Figure 12.13 Analysis of the state of HBV DNA in the hepatocyte using the southern blot hybridization technique. Lane A1: integration of HBV DNA in the PLC/PRF/5 cell line. Lane A6: HBV DNA replicative intermediates in the liver biopsy from an HBeAg-positive patient. Lane B: integration of HBV DNA in an anti-HBe, HBsAg-positive patient without tumour. Lane C: biopsy from an HBeAg-positive patient with both replicative forms and integrated HBV DNA. Lane D: biopsies from an anti-HBe, HBsAg-positive patient with primary liver cancer; 1: biopsy from the nontumourous part of the liver; 2: biopsy from the tumour showing integration of HBV DNA at three sites. All DNA samples were digested with the restriction enzyme Hind III. The leftmost lane shows radioactively-labelled Hind III fragments of bacteriophage DNA as size markers—sizes are given in kilobase pairs alongside. (Source: Modified from (Harrison *et al.*, 1986).)

spleen. Viral DNA in white blood cells usually occurs as monomeric or multimeric episomes, or, rarely, it may be integrated, and there have been reports that replicative forms may also be found. These findings have implications for virus transmission and for the possible recurrence of hepatitis B in individuals who have cleared virus replication from the liver.

Diagnostic Assays

Hepatitis B is usually diagnosed following the detection of HBsAg in serum. Current immunoassays for HBsAg detect 100–200 pg HBsAg/ml of serum, corresponding to roughly 3×10^7 particles/ml. Most HBeAg-positive carriers have more than 10^5 genomes/ml of serum. Detection of HBsAg is the standard assay for infection and is used widely for screening, for example in transfusion centres.

As noted above, HBeAg is a marker of viraemia, but anti-HBe does not necessarily indicate clearance of virus replication. Immunity after infection with HBV is characterized by the presence of anti-HBs together with anti-HBc in serum. Immunity after vaccination is characterized by the presence of anti-HBs alone. Anti-HBc of the IgM class is a valuable marker of acute infection. A simplified guide to the interpretation of the test results is shown in Table 12.3.

Detection of viral DNA is the optimal method for establishing hepatitis B viraemia, and quantitative assays are valuable for monitoring virus loads during antiviral therapy. Tests based on hybridization of labelled probes or branched oligonucleotides are reliably quantitative but lack the sensitivity required to monitor the low virus levels achieved during successful therapy with nucleoside analogues. Currently, commercial assays based on real-time PCR combine the requisite sensitivity with a wide dynamic range and are valuable for monitoring the response to antiviral therapy.

Acute Hepatitis B

The diagnosis of acute hepatitis B generally rests upon the finding of HBsAg and IgM anti-HBc in the serum of a patient with clinical and serum biochemical evidence of acute hepatitis. HBsAg is the first marker to appear in serum, followed by HBV DNA, HBeAg and DNA polymerase, and anti-HBc. Levels of HBV DNA usually reach $10^5 - 10^8$ genome equivalents/ml with the onset of symptoms, after which the levels decrease. In contrast, in patients who develop chronic hepatitis B, levels of HBV DNA remain high. A positive IgM anti-HBc test typically distinguishes acute from chronic hepatitis B (but IgM anti-HBc can also be detected in patients with severe exacerbations of chronic hepatitis B). By the time the patient consults a physician, HBV DNA and HBeAg are often no longer detectable in serum. The loss of HBeAg is a sign that the patient will clear HBsAg. HBsAg may only be present transiently in serum; the only evidence of infection may therefore be the presence of IgM anti-HBc or subsequent development of anti-HBc and anti-HBs.

Serum ALT concentrations typically rise in acute hepatitis B. Serum bilirubin concentrations increase in proportion to the severity of hepatic damage. During this phase, IgM anti-HBc in serum correlates with active hepatitis in patients. Antibodies to pre-S components appear early in the disease and correlate with the disappearance of serological markers of HBV replication, suggesting a role in immunological clearance of HBV. Immune complexes may be responsible for some of the manifestations of the acute disease. A mild decrease in serum C3 and C4 concentrations occurs in acute hepatitis B and may reflect antigen–antibody complex formation. Autoantibodies, including abnormalities in rheumatoid factor and anti-nuclear and anti-smooth-muscle antibodies, are also detectable in acute hepatitis.

Table 12.3 Interpretation of results of serological tests for hepatitis B

HBsAg	HBeAg	Anti-HBe	Anti-IgM	HBc IgG	Anti-HBs	Interpretation
+	+	–	–	–	–	Incubation period
+	+	–	+	+	–	Acute hepatitis B or persistent carrier state
+	+	–	–	+	–	Persistent carrier state
+	–	+	±	+	–	Persistent carrier state
–	–	+	±	+	+	Convalescence
–	–	–	–	+	–	Recovery
–	–	–	+	–	–	Infection with HBV without detectable HBsAg
–	–	–	–	+	–	Recovery with loss of detectable anti-HBs
–	–	–	–	–	+	Immunization without infection. Repeated exposure to antigen without infection, or recovery from infection with loss of anti-HBs

In fulminant hepatitis B (FHB), extremely rapid clearance of HBeAg and HBsAg may occur. FHB is a life-threatening form of acute hepatitis B that is complicated by encephalopathy and bleeding due to liver failure. HBsAg may be negative at the time of presentation, and the diagnosis rests on the detection of IgM anti-HBc. HBeAg-negative variants have frequently been implicated as a cause of FHB.

Chronic Hepatitis B

Many carriers are detected through routine screening for HBsAg or the presence of abnormal liver function tests. Older patients may present for the first time with complications of cirrhosis, or even hepatocellular carcinoma. Typically serum levels of aminotransferases are elevated in patients with HBeAg, HBV DNA-positive chronic hepatitis, but some patients may have normal values. Many patients go on to develop moderate-to-severe HBeAg-positive chronic hepatitis with raised serum ALT after several decades of infection, which can ultimately progress to cirrhosis. The levels of aminotransferases fluctuate with time.

As noted above, several phases of chronic hepatitis B are recognized: typically in the immunotolerant phase, serum HBsAg and HBeAg are detectable; serum HBV DNA levels are high (usually $>10^7$ copies/ml); serum aminotransferases are normal or minimally elevated. This phase is common in young who are infected in the neonatal period, and may last 10–30 years after perinatal HBV infection. The immunotolerant phase is frequently followed by an ‘immunoactive’ phase, when symptoms of hepatitis may be present and serum aminotransferase levels are elevated. Exacerbations in serum aminotransferases may be observed, accompanied by a decrease in serum HBV DNA levels and ultimately followed by HBeAg seroconversion (i.e. HBeAg becomes undetectable, and anti-HBe detectable, in serum). Seroconversion rates are higher in those with raised serum ALT, and in patients with genotype D and (in Asia) genotype B infection. Seroconversion may occur following a sudden, asymptomatic exacerbation in serum aminotransferases. Once HBeAg is cleared, the disease remits temporarily and serum aminotransferases become normal.

Chronic hepatitis also may be observed in HBeAg-negative patients in whom HBsAg and anti-HBe are present in serum and serum HBV DNA is detectable using non-PCR-based methods; serum aminotransferase levels are elevated and liver biopsy shows necro-inflammation. HBeAg is undetectable in these patients because of the predominance of mutant HBV genomes that cannot express HBeAg. Patients with HBeAg-negative (also called

precore mutant) chronic hepatitis tend to be older, male, and to present with severe necroinflammation and cirrhosis (Hadziyannis *et al.*, 1983). HBeAg-negative chronic hepatitis has a variable course, often with fluctuating serum aminotransferases and serum HBV DNA levels. HBeAg may also become detectable transiently in these patients during acute flares. It is now known that such patients are frequently infected with variants of HBV with mutations in the precore region which cannot synthesize HBeAg (reviewed by Harrison, 2006). Precore variants may be selected during the process of seroconversion to anti-HBe, when hepatocytes expressing HBeAg are targeted for lysis by cytotoxic T cells. Different patterns of anti-HBe-positive disease can be discerned, but typically patients tend to have recurrent flares. Precore mutants can be detected in patients with FHB but it is controversial whether infection with these variants *ab initio*, in the absence of wild-type virus, often leads to acute liver failure. Not all patients with FHB are infected with precore mutants, and other mutations, such as in the core promoter (Sterneck *et al.*, 1996), as well as host factors, may be important. HBVs with double mutations in the core promoter have been associated with primary liver cancer in some regions of the world (Fang *et al.*, 2002).

A spontaneous remission in disease activity may occur in approximately 10–15% of HBeAg-positive carriers each year, characterized by a marked reduction in the titre of HBV DNA in the serum, followed by loss of HBeAg and seroconversion to anti-HBe. The inactive carrier state is characterized by very low serum HBV DNA levels in serum ($<10^5$ copies/ml) and normal serum aminotransferases. Liver biopsies are not performed routinely in inactive HBsAg carriers and they usually show little or no necroinflammation and mild or no fibrosis (although inactive cirrhosis may be present if transition to an inactive carrier state has occurred after many years). The prognosis of these patients, if stable without pre-existing advanced disease, is benign. However, active viral replication may reappear in a proportion of patients, leading to raised ALT concentrations. Histologically, chronic hepatitis is always associated with viral replication. If low levels of replication persist, serum HBsAg may become undetectable in serum, and anti-HBs may become detectable. HBsAg may be lost in 1–2% of patients a year. However, a proportion of anti-HBe-positive individuals with low levels of HBV DNA may later develop higher levels of HBV replication and raised ALT and progress to HBeAg-negative chronic hepatitis. The clinical significance of different levels of HBV DNA is incompletely understood. There may be a threshold level of HBV DNA below which the disease is less likely to be progressive. The set point of this concentration is uncertain but a value of 10^5 copies/ml has

been proposed as a threshold to separate high from low levels of serum HBV DNA associated with less active disease.

HBV genotypes A to H have been reported to correlate with spontaneous and IFN-induced HBeAg seroconversion, activity of liver disease and progression to cirrhosis and HCC, but further study is required. In China and Japan, where genotypes B and C circulate, there is evidence for increased pathogenicity, and increased likelihood of development of HCC, of genotype C over B.

Liver-biopsy assessment by an experienced pathologist is considered to be an integral investigation by many hepatologists. The biopsy can be used to assess progression of disease and response to antiviral therapy. Normal concentrations of albumin, ALT and AST can be encountered in patients with established cirrhosis.

In HBeAg-positive patients, progression to cirrhosis occurs at an annual rate of 2–5.5%, with a cumulative five-year incidence of progression of 8–20%. Progression to cirrhosis is generally faster in HBeAg-negative patients, at an annual rate of 8–20%. Recurrent exacerbations and bridging fibrosis with severe necroinflammatory change characterize patients more likely to progress. Recent retrospective studies have examined survival in compensated cirrhosis due to hepatitis B. The reported yearly incidence of hepatic decompensation is about 3%, with a five-year cumulative incidence of 16%. In a European multicentre longitudinal study to assess the survival of 366 cases of HBsAg-positive compensated cirrhosis, death occurred in 23% of patients, mainly due to liver failure or hepatocellular carcinoma. The cumulative probability of survival in this cohort was 84 and 68% at 5 and 10 years, respectively. The worst survival was in HBeAg- and HBV DNA-positive subjects (Fattovich *et al.*, 1997). Chinese patients remaining HBeAg-positive were more likely to develop HCC.

Occult Hepatitis B

Occult hepatitis B (reviewed by Raimondo *et al.*, 2007) is defined as the presence of (usually low levels of) HBV DNA in serum in the absence of detectable HBsAg. Anti-HBc and/or anti-HBs may be present, but these are undetectable in a significant percentage of cases. Occult hepatitis B is of particular concern in individuals who are infected with HCV and in whom there is a high risk of development of advanced fibrosis and cirrhosis. The reported incidence in patients with hepatitis C varies from 0% in the United Kingdom to as high as 70–95% in Japan. There are several reports of HBV genomic sequences from such infections and a variety of mutations have been detected, including in the core promoter and surface ORF.

Pathogenesis of Hepatitis B Infection

HBV is not cytopathic and host immunity plays an important role in cellular injury; there is little correlation between the severity of the illness and the level of HBV replication. Patients with a poor T-cell response may have high concentrations of virus in liver, yet mild disease. The development of chronic infection is due to a failure of an adequate immune response, but the immune response is also responsible for disease pathogenesis during chronic infection. The subsequent expression of the disease involves a poorly-understood interplay between viral and host factors. The nucleocapsid antigens (HBcAg and HBeAg) expressed on the cell membrane contain important targets of the immune response and cytotoxic T cells in acute hepatitis B.

Studies using transgenic mice and of acute hepatitis B in chimpanzees suggest that antiviral mechanisms of clearance of HBV may involve both targeted lysis of infected hepatocytes and inhibition of viral replication by noncytolytic mechanisms (Canvaugh *et al.*, 1997; Guidotti and Chisari, 2001; Guilhot *et al.*, 1993). Inactivation of hepatitis B by CTL-elaborated cytokine expression conceivably occurs via elimination of HBV nucleocapsids and destabilization of HBV RNAs. Natural killer (NK) cells may also contribute to liver inflammation by TRAIL-mediated death of hepatocytes via a non-antigen-specific mechanism, which may be switched on by cytokines produced during active HBV infection (Dunn *et al.*, 2007).

Intense hepatocyte lysis occurs in acute hepatitis B, apparently as a result of a polyclonal, multispecific cytotoxic lymphocyte immune response. The specific epitopes recognized by B as well as T cells are currently being mapped. However, experimental studies in the transgenic mouse model suggest that HBV replication may also be reduced by a complex interplay of cytotoxic T-cell responses, apoptosis and down-regulation of hepatitis B gene expression by several inflammatory cytokines. An association of major histocompatibility complex (MHC) class II alleles (DRB*1302) with lack of persistent HBV infection has been reported (Thursz *et al.*, 1995).

The elimination of virus-infected hepatocytes is dependent on the recognition by cytotoxic T cells of viral determinants, in association with HLA proteins on the infected cells. Data derived from most experimental systems suggest that an acute, polyclonal and vigorous cytolytic T-cell response usually occurs in acute symptomatic icteric hepatitis B and that patients with acute self-limiting hepatitis B develop a polyclonal HLA class I-restricted, cytotoxic T-cell response against numerous epitopes in the HBV envelope, nucleocapsid and polymerase proteins. Several

HLA-A2-restricted cytotoxic T-cell epitopes have been defined.

Perinatal infection is almost always asymptomatic, and chronic infection ensues in 90% of cases. Rarely, infection acquired from an anti-HBe-positive mother may lead to severe hepatitis in the infant. The failure to eradicate HBV reflects an inadequate immune response to the virus, but the precise impairment of humoral and cellular immunity that determines the development and outcome of hepatitis B has not been characterized. Persistent infection is an unusual outcome in those patients with acute icteric hepatitis B and the mechanisms that lead to viral persistence are not well understood. In neonates, specific suppression of the cell-mediated immune response may favour infection, perhaps because intrauterine exposure to HBeAg (at a time when the immune system is ontogenically immature) induces tolerance to epitopes that are usually the target of the cytotoxic T-cell response. Clonal deletion of HBV-specific T cells may occur as a consequence of transplacental infection of the developing fetus or transplacental passage of viral antigens. In contrast to the vigorous polyclonal class I- and class II-restricted T-cell response that can be identified in patients with acute icteric hepatitis B, in chronic disease the response in peripheral blood is relatively weak and focused, and insufficient to clear replicating virus.

Other mechanisms may also operate to prevent clearance of HBV: mutations abrogating the recognition of the wild-type hepatitis, including the natural variants of the HBcAg 18–27 core epitope that interfere with the recognition of the wild-type epitope and act as T-cell receptor antagonists. There is some evidence of a failure of IFN production in patients with chronic hepatitis B.

Prevention and Control of Hepatitis B

Passive Immunization

HBIG is prepared from pools of plasma with high titres of hepatitis B surface antibody and may confer temporary passive immunity under certain defined conditions. The major indication for the administration of HBIG is a single acute exposure to HBV, such as occurs when blood containing HBsAg is inoculated, ingested or splashed on to mucous membranes and the conjunctiva. The optimal dose has not been established but doses in the range of 250–600 IU have been used effectively. HBIG should be administered as early as possible after exposure and preferably within 48 hours, usually 3 ml (containing 200 IU/ml anti-HBs) in adults. It should not be administered seven days or more after exposure. It is generally recommended that two doses of HBIG should be given 30 days apart.

Results following the use of HBIG for prophylaxis in babies at risk of infection with HBV are good if the immunoglobulin is given as soon as possible, and certainly within 12 hours of birth, and the chance of the baby developing persistent infection is reduced by about 70%. More recent studies using combined passive and active immunization indicate an efficacy approaching 90%. The dose of HBIG recommended in the newborn is 1–2 ml (200 IU/ml anti-HBs) combined with a full course of hepatitis B vaccine given at a contralateral site.

Other indications for administration of HBIG include sexual exposure to hepatitis B; prevention of reinfection with HBV after liver transplantation, with or without specific antiviral drugs; and nonresponse to hepatitis B vaccine following acute exposure to HBV (Zuckerman, 2007).

Active Immunization

The major response of recipients of hepatitis B vaccine is to the common *a* determinant, with consequent protection against all subtypes of the virus. First-generation vaccines were prepared from 22 nm HBsAg particles purified from plasma donations from asymptomatic (healthy) chronic carriers. These preparations are safe and immunogenic, but have been superseded in many countries by recombinant vaccines produced by the expression of HBsAg in yeast cells. The expression plasmid contains only the 3' portion of the HBV surface ORF, and only the major surface protein, without pre-S epitopes, is produced. Vaccines containing pre-S2 and pre-S1 as well as the major surface proteins expressed by recombinant DNA technology are available.

In many areas of the world with a high prevalence of HBsAg carriage, such as China and South East Asia, the predominant route of transmission is perinatal. Administration of a course of vaccine with the first dose immediately after birth is effective in preventing transmission from an HBeAg-positive mother in approximately 70% of cases, and this protective efficacy rate may be increased to more than 90% if the vaccine is accompanied by the simultaneous administration of HBIG.

Immunization against hepatitis B is now recognized as a high priority in preventive medicine in all countries, and strategies for immunization are being revised. Universal vaccination of infants and adolescents is recommended as a possible strategy to control the transmission of this infection. More than 160 countries now offer hepatitis B vaccine to all children, including the United States, Canada, Italy, France and most western European countries (Zuckerman *et al.*, 2007).

However, in a number of countries with a low prevalence of hepatitis B immunization is at present only

recommended to groups which are at an increased risk of acquiring this infection. These groups include individuals requiring repeated transfusions of blood or blood products, patients undergoing prolonged in-patient treatment, patients who require frequent tissue penetration or need repeated circulatory access, patients with natural or acquired immune deficiency and patients with malignant diseases. Viral hepatitis is an occupational hazard among health-care personnel, the staff of institutions for the mentally handicapped and in some semi-closed institutions. High rates of infection with hepatitis B occur in narcotic drug addicts and intravenous drug abusers, sexually-active male homosexuals and prostitutes. Individuals working in highly-endemic areas are, however, at an increased risk of infection and should be immunized.

Young infants, children and susceptible persons (including travellers) living in certain tropical and subtropical areas where present socio-economic conditions are poor and the prevalence of hepatitis B is high should also be immunized. It should be noted that in about 30% of patients with hepatitis B the mode of infection is not known, and this is a powerful argument for universal immunization (Zuckerman *et al.* 2007).

Site of Injection for Vaccination and Antibody Response

Hepatitis B vaccination should be given in the upper arm or the anterolateral aspect of the thigh and not in the buttock. Many studies have shown that the antibody response rate is significantly higher in centres using deltoid injection than centres using the buttock. On the basis of antibody tests after vaccination, the Advisory Committee on Immunization Practices of the Centers for Disease Control, United States, recommended that the arm be used as the site for hepatitis B vaccination in adults, as has the Department of Health in the United Kingdom.

Nonresponse to Current Hepatitis B Vaccines

Apart from the site of injection, there are several other factors which are associated with a poor or no antibody response to currently-licensed vaccines. Indeed, all studies of the antibody response to the plasma-derived and recombinant DNA vaccines containing the single antigen only have shown that between 5 and 10%, or more, of healthy immunocompetent subjects do not mount an anti-HBs antibody response to the HBsAg present in these preparations ('nonresponders') or respond poorly ('hyporesponders') (Zuckerman, 1996; Zuckerman *et al.*, 1997). Nonresponders remain susceptible to infection with HBV.

While several factors are known to adversely affect the antibody response to HBsAg, including the site and route of injection, gender, advancing age, body mass (overweight), immunosuppression and immunodeficiency, evidence is accumulating that there is, at least in part, an association between immunogenetics and specific low responsiveness in different populations. Considerable experimental evidence is available suggesting that the ability to produce antibody in response to specific protein antigens is controlled by dominant autosomal class II genes of the MHC in the murine model and in humans. Other mechanisms underlying nonresponsiveness to current hepatitis single-antigen vaccines remain largely unexplained.

The Kinetics of Anti-HBs Response to Immunization and Booster Doses

The titre of vaccine-induced anti-HBs declines, often rapidly, during the months and years following a complete course of primary immunization. The highest anti-HBs titres are generally observed 1 month after booster vaccination, followed by rapid decline during the next 12 months and thereafter. Mathematical models have been designed and an equation was derived consisting of several exponential terms with different half-life periods. It is considered by some researchers that the decline of anti-HBs concentration in an immunized subject can be predicted accurately by such antibody kinetics, with preliminary recommendations on whether or not booster vaccination is necessary (see review by Zuckerman, 1996).

If the minimum protection level is accepted at 10 IU/l, consideration should be given to the diversity of the individual immune response and the decrease in levels of anti-HBs, as well as to possible errors in quantitative anti-HBs determinations. It would then be reasonable to define a level of >10 IU/l and <100 IU/l as an indication for booster immunization. It has been demonstrated that a booster inoculation results in a rapid increase in anti-HBs titres within four days. However, it should be noted that even this time delay might permit infection of hepatocytes.

Several options are therefore under consideration for maintaining protective immunity against hepatitis B infection:

- Relying upon immunological memory to protect against clinical infection and its complications, a view which is supported by *in vitro* studies showing immunological memory for HBsAg in B cells derived from vaccinated subjects who have lost their anti-HBs, but not in B cells from nonresponders, and by post-vaccination surveys.

- Providing booster vaccination to all vaccinated subjects at regular intervals without determination of anti-HBs. This option is not supported by a number of investigators because nonresponders must be detected. While an anti-HBs titre of about 10 IU/l may be protective, this level is not protective from a laboratory point of view, since many serum samples may give nonspecific reactions at this antibody level.
- Testing anti-HBs levels one month after the first booster and administering the next booster before the minimum protective level is reached.

No empirical data are available for the anti-HBs titre required for protection against particular routes of infection or the size of the infectious inoculum. The minimum protective level has been set at 10 IU/l against an international standard. However, the international standard is a preparation of immunoglobulin from pooled plasma of individuals recovered from infection rather than from immunized subjects and the antibody avidity is likely to be different. Furthermore, studies carried out in the 1980s indicated asymptomatic infection after immunization in subjects and health-care workers who had antibody titres below 50 IU/l.

There are studies which show that hepatitis B vaccine provides a high degree of protection against clinical symptomatic disease in immunocompetent persons despite declining levels of anti-HBs. These studies encouraged the Immunization Practices Advisory Committee of the United States, the National Advisory Committee on Immunization of Canada and the European Consensus Group (2000) to recommend that routine booster immunization against hepatitis B is not required. Caution, however, dictates that those at high risk of exposure, such as cardiothoracic surgeons and gynaecologists, would be prudent to maintain a titre of 100 IU/l of anti-HBs by booster inoculations, more so in the absence of an appropriate international antibody reference preparation. Breakthrough infections have been reported and, whereas long-term follow-up of children and adults has indicated that protection is attained for at least nine years after immunization against chronic hepatitis B infection even though anti-HBs levels may have become low or declined below detectable levels (reviewed by the European Consensus Group, 2000), brief periods of viraemia may not have been detected because of infrequent testing. Longer follow-up studies of immunized subjects are required to guide policy. This is illustrated by a cross-sectional study carried out in Gambian children, which showed that the efficacy of hepatitis B vaccination against chronic carriage of HBV 14 years after immunization was 94% in all age groups, whilst the efficacy against infection was 80%, but was

lower (65%) in those aged 15–19 years (Whittle *et al.*, 2002).

Hepatitis B Surface Antigen Variants

Neutralizing antibodies induced by immunization are targeted principally to the conformational epitopes of the *a* determinant, and there is evidence that amino-acid substitutions within this region of the surface antigen can allow replication of HBV in vaccinated persons, since antibodies induced by current vaccine do not recognize critical changes in the surface antigen domain.

The emergence of variants of HBV, possibly due to selection pressure associated with extensive immunization in an endemic area, was suggested by the findings of hepatitis B infection in individuals immunized successfully (Zanetti *et al.*, 1988). These studies were extended subsequently by the finding of noncomplexed HBsAg and anti-HBs and other markers of hepatitis B infection in 32 of 44 vaccinated subjects, and sequence analysis from one of these cases revealed a mutation in the nucleotide encoding the *a* determinant, the consequence of which was a substitution from glycine to arginine at amino-acid position 145 (G145R).

Various mutations and variants of HBsAg have since been reported from many countries. However, the most frequent and stable mutation was reported in the G145R variant. A large study in Singapore of 345 infants born to carrier mothers with HBsAg and HBeAg, who received HBIG at birth and plasma-derived hepatitis B vaccine within 24 hours of birth and then one month and two months later, revealed 41 breakthrough infections with HBV despite the presence of anti-HBs. There was no evidence of infection among 670 immunized children born to carrier mothers with HBsAg and anti-HBe, nor in any of 107 immunized infants born to mothers without HBsAg. The most frequent variant was a virus with the G145R mutation in the *a* determinant. Another study in the United States of serum samples collected between 1981 and 1993 showed that 94 (8.6%) of 1092 infants born to carrier mothers became HBsAg positive despite post-exposure prophylaxis with HBIG and hepatitis B vaccine. Following amplification of HBV DNA, 22 children were found with mutations of the surface antigen, most being in amino acids 142–145; 5 had a mixture of wild-type HBV and variants and 17 had only the 145 variant.

Another important aspect of the identification of surface-antigen variants is the evidence that HBsAg mutants may not be detected by all of the blood-donor screening tests and existing diagnostic reagents. Such variants may therefore enter the blood supply or spread by other means. This is emphasized by the finding

in Singapore, between 1990 and 1992, that 0.8% of 2001 people were carriers of HBV variants in a random population survey (Oon *et al.*, 1996). These findings add to the concern expressed in a study of mathematical models of HBV vaccination, which predict, on the assumption of no cross-immunity against the variant by current vaccines, that the variant will not become dominant over the wild-type virus for at least 50 years—but that the G145R mutant may emerge as the common HBV in 100 (or more) years' time. It is important, therefore, to institute epidemiological monitoring of HBV surface mutants employing test reagents which have been validated for detection of the predominant mutations, and consideration should be given to incorporating into current hepatitis B vaccines antigenic components which will confer protection against infection by the predominant mutant(s).

The Pre-S1 and Pre-S2 Domains and Third-Generation Vaccines

There is evidence that the pre-S1 and pre-S2 domains of the surface antigen have an important immunogenic role in augmenting anti-HBs responses, preventing the attachment of the virus to hepatocytes and eliciting antibodies which are effective in viral clearance, stimulating cellular immune responses and circumventing genetic nonresponsiveness to the S antigen.

These observations led to the development of a new triple-antigen hepatitis B vaccine (Hepacare), a third-generation recombinant DNA vaccine containing pre-S1, pre-S2 and S antigenic components of HBsAg of subtypes *adw* and *ayw*. All three antigenic components are glycosylated, closely mimicking the surface protein of the virus itself, produced in a continuous mammalian cell line, the mouse c127 clonal cell line, after transfection of the cells with recombinant HBsAg DNA. The vaccine is presented as an aluminium hydroxide adjuvant preparation of purified antigenic protein. Animal studies showed that the vaccine was well tolerated and a viral challenge study in chimpanzees demonstrated protective efficacy. This vaccine was evaluated for reactogenicity and immunogenicity in a number of clinical trials (reviewed by Zuckerman and Zuckerman, 2002). The major conclusions from these studies were that the vaccine was safe and immunogenic and overcame the nonresponsiveness to the single S antigen vaccines used widely in some 70% of nonresponders, and that even a single dose of 20 mg of the triple antigen provided significant seroprotective levels of antibody.

However, the anticipated high costs of this triple-antigen vaccine will limit its use initially to the following groups:

- Vaccination of nonresponders to the current single-antigen vaccines, who are at risk of exposure to HBV infection
- Subjects with inadequate humoral immune response to single-antigen hepatitis B vaccines, for example those over the age of 40 years, males, the obese, smokers and other hyporesponders, and
- Persons who require protection rapidly, for example health-care employment involving potential exposure to parenteral procedures involving blood-to-blood contact (current schedules of immunization with single-antigen hepatitis B vaccines involve three doses at zero, one and six months).

Studies are required to determine the efficacy of the triple antigen in patients who are immunocompromised, and also to determine whether the inclusion of pre-S1 and pre-S2 antigenic components in this new vaccine will protect against the emergence of HBV surface-antigen mutants.

Combined Immunoprophylaxis

Whenever immediate protection is required, as, for example, for infants born to HBsAg-positive mothers (see above) or after accidental inoculation, active immunization with the vaccine should be combined with simultaneous administration of HBIG at a different site. It has been shown that passive immunization with up to 3 ml (200 IU/ml anti-HBs) of HBIG does not interfere with an active immune response. A single dose of HBIG (usually 3 ml for adults; 1–2 ml for the newborn) is sufficient for healthy individuals. If infection has already occurred at the time of the first immunization, virus multiplication is unlikely to be inhibited completely, but severe illness and, most importantly, the development of the carrier state of HBV may be prevented in many individuals, particularly in infants born to carrier mothers.

Indications for Immunization against Hepatitis B

The current indications for the use of hepatitis B vaccines in the United Kingdom are given below. Many countries, including the United States and Italy, introduced universal immunization for infants in 1992. The World Health Organization recommended a decade ago that universal immunization should be in place in areas with a prevalence of HBsAg greater than 8% by 1994 and in all countries by 1997, with integration of hepatitis B vaccine into the Expanded Programme of Immunization (EPI). These targets were not met and universal immunization of infants worldwide remains a goal.

In 1996 the Department of Health and other government offices in the United Kingdom recommended immunization for the following risk groups:

Babies born to mothers who are chronic carriers of HBV or to mothers who have had acute hepatitis B during pregnancy.

In addition, babies born to mothers who are HBeAg positive, who are HBsAg positive without e markers (or where HBeAg/anti-HBe status has not been determined) or who have had acute hepatitis B during pregnancy, as well as babies who are low birth weight (<1500 g). Premature babies should receive HBIG as well as active immunization. Currently, vaccine without HBIG is recommended for babies born to mothers who are HBsAg positive but known to be anti-HBe positive.

Parenteral drug misusers.

Close family contacts of a case or carrier.

Sexual contacts of patients with acute hepatitis B should also receive HBIG.

Families adopting children from countries with a high prevalence of hepatitis B.

Particularly some countries in eastern Europe, SE Asia and S America.

Haemophiliacs (and others receiving regular blood transfusions or blood products).

Including carers responsible for the administration of such products.

Patients with chronic renal failure.

Higher doses of vaccine may be required in those who are immunocompromised.

Health-care workers, including students and trainees.

Staff and students of residential accommodation for those with severe learning disabilities.

Other occupational risk groups.

Including certain members of the emergency and prison services.

Inmates of custodial institutions.

Those travelling to areas of high prevalence.

Despite the availability of a vaccine, infection persists worldwide. In populous regions where mass immunization has not started, there has not been a significant decline in HBsAg carrier rates, and therefore the carrier pool has increased with the increase in population. Nonetheless, notable successes through vaccination have emerged, in particular in Taiwan and Alaska. In Taiwan, the long-awaited impact of vaccination on the risk of hepatocellular carcinoma has been discernible (Chang *et al.*, 1997).

Antiviral Therapy for Hepatitis B

Most icteric patients with acute hepatitis B resolve their infection and do not require treatment. FHB is a severe form of acute infection complicated by encephalopathy

and liver failure. Subgroups of FHB, including hyperacute, acute and subacute, are defined by the interval between jaundice and encephalopathy. Subacute hepatic necrosis is characterized by a more protracted acute course and transition to chronic hepatitis with ongoing hepatitis B replication. Patients with fulminant hepatitis and liver failure (including acute and subacute forms) should be considered for liver transplantation, if appropriate. There are no controlled trials of lamivudine or other antiviral agents for patients with acute fulminant or subacute fulminant hepatitis. Historical reports suggest some efficacy of lamivudine in these patients, and carefully-administered antiviral therapy could be tried, if administered early, and if there is evidence of ongoing HBV replication.

Prolonged treatment for years is required for most patients. Several difficulties remain in formulating treatments for HBV infection and areas of disagreement exist. Current guidelines are not always consistent or agreed and will require rapid adjustment as new therapies become available. In general, treatment of chronic hepatitis B should be targeted at patients with active disease and viral replication, preferably at a stage before signs and symptoms of cirrhosis or significant injury have occurred (Dusheiko *et al.*, 1985). Eradication of the infection is possible only in a minority of patients and the optimal treatments for hepatitis B are still being defined. Recombinant IFN- α , pegylated IFN- α and a number of new nucleoside and nucleotides have been licensed. Thus the array of nucleosides may shortly include the licensed therapies (lamivudine, adefovir, entecavir, telbivudine), tenofovir, emtricitabine (licensed for human immunodeficiency virus (HIV)), as well as clevudine, elvucitabine, valtorcitabine, amdoxovir, racivir, MIV 210, β -L-FddC, alamivovir and hepavir B. The licensed agents have to date mostly been used as monotherapies. The efficacies of suitable combination therapies are currently being evaluated. Response rates in HBeAg-positive patients are higher for all currently-licensed agents for those patients with higher baseline ALT. Single measures of ALT (and HBV DNA) are not useful in a disease as dynamic as hepatitis B, and there is controversy regarding the level below which HBV DNA concentrations are indicative of 'inactive' disease, or indicate a threshold for initiating treatment.

HBeAg-positive Chronic Hepatitis B

Patients with mild disease and normal ALT may not require immediate treatment and should be monitored carefully at appropriate intervals. Most clinicians believe that therapy should be considered only if there is evidence of moderate-to-severe activity. HBeAg-positive patients should be followed for a few months to ascertain their status, and antiviral therapy should be considered if there is active HBV replication (HBV DNA above

1×10^5 copies/ml (20 000 IU/ml)) and elevated ALT after observation, with a biopsy showing active hepatitis, that is inflammation, necrosis or accumulating fibrosis. HBeAg-positive patients with greater disease activity may be more likely to seroconvert to anti-HBe within one year of treatment.

HBeAg-negative patients should be considered for antiviral therapy when the serum ALT is raised and there is active viral replication (HBV DNA above 1×10^4 – 1×10^5 copies/ml (2000–20 000 IU/ml)) and active hepatitis, that is inflammation, necrosis or accumulating fibrosis. The threshold for treatment remains debated. No definite consensus criteria have been established for initiating treatment in this group. Anti-HBe-positive patients with normal ALT and low levels of DNA ($< 10^4$ copies/ml, < 2000 IU/ml) should be monitored. Indeed, both HBeAg-positive and -negative patients require careful and regular monitoring to ascertain a change in the pattern of disease.

Many clinicians consider a liver biopsy helpful for ascertaining the degree of necroinflammation and fibrosis in HBeAg-positive and -negative disease. In many centres a biopsy would be considered to assess the stage and grade of inflammation, as hepatic morphology can assist the decision to treat. There are several established methods for scoring histology, measuring activity (necroinflammation) separately from stage (fibrosis). There are, however, several limitations of biopsy, including sampling error, subjectivity and reproducibility, and of course costs, risks and discomfort to the patient. Progression of disease is often punctuated by episodes of activity which are injurious to the liver. There is growing interest in the use of non-invasive methods, including serum markers and transient elastography to assess hepatic fibrosis.

Goals of Treatment

The major goals of therapy for hepatitis B are to prevent progression of the disease to cirrhosis, end-stage liver disease or HCC. If HBV replication can be suppressed, the accompanying reduction in histological chronic active hepatitis lessens the risk of cirrhosis and hepatocellular carcinoma. Patients may request treatment to reduce infectivity. Extra-hepatic manifestations of hepatitis B such as glomerulonephritis or polyarteritis nodosa require treatment. The immediate objectives depend upon the stage of disease, but cirrhosis can be prevented, or decompensation can be prevented in patients with established cirrhosis.

End Points

The end points of treatment are not clearly defined, and differ in HBeAg-positive and -negative disease. However,

it is reasonable to infer improvement in disease outcome if HBV replication is suppressed with an accompanying improvement in serum ALT and hepatic necroinflammatory disease. The accompanying reduction in histological chronic active hepatitis lessens the risk of cirrhosis and hepatocellular carcinoma (Niederau *et al.*, 1996). Newer potent agents are capable of suppressing most patients to less than 1×10^3 (200 IU/ml), or even to levels undetectable by current PCR assays; that is, < 200 copies/ml (50 IU/ml), which may become the important benchmark.

HBeAg-positive Disease

In our current state of knowledge, antiviral therapy for HBeAg-positive disease is directed to attaining loss of HBeAg and, ideally, durable seroconversion to anti-HBe. In HBeAg-positive disease, reduction in HBV replication leads to an accompanying reduction in ALT. A reduction of HBV DNA concentrations within six months to less than 10^4 copies/ml (2000 IU/ml), or preferably levels undetectable by sensitive PCR to levels of around 200 copies/ml (or 50 IU/ml) or lower, may enhance the rate of loss of HBeAg and reduce the rate of resistance. Loss of HBeAg and associated suppression of viral replication leads to biochemical remission, histological improvement and, in a small percentage, loss of HBsAg. Histological improvement follows suppression of necroinflammatory disease.

Loss of HBeAg and seroconversion to anti-HBe is a potential stopping point in HBeAg-positive patients, although treatment with nucleoside analogues should be prolonged for at least six months after loss of HBeAg. Unfortunately, a variable T-cell response and antiviral resistance suggest that finite courses with such circumscribed responses occur in only a minority of HBeAg-positive patients, and the majority still require long-term maintenance suppressive therapy. Categorical analysis has not clarified what relative or absolute reduction in ALT and DNA predicts histological improvement and HBeAg seroconversion and will alter the natural history of the disease, although there are some pointers. It is not clear whether profound reductions in DNA titre (for example 6–7 \log_{10}) are critical for long-term therapy. However, the rapidity and efficacy of HBV DNA reduction to less than 10^3 copies/ml or to undetectable by sensitive PCR (< 200 – 300 copies/ml) clearly has implications for development of resistance and loss of HBeAg.

HBeAg-negative Disease

Because such patients are already HBeAg-negative, reduction in ALT and HBV DNA, and the accompanying reduction in cccDNA and histological improvement, are the end points. Stopping points and finite courses of

treatment are less commonly achieved because of higher rates of relapse in these patients, but progression can be halted if HBV DNA remains suppressed and resistance or relapse does not occur. Successful treatment with nucleoside(s) or nucleotide is characterized by a decline in HBV DNA to less than 10^4 copies/ml (<2000 IU/ml) or preferably levels undetectable by sensitive PCR. Maintaining the response is the objective, defined by a low viral load during therapy. As for HBeAg-positive disease, a rapid lowering of viral load lessens the risk of developing viral drug resistance.

Treatment of Cirrhosis

Immediate, prolonged and profound suppression of viraemia confers benefit, reducing the risk of progression to decompensated liver disease and, possibly, HCC. Clinical studies indicate that prolonged and adequate suppression of viraemia may stabilize patients and delay or even obviate the need for transplantation. Recent longer-term studies have suggested the clinical utility of HBV DNA suppression with lamivudine; Kaplan–Meier estimates of disease progression after three-year treatment of patients with cirrhosis are reduced to 5% in treated versus 21% in placebo recipients. However, the development of lamivudine resistance significantly reduces the clinical benefit in treated patients. HCC remains a persistent risk, albeit a lessened one. Hepatic decompensation may occur, with exacerbations of disease occurring after instituting nucleoside analogues, and these patients should be monitored carefully.

Prophylactic therapy with a nucleoside is recommended for all patients undergoing liver transplantation for end-stage hepatitis B, to reduce levels of HBV DNA to at least less than 10^5 copies/ml before transplantation. End-stage liver disease should be treated as a matter of urgency. Patients may show slow clinical improvement over a period of three to six months. However, some patients with advanced hepatic disease with a high Child–Pugh score and jaundice will not benefit. All such patients require long-term therapy, with careful monitoring for resistance and flares. Regression of fibrosis has been reported. There is less data for the efficacy and safety of newer potent agents such as entecavir, telbivudine and tenofovir in this group, but these could be utilized.

Recurrent HBV infection in the transplanted liver has previously been a major problem. Lamivudine for pre-transplant prophylaxis, in combination with HBIG, reduces the risk of graft infection to less than 10%, as long as the HBV is suppressed before transplantation. With the advent of lamivudine and adefovir, outcomes have improved further. Currently both HBIG and lamivudine and/or adefovir are used prophylactically and recurrent HBV is now rare. Other licensed nucleosides

could also be considered; profound suppression and low rates of resistance are advantageous.

Specific Therapies

Alpha Interferons

IFN remains a benchmark therapy for chronic hepatitis B. IFN- α s are naturally-occurring intercellular signalling proteins used therapeutically for their properties of inducing an antiviral state in cells, inhibiting cellular proliferation and immunomodulation. The IFNs have been classified into two types, based on receptor specificity. Type 1 IFNs (viral IFNs) include IFN- α (leukocyte), IFN- β (fibroblast) and IFN omega. Type 2 IFN is also known as immune IFN, that is IFN- γ . The cellular activities of IFN- α are mediated by the products of the IFN inducible genes. The natural IFN- α -producing cell is the precursor dendritic cell. IFN- γ is produced by cells of the immune system, including NK cells, CD4 Th1 cells and CD8T cells following antigen-specific stimulation, and acts via a separate cell receptor. IFN- γ is critical for innate and adaptive immunity.

The main advantages of IFN- α over nucleoside analogues are the absence of resistance and the possibility of immune-mediated clearance of hepatitis B. A meta-analysis of 15 randomized controlled trials in HBeAg-positive patients showed a 33% HBeAg seroconversion rate after 16 weeks of IFN- α treatment, compared with 12% in untreated control patients. The incidences of HBsAg loss were 7.8% and 1.8% respectively. The recommended regimen for HBeAg-positive adult patients is 5 MU daily or 10 MU thrice weekly for four to six months (Lok and McMahon, 2004). Pre-treatment factors predictive of response to IFN- α are low viral load, high serum ALT levels, increased activity scores on liver biopsy and shorter duration of infection. The HBeAg seroconversion rate correlates with baseline ALT levels and reaches 30–40% for baseline ALT greater than five times the upper limit of normal (ULN). An increase of ALT levels during the second or third month of therapy can be observed.

Relative or absolute contraindications to IFN- α therapy include severe depression, Childs B/C cirrhosis, cirrhosis and hypersplenism, autoimmune hepatitis, hyperthyroidism, coronary artery disease, renal transplant, pregnancy, seizures, concomitant drugs including several herbal remedies, diabetes/hypertension and retinopathy, thrombocytopenia, leucopenia, anaemia, high titre autoantibodies and hyperthyroidism. The side effects of IFN- α are relatively common, but are acceptable in most patients. Toxicity can be predicted in patients with low baseline white cell counts or thrombocytopenia, or pre-existing

thyroid disease (Dusheiko, 1997). The risk of serious complications from IFN- α is rare. However, serious idiosyncratic complications such as immune disorders, pneumonitis, retinal disease, renal disease or deafness can occur and the drug must always be prescribed with caution. Close monitoring is required throughout therapy. Monitoring requires regular clinical examinations, vital signs, urinalysis and usually monthly measurement of serum chemistries, complete blood counts and thyroid function tests including thyroid-stimulating hormone. A serum pregnancy test should be done to exclude pregnancy before starting treatment.

Polyethylene Glycol-linked Interferons (PEG IFNs)

The efficacy of IFN- α therapy is restricted by protein characteristics including poor stability, a short half-life and immunogenicity. The half-life varies from 4 to 16 hours with peak concentrations 3–8 hours following IM or SC injection. By 24 hours, little IFN- α remains in the circulation. Thus frequent dosing is required to achieve effective therapeutic concentrations of drug in the plasma. Large fluctuations in serum concentrations occur, resulting in peaks and troughs in the drug concentration. Polyethylene glycol (PEG) is an inert nontoxic polymer which can be used to modify the pharmacological properties of biologically-active proteins without completely inactivating their intrinsic biological activity. Recent technology has enabled activation of the PEG moiety through substitution of the hydroxyl group by an electrophilic functional group. The reactive functional group of activated PEG can be attached to a specific site, for example amine, sulfhydryl group or other nucleophile on the protein. Until recently, monofunctional PEG derivatives used for protein pegylation were linear with molecular weights of 12 kDa. New advances in pegylation technology have resulted in the development of branched PEGs of high molecular weights up to 60 000 kDa. The PEG polymer increases the half-life of the conjugated protein, protecting the protein from proteolysis and reducing renal clearance. This enables once per week dosing.

Reduced clearance of pegylated interferon results in increased circulation time and sustained systemic exposure of the pegylated compound. When the biological activity of a pegylated protein is measured *in vivo*, a direct relationship between the mass of the PEG conjugate and its biological activity is frequently observed, with increasing mass associated with increased activity.

HBeAg-positive Patients A study in HBeAg-positive patients compared treatment for 48 weeks with polyethylene glycol-linked interferon (PEG IFN)- α 2a versus PEG IFN- α 2a plus lamivudine versus lamivudine monotherapy. At the end of 24 weeks follow-up, HBeAg seroconversion

rates were 32%, 27% and 19% respectively. ALT normalization occurred in 41%, 39% and 28% of the same groups. HBeAg levels higher than 100 IU/ml at weeks 12 and 24 was highly predictive of failure to achieve seroconversion. Conversely, low HBeAg levels at baseline, week 12 and week 24 correlated with improved rates of seroconversion.

PEG IFN- α 2b has also been shown to be active in HBeAg-positive patients, with similar seroconversion rates. There may be an effect of genotype and other baseline factors on response to PEG IFN- α 2a in HBeAg-positive chronic hepatitis B: patients with genotype A and B tend to respond better than patients with genotype C and D.

HBeAg-negative Patients In a similar study of HBeAg-negative patients, at the end of 48 weeks follow-up HBV DNA less than 400 copies/ml was maintained in 17% of an observational subset of PEG IFN- α 2a-treated patients followed for three years post-treatment. Baseline ALT and HBV DNA levels, patient age, gender and infecting HBV genotype significantly influenced response at 24 weeks post-treatment (Bonino *et al.*, 2007). HBsAg loss occurred in 8% after three years.

Frequent side effects and the necessity for monitoring patients closely are the main disadvantage of PEG IFN treatment. There is no role for IFN- α in the treatment of acute or fulminant hepatitis B. The role of IFN in patients with decompensated hepatitis B is more problematic, given the effect on platelets and neutrophils, and the pro-inflammatory effects. IFN should be used with caution and regular monitoring in patients with compensated cirrhosis, since there is a risk of hepatic decompensation with prolonged treatment (Keeffe *et al.*, 2004). Moreover, serious bacterial infections have been reported in this group of patients.

Nucleoside Analogues

The patterns of response observed with nucleosides are broadly similar, although these agents have different structures and inhibit different phases of hepatitis B replication. Nucleosides and nucleotides have variable mechanisms of action and their pharmacokinetics, inhibitory capacity, onset of action, resistance patterns and rates of HBeAg seroconversion vary during the first year of treatment. Nucleoside analogues have similar structures to the natural nucleotides and compete at the HBV polymerase catalytic site during the synthesis of viral DNA. They lack a hydroxyl group, preventing the formation of a covalent bond with the adjoining nucleotide, causing chain termination during DNA synthesis. Although all nucleotide analogues act on HBV polymerase, their mechanism differs; thus adefovir inhibits the priming of

reverse transcription, lamivudine and emtricitabine inhibit the synthesis of the viral minus-strand DNA, entecavir inhibits three major stages of HBV replication, and clevudine inhibits the elongation of the plus-strand DNA and has a weaker effect on priming, and may have additional effects of cccDNA. Nucleic acids in general are less effective against cccDNA formation after viral entry to the hepatocyte, and thus residual viraemia persists during antiviral treatment.

Lamivudine

Lamivudine (2',3'-dideoxy-3'thiacytidine or 3TC) is a cytidine analogue and competes for cytosine in the synthesis of viral DNA. It is a (-) enantiomer and a phosphorylation step is required for the transformation to active drug. The drug has a strong track and safety record, and reliably reduces HBV DNA concentrations in serum by 2–4 log₁₀. Elevated serum ALT levels predict a higher likelihood of HBeAg loss in patients with chronic hepatitis B treated with lamivudine. Lamivudine is a relatively inexpensive drug, and the lack of side effects in patients with advanced disease is attractive. As a result lamivudine has become a widely used first-line drug for the treatment of HBeAg and anti-HBe-positive disease. The major disadvantage of lamivudine treatment is the high rate of resistance observed in both HBeAg and anti-HBe-positive patients. Elimination of lamivudine occurs mainly by renal elimination and dosages should be adapted to creatinine clearance.

Lamivudine in Acute Hepatitis B Although 95% of immune-competent adults clear HBsAg spontaneously, lamivudine may have a role in acute HBV infection, preventing progression to fulminant hepatic failure in small uncontrolled studies of patients.

Lamivudine in Chronic Hepatitis B Much information has been gleaned from the early controlled trials of lamivudine and by the use of lamivudine as the control arm in trials of newer agents. Longer-term studies have also been informative.

In HBeAg-positive patients reductions in HBV DNA concentration, HBeAg seroconversion, ALT normalization and histological improvement observed after one year of treatment reach 44%, 17%, 41% and 52% respectively. Pre-treatment factors predictive of response are high baseline serum ALT levels and high degree of histological necroinflammation. Several factors, including genotype and the presence of cirrhosis, may predict the durability of response to lamivudine; higher rates of resistance have been reported in serotype *adw* (genotype A) than *ayw* (genotype D) (54% vs. 8%, Zollner *et al.*, 2004). Early viral suppression, in particular HBV DNA levels either

below 200 copies/ml or less than 3 log₁₀ after six months of treatment predict a lower risk of resistance after one year of treatment (Lai *et al.*, 2005b; Yuen *et al.*, 2001).

In HBeAg-negative patients after one year of treatment with lamivudine, HBV DNA became 'undetectable' with a nonstandardized assay in 70% of patients, serum ALT normalized in 75% and histological improvement was noticed in 60% (Tassopoulos *et al.*, 1999). However, the overwhelming majority of patients relapsed after treatment cessation.

Long-term lamivudine therapy can prevent complications of HBV-related liver disease as long as viral suppression is maintained. There has been wide experience of lamivudine to prevent chemotherapy-associated exacerbations of hepatitis B. The argument for 'deferred' or 'pre-emptive therapy' is probably weighted in favour of early treatment and prolonged therapy.

Resistance to Lamivudine Lamivudine resistance is conferred through acquired selection of HBV with mutations of the HBV DNA polymerase gene. Variants emerging during lamivudine therapy display mutations in the viral polymerase, within the catalytic domain (C domain)—which includes the YMDD motif (e.g. M204V or M204I)—and within the B domain (e.g. L180M or V173L). These mutants have a reduced replicative capacity compared to the wild-type virus. Four major patterns have been observed: L180M + M204V; M204I; L180M + M204I; V173L + L180M + M204V; and occasionally L180M + M204V/I. The L180M + M204V pattern occurs most frequently. The incidence of lamivudine resistance is 15–20%/year, with 70% of patients becoming resistant after five years of treatment. In patients treated with lamivudine monotherapy and decompensated cirrhosis, early detection of viral breakthrough is critical. A 1 log₁₀ rise of previously undetectable HBV DNA levels is taken as indicative of phenotypic resistance; adding adefovir before waiting for an ALT rise would be helpful for those patients (see below). The value of lamivudine monotherapy is being questioned because of the likelihood of subsequent resistance to a lineage of drugs including entecavir, telbivudine and possibly adefovir. Lamivudine could form (as will other nucleosides with even lower rates of resistance) the backbone of maintenance combination therapies. Lamivudine resistance has typically been managed by sequential treatment with adefovir (or more recently entecavir).

Adefovir Dipivoxil

Adefovir dipivoxil is an orally-bioavailable prodrug of adefovir, a phosphonate-acyclic nucleotide analogue of adenosine monophosphate. Adefovir diphosphate acts by selectively inhibiting the RT-DNA polymerase of HBV

by direct binding in competition with the endogenous substrate deoxyadenosine triphosphate (dATP). Adefovir is monophosphorylated and is not dependent on initial phosphorylation by viral nucleoside kinases to exert its antiviral effect. Clearance of adefovir is by renal excretion. Nephrotoxicity is the major side effect of higher doses of adefovir. However, in the two largest hepatitis B phase III trials involving 695 patients, no renal toxicity was found at the 10 mg dose.

Adefovir in HBeAg-positive Disease Adefovir 10 mg daily resulted in significant improvement when compared with placebo: improvement in liver histology (53% vs. 25%), reductions in HBV DNA (3.52 vs. 0.55 log₁₀ copies/ml), normalization of ALT (48% vs. 16%) and HBeAg seroconversion (12% vs. 6%). The 10 mg dose was chosen because of the more favourable risk–benefit ratio, but this dose is not optimal for a proportion of patients. A variable proportion of patients, particularly HBeAg-positive patients with higher body mass index and high viral load, have slower and poor primary responses.

Adefovir in HBeAg-negative Chronic Hepatitis B These patients require long-term treatment to suppress viraemia. In the pivotal anti-HBe-positive adefovir study (Hadziyannis *et al.*, 2003), 185 patients were randomized to placebo or adefovir 10 mg daily for 48 weeks. At 48 weeks the adefovir-treated group had significant improvement compared to placebo: improvement in liver histology (64% vs. 33%), reduction in HBV DNA (3.91 vs. 1.35 log₁₀ copies/ml), an undetectable HBV DNA (< 400 copies/ml, 51% vs. 0%) and normalization of ALT (72% vs. 29%). Adefovir may have particular benefit in HBeAg-negative HBV infection. Longer-term results indicate that continued therapy with adefovir resulted in suppression of HBV DNA to levels of < 1000 copies/ml in 79% of patients after 144 weeks of treatment. Resistance mutations were noted in 5.9% of patients after 144 weeks.

Adefovir-resistant Mutations Development of resistant mutations has been reported with adefovir monotherapy in both HBeAg-positive and HBeAg-negative patients. Sequencing of the RT domain of the HBV polymerase suggests that mutations rtA181V/T (the B domain) and rtN236T in the D domain confer resistance to adefovir. The reported mutations correlate with HBV DNA rebounds of >1 log above nadir, suggesting phenotypic resistance.

Life table analysis has suggested a cumulative incidence of 3.9%–5.9% (in naïve patients) after three years of treatment. A figure of 18% has been reported at four years of therapy. Adefovir resistance is apparently uncommon in treatment-naïve patients treated with adefovir and

emtricitabine (2′3′-dideoxy-5′ fluoro-3′ thiacytidine (FTC)) or adefovir and lamivudine in combination.

Adefovir mutants remain sensitive to lamivudine, emtricitabine, telbivudine and entecavir. Adefovir is an important drug for the treatment of lamivudine-resistant HBV infection. The early addition of adefovir at the time of detection of a log rise in HBV DNA is advocated. Resistance and clinical events are reduced if adefovir is added at lower concentrations of HBV DNA.

Entecavir

Entecavir is a cyclopentyl guanosine analogue. Recently activity against HIV has been suggested. Entecavir inhibits base priming, reverse transcription of the negative strand from the pregenomic messenger RNA and synthesis of the positive strand of HBV DNA. Phase III trials have been completed.

HBeAg-positive Patients A randomized study of entecavir 0.5 mg daily vs. lamivudine 100 mg daily for 52 weeks in 715 naïve patients showed histological improvement in 72% of entecavir- and 62% of lamivudine-treated patients. HBV DNA was suppressed to <300 copies/ml in 67% and 36% of entecavir- and lamivudine-treated patients (Chang *et al.*, 2006). The mean changes from baseline were −6.9 log and −5.4 log, respectively. HBeAg seroconversion occurred in 21% and 18% of entecavir- and lamivudine-treated patients.

HBeAg-negative Patients A phase III trial of 638 patients treated with entecavir 0.5 mg daily or lamivudine 100 mg for 52 weeks showed histological improvement in 7% and 61% of entecavir- and lamivudine-treated patients. HBV DNA suppression to less than 300 copies/ml occurred on treatment in 90% of entecavir- and 72% of lamivudine-treated patients. The mean changes of HBV DNA from baseline were −5.0 log₁₀ and −4.5 log₁₀, and ALT normalized in 78% and 71%, respectively. Rebound to levels detectable by PCR occurred in the majority of patients after cessation of treatment (Lai *et al.*, 2006).

Entecavir for Lamivudine-resistant Patients Entecavir shows some efficacy against lamivudine-resistant HBV, but the effect is partial, and higher doses of entecavir (1.0 mg) are required.

Entecavir Resistance After four years of follow-up, a cumulative resistance rate of approximately 1.2% of a subset of naïve treated and monitored patients has been reported. Virological responders however have not been evaluated in this protocol analysis. Virological rebound and resistance has been reported in 43% of

lamivudine-resistant patients after four years of switching treatment to entecavir. A complex picture of entecavir resistance is emerging, suggesting a requirement for new changes in the *rt* domain, in combination with those conferring lamivudine resistance, to reduce susceptibility to entecavir. Entecavir resistance requires M204V/I + L180M mutations + T184, S202 or M250 mutations (Tenney *et al.*, 2004).

Although entecavir is superior to lamivudine in terms of viral suppression, HBeAg seroconversion rates are not different between the two analogues at one year (21% and 18% respectively), although these could change with time (but not at a linear rate, apparently) given the lower rates of resistance to entecavir. Carcinogenicity after exposure to levels more than 35-fold greater than the dose administered in humans has been reported in rodents. These lesions include lung adenomas and carcinomas, and liver adenomas and carcinomas. The cumulative human risk will require post-marketing surveillance.

Telbivudine

Telbivudine is a thymidine analogue and belongs to a new class of β -L-configuration nucleoside analogues with specific activity against *Hepadnaviruses*. Preliminary studies have shown a pronounced inhibition of HBV replication with a safe profile and no effect on mitochondrial metabolism. Pharmacokinetic studies indicate once-daily dosing. Telbivudine is cleared by the kidneys, and dosing adjustments are recommended for patients with estimated creatinine clearance <50 ml/minute.

HBeAg-positive Patients A greater therapeutic response with telbivudine at week 104 has been noted in HBeAg-positive patients (64% of those receiving telbivudine vs. 48% of those on lamivudine). The mean decline was $-5.7\log_{10}$ vs. $-4.4\log_{10}$ in recipients of telbivudine versus lamivudine. In particular, HBV DNA was not detectable by PCR in 56% of the HBeAg-positive patients receiving telbivudine versus 39% of those on lamivudine. HBeAg seroconversion occurred in 30% at year 2, and 70% had normal ALT.

Histological responses, ALT normalization and HBeAg loss were greatest for patients whose week 24 HBV DNA was below $3\log_{10}$ (46% in the combined lamivudine/telbivudine groups) compared to those whose level of HBV DNA was $>4\log_{10}$ (45% of HBeAg-positive patients and 80% of the HBeAg-negative patients treated with telbivudine had undetectable HBV DNA by 24 weeks of treatment). Limited follow-up information suggests that patients may discontinue treatment after HBeAg seroconversion; responses are durable in approximately 80% (Lai *et al.*, 2006). Other studies have been completed (Chan *et al.*, 2006).

Anti-HBe-negative Patients The mean \log_{10} decline was -5.0 and -4.2 in telbivudine and lamivudine recipients, respectively. At two years, HBV DNA was undetectable by PCR in 82% of HBeAg-negative patients versus 52% of lamivudine recipients. 78% had normal ALT.

Viral resistance (defined as resistance mutations documented in HBV DNA amplified from serum from patients with viral breakthrough) was found in 17.8% and 7.3% of the HBeAg-positive and -negative patients after two years. Telbivudine-associated resistant mutations were all M204I or M204I + L180I/V. Lamivudine-associated resistant mutations were a mixture of M204V, M204I and + L180M double mutants. The explanation for this lies in the pathways of selection for YMDD-mediated HBV resistance and the fact that telbivudine is active against M204V, whereas lamivudine has reduced activity against both M204V and M204I mutants. Response at week 24 also predicted resistance. Resistance at two years was observed in 4% of HBeAg-positive patients and 2% of HBeAg-negative patients who had undetectable HBV DNA at 24 weeks, but the rates of resistance increased substantially in patients with higher levels of viraemia at this time point.

Tenofovir

Tenofovir is related to adefovir and has a similar mechanism of action. Tenofovir disoproxil fumarate is the prodrug of tenofovir. Tenofovir diphosphate inhibits the activity of RT by competing with the natural substrate deoxyadenosine 5' triphosphate, and after incorporation into DNA by DNA chain termination. The drug is approved at a dose of 300 mg for the treatment of HIV. There is emerging clinical evidence of the efficacy of tenofovir in chronic hepatitis B, with less nephrotoxicity. The drug is active against wild-type and precore mutant hepatitis B, as well as lamivudine-resistant HBV *in vitro* (Benhamou *et al.*, 2003; Bruno *et al.*, 2003; Dore *et al.*, 2004; Lacombe *et al.*, 2005; Nelson *et al.*, 2003; Van Bommel *et al.*, 2006). It is possible that the greater efficacy of tenofovir is a result of the higher active dose. A large-scale randomized phase III controlled trial comparing the efficacy of adefovir and tenofovir disoproxil in HBeAg-positive and -negative patients is underway.

Small sub-studies in HBV mono-infected and HIV-HBV co-infected patients have demonstrated the activity of tenofovir against HBV. In the ACTG 5127 study, 51 HIV-HBV co-infected patients with lamivudine resistance were randomized to tenofovir versus adefovir. Tenofovir-treated patients showed a greater time-weighted average DNA change and log suppression of HBV with tenofovir 300 mg (4.0 log vs. -3.1 log with adefovir 10 mg). Recent trials show a favourable effect of tenofovir

(72–130 weeks) and adefovir (60–80 weeks) in patients with lamivudine-resistant HBV infection and high baseline HBV DNA ($>10^6$ copies/ml) (Benhamou *et al.*, 2006; Van Bommel *et al.*, 2004). Patients treated with tenofovir (300 mg/day) had a faster and greater suppression of HBV DNA than those treated with adefovir (10 mg/day). The absence of phenotypic HBV resistance to tenofovir suggests a favourable resistance profile.

Although tenofovir has not yet been licensed for HBV treatment, it may become an important treatment of highly-replicative HBV infection and HIV–HBV co-infection. Phase III results are awaited. The pharmacokinetics of tenofovir are altered in patients with renal impairment, and it is recommended that the dosing intervals of tenofovir be adjusted for creatinine clearance. Lactic acidosis, hepatomegaly, steatosis and renal impairment have rarely been reported in patients with HIV infection treated with antiretrovirals and tenofovir. Exacerbations of hepatitis B may occur. Decreases in bone mineral density have rarely been reported in HIV-positive patients.

Emtricitabine

Emtricitabine (FTC) is a 5-fluoro oxathiolane derivative, closely related to lamivudine. Like lamivudine, emtricitabine is a cytosine nucleoside analogue. Early studies indicate that it is effective in both HBeAg-positive and HBeAg-negative patients (Gish *et al.*, 2005; Korba *et al.*, 2000). Emtricitabine shows a dose-related efficacy with an average 3 \log_{10} decrease of HBV DNA levels after eight weeks of treatment on the highest doses. In a randomized study of 48 weeks assessing treatment with 25, 100 or 200 mg for the first year and 200 mg for the second year, the median decreases in viral load were 2.6 \log_{10} , 3.1 \log_{10} and 2.9 \log_{10} respectively. HBeAg seroconversion rates were 23%, 24% and 23% respectively.

A major double-blind study has been completed. Patients were treated with 200 mg of emtricitabine or placebo once daily for 48 weeks. At the end of treatment, 62% of 167 patients receiving emtricitabine had improved hepatic histological findings versus 25% of 81 receiving placebo. HBV DNA concentrations were less than 400 copies/ml in 54% of patients in the emtricitabine group versus 2% in the placebo group. At week 48, 13% patients in the emtricitabine group with HBV DNA measured at the end of treatment had detectable virus with resistance mutations. The rate of seroconversion to anti-HBe (12%) and the HBe antigen loss were not different between arms (Lim *et al.*, 2006).

Due to its resemblance to lamivudine, emtricitabine selects the same HBV polymerase mutations associated with resistance. These rates of resistance are relatively high and limit its role as a monotherapy regimen. Like

lamivudine, emtricitabine may form a backbone of combination therapy.

Clevudine

Clevudine (L-FMAU or 2'-fluoro-5-methyl- β -L-arabino-furanosyluracil) is a novel L-nucleoside analogue derived from deoxythymidine (Chong and Chu, 2002; Marcellin *et al.*, 2004). Clevudine has potent anti-HBV activity. The mechanism of action is mainly inhibition of viral plus-strand DNA synthesis. A marked decrease of 9 \log_{10} in the viral load was observed in the woodchuck model. In the same model, clevudine combined with emtricitabine resulted in a sharp decrease in viraemia levels that was more pronounced than with the single drugs alone (Jacquard *et al.*, 2004). A unique characteristic of clevudine is the slow rebound of viraemia after cessation of treatment, which was observed also in patients; in an early phase II trial, the median decrease of HBV DNA levels varied from 2.48 to 2.95 \log_{10} with three different doses after 28 days of treatment. At the end of the 20 week follow-up period, a slow increase of HBV DNA levels was noticed. The drug does not accumulate.

In a further study, HBeAg-positive patients were randomized to placebo 30 mg or 50 mg clevudine (Lee *et al.*, 2006). Patients were followed up after 12 weeks of treatment for a further 24 weeks off-therapy. Median serum HBV DNA reductions from baseline at week 12 were 0.20, 4.49 and 4.45 \log_{10} copies/ml in the placebo, 30 mg clevudine and 50 mg clevudine groups, respectively.

Phase III placebo-controlled trials of 24 weeks duration have been completed. HBV DNA levels were significantly lower at the end of treatment ($-4.2\log_{10}$) and at 24 weeks follow-up for patients receiving clevudine. HBV DNA was undetectable at the end of treatment in 59% of patients receiving clevudine.

In vitro studies suggest that there may be cross-resistance with lamivudine-resistant HBV mutants. In animal studies resistance occurred in the B domain of the polymerase gene within 12 months of treatment.

Other New Agents

LB80380 is an oral nucleotide prodrug which is chemically similar to adefovir dipivoxil and tenofovir disoproxil fumarate. LB80380 is rapidly converted to the parent drug LB80331 in the liver and intestine. LB80331 is further metabolized to LB80317, a nucleotide analogue of guanosine monophosphate. After phosphorylation to the di- and triphosphate forms, the molecule inhibits viral replication following incorporation into viral DNA. The antiviral efficacy of a four-week course of LB80380 has been reported. The mean maximal HBV DNA changes during the four-week treatment period were

–3.02 to –3.80log₁₀ copies/ml for doses ranging from 30 to 240 mg daily. LB80380 has also been shown to be effective against YMDD mutants, including rtM204I and rtM204V in both *in vitro* and *in vivo* study. Further trials of LB80380 are being conducted for efficacy in patients with lamivudine-resistant HBV (Lai *et al.*, 2005a). Val LdC and LdA are also small molecule inhibitors of the hepatitis B DNA polymerase. These agents induce marked viral-load reduction in the woodchuck infected with woodchuck hepatitis B.

Pradefovir mesylate, a PMEA prodrug that was formerly known as remofovir, is preferentially metabolized into its active form in the liver, resulting in targeting to the liver and higher PMEA concentrations in that organ. The lower concentrations in the kidneys result in a potentially lower risk of nephrotoxicity than with adefovir. The efficacy, pharmacokinetics and safety of pradefovir at various doses compared with adefovir in patients with chronic hepatitis B has been evaluated in 242 patients randomized to receive pradefovir 5, 10, 20 or 30 mg/day or adefovir 10 mg/day. The declines in HBV DNA were greater for pradefovir 30 mg than for adefovir. In addition, metabolism of pradefovir in the liver was associated with less systemic drug exposure than with adefovir. HBV DNA viral-load reduction was 5 log₁₀ with the highest dose of 30 mg/day.

PMEO-DAPym, a novel acyclic pyrimidine analogue, has been assessed *in vitro*. Most drug-resistant mutants, including multidrug-resistant strains, remained sensitive to tenofovir and PME0-DAPym. Therefore, the latter molecule deserves further evaluation for the treatment of HBV infection, given the need for agents that do not share cross-resistance (Brunelle *et al.*, 2007).

Biological Response Modifiers

Relatively little progress has been made with biological response modifiers. Interleukin-12 (IL-12) is a 75-kDa heterodimeric protein and a member of the tumour necrosis factor (TNF) receptor superfamily. This cytokine is a product of macrophages and dendritic cells (i.e. antigen-presenting cells). IL-12 promotes Th-1 and suppresses Th-2 cell development, suggesting that IL-12 may be useful therapeutically to promote a cellular immune response. The compound induces secretion of IFN- γ from T and NK cells, increases the lytic activity of NK cells and facilitates specific CTL responses.

Previous studies suggest that a predominance of HBeAg-specific Th-2-type cells may contribute to chronicity in HBV infection. Pilot studies in HBV-infected patients have been conducted but this agent is not in clinical use.

Thymosin is an immune stimulant which is known to enhance suppressor T-cell activity and B-cell synthesis

of IgG *in vitro*. Peptide preparations of thymosin have been evaluated in small controlled trials and HBV DNA has been noted to become undetectable in some of these patients and in chimpanzees with chronic hepatitis. It is given parenterally by subcutaneous injection. This agent's possible therapeutic role was evaluated in a larger controlled trial, in which HBeAg seroconversion rates were not significantly different from recipients given placebo. Thymosin α 1, a synthetic 28 amino acid peptide with multiple biological activities primarily directed towards immune-response enhancement, has been developed for the treatment of HBV infection. Although approval for the treatment of hepatitis B has been granted in a few countries, the place of this drug is still being appraised. Few side effects are observed with thymosin injected subcutaneously.

Immunotherapy

It remains important to consider the possibility of therapeutic immunization of chronically-infected, HBV-positive patients to activate an immune response and eradicate viraemia. Progress remains slow however, and no therapeutic vaccination strategy has advanced beyond phase III assessment or to licensing. In a study of 32 patients with HBeAg-positive, HBV-DNA-positive chronic hepatitis B, three standard doses of GenHevac vaccine were given at monthly intervals. Six months after the first injection, 37% had undetectable DNA. Eight responders were given IFN- α to maintain virus inhibition (Pol, 1995). More recent results have not suggested successful immunotherapy using HBV vaccine.

Because of the interest in the possibility of provoking a cytotoxic or noncytolytic immune response to HBV core epitopes, attempts are continuing to invoke a strong response to decrease HBV DNA replication. An immune response has been observed in HLA A2.1 individuals with acute hepatitis B against an epitope (FLPSDFF-PSV) that contains an HLA A2 binding motif located between residues 18–27 of the hepatitis B nucleocapsid protein. This identification has led to development of an experimental therapeutic vaccine (Theradigm HBV tm), in which the CTL epitope derived from the hepatitis B core protein amino acids 18–27 has been incorporated into a vaccine also comprised of a T-helper cell epitope and two palmitic acid residues (CY-1899). In a pilot study, 90 patients with chronic hepatitis B infection received CY-1899. No significant changes in liver biochemistry or viral serology were observed during follow-up. Thus administration of the single-epitope vaccine, CY-1899, initiated CTL activity, but of a magnitude lower than that observed during spontaneous HBV clearance, which did lead to viral clearance (Heathcote *et al.*, 1999).

Recently, DNA-based immunization was tested using purified plasmid DNA containing protein coding sequences and the regulatory elements required for their expression. The DNA was introduced by means of a parenteral injection and the number of cells transfected and the amount of protein produced were sufficient to produce a broad-based immune response to a wide variety of foreign proteins. A response to HBsAg was achieved using this form of antigen presentation. A CD8+ CTL response was induced in BALB/C mice, suggesting a pathway for exogenous presentation of hepatitis B envelope protein via MHC class I expression (Davis *et al.*, 1995). A Pekin duck infected by DHBV was immunized with a plasmid encoding DHBV large (L), leading to sustained decrease in viral replication and even to clearance of intrahepatic viral cccDNA pool in some animals. Combination therapy data with lamivudine showed a pronounced antiviral effect of DNA vaccine to DHBV envelope protein (Thermet, 2003). If the safety of DNA-mediated immunization can be assured, this form of vaccination may also have therapeutic potential (Whalen and Davis, 1995).

The combination of nucleoside analogues to inhibit viral production with immunomodulatory approaches may prove useful. Phase I–II trials are in progress. At present, immunomodulatory therapy remains an unrealized goal of treatment. Use of a DNA vaccine approach relying on the administration of a DNA plasmid encoding the viral envelope gene with a prime boost strategy is perhaps most advanced.

HIV–HBV Co-infection

The management of hepatitis B is made more complex with co-infection with HIV. HIV and HIV treatment profoundly affect the natural history of HBV. HIV–HBV co-infected patients whose immune status is preserved on highly active antiretroviral therapy (HAART) should be considered for anti-HBV therapy, with appropriate therapy for HIV infection if indicated in order to minimize resistance. If HAART is indicated for a patient co-infected with HIV, lamivudine can be utilized, as lamivudine is active against HIV and HBV. Tenofovir is also active against HBV and HIV. Thus treatment of co-infection offers the opportunity to utilize combination treatments for hepatitis B. Adefovir is a useful agent for the treatment of hepatitis B in HIV co-infected patients where treatment of HIV is not deemed necessary as the drug has no effect on HIV at a dose of 10 mg/day, or for lamivudine-resistant HBV. Entecavir has anti-HIV activity.

Hepatitis B Virus and Primary Liver Cancer

Persistent infection with HBV is associated with a high risk of developing HCC. However, the precise role played

by the virus in causing this tumour remains to be elucidated (Kremsdorf *et al.*, 2006).

Epidemiological Evidence

Hepatocellular carcinoma is one of the 10 most common cancers in the world, with over 250 000 new cases each year. In areas where the tumour is particularly common, for example some regions of sub-Saharan Africa, China and South East Asia, the age-adjusted incidence of HCC is over 30 new cases per 100 000 population each year, whereas it is less than 5 cases per 100 000 per year in western Europe and North America. Primary liver cancer is more common in males than among females and the incidence of the tumour increases with age, reaching a peak in the 30–50 age group.

When specific tests for the serological markers of HBV infection became available, it was clear that the geographical areas with a high incidence of primary liver cancer were coincident with those with a high prevalence of seropositivity for HBsAg. Furthermore, most patients from high-risk areas presenting with primary liver cancer proved to be HBsAg-positive or to have high titres of anti-HBc. There is often a considerable interval between the initial virus infection and development of HCC, although the tumour does occur in younger age groups in high-risk populations.

The relative risk of developing primary liver cancer was estimated in an elegant prospective study carried out in Taiwan by Beasley and coworkers. Over 22 000 men, including more than 3000 HBsAg carriers, were followed for 75 000 man-years. The HBsAg carriers proved more than 200-fold more likely to develop HCC than members of the noncarrier group and more than 50% of the deaths in the former group were due to the tumour or to cirrhosis, another consequence of long-term HBV infection (Beasley and Hwang, 1991).

HBV DNA in Primary Liver Tumours

If HBV does indeed play a causal role in the development of primary liver cancer then the tumours might be expected to contain virus-specific nucleic acid and perhaps also to express viral proteins. Immunohistochemistry of HBV-associated tumours shows that a minority produce HBsAg, but production of HBcAg seems to be rather less common. Following the molecular cloning of the HBV genome, which made available hybridization probes of high specific activity, the PLC/PRF/5 cell line was shown to contain HBV DNA integrated chromosomally at several sites (Figure 12.13, lane A1). Further analysis reveals considerable rearrangement of the viral DNA, although it is not clear whether these rearrangements were present

in the original tumour or occurred during the establishment of the cell line (the genotype of the cultured cells appears to be stable with respect to the integrated HBV sequences). Other cell lines derived from HBV-associated primary liver tumours have also been shown to contain integrated HBV DNA.

Analysis by southern hybridization reveals integrated HBV DNA in approximately 80% of primary liver tumours from HBsAg carriers. An example is illustrated in Figure 12.13, lane D2. It is possible that more sensitive techniques, such as PCR, may enable the detection of viral DNA sequences in tumours which test negative by hybridization. In most cases, the tumours seem to be clonal with respect to the integrated viral DNA and seem to have arisen from a single cell. There is, however, considerable variation between tumours in terms of the number of integrants and their location. Although HBV DNA is often found integrated into Alu or other satellite sequences, this is likely to reflect the size of such targets and it seems that the sites of integration in the cellular chromosomes are random.

Nucleic-acid sequencing of the junctions between viral and cellular DNA shows that the direct repeats in the virus genome (see the account of the replication of the viral genome above) are frequently located close to these junctions and may be hot spots for recombination with host DNA. Because synthesis of progeny viral DNA in infected cells is cytoplasmic, it is likely that it is an intermediate in the process of conversion of virion DNA, or progeny HBV DNA recycled from the cytoplasm to the nucleus, which is involved in the recombination.

Mechanisms of Oncogenesis

Because primary liver cancer most often develops in a liver which is affected by chronic hepatitis or cirrhosis (or both) it has been suggested that the involvement of HBV is mediated through these pathological changes and subsequent regeneration. However, it is clear that HCC is much more likely to develop in an HBV-infected cirrhotic liver than in the cirrhotic liver of an uninfected patient and that tumours often develop in the livers of HBV-infected patients without an intermediate cirrhotic stage. The common finding of integrated viral DNA in tumours implies a more direct role of viral oncogenesis. However, the fact that up to 20% of tumours from HBsAg carriers may be negative for viral DNA suggests that other mechanisms may sometimes be involved.

Integrated viral DNA may also be detected in the livers of some HBsAg-carriers without tumour, both in patients with ongoing virus replication and in those who have cleared replicating virus. The hybridization techniques used are able to detect these integration events only if they occur at the same site in many cells and, because

the sites of integration in the chromosomes appear to be random, this implies the clonal expansion of a cell with integrated viral DNA. The establishment of such clones in the liver may be the first step in a multistage process leading to carcinoma and there may be a role for other environmental factors (such as mycotoxins in the diet) in such a process. The long interval often seen between the initial virus infection and tumour development fits with this concept. Furthermore, because integration seems to occur repeatedly throughout the period of virus replication, the continuing accumulation of pre-neoplastic clones within the liver might increase the probability of progression to tumour for patients with long-term chronic infection.

Production of HBsAg in the livers of carriers who have cleared virus replication, as well as by some tumours, indicates that the surface mRNA may be actively transcribed from integrated viral DNA. The activity of this promoter makes attractive the promoter-insertion hypothesis that aberrant transcription of cellular genes may result in loss of growth control. Recent studies suggest that, in up to 90% of tumours with integrated HBV DNA, the viral genome may be integrated into cellular genes, potentially providing cell-growth advantage (Murakami *et al.*, 2005). Another mechanism whereby viruses cause neoplastic transformation of cells is via the expression of a transforming gene introduced in the integrated viral genome. The HBV genome does not seem to contain such a gene and it has not been possible to transform cells *in vitro* using virus or viral DNA. Nevertheless, the X gene of HBV encodes a transcriptional transactivator and the possibility that this protein plays a role in disrupting the normal transcriptional control of the cell cannot be ruled out. Transactivation by the X protein seems to be through responsive elements such as the transcription factor AP-1 (*jun/fos*), AP-2, NF- κ B and the CRE site. It has been shown that truncated pre-S proteins may also have transactivating properties. These may be produced in tumour cells by expression of integrated HBV DNA with virus/host junctions in the surface ORF.

HEPATITIS D

Delta hepatitis was first recognized following detection of a novel protein, delta antigen (HDAg), by immunofluorescent staining, in the nuclei of hepatocytes from patients with hepatitis B (Rizzetto *et al.*, 1977). HDV is now known to be defective and to require a helper function from HBV for its transmission. HDV is coated with HBsAg, which is needed for release from the host hepatocyte and for entry in the next round of infection. The agent is unique among human viruses and consists of a particle

measuring 35–37 nm in diameter, with an internal nucleocapsid comprising the genome surrounded by the delta antigen and enveloped by an outer protein coat of HBsAg. The genome consists of a single-stranded, circular RNA of around 1700 nucleotides, the delta antigen being encoded by antigenomic RNA (reviewed by Taylor, 1997).

Two major modes of delta hepatitis infection are known (Hadziyannis, 1997). In the first, a susceptible individual is co-infected with HBV and HDV, often leading to a more severe form of acute hepatitis caused by HBV. Vaccination against HBV prevents such infections. In the second, an individual infected chronically with HBV becomes superinfected with HDV. This may accelerate the course of the chronic liver disease and cause overt disease in asymptomatic HBsAg carriers. HDV may be cytopathic, and HDAg directly cytotoxic. A less common type of infection has been seen in HDAg-positive patients who have received liver transplants. Hepatocytes in the graft become infected with HDV circulating at the time of transplantation. In the absence of HBsAg there is no cell-to-cell spread of the virus but HDV replication persists in isolated hepatocytes.

Epidemiology

Limited serological studies indicate a worldwide distribution of hepatitis D in association with HBV. The infection is important epidemiologically in southern Europe, the Middle East (the Gulf States and Saudi Arabia), Japan and Taiwan, and parts of Africa and South America. However, there is evidence that the prevalence of delta infection is declining in southern Europe, particularly Italy (Gaeta *et al.*, 2000). It has been estimated that 5% of HBsAg carriers worldwide (approximately 15 million people) are also infected with HDV. In areas of low prevalence of HBV, those at risk of hepatitis B, particularly intravenous drug users, are also at risk of HDV infection. Delta infection is associated with acute and chronic hepatitis, always in the presence of hepatitis B, and superinfection in a carrier of HBV often leads to exacerbation of severe hepatitis. Epidemics with high mortality have been described in South America in association with severe hepatitis B. Three genotypes (I to III) of HDV are recognized on the basis of phylogenetic analysis, and these are found, respectively, in southern Europe, Japan and Taiwan, and South America.

The mode of transmission of hepatitis D is similar to parenteral spread of hepatitis B, so serological evidence of infection is found most frequently in western Europe and North America in multiply-transfused individuals, such as patients with haemophilia, and in drug addicts, and is endemic in the Mediterranean Basin, the Middle East, West Africa, the Amazon Basin and some South Pacific Islands.

Structure and Replication of HDV

The HDV particle is approximately 36 nm in diameter and composed of an RNA genome associated with HDAg, surrounded by an envelope of HBsAg. The virus reaches higher concentrations in the circulation than HBV; up to 10^{12} particles/ml have been recorded. The HDV genome is a closed circular RNA molecule of 1679 nucleotides with extensive sequence complementarity that permits pairing of approximately 70% of the bases to form an unbranched rod structure. The genome thus resembles those of the satellite viroids and virusoids of plants and, similarly, seems to be replicated by the host RNA polymerase II, with autocatalytic cleavage and circularization of the progeny genomes via *trans*-esterification reactions (ribozyme activity). Consensus sequences of viroids, which are believed to be involved in these processes, also are conserved in HDV.

Unlike the plant viroids, HDV codes for a protein, HDAg, in an ORF in the antigenomic RNA. Around 600 copies of a polyadenylated mRNA, approximately 800 nt in length, may be detected in the cytoplasm of infected hepatocytes. The antigen, which contains a nuclear localization signal, was originally detected in the nuclei of infected hepatocytes and may be detected in serum only after stripping off the outer envelope of the virion with detergent. The delta antigen is detectable in two forms in the infected hepatocyte. The 195 aa (small) form is required for HDV RNA replication and binds to the rod-like structures of the genome and genome complement. The larger (214 aa) form, which is structural, and therefore required for virion assembly, seems to be synthesized following RNA editing. This process converts the termination codon at the end next to the ORF in the short form to a tryptophan codon, resulting in a 19 aa carboxyl-terminal extension.

Laboratory Diagnosis of HDV Infection

Specific serological tests are available to detect antibody to HDV—anti-HD IgM and anti-HD IgG—and HDV RNA and HDAg. Co-infection and superinfection can be distinguished by correlation of the results of these tests with those for markers of HBV infection. Thus, in co-infection, HBsAg, HBeAg and HBV DNA become detectable in serum, along with HDAg and HDV RNA. Co-existence of anti-HBc IgM with markers of HDV infection is a reliable indication of co-infection; anti-HD IgM becomes detectable, followed by anti-HD IgG. Markers of virus replication usually become undetectable during convalescence.

Superinfection of HBV carriers with HDV frequently results in persistent HDV infection. HD viraemia is followed by an anti-HD IgM, and then IgG, response.

Markers of HBV replication may be suppressed during acute HDV infection. Anti-HD IgM persists with HDAG and HDV RNA in serum in chronic delta hepatitis.

Pathogenesis

The pathogenesis of the disease is uncertain. It was believed that HDV is pathogenic and that the liver injury in hepatitis D is related to HDV itself, but this concept has been challenged with the observation that HDV re-occurs in liver-transplanted patients soon after grafting, but without signs of HBV recurrence or evidence of liver damage. In these individuals, HDV may establish latent infection that is not dependent upon HBV for replication, and which is only associated with recrudescence liver injury after the acquisition of HBV. Hepatitis D virions cannot be released from the infected hepatocytes without an envelope supplied by HBV. There is also a large body of data to suggest that the pathogenesis of HDV hepatitis is in part immunologically mediated.

Fulminant hepatitis may occur in acute HDV and HBV infection, and outbreaks of severe hepatitis have been reported in Indians of the Amazon basin and in areas of Central Africa. Degenerative changes were observed in these patients, characterized by fine steatotic vacuolization of hepatocytes, in keeping with a cytotoxic inflammatory lesion. It is known that hepatitis D may interfere with HBV replication but the molecular mechanism has not been established. An increase in HDV replication has been noted in concurrent HDV and HIV replication, but this may not necessarily cause more severe hepatitis.

Clinical Features

Hepatitis D causes acute, fulminant and chronic hepatitis either as a co-infection with hepatitis B or as a superinfection in patients with chronic hepatitis B. Clinically there is a spectrum of disease, and in some co-infected or superinfected persons HDV appears to be a pathogenic agent and to aggravate the underlying HBV infection. There is much interest in the study of pathogenicity caused by this agent.

Treatment

The mainstay of treatment remains long-term IFN or PEG IFN. A proportion of patients become HDV RNA-negative, or even HBsAg-negative, with accompanying improvement in histology. To date, treatment with nucleoside analogues has proven disappointing. IFN remains the only feasible treatment. Newer agents, such as clevudine or prenylation inhibitors, may prove useful (Andreone *et al.*, 2000; Farci, 2006). Patients with decompensated liver disease should be considered for transplantation with prophylaxis against reinfection with HBV.

Prevention and Control

Prevention and control measures of HDV are similar to those for HBV. Immunization with hepatitis B vaccine protects against HDV. There is, however, the problem of protecting many millions of carriers of HBV against superinfection with HDV. Studies are in progress to develop specific immunization of hepatitis B carriers against HDV using vaccines based on HDAG.

HEPATITIS C

Hepatitis C Virus is Responsible for Almost All Cases of Parenterally-transmitted Non-A, Non-B Hepatitis

The specific diagnosis of hepatitis types A, B and D revealed a previously-unrecognized form of hepatitis which was clearly unrelated to any of these three types. Results obtained from several surveys of post-transfusion hepatitis in the United States and elsewhere provided strong epidemiological evidence of an infection of the liver termed non-A, non-B hepatitis. This was the most common form of hepatitis occurring after blood transfusion in some areas of the world following the introduction of tests for HBsAg. Studies also showed that this infection was common in haemodialysis and other specialized units, that it occurs in a sporadic form in the general population and that it can be transmitted by therapeutic plasma components. There was also considerable evidence that the parenterally-transmitted infection, like hepatitis B, may become persistent and progress to chronic liver disease, cirrhosis and HCC. Transmission studies in chimpanzees helped establish that the main agent of parenterally-acquired non-A, non-B hepatitis was likely to be an enveloped virus with a diameter of 30–60 nm. These studies made available a pool of plasma known to contain a relatively high titre of the agent and enabled the molecular cloning of the genome of what we now know as HCV (Choo *et al.*, 1989).

The Organization of the HCV Genome

The genome of HCV (Figure 12.14) resembles those of other members of the family *Flaviviridae*, the *Pestiviruses* and *Flaviviruses*. It comprises around 9400 nt of positive-sense RNA, lacks a 3' poly(A) tract and has a similar gene organization. It has been proposed that HCV should be designated the prototype of a third genus in the family *Flaviviridae*, *Hepacivirus*. All of the viral genomes from this family contain a single large ORF which is translated to yield a polyprotein (of around 3000 amino acids in the case of HCV) from which the viral proteins are derived by post-translational cleavage and other modifications.

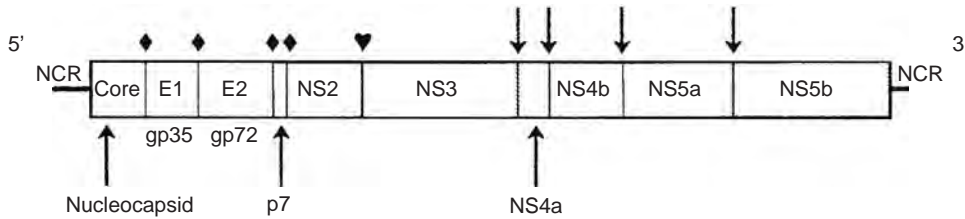


Figure 12.14 Organization of the hepatitis C virus genome. See text for details.

There is a short, untranslated region (UTR) at the 5' end of the genome of HCV and a further UTR at the 3' end, the large ORF accounting for over 95% of the sequence. The genome is not capped and translation is mediated by an internal ribosome entry site (IRES) in the 5' UTR. The structural proteins are encoded towards the 5' end and the nonstructural proteins towards the 3' end of the ORF. The first product of the polyprotein is the nonglycosylated capsid protein, C, which complexes with the genomic RNA to form the nucleocapsid. A hydrophobic domain anchors the growing polypeptide in the ER and leads to cleavage by a cellular signal peptidase. The amino acid sequence of the nucleocapsid protein seems to be highly conserved among different isolates of HCV.

The next two domains in the polyprotein also have signal sequences at their carboxyl termini and are processed in a similar fashion. The products are two glycoproteins, E1 or gp35 and E2 or gp70, which are found in the viral envelope. These glycoproteins have not been visualized *in vivo* and the molecular sizes are estimated from sequence data and expression studies *in vitro*. These envelope proteins are the focus of considerable interest as potential targets in tests for the direct detection of viral proteins and for anti-HCV vaccines. As with many other RNA viruses, replication of the HCV genome is an error-prone process and the resulting mutations lead to the generation, within an infected individual, of a population of viruses with closely-related, but different, nucleotide sequences (quasispecies). The effect is particularly noticeable in the region of the genome (the hypervariable region, HVR-1) which encodes a domain at the amino terminus of E2. Sequences within this region are highly variable within each individual, as well as between isolates of the virus, and divergence seems to be driven by the generation of neutralizing antibodies which are targeted to that domain of E2. This hypothesis is supported by the observation that much less variability occurs in agammaglobulinaemic individuals. Thus, efforts to develop hepatitis C vaccines are hampered by variability of the most obvious target molecule and the efficacy of candidate vaccines is likely to be impaired by the rapid evolution of antibody escape mutants.

Cleavage at the carboxyl terminus of E2 generates a small protein, p7, which oligomerizes to form an ion

channel. The remainder of the nonstructural region of the HCV genome is divided into regions NS2 to NS5 (Figure 12.14). HCV encodes a protease activity at the NS2/NS3 junction which cleaves that site *in cis*. Following cleavage, the nonstructural proteins remain associated with each other and with cellular membranes (so-called membranous web), forming a replication complex. NS3 has two functional domains, a protease which is involved in cleavage of the remainder of the nonstructural region of the polyprotein and a helicase which is assumed to be involved in RNA replication. The HCV protease, which uses NS4a as a cofactor, is a major target of efforts to develop specific antiviral agents. The HCV NS5, unlike that of the *Flaviviruses*, is cleaved to yield NS5a and NS5b. NS5b contains the gly-asp (GDD) motif common to viral RNA-dependent RNA polymerases and so is likely to be the HCV replicase, and NS5a may also be involved in genome replication. The biology of HCV was reviewed recently by Lindenbach and Rice (2005).

Recent progress in cell culture of HCV has helped advance our understanding of the biology of the virus. Subgenomic replicons comprising a selectable marker (such as the gene for neomycin resistance) driven by the HCV IRES coupled to the nonstructural region of the HCV genome, driven by a heterologous IRES, are able to replicate when transfected into Huh7 cells (derived from a human HCC) (Lohmann *et al.*, 1999). One strain of HCV, JFH-1 (for Japanese fulminant hepatitis), has been shown to be able to replicate in Huh7 cells (Wakita *et al.*, 2005). In addition, pseudoviruses enveloped with the HCV glycoproteins, E1 and E2, have been valuable in the analysis of early events in HCV replication. Several candidate receptors have been identified, including the tetraspannin CD81, the low-density lipoprotein receptor, scavenger receptor class B type I and heparin; and Claudin-1 (a tight junction component) has been shown to be an important co-receptor (Evans *et al.*, 2007).

Epidemiology of Hepatitis C

Infection with HCV occurs throughout the world, currently estimated at 170 million people. Many of the seroprevalence data are based on blood donors, who represent

a carefully-selected population in many countries. Almost 4 million Americans (1.8% of the population of the United States) have antibody to HCV, indicating ongoing or previous infection with this virus. Higher rates have been found in southern Italy, Spain, central Europe, Japan and parts of the Middle East, with as many as 19% in Egyptian blood donors. Until screening of blood donors was introduced, hepatitis C accounted for the vast majority of non-A, non-B post-transfusion hepatitis. However, it is clear that while blood transfusion and the transfusion of blood products are efficient routes of transmission of HCV, these represent a small proportion (about 15%) of cases of acute clinical hepatitis in the United States and a number of other countries (with the exception of patients with haemophilia). Current data indicate that in some 40–50% of patients in industrialized countries the source of infection cannot be identified; 35% or more of patients have a history of intravenous drug misuse; household contact and sexual exposure appear not to be major factors in the epidemiology of this common infection; and occupational exposure in the health-care setting accounts for about 2% of cases. Transmission of HCV from mother to infant occurs in about 10% of viraemic mothers and the risk appears to be related to the level of viraemia. HCV can be transmitted by organ transplantation.

Diagnosis of HCV Infection

Cloning of the HCV genome made possible the development of specific diagnostic tests, including EIAs for antibody and RT-PCR for viraemia. Because expression of the original clone was detected by antibodies from the serum of an infected patient, it was an obvious candidate for development of an EIA to detect anti-HCV antibodies. A larger clone, C100-3, was assembled from a number of overlapping clones and expressed in yeast as a fusion protein using human superoxide dismutase sequences to facilitate expression. This fusion protein formed the basis of first-generation tests for HCV infection. It is now known that antibodies to C100 are detected relatively late following an acute infection. Furthermore, the first-generation assays were associated with a high rate of false positivity when applied to low-incidence populations and there were further problems with some retrospective studies on stored sera. Data based on this test alone should, therefore, be interpreted with caution.

Second- and later generation tests include antigens from the nucleocapsid and further nonstructural regions of the genome. The former (C22) is particularly useful; the sequence of the HCV core protein is relatively highly conserved compared to other HCV proteins, and antibodies appear relatively early during infection. Supplementary tests involving several viral antigens bound to a solid substrate, RIBAs, are available and give a more detailed

evaluation of the antibody profile of the patient. Antibody tests based on synthetic peptides also are available.

Routine testing of blood donations is now in place in most industrialized countries and prevalence rates vary from 0.2–0.5% in northern Europe to 1.2–1.5% in southern Europe and Japan. Many of those who are found to be antibody-positive have a history of parenteral risk such as transfusion or administration of blood products or intravenous drug use. Sexual and perinatal transmission of HCV are uncommon, so it is not clear what the 'natural' routes of transmission are.

The availability of the nucleotide sequence of HCV made possible the use of PCR as a direct test for the (genome of the) virus itself. The first step is the synthesis of a complementary DNA copy of the target region of the RNA genome using RT primed by the antigenomic PCR primer or random hexadeoxyribonucleotides. The product of this reaction is a suitable target for amplification (RT-PCR). The concentration of virus in serum samples is often very low, so that the mass of product from the PCR reaction is insufficient for visualization on a stained gel. Therefore, a second round of amplification (with nested primers) or detection of the primary product by southern hybridization is required. There is considerable variation in nucleotide sequences among different isolates of HCV, and the 5' UTR, which seems to be highly conserved, is the preferred target for diagnostic PCR. Hepatitis C viral load may be estimated by quantitative RT-PCR or by using a hybridization assay based on branched oligonucleotides (branched-chain deoxyribonucleic acid (bDNA) assay). An immunoassay for the direct detection of the HCV core protein is also available and provides an alternative means of quantifying viraemia in patients with relatively high viral loads.

Interpretation of Serological Tests for HCV

A negative EIA test is sufficient to rule out infection in individuals, such as blood donors, without risk factors for HCV infection. For those of low risk, a positive EIA requires confirmation, and a supplementary assay for antibodies, such as RIBA, is valuable. Where the RIBA is negative, the EIA result is likely to have been a false positive. If the RIBA is positive, the patient is likely to have (or to have had) hepatitis C. RT-PCR may then be used to determine whether the patient is viraemic. Follow-up RT-PCR also is indicated where the result of the RIBA is indeterminate.

Individuals with even slightly raised serum aminotransferases should be tested for anti-HCV by EIA and any positive results should be confirmed by RT-PCR. In patients with biochemical or clinical evidence of liver disease, a positive EIA is sufficient to diagnose hepatitis C,

though testing for HCV RNA is valuable for confirmation. Quantitative assays for HCV RNA are valuable in monitoring the efficacy of antiviral therapy and may help predict long-term outcome.

Persistence of HCV Infection is Common

Current data suggest that 60–80% of infections with HCV progress to chronicity. Thus, in contrast to HBV infection, where persistent infections of immunocompetent adults is rare, persistent HCV infection seems to be the norm. The morbidity of chronic hepatitis C is affected by many interactive factors, including age of acquisition, concomitant alcohol abuse, gender, co-existing viral disease and the host immune response. Histological examinations of liver biopsies from ‘healthy’ HCV-carriers (blood donors) reveal that none has normal histology and that up to 70% have chronic active hepatitis and/or cirrhosis. It is not clear whether pathological changes result from direct cytopathology of the virus or are mediated by the immune response to infection. HCV infection is also associated with progression to HCC. For example, in Japan, where the incidence of HCC has been increasing despite a decrease in the prevalence of HBsAg, HCV is the major risk factor. There is no DNA intermediate in the replication of the HCV genome or the integration of viral nucleic acid, and viral pathology may contribute to oncogenesis through cirrhosis and regeneration of liver cells. HCV infection rarely seems to cause acute liver failure. In the United States, HCV accounts for 60–70% of chronic hepatitis, up to 50% of cirrhosis, end-stage liver disease and HCC, and causes an estimated 8000–10 000 deaths annually. Chronic HCV is the major reason for requiring liver transplantation in most countries.

Genotypes

The tremendous variation in the sequence of the genomes of various isolates of HCV has led to their classification into types and subtypes (Simmonds *et al.*, 2005), and six major genotypes are recognized (Figure 12.15). There is some evidence of variation between genotypes of viral pathogenicity and responsiveness to treatment (see below) but data are incomplete for most genotypes and other variables such as the age of the patient and the duration of infection confound interpretation.

Infections with types 1b and 1a are relatively common in Europe; infection with type 1b is frequent in southern Europe. Epidemiological differences in age distribution of major types and the risk factors associated with particular genotypes have become apparent. In Europe, types 3a and 1a are relatively more common in young individuals with a history of intravenous drug use. Type 1b accounts for most infections in those aged 50 or more. Type 4 infection

is the most prevalent infection in Egypt, and in many parts of the Middle East and Africa.

Although an inherently greater pathogenicity of type 1 HCV has been implied, these studies have not always been based on prospective follow-up, or appropriately controlled to account for the influences of several interdependent parameters and cofactors. Moreover, several clinical investigations have documented severe and progressive liver disease after infection with each of the well-characterized genotypes (1a, 1b, 2a, 2b, 3a, 4a, 5 and 6) so there is little evidence so far for variants of HCV that are completely nonpathogenic.

HCV genotypes differ from each other by 31–33%, and subtypes by 20–25%, at the nucleotide level (Simmonds *et al.*, 2005). Typically, genotypes may be assigned by analysis of amplicons derived from the 5′ UTR, which is frequently targeted in diagnostic assays, by detecting particular nucleotide polymorphisms through direct sequencing, or indirectly by using hybridization probes or changes in restriction sites. These approaches are sufficient for most clinical purposes, such as prediction of likely responses to treatment (see below), but do not provide a definitive assignment of genotype and subtype; this requires sequence and phylogenetic analysis of other regions, such as core/E1 and NS5b.

Treatment of Hepatitis C

Acute Hepatitis C

Early identification of acute hepatitis C is important, but may be difficult as the disease may be relatively silent in the acute phase; 75% of patients are not jaundiced and have nonspecific symptoms. Management of acute sporadic hepatitis C includes conventional supportive treatment and specific antiviral therapy. Therapeutic trials of IFN- α have been undertaken. Recent studies have indicated that treatment benefits those patients who have been treated early, but it may be reasonable to allow one to three months to determine which patients might convalesce spontaneously (Jaeckel *et al.*, 2001). In those who do not appear to be convalescing two to four months after onset of the disease, antiviral treatment should be considered, as a high percentage of patients (>80%) may respond. The optimal form of treatment for acute hepatitis C is not yet determined but weekly pegylated IFN- α and ribavirin can be considered. Studies are in progress to determine whether a wait-and-see strategy is detrimental compared to immediate treatment.

Chronic Hepatitis C

There is evidence that alcohol and hepatitis C may synergistically aggravate hepatic injury and the drinking of excess alcohol is discouraged because of this (Miyakawa

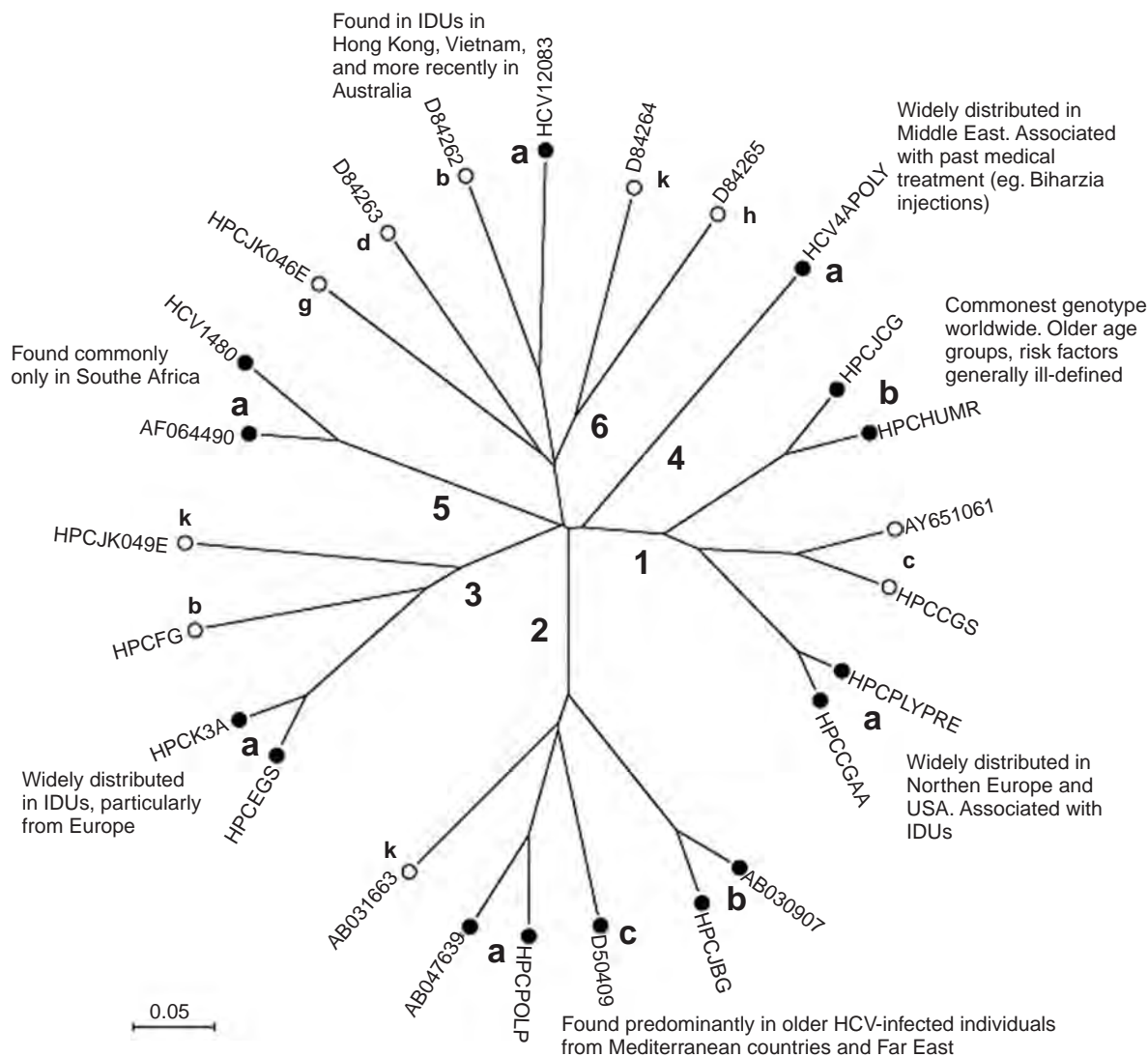


Figure 12.15 Evolutionary tree of complete ORF sequences for each HCV genotype. (Source: (Simmonds *et al.*, 2005).)

et al., 1993; Sawada *et al.*, 1993). The patient should be advised not to donate blood. Patients can be told that the parenteral route is the most important route of transmission and that the virus is not easily transmitted except by this route.

Treatment of hepatitis C has improved considerably. The aim of therapy is to achieve an undetectable HCV RNA six months following therapy (sustained virological response, SVR). A sustained response is associated with reduction in inflammation and severity of fibrosis. A substantial proportion of patients with chronic hepatitis C can be cured, although current treatments have limitations. HCV RNA should be measured in all patients

to confirm viraemia. If the test is reproducibly positive, serum aminotransferases, bilirubin, alkaline phosphatase and prothrombin time should be measured. In patients whose lifestyle or geographic origin suggest they are at risk of other forms of viral hepatitis, HBsAg and HIV infection must also be considered. Because autoimmune hepatitis is treated differently, it is particularly advisable to exclude this diagnosis by measuring titres of anti-smooth muscle and anti-liver-kidney microsomal antibodies, even in patients with a positive anti-HCV test.

A liver biopsy is helpful in grading the degree of inflammation and staging the degree of fibrosis. Earlier guidelines recommended antiviral therapy for those

patients with chronic hepatitis C who were deemed to be at highest risk of developing cirrhosis; that is, patients with chronic hepatitis C who had persistently increased serum ALT levels, detectable levels of HCV RNA and histological evidence of portal or bridging fibrosis or inflammation and necrosis. However, recent guidelines have been modified in the light of improved treatment responses, so that all patients with hepatitis C are potential candidates for treatment; a liver biopsy can be informative and provides unique clinical information, but may not be mandatory for all patients.

Careful clinical monitoring is suggested as an alternative to antiviral therapy for patients with less severe histological changes in whom cirrhosis may not develop.

IFN- α is difficult to apply in decompensated cirrhosis and may precipitate deterioration. These patients should be considered for liver transplantation.

Treatment with Interferon- α and Ribavirin

As noted above for HBV, IFN acts via species-specific surface target cell receptors. The cellular activities of IFN- α are mediated by the products of the IFN-inducible genes.

Ribavirin is a guanosine nucleoside analogue. This agent shows only modest activity against hepatitis C, but it increases the activity of IFN- α when the two agents are used in combination. The drug exerts its action after intracellular phosphorylation to mono-, di- and triphosphate nucleotides. The precise mode of action probably includes perturbation of intracellular nucleoside triphosphate pools, interference with the formation of the 5' cap structure of viral mRNA by competitive inhibition of both guanylttransferase- and methyltransferase-capping enzymes (although this mechanism does not apply to HCV), direct inhibition of the viral mRNA polymerase complex and possibly enhancement of macrophage inhibition of viral replication. Ribavirin may also induce mutations in the hepatitis C genome, inducing error catastrophe and thus affecting HCV replication. The combination of ribavirin and IFN- α has been shown to produce SVRs in about 40–50% of patients (Khakoo *et al.*, 1998).

Pegylated Interferons (See Above)

Twelve-kilodalton pegylated α 2b (Viraferon PEG, Schering) and forty-kilodalton PEG IFN- α 2a (Pegasys, Roche) have been licensed for the treatment of hepatitis C. There do not appear to be major differences in efficacy between the two IFNs, although they have different pharmacokinetic profiles and molecular structures. The efficacy of pegylated α -2a has been investigated in several controlled trials, and the outcome compared to standard IFN or IFN

plus ribavirin, based on virological, biochemical and histological responses.

Forty-eight weeks of PEG IFN- α -2b 1.5 μ g kg⁻¹ QW in combination with weight-based ribavirin 800 mg/day achieved a 54% SVR overall (42% for genotype 1, 80% for genotypes 2 and 3), compared to 47% for IFN α -2b plus ribavirin. Analysis of response by patient weight demonstrated that the optimum dose of ribavirin is 10.6 mg/kg/day, and those receiving this dose achieved SVR of 48% for genotype 1 and 88% for genotypes 2 and 3 (Manns *et al.*, 2001).

Similar results have been obtained with PEG IFN- α 2a plus ribavirin (Fried *et al.*, 2002). However, a fixed ribavirin dose of 800 mg/day was shown to be adequate for those with genotypes 2 and 3. Additionally, 24 weeks of combination therapy was adequate for those with genotypes 2 and 3, a finding which has generally been extrapolated and applied to the use of PEG IFN- α 2b and ribavirin.

Current Treatment Protocols PEG IFN- α 2b is administered at a dose of 1.5 μ g/kg/week by subcutaneous injection. Ribavirin is administered according to body weight of the patient, for patients infected with genotypes 1–6. For patients less than 65 kg the dose of ribavirin is 800 mg orally/day. For patients between 65 and 85 kg the dose of ribavirin is 1000 mg/day. For patients more than 85 kg the dose of ribavirin is 1200 mg. Pegylated IFN- α 2a is given at a fixed dose of 180 μ g/week. Ribavirin is given at a dose of 1000 mg if the patient weighs less than 75 kg or at a dose of 1200 mg for patients greater than 75 kg in all patients with genotype 1 infection. However, patients with genotypes 2 and 3 are treated with a dose of 800 mg daily of ribavirin.

The pivotal studies suggested that a 24-week schedule for HCV genotype 2 or 3 is sufficient, whereas patients with HCV genotype 1 require 48 weeks of therapy. Thus patients with genotypes 1 and 4 are treated for 12 months whereas patients with genotypes 2 and 3 are treated for 6 months. Few patients with genotype 4 have been studied. However, an SVR of 79% may be achieved following a 48-week course of PEG IFN- α plus ribavirin. There is limited published data on treatment outcome in patients with genotypes 5 and 6.

Patients with genotype 1 who do not show an HCV RNA decline of at least 2 log₁₀ after 12 weeks of therapy have no opportunity of achieving an SVR and therapy should be discontinued in these patients. At week 24 HCV RNA should be measured by a sensitive qualitative PCR. Patients who are HCV RNA-positive at six months should stop therapy. As patients with genotype 2 or 3 infrequently fail to respond to treatment (4%), it is unnecessary to check the viral load at 12 weeks.

The major early side effects of IFN include influenza-like syndrome, chills, fever, malaise, muscle aches and headaches. These symptoms may be ameliorated by paracetamol. Headaches, poor appetite, weight loss, increased need for sleep, psychological effects (irritability, anxiety and depression), hair loss, thrombocytopenia and leukopenia are also common side effects. Depression can be modified by serotonin uptake inhibitors. Dose modifications may be necessary, particularly in patients with cirrhosis who have low white cell counts and platelets due to portal hypertension. Unusual or severe side effects include seizures, acute psychosis, bacterial infections, autoimmune reactions and thyroid disease. Hyperthyroidism or hypothyroidism is relatively common and can be seen in up to 5% of patients, particularly in those with pre-existing antithyroid antibodies. Proteinuria, myocardopathy, skin rashes and IFN antibodies may also occur. There have been reports of patients developing interstitial lung disease with IFN- α , though this is rare, and a neuroretinitis may occur, particularly in patients with diabetes. This is a medical emergency and treatment must be stopped immediately if patients report visual symptoms or visual field abnormalities. Careful retinal examination is mandatory. There are rare reports of bone marrow suppression resulting in bone marrow aplasia.

The major side effects of ribavirin are haemolytic anaemia, myalgia (muscle pain), hyperuricaemia, some gastrointestinal upset and dyspepsia. Some patients also report irritability. Patients should be appropriately clinically monitored for the above side effects, and white cells, haemoglobin and platelets, as well as AST, ALT, uric acid, albumin, bilirubin and thyroid function tests should be measured every four weeks.

Adherence to therapy is an important factor for improving SVR. Unfortunately, side effects frequently necessitate dose reductions, and discontinuation of therapy in 14%. It is important to motivate patients and to provide appropriate support. Patients with hepatitis C should be advised to minimize their intake of alcohol. Those who are not immune should be vaccinated against hepatitis A and B. The risk of sexual transmission in monogamous partners is low.

Supportive therapies such as antidepressants, erythropoietin and GCSF have been demonstrated to reduce the incidence of IFN-induced depression, anaemia and neutropenia respectively, and to enhance adherence to therapy, but have not yet been shown to benefit the SVR rate. These treatments add to the expense of therapy.

Nonresponders to PEG IFN and ribavirin combination therapy have a poor response to retreatment, and at present no treatment regimen is generally recommended. Those who relapse after IFN monotherapy, or standard IFN and ribavirin therapy, may achieve an SVR with PEG IFN

and ribavirin, particularly if they do not have genotype 1. However, treatment should be stopped if patients do not show an early viral response with retreatment. Several major trials including EPIC3 and HALT-C are currently investigating the long-term use of low-dose PEG IFN in nonresponders and relapsers.

There is an attempt to abbreviate courses of treatment for patients with genotype 1 and low viral loads (600 000 IU/ML) who show a rapid viral response; that is, those who are negative for HCV RNA by PCR at one month. Similarly, it may be possible to stop treatment at 16 weeks for patients with genotypes 2 and 3 who show a rapid viral response and are negative by PCR at 4 weeks. Optimal dosing of ribavirin may be necessary to achieve a rapid viral response in order to shorten treatment. HCV RNA levels higher than 800 000 IU/ML in genotype 3 may be disadvantageous.

Several small trials have suggested that IFN therapy improves liver function and reduces the incidence of HCC in patients with cirrhosis due to hepatitis B or C (Nishiguchi *et al.*, 1995). A retrospective analysis of data for 913 patients from Italy and Argentina with chronic viral hepatitis and cirrhosis showed that IFN treatment lowered the rate of progression to HCC twofold (International Interferon α -Hepatocellular Carcinoma Study Group, 1998). The risk reduction was apparently greater for patients with chronic hepatitis C and no evidence of infection with HBV.

New Treatments on the Horizon

Important progress is being made in the development of new treatments, in particular new specific inhibitors of hepatitis C. To date, most of the early trial results have only been published in abstract form. Infergen (interferon alphacon 1), a consensus IFN, is more potent than natural IFN- α , and could provide increased efficacy. IFN- β has been used for retreatment for HCV relapsers after failure of IFN- α . Viroimidine is a prodrug of ribavirin which may cause less haemolytic anaemia. Pharmacokinetic studies in animals have shown the drug to be preferentially taken up by the liver, with reduced exposure of ribavirin in plasma and red blood cells. Phase III studies showed that a fixed dose of viroimidine (600 mg twice daily) resulted in lower efficacy but less anaemia than a weight-based dose of ribavirin.

Albuferon (alphaferon) is a novel 85.7 kDa protein consisting of IFN- α genetically fused to human serum albumin. The fusion with serum albumin extends the half-life of the IFN. Albuferon may be dosed at intervals of two and possibly four weeks. Phase III studies are in progress.

Pilot studies with amantadine, IFN and ribavirin have been completed. The role of triple therapy with pegylated IFN is being assessed but a marked effect is not immediately apparent. Thymalfasin plus pegylated IFN- α 2a plus ribavirin has been suggested to add to the efficacy of IFN and ribavirin treatment in nonresponders. There is no advantage to treating nonresponders with pegylated IFN- α 2a plus mycophenolate mofetil.

Several new polymerase and protease inhibitors of HCV show some promise and are in phase I or phase II study. MN283 is a prodrug of MN107, the triphosphate of which competitively inhibits NS5b RNA polymerase. The activity of NM283 when orally administered once a day has been examined in chimpanzees and in phase II studies. In treated animals HCV RNA concentrations dropped rapidly, with a mean viral load reduction of 1.05 and 0.83 log at day 7 of therapy in the high- and low-dose groups. Phase II studies in humans in combination with IFN in both naïve and nonresponder patients have resulted in unacceptable gastrointestinal toxicity. VX-950 is a peptidomimetic protease inhibitor of the hepatitis C NS3-4A protease. Encouraging results in phase I and phase II studies have been reported in a small number of patients. Genomic analysis of HCV sequences attained after a few weeks of monotherapy have shown that the emergence of resistance develops relatively quickly. A 28-day, phase II study of VX-950 in combination with PEG IFN and ribavirin showed that plasma HCV RNA levels were below the limit of detection (10 IU/ml) in 12 of 12 patients at the end of 28 days of treatment. Further studies are planned; phase II studies assessing the efficacy of 12 weeks and 24 weeks of treatment with VX950 (750 mg every 8 hours) together with PEG IFN- α or in combination with PEG IFN- α and ribavirin have begun. In addition, VX-950 is being studied in other treatment regimens and patient populations, including nonresponders. The further development of BILN-2061, an HCV protease inhibitor, has been stopped because of preclinical cardiac toxicity. SCH 503034 is a novel ketoamide, peptidomimetic protease inhibitor of HCV replication *in vitro*. Phase II studies in combination with IFN- α 2b have begun. Other protease inhibitors are in development.

Isatoribine is a toll-like receptor 7 agonist. It has no direct *in vitro* activity but induces hepatic 2'/5'-oligoadenylate synthetase, leading to activation of the innate immune response. Some change in viral load has been noted in a varying dose study. CPG 10101 is a toll-like receptor agonist; a preliminary dose-finding study indicates suppression of viraemia. Nuclease-resistant ribozymes targeting the minus-strand complement of the highly-conserved 5' UTR of HCV RNA are an experimental treatment of chronic HCV infection. Antisense inhibitors, for example ISIS 14803, a

20-base phosphorothioate antisense oligodeoxynucleotide that inhibits HCV replication and protein expression, are being tested in cell culture and mouse models.

Prevention of Hepatitis C

There are no vaccines available to protect contacts of individuals with hepatitis C. Difficulties in vaccine development include the sequence diversity between different viral groups and the substantial sequence heterogeneity among isolates in the N-terminal region of the E2 glycoprotein. However, secondary transmission should be relatively easy to prevent because it is inefficient. The role of intra-familial transmission requires clarification, but is relatively infrequent. Sexual transmission is possible and has been described, but fortunately this is a relatively inefficient and infrequent route. Young sexually-active adults should be told of the advisability of condom use in general for casual sexual contact.

Mother-to-infant transmission has been observed, but appears to be unusual. Differences in the rates of maternal-infant transmission in different countries remain unexplained, and the importance of this route in perpetuating the reservoir of human infection is unknown, but could be relevant. Maternal-infant transmission is more likely in mothers with HCV RNA concentrations higher than 10^7 genomes/ml. Transmission from infected surgeons to their patients has been documented and verified by molecular epidemiological evidence (Esteban *et al.*, 1996).

Immunization

There are currently no vaccines for the prevention of HCV infection. Physicians should consider vaccinating patients with chronic hepatitis C against HAV and HBV.

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GB Virus C (GBV-C) and Torque Teno Virus (TTV)

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INTRODUCTION

Most blood-borne hepatitis viruses have been discovered with hepatitis A–E, but researchers are still striving to find additional examples. Toward the end of the last decade, newly-established molecular biological techniques identified two candidate viruses, known as GB virus C (GBV-C) and torque teno virus (TTV). The real impact of these viruses on liver diseases is still unclear and their biology remains obscure due to a lack of suitable cell culture systems.

While GBV-C is a member of the family *Flaviviridae*, it has not been assigned a specified genus. Closely related hepatitis C virus (HCV) is classified within the genus *Hepacivirus*. TTV is the first human virus to be found with a circular single-stranded (ss) DNA genome with an anti-sense orientation, similar to that of chicken anaemia virus (CAV). The prevalence of GBV-C is 1–4% in the general population, while that of TTV is over 90%. Animals were found to harbour similar viruses specific to each species. Where donors are screened for hepatitis B and C viruses, the incidence of post-transfusion hepatitis is not high enough to be explained by these highly-prevalent viruses.

GB VIRUS C (GBV-C)

History of GBV-C

In 1967, Deinhardt *et al.* (1967) reported four tamarins (*Saguinus* spp.) inoculated with a day 3 acute-phase serum sample from a surgeon (whose initials were G.B.).

All four contracted acute hepatitis with elevation of serum transaminase levels 14–53 days post-inoculation, and one developed hyperbilirubinemia. The agent (GBV) remained hepatogenic in tamarins after passages (Table 13.1). Tamarins have proved susceptible to the GB agent, but common marmosets (*Callithrix jacchus*) and baboons (*Papio* spp.) were not. Presence of indigenous pathogens in tamarins created ambiguous results. In early phases of the investigations it was not easy to distinguish GBV from hepatitis A virus (HAV), even though GBV was reported to be small, heat labile and ether sensitive, unlike HAV. GBV was suggested to be of tamarin origin, because some control animals exhibited similar symptoms and the disease associated with the GB agent was not easily transmissible between tamarins reared in the same cage. However, the conclusions remained controversial. Only the serum and liver extracts of an infected tamarin were infectious, whereas its faeces were not (Reviewed in Hino and Miyata, 2007).

In 1995, almost two decades after the first observation of this transmissible hepatitis, representational difference analysis identified two related RNA viral sequences in the GB agent, which came to be designated GBV-A and GBV-B (Simons *et al.*, 1995). The genome of each virus was over 9 kb long and had a limited sequence similarity to the isolates of HCV. The original serum of GB did not contain either virus, but instead harboured another related virus, called GBV-C. Another group of investigators independently discovered a viral sequence they designated 'hepatitis G virus', which was closely related, if not identical, to GBV-C (Linnen *et al.*, 1996).

Table 13.1 Summary of GBV

Source	Remarks	Virus
Human serum of G.B.	Hepatitis in tamarin, transmissible, but not in marmoset	GBV
Serum of tamarin with hepatitis	Nonpathogenic in tamarin, transmissible. Found also in mystax, owl monkey and marmoset Acute hepatitis in tamarin, transmissible. Found transmissible to marmoset	GBV-A GBV-B
Human serum (1–4%) (G.B. serum)	Nonpathogenic. Transmissible to cause persistent infection. Not transmissible to tamarin. Found also in chimpanzee.	GBV-C (HGV) (Not GBV-A or -B)

GB Agents in Animals

GBV-A was found in tamarins (*Saguinus labiatus*), mystax (*Saguinus mystax*), owl monkey (*Aotus trivirgatus*) and common marmoset (*Saimiri* spp.), while GBV-B was detected in tamarins (Linnen *et al.*, 1996). GBVs from different species are divergent from each other and cluster according to the genetic similarity of their original animal species, suggesting that GBV-A and GBV-B originated in New World monkeys. GBV-C was also identified in chimpanzees (*Pan troglodytes*) and similarly clustered within the host species but was divergent from human GBV-C (Adams *et al.*, 1998).

GBV-A does not cause a significant elevation of alanine aminotransferase (ALT), even in tamarins. The natural course of GBV-B infection in tamarins has been investigated extensively. In tamarins, transient hepatitis caused by GBV-B was confirmed by elevation of liver enzyme levels as well as inflammation and focal necrosis in liver biopsies. Peak viraemia levels exceeded 10^9 copies/ml, after which the virus was cleared within 14–16 weeks. A strong protective immune response after the initial infection was suggested, because challenge with the same virus induced only a brief period of viraemia. After intrahepatic inoculation of infectious synthetic RNA, one of two healthy tamarins (*Saguinus oedipus*) was persistently infected with GBV-B for more than two years. High-titre viraemia (10^8 – 10^9 copies/ml) and transiently elevated serum ALT levels were detected at 4–12 weeks post-inoculation in both animals. While some tamarins are infected persistently, the induction of chronic liver disease has not been reported in them (Reviewed in Hino and Miyata, 2007).

A tamarin became persistently infected after transfection with the poly(U) deletion mutant of GBV-B (Nam *et al.*, 2004). This animal developed viraemia and hepatitis, and died at week 90; the recovered virus contained eight amino acid (aa) changes in the genome. Tamarins inoculated with serum of this animal developed an acute resolving infection. Jacob *et al.* (2004) found that a widely

available, non-endangered primate species, the common marmoset (*C. jacchus*), is susceptible to GBV-B infection and develops a characteristic acute hepatitis. Thus, the questions raised in the original experiments performed by Deinhardt *et al.* (1967) remain unanswered: If GBV-C does not replicate in tamarins, why did all the tamarins inoculated with the GB serum develop hepatitis? If GBV-C can replicate in tamarins, why did it disappear from the GB agent after several passages?

The GBV Genome

GBVs have genomes consisting of $\sim 9.3 \times 10^3$ nt, and a single large open reading frame (ORF) that encodes a precursor polyprotein of around 2850 aa. The genome is organized much like that of HCV, with genes encoding the structural and nonstructural proteins located towards the 5' and 3' ends, respectively (Figure 13.1) (Linnen *et al.*, 1996; Simons *et al.*, 1995). The core protein of GBV-B and GBV-C seems to be truncated, while that of GBV-A is almost missing (Linnen *et al.*, 1996; Simons *et al.*, 1996). The 5' termini of GBVs contain a 5' noncoding region (nontranslated region (NTR)) with an internal ribosome entry site (IRES) as in HCV (Simons *et al.*, 1996). The core-coding sequences of GBV-B are not only found to be a part of the IRES but also take part in the replication process. The C-terminal half of p13 of GBV-B, located between E2 and NS2, showed clear similarity to p7 of HCV, a small membrane-spanning protein (Ghibaudo *et al.*, 2004).

Schleicher and Flehmig (2003) reported that genotyping by restriction fragment length polymorphism (RFLP) for the 5' NTR is capable of identifying known genotypes (types 1–5) with a 99.6% accuracy. The phylogenetic analysis of human GBV-Cs has revealed that they cluster into six groups: genotype 1 (West Africa), genotype 2 (USA/Europe), genotype 3 (Asia), genotype 4 (South East Asia), genotype 5 (South Africa) and genotype 6 (Indonesia) (Muerhoff *et al.*, 2006). The hypervariability of the HCV E2 protein is one of the main factors in maintaining chronic HCV infection. However, GBV-C E2 seems

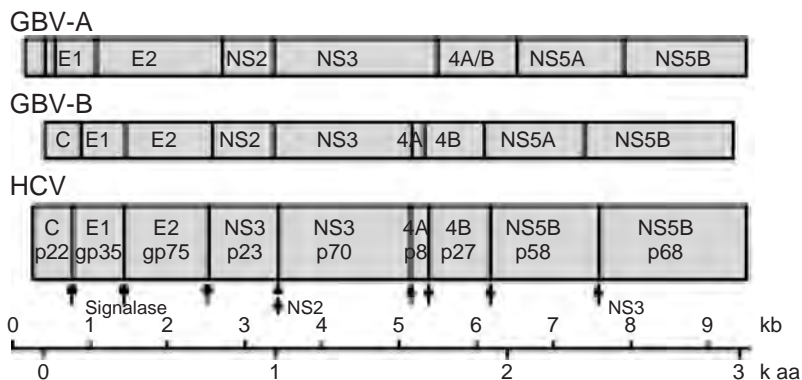


Figure 13.1 Genomic structure of GBV-A, GBV-B and HCV. Untranslated regions at 3' and 5' termini are not shown in the figure.

to be 100 times more stable than HCV E2 in six patients with acute hepatitis (Reviewed in Hino and Miyata, 2007). No aa substitutions in the loop domain were observed in seven additional patients with persistent GBV-C viraemia over a course of more than two years. How does GBV-C maintain persistent viraemia in some individuals without substantial variations in the envelope protein?

GBV in Cell Culture

GBV-C replicates in cells that originate from CD4⁺ T cells, B cells and hepatocytes. In every case, however, the replication of GBV-C was not efficient enough to serve for classic virological analyses (Reviewed in Hino and Miyata, 2007). In contrast, GBV-B replicates in primary cultures of normal tamarin hepatocytes with rapid amplification of the cell-associated viral RNA and secretion of $\sim 10^7$ copies/ml. In addition, the virus passage could be monitored by immunofluorescence staining of the NS3 protein. Despite reducing GBV-B replication in cell culture, ribavirin exhibited no significant decrease of viraemic titres in tamarins. GBV-B replication was reduced by more than two logs in hepatocyte cultures supplemented with an inhibitor of HCV NS3 protease (pyrrolidine-5,5-trans-lactams) and by three logs in marmosets given it *in vivo* (Bright *et al.*, 2004).

In a quest for gene therapies, HD-TET-tIFN, a helper-dependent adenovirus vector expressing tamarin IFN- α (tIFN- α) under the control of the tetracycline-inducible transactivator (rtTA2s-S2) was constructed (Aurisicchio *et al.*, 2005). tIFN- α efficiently inhibited a GBV-B replicon in an Huh-7 hepatoma cell line at low HD-TET-tIFN- α doses. A certain degree of transcriptional control of tIFN- α was also achieved in tamarins. In spite of this progress with GBV-B, the recent success in robust replications of the entire HCV in culture

(Lindenbach *et al.*, 2005) may make this GBV-B system in cell culture less attractive as a surrogate for HCV research.

Replication Site of GBV-C

The *in vivo* replication site of GBV-C is of interest because it may provide an insight into the possible pathogenicity of GBV-C. Surveillance of 17 liver-transplantation patients revealed that 70% had significantly lower GBV-C RNA titres in the liver than in the serum, while the remainder had viral RNA only in the serum. In another study, GBV-C RNA was undetectable in liver, colon or gall bladder, but detectable in serum and appendix. In a study to investigate the negative-strand RNA in serum and 23 tissue samples taken from 4 individuals who had died in accidents, the spleen and bone marrow were invariably positive. Individual instances of positive kidney and liver were also found. No negative strands were detected elsewhere. Still another study for detection of the negative-strand RNA found that five bone marrow samples were positive. Although most of these studies suggest that GBV-C replicates mainly in extrahepatic tissues, samples were probably obtained from chronically-infected people. The negative-strand RNA was detected in four of six (67%) explanted liver specimens, as well as hepatocyte-restricted infection *in situ* hybridization. Given that the hepatitis induced by GBV-B in tamarins is acute and transient, significant intrahepatic replication may occur only in the acute phase of infection (Reviewed in Hino and Miyata, 2007).

GBV-C in the Healthy Population

Most studies of GBV-C in the healthy population have been based on volunteer blood donors, using reverse transcriptase polymerase chain reaction (RT-PCR) for

the 5' NTR of GBV-C to detect GBV-C viraemia and anti-E2 antibody. Most individuals exposed to GBV-C (GBV-C-positive) were either RNA-positive or antibody-positive. The prevalence of GBV-C viraemia is reported to be 1–4% in most countries; a high rate of GBV-C viraemia is usually associated with frequent HCV-positive donors in the various regions. Although the viraemic population was within a 1–4% range throughout generations, the antibody-positive population was age-dependent: <1, 9, 12 and 13% in age groups of <15, 18–29, 30–39 and 40–49 respectively. This suggests that most children who acquire GBV-C at birth or in early childhood enter a long period of viraemia, whereas most adults tend to have acute infections with a relatively short period of viraemia. Among injecting drug users, the viraemic population exceeded 20% (Reviewed in Hino and Miyata, 2007).

Transmission of GBV-C

In a multicentre study, 34/175 (19%) anti-HCV-positive mothers were positive for GBV-C RNA. All 21 (62%) of the 34 babies to whom GBV-C had been transmitted remained persistently viraemic during 3–19 months after birth, except for one who seroconverted at 18 months. Seven of them (35%) developed marginally elevated ALT, excluding the one co-infected with HCV. Elective Caesarean section did not significantly reduce the rate of mother-to-child infection. An even higher transmission rate has been reported, in which 7/11 (64%) children born to mothers co-infected with HCV and GBV-C were infected, and their mothers' GBV-C RNA loads were significantly higher than those without transmission. In another study, 26/34 (77%) babies born to viraemic mothers were infected, including 23/24 (96%) babies born to mothers with viral loads of $>10^6$ copies/ml. They claimed that elective Caesarean sections reduced the transmission rate, but carrying these out solely to avoid nonpathogenic GBV-C transmission would not be justified. In regard to the possibility of transmission through saliva, the titre of GBV-C RNA in saliva was 10^{-2} – 10^{-4} -fold of that in the corresponding serum (Reviewed in Hino and Miyata, 2007).

Among 98 relatives of RNA-positive donors, 14 (14%) were viraemic and 29 (30%) were antibody-positive, whereas among the relatives of RNA-negative donors, only 1/95 (1.1%) was viraemic and 8/94 (8.5%) were antibody-positive. The prevalences of viraemia were high among the sexual partners of viraemic individuals: 7/32 (22%) and 4/12 (33%). However, 5/7 (71%) spouses in the former series exhibited parenteral risk factors, such as blood transfusions, acupuncture and major surgery. Even in the apparently healthy population, people at risk of parenteral infection exhibited a much higher prevalence,

usually in excess of 20%. Thus, GBV-C does not seem to be efficiently transmitted via sexual intercourse (Reviewed in Hino and Miyata, 2007).

Blood transfusion is one of the major transmission routes for GBV-C. RNA-positive blood products were infectious at the rate of 40–60%. None of 37 vials of clotting factor concentrates manufactured in the United States from 1981 to 1995 treated by pasteurization, solvent detergent or dry heat for 144 hours were negative for GBV-C RNA (Alonso-Rubiano *et al.*, 2003).

GBV-C in Patients

Among patients with acute non-A–E hepatitis, most studies found the prevalence of GBV-C viraemia to be less than 6%; not significantly higher than that in the general population. Most studies could not find evidence of significant ALT elevation, either. However, in a series of 62 patients with acute non-A–E hepatitis, 19 (31%) were found with GBV-C viraemia. Mild portal inflammatory lesions as well as steatosis were reported more frequently in the patients with persistent and isolated GBV-C viraemia (Reviewed in Hino and Miyata, 2007).

Potential technical bias may affect the interpretation of obtained results. Souza *et al.* (2006) pointed out that the specificity of RT-PCR with their E2 primers was 100%, but the sensitivity was only 77%. Assays utilizing additional three-primer sets deduced from conserved regions of other genes were more sensitive, but associated with higher false-positive rates. Recently, Ruiz *et al.* (2006) applied a competitive RT-PCR, which can discriminate between positive and negative strands, and measure viral loads over a wide range.

Co-infection with GBV-C might moderate the course of HCV infection, since HCV RNA levels were significantly lower in the 15 HCV/GBV-C co-infected patients than in the 48 patients with HCV infection alone (2.2 vs 10.8×10^6 copies/ml, $P = 0.02$). The role of GBV-C in patients infected with human immunodeficiency virus (HIV) is more interesting. GBV-C viraemia did not aggravate the course of patients with HIV infections, but was associated with slower progression of HIV infections. All parameters (survival, CDC stage B/C, HIV RNA load and CD4 T-cell count) were significantly better in terms of the cumulative progression rate (Reviewed in Hino and Miyata, 2007).

In evaluating participants infected with HIV for five to six years, GBV-C viraemia was significantly associated with a prolonged survival among the long-follow-up group, but not among the short-follow-up group, and the loss of GBV-C RNA was associated with the poorest prognosis in the long-follow-up group (Williams *et al.*, 2004). African patients with acquired immunodeficiency syndrome (AIDS) treated with highly active anti-retroviral

therapy (HAART) for 52 weeks were also studied. The patients co-infected with GBV-C showed a faster decline in HIV viral load ($P = 0.03$) and fewer opportunistic infections (14.3% vs 50%, $P = 0.18$) without any serious adverse events (none vs 61%, $P = 0.008$) after HAART (Mosam *et al.*, 2007). Patients viraemic with GBV-C ran a significantly lower risk of HIV rebound than those negative for GBV-C ($P = 0.03$) (Antonucci *et al.*, 2005). GBV-C RNA was cleared in 50% of patients after 24 weeks of HCV therapy, although this was not associated with changes in HIV load or CD4⁺ cell counts (Schwarze-Zander *et al.*, 2006). If an HIV patient is co-infected with HCV and GBV-C, how should their HCV be treated—with IFN?

Among patients infected with HIV, while IL-2 and IL-12 levels decreased in association with significant increase of IL-4 and IL-10 levels in the GBV-negative group, they stayed constant in the GBV-viraemic group, suggesting that the co-infection with GBV-C may help maintain the T-helper population (Nunnari *et al.*, 2003). On the other hand, an enhanced activation of the IFN system (2-5-OAS, MxA, IFN AR-1 and PKR in peripheral blood mononuclear cells (PBMCs) was observed in the GBV-viraemic and HIV-positive group (Capobianchi *et al.*, 2006).

Jung *et al.* (2005) have shown that GBV-C infection of PBMC leads to significant reduction of HIV mediated by expression of GBV-C structural glycoproteins and/or nonstructural proteins NS2/NS3. Upon GBV-C infection, CD4 and CD8 T lymphocytes are stimulated by GBV-C to secrete anti-retroviral factors, such as stromal cell-derived factor, SDF-1. The glycoprotein E2 of GBV-C interferes with the early replication steps of both X4- and R5-tropic HIV strains, and the inhibitory effect is abrogated by pre-incubation with anti-E2 antibody (Jung *et al.*, 2007). This was confirmed by the *in vitro* expression of E1/E2 encoding GBV-C RNA. GBV-C NS5A protein expressed in Jurkat induced a dose-dependent inhibition of HIV replication, independent of PKR inhibition (Xiang *et al.*, 2006). NS5A induced release of SDF-1 and decreased surface expression of HIV co-receptor CXCR4, potentially explaining the HIV inhibition. Deletion mapping of the NS5A protein identified an 85 aa region (152–237) that inhibits HIV-1 replication.

TORQUE TENO VIRUS (TTV)

History of TTV

TTV was found in 1997 as a naked ssDNA virus by the use of differential display technology (Nishizawa *et al.*, 1997). The index serum was obtained from a hepatitis patient with initials T.T. who did not possess any

markers of known hepatitis viruses. Okamoto *et al.* (1998) reported a linear 3739 nt sequence of TTV (TA278). A 113 nt GC-rich stretch was added, to complete its circular genome to 3852 nt (Miyata *et al.*, 1999; Mushahwar *et al.*, 1999). It was the first human virus with a circular ssDNA genome, and was tentatively classified as a *Circovirus*.

Despite intensive studies to define the pathogenicity of TTV, the real impact of TTV on liver disease still remains uncertain (Reviewed in Hino and Miyata, 2007). The vast majority of virological efforts have focused on the molecular epidemiology of diverse spectra of the TTV genome (Peng *et al.*, 2002). The considerable diversity of TTVs and lack of suitable conventional virological systems to study them are still limiting our knowledge about these viruses.

Classification of TTV

Viruses possessing circular ssDNA genomes have been well known for some time, even though their classifications have been reformed recently, such as the bacterial viruses *Inoviridae* and *Microviridae*, the plant viruses *Geminiviridae* and *Nanoviridae* and the animal viruses *Circoviridae* (Table 13.2). Members of the *Geminiviridae* and *Nanoviridae* have more than one circular DNA genome. Within the *Circoviridae*, the genus *Circovirus* includes animal viruses such as the porcine circovirus (PCV) and psittasine beak and feather disease virus (PBFDV). The genome of *Circovirus* is ambisense and has a common 9 nt stem-loop structure at its replication origin. The largest rep protein of *Circovirus* is coded for by the genomic strand, with the other two proteins encoded by the anti-genomic strand (Figure 13.2) (Niagro *et al.*, 1998). Plant viruses, such as banana bunchy top virus (BBTV), have been moved into a new family, *Nanoviridae*, whose genome has six segments of sense-strand circular DNA (International Committee on Taxonomy of Viruses, 2007).

TTV has a 3.8 kb circular genome of ssDNA. One third of the genome is an NTR and has a high degree of similarity within extremely divergent TTVs (Takahashi *et al.*, 1998). The 36 nt region (nt 3816–3851 of TA278) in the GC-rich stretch and its immediate vicinity constitutes a stem-loop structure, serving for the origin of DNA replication (Mushahwar *et al.*, 1999). Moreover, multiple transcription modifier motifs, such as ATF/CREB, AP-2, SP-1 and NF- κ B binding sites, are found in this region (Miyata *et al.*, 1999).

Some researchers still refer to TTV as ‘transfusion-transmitted virus’, even though it has been made explicit that the acronym TTV is based on the initials of the original patient (Takahashi *et al.*, 2000). Current spelled-out names for TTV and TTV-like mini virus (TLMV) were approved as ‘torque teno virus’ and ‘torque teno mini

Table 13.2 Viruses with a single-stranded circular DNA genome

Family	Genus	Representative viruses	Strandedness	Number of circles
Unassigned ^a	<i>Anellovirus</i>	TT virus (human, nonhuman primates and other animals), TTV-like mini virus (TTMV), Small anellovirus (SAV)	Antisense	1
<i>Circoviridae</i>	<i>Circovirus</i>	Porcine circovirus (PCV) Psittasine beak and feather disease virus (BFDV) Pigeon circovirus (PiCV) Goose circovirus (GoCV) Canary circovirus (CaCV)	Ambisense	1
<i>Geminiviridae</i>	<i>Gyrovirus</i>	Chicken anaemia virus (CAV)	Antisense	1
	<i>Mastrevirus</i>	Maize streak virus (plant)	Ambisense	1–2
	<i>Curtovirus</i>	Beet curly top virus		
	<i>Begomovirus</i>	Bean golden yellow mosaic virus		
	<i>Topocuvirus</i> Unclassified	Tomato pseudo-curly top virus		
<i>Inoviridae</i>	<i>Inovirus</i>	(Bacteria)	Sense	1
	<i>Plectrovirus</i>			
<i>Microviridae</i>	<i>Microvirus</i>	φX174 (Bacteria)	Sense	1
	<i>Spiromicrovirus</i>			
	<i>Chlamydiamicrovirus</i>			
<i>Nanoviridae</i>	<i>Babuvirus</i>	Banana bunchy top virus (BBTV); Coconut foliar decay virus (plant)	Sense	6
	<i>Nanovirus</i>			

^aA new family, *Anelloviridae*, is proposed, and the National Center for Biotechnology Information has incorporated the family name into the taxonomic table.

virus' (TTMV) by the International Committee on Taxonomy of Viruses (ICTV). These acronyms were devised to incorporate 'torques' (necklace) and 'tenuis'/'teno' (thin) into the established TTV name. TTV is currently classified in the unassigned genus *Anellovirus* (ring) within ssDNA viruses, but a family *Anelloviridae* has been proposed; the new family name is widely used already, since the Third International ssDNA Comparative Virology Workshop at Ouro Preto, Brazil, held over 20–26 May 2007 (Table 13.3).

The TTV Group

TTVs are composed of a wide variety of viruses (Figure 13.3) (Peng *et al.*, 2002). According to the proposed classification, all human TTVs with the 3.8 kb genome belong to the genus *Alphatorquevirus*, and they are divided into six groups (Table 13.3). Human TTMVs with the 2.9 kb genome (Takahashi *et al.*, 2000) and small anelloviruses (SAVs) with 2.2 and 2.6 kb genomes (Jones *et al.*, 2005) (Figure 13.2) are classified into separate genera, *Beta-* and *Gammatorquevirus* respectively (Table 13.3). The diversity is dependent not only on the mutations but also on frequent recombinations among

TTVs (Reviewed in Hino and Miyata, 2007; Manni *et al.*, 2002). Why and how these viruses can survive with such a wide spectrum of diversity is still unclear. A single host can be infected with different species of TTV. Do TTVs and TTMVs continue to mutate within a given host to escape its immunosurveillance system, as is the case for HCV or HIV?

Although the genomic sizes of TTV and TTMV are different, they share several common features. The NTRs occupying the approximately one third of the genome inclusive of the GC-rich stretch are similar to each other. The structures of the coding regions in these two virus groups are also similar to each other (Figure 13.4). The largest, ORF1, which accounts for approximately two thirds of the viral genome, is common to all these viruses. Two other double-spliced mRNAs have been found in TTV and TTMV. A single host can be infected with both virus groups, but there are some hosts with only one virus, indicating that the replications of these viruses are not dependent upon each other. Recently isolated 3.2 kb TTVs exhibiting 76–99% identity with SAVs were phylogenetically distinguishable from all reported TTVs and TTMVs. These small viruses are proposed as deletion mutants (Ninomiya *et al.*, 2007).

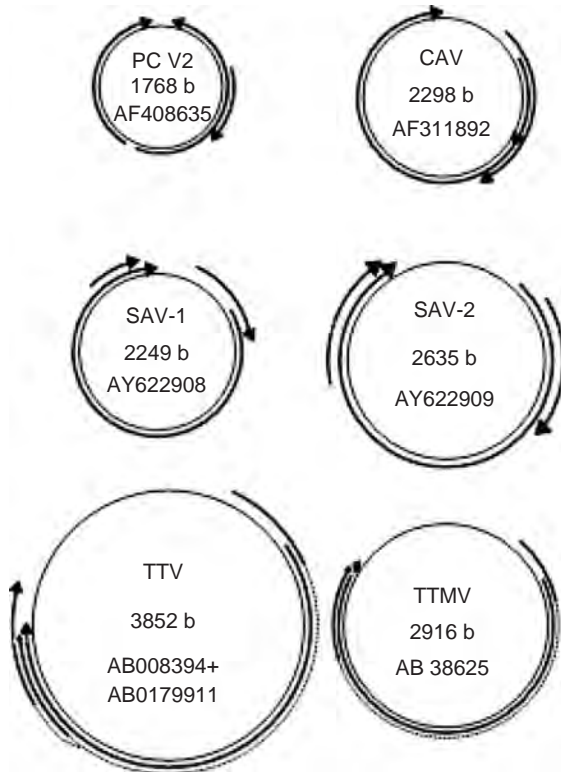


Figure 13.2 Strands of TTV, TTMV and SAVs in comparison to CAV (genus *Gyrovirus*) and porcine circovirus type 2 (PCV-2, genus *Circovirus*). The sizes of the circles correspond to the genome size of each virus. Clockwise arrows, translations on the anti-genomic strand; counter-clockwise arrows, those on the genomic strand.

TTV in Nonhuman Primates

Primates also have TTVs and TTMVs. TTV DNA was detected in most chimpanzees, Japanese macaques, red-bellied tamarins, cotton-top tamarins and owl monkeys examined. In 46% of the 104 chimpanzees, species-specific TTVs in four genetic groups were detected by analysis of the N22 region. Shortened N22 primers detected a diverse spectrum of chimpanzee TTVs, and UTR primers detected TTV/TTMVs in a wide range of apes (gorillas, orangutans, gibbons) and African monkey species (mangabeys, drills, mandrills). (Reviewed in Hino and Miyata, 2007).

TTV is prevalent even in lower primates, such as tupaia (*Tupaia belangeri chinensis*) (Okamoto *et al.*, 2001). Although the genome length of Tbc-TTV14 was short at 2.2kb, it clustered with TTVs and TTMVs in regard to putative genomic organization and transcription profile. Conserved motifs were commonly observed in coding and

Table 13.3 Viruses in torque teno virus family (putative *Anelloviridae*)

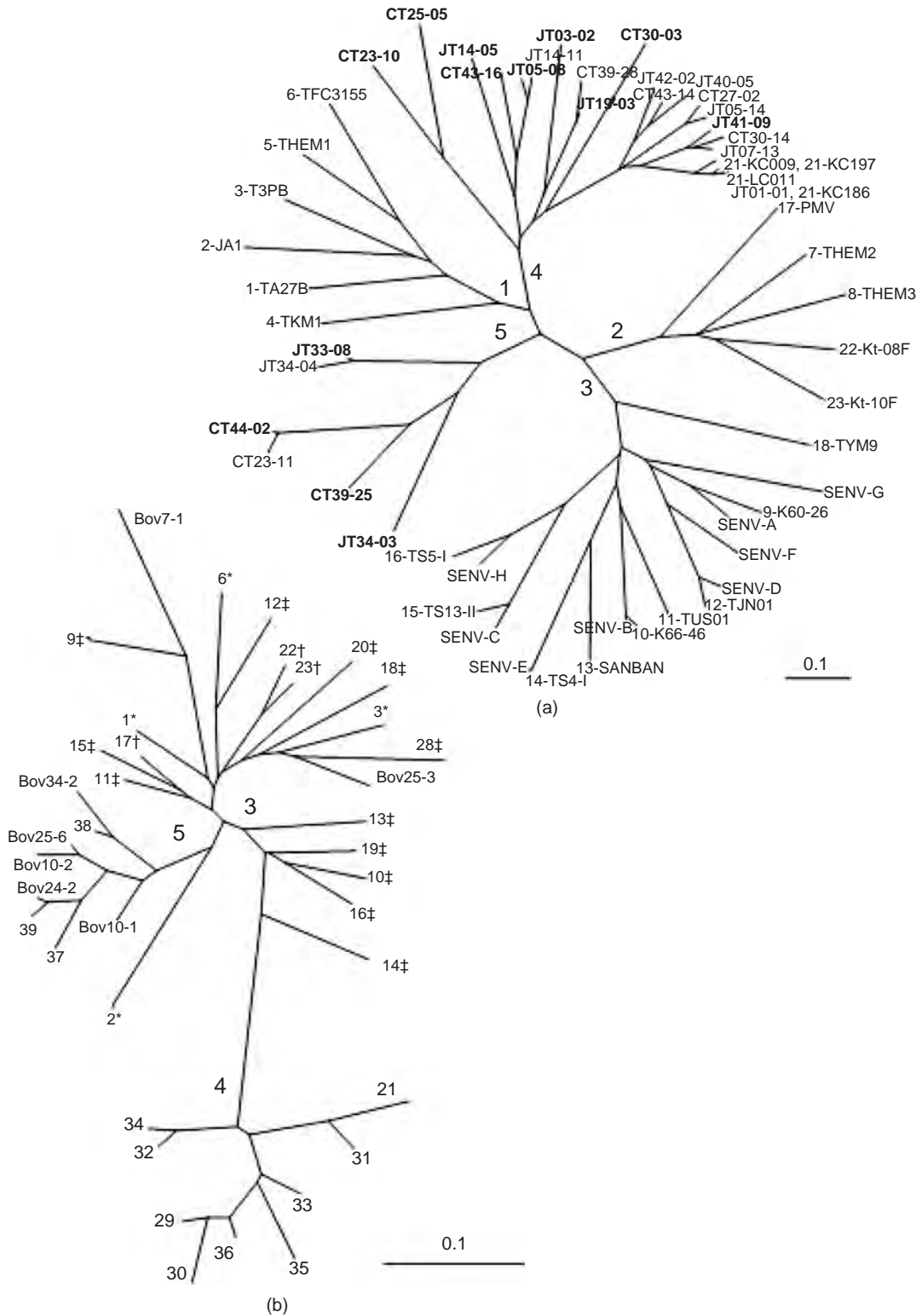
	Genus	Species originated
<i>Alphatorquevirus</i>	Group 1, Torque teno virus 1–4	Human
	Group 2, Torque teno virus 7–9	
	Group 3, Torque teno virus 10–18	
	Group 4, Torque teno virus 19, 20	
	Group 5, Torque teno virus 5, 6	
	Group 6, Torque teno virus 21, 22	
<i>Betatorquevirus</i>	Torque teno mini virus 1–9	Human
<i>Gammatorquevirus</i>	Torque teno vario virus 1, 2	Human
<i>Deltatorquevirus</i>		Tupaia
<i>Epsilontorquevirus</i>		Tamarin
<i>Zetatorquevirus</i>		Owl monkey
<i>Etatorquevirus</i>		Cat
<i>Thetatorquevirus</i>		Dog
<i>Iotatorquevirus</i>		Boar

noncoding regions of the Tbc-TTV14 genome and in all TTV and TTMV genomes. Phylogenetic analysis revealed that Tbc-TTV14 is more closely related to TTMVs, as well as those isolated from tamarins and douroucoulis, than to those from humans and chimpanzees.

Viraemia developed in rhesus monkeys 4–7 days and 7–10 days after intravenous and oral inoculation of human TTV, respectively (Luo *et al.*, 2000). The virus was excreted in faeces for over six months. These data imply that TTVs isolated in animals can be of human origin, and that recombination events between human and animal TTVs may take place. TTVs in faeces of wild animals were surveyed in an attempt to test them through noninvasive methods (Barnett *et al.*, 2004). Although TTVs in each host species are more closely related to each other than to those of other host species, the diversity of human TTVs is broad enough to overlap with those of other animals. These patterns suggest interspecies transmission, probably by faeco-oral transmission.

TTV in Lower Animals

TTVs are also found in chickens, pigs, cows, cats, dogs and sheep (Okamoto *et al.*, 2002). Although porcine TTV is not known to be associated with any swine disease,



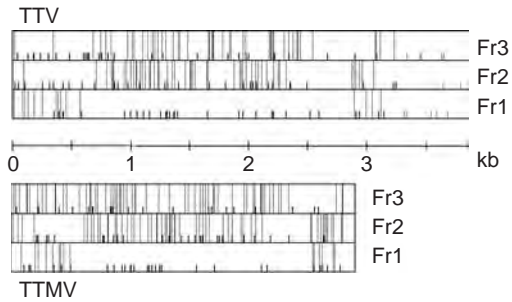


Figure 13.4 Frames and ORF structures of TTV (AB008394 + AB017911) and TTMV (AB38625). Short vertical lines represent ATG while long vertical lines correspond to stop codons. Fr, reading frame.

there is a potential risk of human infection during xenotransplantation. Porcine TTVs are prevalent in the pig populations of various countries (Table 13.4). These isolates were similar to each other, and the clustering of porcine TTV isolates was independent of the geographic origin. Recently, a novel member of swine TTV was identified using bacteriophage phi29 DNA polymerase to amplify circular DNA molecules through rolling circular amplification with random primers (Niel *et al.*, 2005). Camel TTVs ($n = 23$) were classified in genotype 11 (48%), group 5 (43%) and genotype 16 (9%), which are among the predominant genotypes found in humans in the United Arab Emirates (Al-Moslih, Perkins and Hu, 2007). Bovine isolates, on the basis of NTR sequences, are scattered within the human TTV phylogenetic tree (Figure 13.3b) (Hino and Miyata, 2007). The taxonomic divergence of these animal viruses relative to human TTVs and their pathogenicity are yet to be clarified.

Target Cells of TTV

The concentration of TTV is 10–100 times greater in bile than in peripheral blood, suggesting replication of TTV in the liver. The replicative form of TTV was found in liver and bone marrow. TTV loads in nasal secretion were 10–100 times higher than those in circulating blood, suggesting robust TTV replication in the nasal epithelium. The replication of TTV in respiratory epithelial cells was also suggested by a high concentration of TTV in nasal secretions of 1- to 24-month-old hospitalized

Table 13.4 Prevalence of TTV in pigs (McKeown *et al.*, 2004)

Area	<i>n</i>	Prevalence (%)
Iowa, USA	36	33
Beijing, China	20	80
Thailand	20	40
Korea	20	85
Ontario, Canada	13	46
Quebec, Canada	10	100
Saskatchewan, Canada	15	100
Spain	20	90

children with acute respiratory diseases (ARDs) (Maggi *et al.*, 2003). Low TTV titres in cerebrospinal fluid (CSF) suggested that the central nervous system would be less likely to be a target of TTV replication. (Reviewed in Hino and Miyata, 2007).

Although TTV was originally found in serum, the detection of TTV in stool has suggested that it is transmitted by the faeco-oral route. TTV was detected in water in rivers at a level comparable with those of enteroviruses, rotaviruses and noroviruses (Haramoto *et al.*, 2005; Verani *et al.*, 2006). TTV can be transmitted by mother-to-child infection. The presence of TTV in cord blood is reportedly 0–1% using the N22 PCR (Kazi *et al.*, 2000). Within six months of birth, the prevalence of TTV in children born to TTV-positive mothers was significantly higher than that of children born to TTV-negative mothers. However, at one year of age, the prevalence in children born to TTV-negative mothers caught up with that of children born to TTV-positive mothers and of adults, even without breast-feeding. This implies that a milk-borne transmission of TTV is insignificant.

Clear morphological changes in Chang liver cells have been reported following *in vitro* infection with genotype 1a TTV, and the infectivity is transferable by the culture supernatant (Desai *et al.*, 2005). However, this data contained no quantitative analysis, even if photos suggested the induction of a clear cytopathic effect. Although TTV production in the culture fluid lasted for at least six days, the amount of signalling seen was merely 1/100 that in stimulated PBMCs in which TTV replication aborted within a short period of time.

Figure 13.3 Phylogenetic analysis of TTVs. (A) Phylogenetic tree was constructed on the full genome of TTVs by Peng *et al.* (2002) (with permission of publisher and authors). Group 6 is not shown in the figure. (B) Tree based on NTR of TTVs, including those included in (A) and several newly found bovine sequences (Hino and Miyata, 2007). Strain names starting with ‘Bov’ are of bovine origin. Numbers in italics indicate branch in groups. Genotypes are indicated in accordance with group markers in (B) (*group 1, †group 2, ‡group 3).

In the bone marrow, TTV may prefer the erythroid series, because the promoter activity of TTV was most significant in K562 cells of the erythroid origin (Kamada *et al.*, 2004), and/or the megakaryocyte series, as thrombocytopenia has been reported in patients infected with TTV. However, TTV replication in PBMC may not be so important, because the highest TTV load in granulocytes was no more than 0.3 copies per cell. Moreover, the TTV-positive signal in PBMCs disappeared after degradation of ssDNA by mung bean nuclease. Transient replication of TTV in PBMCs stimulated with phytohaemagglutinin and Raji cells was reported, but, as elsewhere, quantitative analysis of these observations was lacking. (Reviewed in Hino and Miyata, 2007).

Since most small DNA viruses need cellular factors for initiating DNA replication, the replication of TTVs in PBMC only after stimulation is consistent with the hypothesis that TTV needs a cellular S phase. In this context, higher titres of TTV in nasal secretions of children with ARD may be correlated with the presence of regenerating epithelial cells and/or accumulating inflammatory cells. However, this assumption may be too simple. On the other hand, TTV replication is not sensitive to lamivudine in patients infected with hepatitis B virus (HBV) (Garcia *et al.*, 2003). No sustained responses of TTV can be achieved in any patient after PEG-IFN plus ribavirin treatment of those infected with HCV (Moreno *et al.*, 2004).

Association with Diseases

At present, the pathogenicity of TTV and TTMV remains in question. Most reports have not suggested any associations with significant hepatic diseases. Several reports suggest potential aggravation of underlying hepatic diseases by TTV viraemia. Co-infected patients have been reported with a higher histological score, such as TTV-positive/HCV-positive patients and TTV-positive/HBV-positive children, although there were no significant differences in ALT levels. Among patients with alcoholic liver disease, the improvement rates in ALT, gamma-glutamyl transpeptidase and alkaline phosphatase levels were lower in the TTV-positive group. The same group proposed that a high TTV viral load was a significant risk factor for hepatocellular carcinoma (HCC) in patients infected with HCV. None of these reports has been confirmed by further studies, even by the same groups. (Reviewed in Hino and Miyata, 2007).

Bando *et al.* (2001) found that serum lactate dehydrogenase was significantly higher in the 12/33 (39%) patients with idiopathic pulmonary fibrosis who had been infected with TTV. Additionally, the three-year survival rate was significantly lower in the TTV-positive group. Active TTV replication in ARD in 1- to 24-month-old hospitalized children has been documented (Maggi *et al.*,

2003) but prevalence and viral load of TTV in nasal fluid were similar to those of controls. However, in the children with asthma, three important indices of pulmonary function were inversely correlated with high nasal TTV load. Furthermore, signs of reduced airflow were more frequent in the children with high nasal TTV loads ($\geq 10^6$ DNA copies/ml) (Pifferi *et al.*, 2006). Presence of TTV in the gingival tissue was significantly associated with periodontitis, but it may be derived from the infiltrating inflammatory cells (Rotundo *et al.*, 2004).

Co-infection with other viruses may lead to certain clinical consequences. TTV/TTMV virus loads in both bone marrow and spleen were significantly higher among AIDS patients than among HIV-positive or -negative non-AIDS patients (Thom and Petrik, 2007). TTV/TTMV titre and CD4 T-lymphocyte count had a significant inverse correlation. In the series of Szladek *et al.* (2005), 8/11 (73%) patients with metastasizing or relapsing laryngeal carcinoma were co-infected with genogroup 1 TTV and HPV, in contrast to none of the 14 without tumour progression ($P < 0.001$). Furthermore, 4/5 (80%) papillomatosis patients with malignant transformation were co-infected. Similar studies on the TTV behaviour in patients co-infected with HPV in gynaecology and urology departments would be of interest.

Gergely *et al.* (2005) found that the prevalence of TTV was significantly higher in the patients with severe idiopathic inflammatory myopathy who needed immunosuppressive treatment in addition to corticosteroids than in those with a milder form of this disease. They reported another potentially interesting scenario. Patients with systemic lupus erythematosus produce autoantibodies to HRES-1/p28, an internal protein of human endogenous retrovirus. Computer searching for viral epitopes cross-reactive to the HRES-1/p28 by BLAST yielded ten of them, including four TTV epitopes, two on VP1 and another two on VP2. Prevalence of TTV DNA increased in lupus patients (120/211 (57%)) in comparison with healthy (66/199 (33%); $P < 0.0001$) and rheumatoid arthritis (23/91 (25%); $P < 0.0001$) controls.

Even though the pathogenicity of TTVs is not defined, they might be transmitted with blood transfusions, and possibly by blood products. Oza *et al.* (2004) found no evidence of TTV infections in the commercially-available plasma-derived concentrates of factor IX that are most commonly used for treatment of haemophilia B. Information on the methods needed to inactivate TTV is lacking, but the marked stability of PCV-2 and CAV should be kept in mind. Infectivity of PCV-2 or CAV was almost completely resistant to dry heat up to 120°C for 30 minutes. Their titres were reduced only to 1/20 after pasteurization at 60°C for 24 hours, and to 1/40–1/400 after pasteurization at 70°C for 30 minutes (Welch *et al.*, 2006).

Transcriptional Control

Kamada *et al.* (2004) studied the transcriptional regulation of TTV (group 1) by dual luciferase assays. They found that the region -154/-76 contains the critical regulatory element for the functioning of the TTV promoter (Figure 13.5). Similar results were observed with the SANBAN TTV (group 3). Interestingly, the promoter was more active in HepG2 cells than Huh7 cells for the group 1 TTV, while more active in Huh7 cells than HepG2 cells for the group 3 TTV, possibly suggesting distinct cell tropisms of these two TTVs. (Reviewed in Hino and Miyata, 2007).

Proteins of TTV

On the anti-genomic strand of TTV, three ORFs in each frame are evident with putative initiation codons (Figure 13.6). The largest ORF of TTV in frame 1 resides in association with a TATA box (nt 85–90 of TA278) and a polyA signal (nt 3073–3079). There are additional ORFs on the anti-genomic strand without legitimate initiation codons.

Kamahora *et al.* (2000) constructed a plasmid containing the promoter region through to the polyA signal continuously (nt 2762–3852 connected in tandem with nt 1–3770) to transcribe mRNA in eukaryotic cells. Three mRNA species sized 3.0, 1.2 and 1.0kb, respectively, were expressed in transfected COS1 cells (Figure 13.7). Each mRNA had the 5' terminus at nt 114, adjacent to the TATA box, and the 3' terminus at nt 3087, 6–9 nt downstream of the polyA signal. All three species of mRNA exhibited a common splicing at nt 186–276.

The 3.0kb mRNA has an initiation codon at A⁵⁸⁹TG and codes for the rep protein with 770 aa. The other two mRNA species of 1.2 and 1.0kb have a second splicing, connecting an ORF2 in frame 2 to the other ORF in frames 2 and 3 respectively. The first choice-initiation

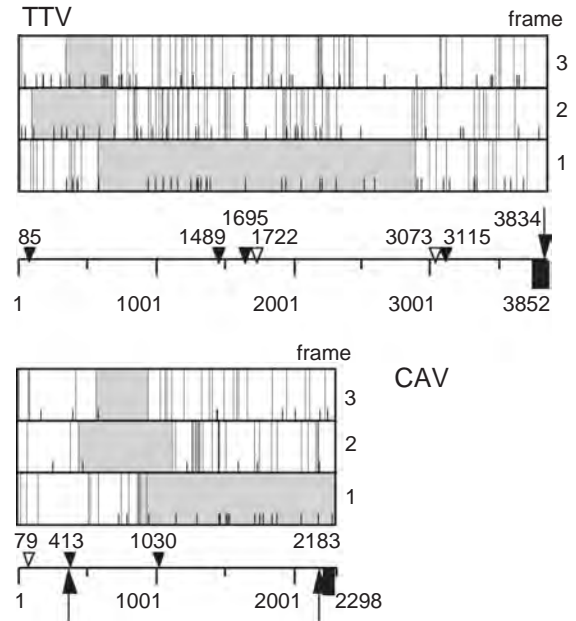


Figure 13.6 Comparative arrangement of ORFs expected from the genomes of TTV (AB008394 + AB017911) and CAV (AF372658). SP-1 site (arrow), TATA box (closed triangle), PolyA signal (open triangle) and GC-rich region (closed box) are indicated.

codon for all three mRNAs is common A³⁵³TG. These three mRNAs potentially code for six different proteins (Figure 13.7) (Qiu *et al.*, 2005). The real nature of these proteins has to be elucidated.

The ORF1 protein probably serves as replicase and represents the major structural protein of TTV, because its N-terminus contains a highly basic stretch consistent with other circoviral capsid proteins and several conserved rep protein motifs (FTL and YXXK) (Niagro

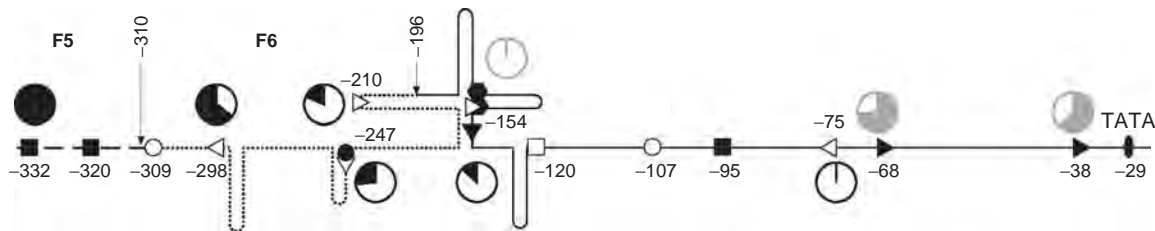


Figure 13.5 Enhancer and promoter region of TTV in relation to the tertiary structure in the NCR. Binding sites for transcription factors conserved in various TTVs (open circle, CREB; closed circle, E2F; open square, c-Ets; closed square, USF; hexagon, SP1), and their positions in italics, are shown. Open triangles indicate the boundary of 5' deletion mutants, and closed triangles indicate the boundary of 3' deletion mutants. Their luciferase signals relative to that of the pGL3B (1.2kb NCR) construct are shown on graphs as circles (black for 5' deletions and grey for 3' deletions). The initiation site (nt114) is denoted as +1.

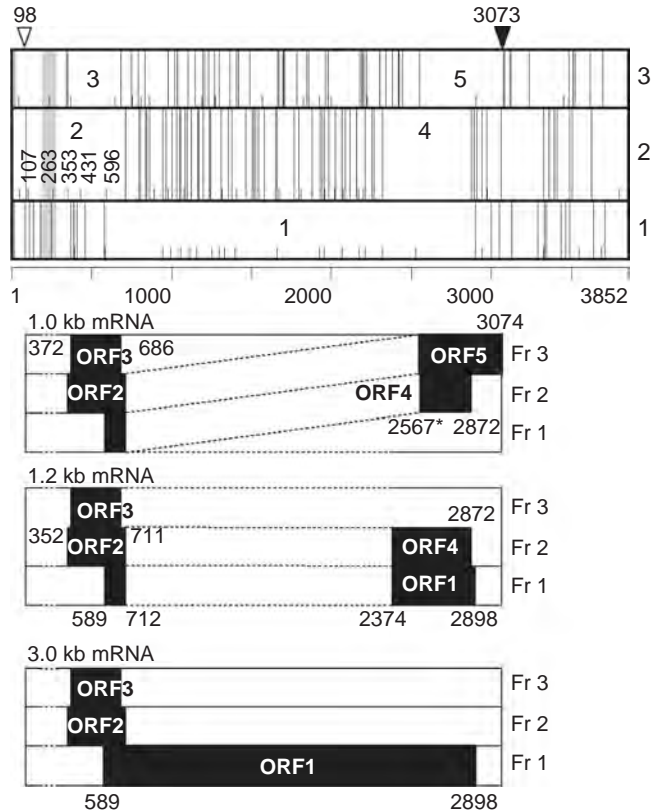


Figure 13.7 Schematic diagram of the TTV genome (AB008394 + AB017911) and its mRNAs. Three reading frames of the genome are shown in the upper panel. The open triangle indicates the position of the cap site while the closed triangle indicates that of the polyA signal. The short and long vertical lines indicate ATGs and stop codons, respectively. Predicted ORFs are indicated by numbers. The shaded area represents the first splicing common to all mRNAs of three different sizes. The lower panel indicates the frames used and configurations of 3.0, 1.2 and 1.0 kb mRNAs. The solid lines indicate exons, the dotted lines represent introns, and the boxes indicate coding regions. Because of the alternative splicing for the 1.0 kb mRNA, the 5' terminal nt 2567 is labelled with an asterisk.

et al., 1998; Takahashi *et al.*, 1998). Post-translational modifications should be investigated further.

Phosphorylation of the ORF 2–4 product on frame 2, derived from the 1.2 kb spliced mRNA using an expression plasmid, has a similarity to NS5A of HCV. Apoptotic ORF3 protein will be discussed later, with CAV protein. A transgenic mouse expressing a TTV product mainly in kidneys and suffering from a nephrotic syndrome has been reported (Reviewed in Hino and Miyata, 2007). It transcribed 1.1 kb mRNA corresponding to the 1.0 kb mRNA, and expressed a 143 aa protein, however the ORF2–5 product derived by the second splicing in the 1.0 kb mRNA species is an artificial protein using A⁵⁸⁹TG instead of A³⁵³TG, and thus is truncated of most N terminus in the ORF2 region (Figure 13.7).

Chicken Anaemia Virus

Although CAV is not a direct subject of this chapter, I would like to refer to it briefly. CAV is currently classified in the family *Circoviridae*, genus *Gyrovirus*. Both CAV and TTV have antisense genomes, not the ambisense genome of *Circovirus*, and lack the 9 nt stem-loop structure common in *Circovirus*. Configurations of ORF in CAV and TTV were similar in spite of the difference in genome size (Figures 13.2 and 13.6). CAV also produces multiple mRNAs (Figure 13.8) (Kamada *et al.*, 2006). A 36 nt stretch in the region at their replication origins has ~80% identity (Miyata *et al.*, 1999). However, no other parts of the genomes show any significant similarities in their nucleotide sequences. The genomic size of CAV, 2.3 kb, is distinctly smaller than that of typical

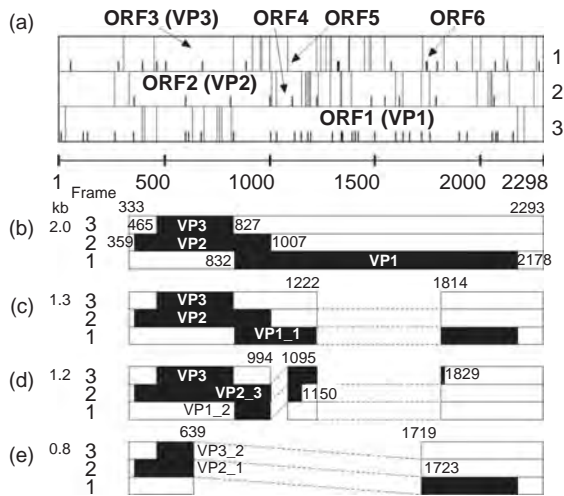


Figure 13.8 Schematic representation of the CAV transcripts and their candidate ORFs. (a) Six open reading frames of the CAV genome. Short and long vertical lines indicate ATG start and stop codons, respectively. Putative ORFs encoded by the transcripts are indicated by arrows. ORFs evident in the 2.0, 1.3, 1.2 and 0.8 kb transcripts are shown in panels b–e, respectively. Dotted lines indicate introns or deleted regions. Black boxes represent putative coding regions. Nucleotide numbers indicate the positions of ORF junctions or boundaries of ORFs.

TTV, 3.8 kb. TTV lacks a unique region of CAV, possessing four or five near-perfect direct repeats of 21 bp (Noteborn *et al.*, 1994). While TTVs are extraordinarily diverse, CAVs are strikingly homogeneous. Considering these features, CAV might be transferred into a new family, *Gyroviridae*, or into *Anelloviridae*, in the future.

In cells infected with CAV, three major proteins, VP1, VP2 and VP3, are expressed, and its capsid contains only the VP1 protein (Noteborn *et al.*, 1998). A single aa change in VP1, Q394H, abrogates the pathogenicity of CAV. Cells expressing either VP1 or VP2 alone are not recognized by neutralization antibody, suggesting that the nonstructural protein VP2 may act as a scaffold in the virion assembly. VP2 and VP3 proteins are probably early proteins, because they appear at 12 hours post-infection, while VP1 appears only after 24 hours (Reviewed in Hino and Miyata, 2007).

VP2, both of CAV and TTV, possesses a common unique feature of both tyrosine and serine/threonine phosphatases. Mutation of the cysteine residue (C95 or C97) in CAV VP2 greatly reduced the virus titre. C95 mutation abrogated both phosphatase activities. However, while C97 mutation slightly reduced the tyrosine phosphatase activity, it significantly increased serine/threonine phosphatase

activity sevenfold. By a number of site-directed mutations introduced into the VP2 genes, discrepancy was observed among virus replication, cytopathogenicity and downregulation of MHC I in infected cells (Peters *et al.*, 2006).

VP3 of CAV, apoptin, induces apoptosis by itself in various transformed and/or tumorigenic cell lines, but not in normal diploid cells. Apoptin is located mainly in the heterochromatic regions of tumour cells, whereas it stays in perinuclear structures of normal cells. Apoptin-induced apoptosis is independent of p53 and Bcl-2. A number of proteins were shown to interact with apoptin in transformed cells (Noteborn, 2004). VP3 of TTV, named TTV-derived apoptosis-inducing protein (TAIP), also has an apoptotic character, but induced only low-level apoptosis in several non-HCC human cancer cell lines (Kooistra *et al.*, 2004). VP3 of CAV is found indispensable in the replication of CAV, and its knockout virus is complemented by VP3 of TTV (Prasetyo *et al.*, 2009).

Problems with the Detection of TTV

In spite of the introduction of TTV as a putative hepatitis virus, there is little evidence for the pathogenicity of TTV. No TTVs may be pathogenic, or else only a limited type of TTV may be pathogenic. While hemi-nested PCR on the N22 region (nt 1914–2185) detects genotype 1–6 TTVs (Okamoto *et al.*, 1998), PCR on the 5' NTR (nt 26–184) detects most genotypes of TTV (Takahashi *et al.*, 1998). Most studies involving NTR PCR are probably less informative in assigning a possible pathogenicity to TTV. This is reminiscent of the history of PCV, where more than 15 years elapsed from the discovery of the original PCV (Tischer *et al.*, 1982) to that of pathogenic PCV-2 (Allan *et al.*, 1998).

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Rotaviruses

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INTRODUCTION

Rotaviruses are a major cause of gastroenteritis among children of less than five years of age worldwide (Bern *et al.*, 1992; Parashar *et al.*, 2003, 2006b) and of acute diarrhoea in the young of many other mammalian species (calves, piglets, lambs, rabbits, etc.). They cause more than 600 000 deaths each year, mostly of infants and young children in developing countries (Parashar *et al.*, 2006b). Since the 1960s rotaviruses have been known as the cause of diarrhoeal disease in mice (Adams and Kraft, 1963), monkeys (Malherbe and Harwin, 1963) and calves (Mebus *et al.*, 1969). They were discovered as the cause of the human disease in 1973 (Bishop *et al.*, 1973; Flewett *et al.*, 1973).

ROTAVIRUS STRUCTURE, GENOME AND GENE-PROTEIN ASSIGNMENT

The wheel-like structure of the particle as seen by electron microscopy (EM) is characteristic (Figure 14.1) and has provided the name of the virus (Latin *rota* = wheel). Rotaviruses possess a genome consisting of 11 segments of double-stranded (ds) RNA encoding six structural proteins (VP1–4, 6 and 7) and six nonstructural proteins (NSP1–6) (Figure 14.2a). The genomic RNA segments are between 667 and 3302 bp in size and have short conserved 3' and 5' termini. The structural proteins of the particle constitute an inner layer (VP1–VP3; 'the core') enclosing the genome (Figure 14.2a–c), an intermediate layer (VP6; 'the inner capsid') and an outer layer (VP4, 7; 'the outer

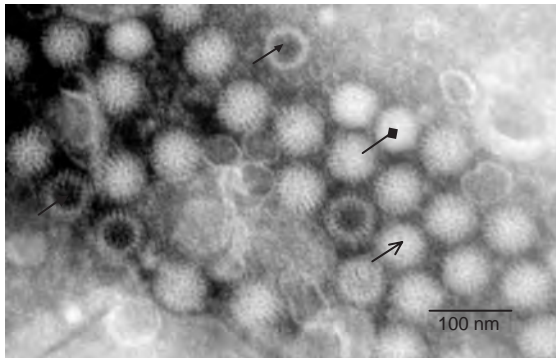
capsid') (Figure 14.2c). Details of the three-dimensional structure have been elucidated in a number of excellent studies by the groups of Prasad, Chiu and Estes (Crawford *et al.*, 2001; Lawton *et al.*, 1997a, 1997b, 1999, 2000; Pesavento *et al.*, 2001; Prasad and Estes, 1997; Prasad *et al.*, 1988, 1990, 1996; Shaw *et al.*, 1993), Yeager (Yeager *et al.*, 1990, 1994) and Cohen and Rey (Lepault *et al.*, 2001; Mathieu *et al.*, 2001; Petitpas *et al.*, 1998). According to their findings, the triple-layered capsid is ordered in 5-, 3- and 2-fold symmetry axes (Figure 14.2b–e) and is perforated by 132 aqueous channels (classes I–III, in three symmetry arrangements; Figure 14.2b). The class I channels have functions in transcription from subviral particles (see below).

Complete gene–protein assignments have been established for several strains; the protein–function correlations are only partially known. Details describing sizes of RNA segments and their products, as well as on post-translational modification and identified or likely functions of the virus-encoded proteins, are summarized in Table 14.1. Functions are reviewed in more detail below.

CLASSIFICATION

A classification scheme for rotaviruses has been derived from the immunological reactivities of three of its components as well as from genomic sequence comparisons and has been established as follows (Estes and Kapikian, 2007):

1. According to the serological cross-reactivity of the inner capsid protein VP6, five groups (A–E) have



▀ DLP; ↗ TLP; ↗ EDLP; ↗ ETLP

Figure 14.1 Electron micrograph of rotavirus particles in the faecal suspension of an infant with acute gastroenteritis, London, 2005. A mixture of triple-layered particles (TLPs = virion, fully-infectious particle), double-layered particles (DLPs containing RNA), empty TLPs (ETLPs, lacking RNA) and empty DLPs (EDLPs, lacking RNA) is seen. (Courtesy of Dr Hazel Appleton and Brian Megson, Health Protection Agency, London.)

been firmly established, and two more groups (F, G) are likely to exist. Within group A rotaviruses there are subgroups (I, II, I + II, nonI, nonII) according to exclusive reactivities with two VP6-specific monoclonal antibodies. More recently, reverse polymerase transcription chain reaction (RT-PCR) was applied to the subgroup classification of human rotaviruses and only two major subgroups were identified (Iturriza-Gómara *et al.*, 2002c).

- Both surface proteins, VP4 and VP7, elicit antibodies which neutralize *in vitro* and *in vivo* (Green and Kapikian, 1992; Offit *et al.*, 1986) and are therefore considered to be involved in protection. In order to characterize strains, a binary classification scheme distinguishing types, similar to that developed for influenza viruses, has been established (so far only for group A rotaviruses). The system differentiates G types (VP7-specific; G for glycoprotein) and P types (VP4-specific; P for protease-sensitive protein). So far, 16 different G types and 27 P types have been detected (and more are likely to be identified), indicating extensive genomic diversity within group A rotaviruses. Whereas the correlation of G serotypes with their genotypes is practically complete, for many P genotypes no serotype has yet been established, due to the lack of P-type-specific monoclonal antibodies and monospecific sera. Thus, it has been agreed to designate the P serotype and genotype individually but jointly, the latter in square brackets; for example,

the human Wa strain is classified as G1P1A[8], the human DS-1 strain as G2 P1B[4], the ovine strain Lp14 as G10P[15] and so on (Estes and Kapikian, 2007).

VP4 and VP7 are coded for by different RNA segments (segment 4 and segment 7, 8 or 9 depending on strain, respectively; see Table 14.1). As rotaviruses were found to reassort readily in doubly-infected cells *in vitro* and *in vivo* (Garbarg-Chenon *et al.*, 1986; Gombold and Ramig, 1986), various combinations of VP4 and VP7 types have been observed in natural rotavirus isolates (Estes and Kapikian, 2007).

REPLICATION

Rotaviruses spread via the faeco-oral route and infect the small intestine after oral ingestion. Viral replication occurs in the mature epithelial cells at the tips of the villi of the small intestine. In the presence of trypsin, rotaviruses grow well in secondary monkey kidney cells, in immortalized monkey kidney cell lines (MA104, BS-C1) and in differentiated human intestinal cell lines (e.g. Caco-2, derived from a colon tumour). Their replication *in vitro* can therefore be studied in detail (Estes and Kapikian, 2007). Triple-layered particles (TLPs) (i.e. the infectious virions) attach to the host cells via the outer-layer protein VP4 (Ludert *et al.*, 1996). Virus entry is by receptor-mediated endocytosis or direct penetration. The cellular receptors of rotaviruses have not been fully characterized. Some animal strains use sialic acid on glycolipids (Ciarlet and Estes, 1999; Ciarlet *et al.*, 2002). Other strains seem to recognize galactose (Jolly *et al.*, 2001). In addition to glycolipids, several integrins have been proposed to mediate internalisation, possibly in a post-attachment step, acting as co-receptors (Coulson *et al.*, 1997; Guerrero *et al.*, 2000; Hewish *et al.*, 2000). The heat shock cognate protein (hsc70) may also be involved as a co-receptor (Guerrero *et al.*, 2002). Human rotavirus strains appear to infect the polarized epithelial cells of the gut via their basolateral membranes (Ciarlet and Estes, 1999).

Replication is exclusively in the cytoplasm. After removal of the outer capsid from TLPs by lysosomal enzymes the viral RNA-dependent RNA polymerase (coded for by RNA 1) is activated in double-layered particles (DLPs), and by use of the VP1/VP3 transcription complex inside the VP2 layer at all fivefold axes of symmetry (Lawton *et al.*, 2000) (Figure 14.2c) large numbers of positive-stranded RNA molecules are transcribed and exit the DLPs via the twelve aqueous class I channels located on the edges of the fivefold axes (Figure 14.2f). This process is adenosine triphosphate (ATP)-dependent. The new RNA molecules (capped, but not polyadenylated) act as

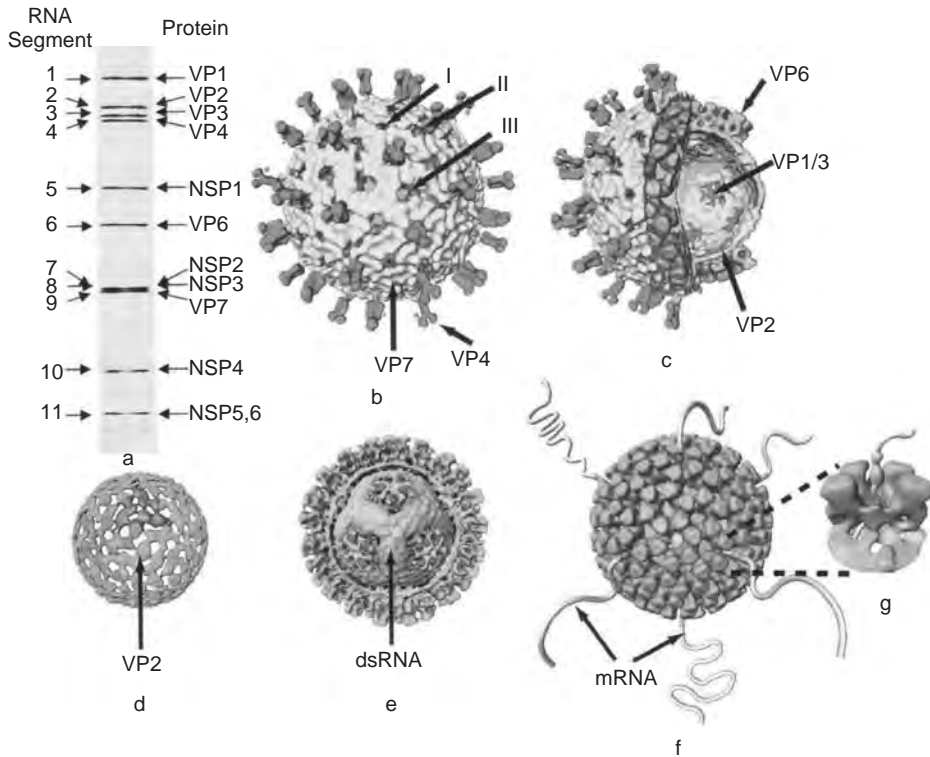


Figure 14.2 Structural organization of rotavirus. (a) Polyacrylamide gel showing rotavirus RNAs 1–11 with gene–protein assignment on the right. (b) Surface representation of rotavirus structure. Channels of classes I–III are indicated. (c) Cut-away of the rotavirus structure showing the intermediate layer (VP6), the core (VP2) and the flower-shaped VP1/VP3 complexes at the inside of VP2 opposite of class I channels. (d) Structural organization of the VP2 layer (60 dimers are shown). (e) Genomic RNA in the rotavirus structure. The VP6 and VP2 layers are partially cut away to expose the RNAs, which at the outside have dodecahedral appearance. (f) Structure of the actively transcribing DLPs with nascent mRNAs exiting through the class I channels. (g) A close-up cut-away view of the exit pathway in one of the channels. The bowling pin-shaped density of the exiting transcript is seen in actively transcribing DLPs. Panels (b) to (g) are delineated from image reconstructions of cryo-electron micrographs. (Source: Pesavento *et al.*, 2003; with permission from Dr B.V. Venkataram Prasad.)

mRNAs, and their translation products start to accumulate in the cytoplasm. NSP3 is intimately involved in translation by binding to the 3' end of mRNA and the eukaryotic translation factor eIF4G (Piron *et al.*, 1998; Vende *et al.*, 2000). However, translation may also occur independent of NSP3 (Montero *et al.*, 2006).

For replication, mRNA molecules may be pulled through VP2 oligomers (the nascent 'core') by means of a complex formation with NSP3, which has NTPase activity and likely acts as a molecular motor (Patton, 2001; Schuck *et al.*, 2001; Taraporewala and Patton, 2001; Taraporewala *et al.*, 1999), and NSP5, a hyperphosphorylated protein in pseudocrystalline aggregates termed 'viroplasm' (intracytoplasmic inclusion bodies). NSP2 and NSP5, when co-expressed in noninfected cells, form viroplasm-like particles (Fabbretti *et al.*, 1999) and have been shown to be essential for RNA replication and

morphogenesis (Campagna *et al.*, 2005; Silvestri *et al.*, 2004; Vascotto *et al.*, 2004). NSP5 interacts with both VP2 (Berois *et al.*, 2003) and VP1 (Arnoldi *et al.*, 2007). The exact order of events in early morphogenesis and the molecular reactions by which the packaging and reassortment of RNA segments into cores (Figure 14.2c,e) are controlled are unknown. Cryo-EM studies have shown the arrangements of dsRNA inside the core as concentric rings (Pesavento *et al.*, 2001, 2003). DLPs form in the viroplasm, consisting of VP1, VP2, VP3 and VP6 and several of the nonstructural proteins, and containing one genome equivalent of packaged single-stranded RNA (which is then replicated to form dsRNA). The DLPs bud through the rough endoplasmic reticulum (RER), with NSP4 (at its C-terminal, cytoplasmic domain) acting as an intracellular receptor for VP6 (Au *et al.*, 1993; Taylor *et al.*, 1993). There, DLPs attract a third,

Table 14.1 Genes, gene-protein assignments and functions of the proteins of group A rotavirus

RNA segment	Protein product						
	No.	Size (bp)	Description	Deduced MW (kDa)	Location (oligomeric state)	No. of molecules per virion	Post-translational modification
1	3302	VP1	125.0	Inner core	12	–	RNA-dependent RNA polymerase; ssRNA binding; complex with VP3
2	2690	VP2	94.0	Core	120	Myristylation	RNA binding; required for replicase activity of VP1
3	2591	VP3	88.0	Inner core	12	–	Guanylyl transferase; methyl transferase; ssRNA binding; complex with VP1
4	2362	VP4	86.8	Outer capsid (trimer)	120	Proteolytic cleavage to VP5* and VP8*	Haemagglutinin; cell attachment; neutralization antigen (ab protective); fusogenic; protease-enhanced infectivity; virulence (mice, piglets)
5	1611	NSP1 (VP5)	58.7	Nonstructural	NA ^c	–	RNA binding; virulence (mice); interacting with and degrading IRF-3; non-essential for replication of some strains
6	1356	VP6	44.8	Inner capsid (trimer)	780	Myristylation	Group- and subgroup-specific antigen; ab protective (by intracellular neutralization?); required for transcription

7 ^a	1059	NSP3 (VP9)	34.6	Nonstructural (dimer)	NA	–	RNA binding (3' end); competing with cellular PABP for interaction with EIF4G1 (translation); inhibiting host-cell translation
8 ^a	1104	NSP2 (VP8)	36.7	Nonstructural (octamer)	NA	–	RNA binding; NTPase; helicase; +strand RNA packaging; forms viroplasm with NSP5; virulence (mice)
9 ^a	1062	VP7	37.4 ^b	Outer capsid (trimer)	780	Cleavage of signal sequence; glycosylation	Neutralization antigen (ab protective); Ca ⁺⁺ binding
10	751	NSP4 (VP12)	20.3	Nonstructural	NA	Glycosylation (→VP10, NS28); Trimming	Intracellular receptor for DLPs; role in morphogenesis; interacting with viroplasm; modulating transcription; viral enterotoxin (secreted cleavage product); virulence (mice, piglets)
11	667	NSP5 (VP11)	21.7	Nonstructural (dimer)	NA	0-glycosylation, phosphorylation	RNA binding; protein kinase; forming viroplasm with NSP2; interacting with VP1 and NSP6
		NSP6	12.0	Nonstructural	NA	–	Interacting with NSP5

^aThis gene–protein assignment is of the SA11 rotavirus strain.

^bSecond in-frame initiation codon located 30 codons downstream (deduced MW33.9 kDa).

^cNA, not applicable. Modified from Estes and Kapikian (2007).

transient, RER-derived envelope layer, which is shed before complete maturation, and incorporate VP7 and VP4. VP4 may be added late in the process in 'raft'-like structures near the plasma membrane (Delmas *et al.*, 2004). Triple-layered infectious virions are released by the lysis of nonpolarized cells (e.g. MA104), and also from polarized cells before a CPE becomes obvious, probably by other mechanisms. In faecal specimens, TLPs and DLPs (also as 'empty particles'; that is, not containing genomic RNA) may often be observed to occur side by side (Figure 14.1). (For further details of structure–function correlations and replication see Estes and Kapikian, 2007; Lawton *et al.*, 2000; Prasad and Estes, 1997). In immunodeficient hosts and under certain experimental conditions, rotaviruses undergo genome rearrangements (for review see Desselberger, 1996).

PATHOGENESIS

Extensive cellular necrosis of the gut epithelium leads to villous atrophy, loss of digestive enzymes, a reduction of absorption and increased osmotic pressure in the gut lumen, resulting in the onset of diarrhoea. This is followed by a reactive crypt-cell hyperplasia, accompanied by increased fluid secretion, which also contributes to the severity of diarrhoea. Local pathogenesis is shown diagrammatically in Figure 14.3.

Viral factors determining pathogenicity of rotaviruses have been investigated in several animal models (piglets, mice, rabbits; for review see Burke and Desselberger, 1996). The protein product of RNA segment 4, VP4, has been found to be a major determinant of pathogenicity in

several systems, but products of other structural genes (VP3, VP7) and of nonstructural genes (NSP1, NSP2, NSP4) have also been implicated (Broome *et al.*, 1993; Hoshino *et al.*, 1995).

The seminal discovery of NSP4 acting as a viral enterotoxin (Ball *et al.*, 1996; Tian *et al.*, 1994, 1995) has explained the well-known observation that rotavirus-infected animals exhibit profuse diarrhoea prior to the detection of histologic lesions. NSP4 or a peptide thereof (aa 114–135) induces dose- and age-dependent diarrhoea in laboratory animals (mice, rats) *in the absence of histological changes* (Ball *et al.*, 1996). NSP4 produces an increase in intracellular Ca^{++} concentration (Tian *et al.*, 1994), disturbing the cellular electrolyte homeostasis. A peptide of NSP4 which is active as an enterotoxin is secreted from infected cells (Zhang *et al.*, 2000) and is able to induce intracellular Ca^{++} elevation and diarrhoea in mice. It is thought that the secreted protein binds to a (still hypothetical) receptor and thus affects uninfected cells (Tafazoli *et al.*, 2001; Zhang *et al.*, 2000). Silencing of NSP4 expression in rotavirus-infected cells by specific siRNA affects mRNA production and the formation of viroplasms, suggesting a significant involvement of NSP4 in rotavirus replication and morphogenesis as well (Lopez *et al.*, 2005; Silvestri *et al.*, 2005). Immunization of mice with an NSP4-based vaccine, followed by specific antibody production, attenuates the symptoms of rotavirus-induced diarrhoea (Choi *et al.*, 2005). Whether NSP4 antibodies exert a protective function in humans remains to be explored. The enteric nervous system may be involved in rotavirus diarrhoea (and diarrhoea of other causes) as drugs blocking this system were shown to alleviate the diarrhoea (Kordasti *et al.*, 2004; Lundgren *et al.*, 2000; Salazar-Lindo *et al.*, 2000).

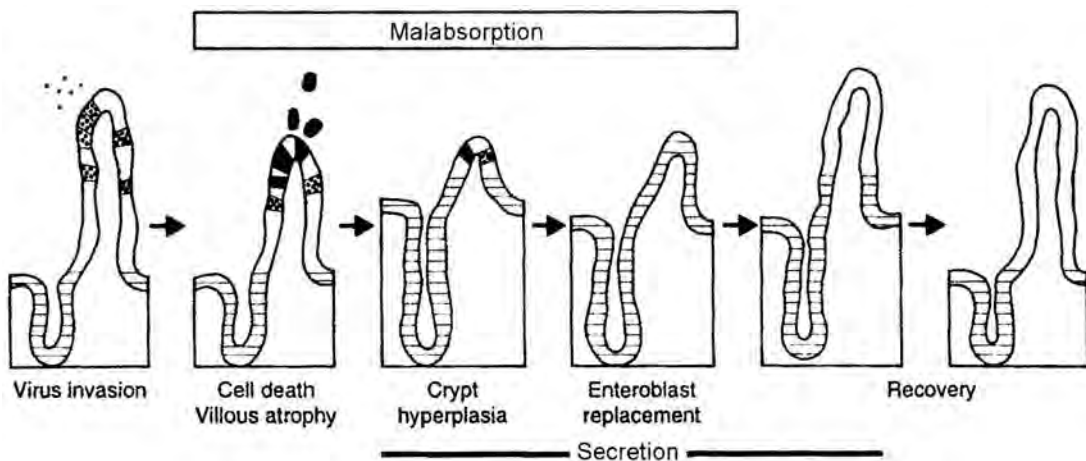


Figure 14.3 Rotavirus pathogenesis. Development of damage to gut mucosa, diarrhoea and repair. (Source: Phillips, 1989; with permission of author and publisher.)



Figure 14.4 Rotavirus deaths worldwide (1 dot = 1000 deaths). (Source: Parashar *et al.*, (2003); with permission from Emerging Infectious Diseases.)

Rotavirus RNA and antigen have been detected in the sera of children with acute rotavirus gastroenteritis (Blutt *et al.*, 2003, 2007; Fischer *et al.*, 2005). Whilst viremia in rotavirus infection appears to be relatively frequent, systemic disease is rare (see below), suggesting that spread of rotavirus may be co-incidental with systemic disease of different origin (Dormitzer, 2005; Estes and Kapikian, 2007; Ramig, 2007).

IMMUNE RESPONSES AND CORRELATES OF PROTECTION

After neonatal or primary rotavirus infection a mainly serotype-specific humoral immune response is elicited, providing homotypic immunity, but there is also partial protection against subsequent rotavirus infections by other serotypes, and the heterotypic protective capacity increases with the number of re-infections (Velasquez *et al.*, 1996). The exact correlates of protection remain to be determined (Offit, 1994), but levels of rotavirus-specific coproantibodies of the IgA subclass seem to correlate best with protection (Coulson *et al.*, 1992; Feng *et al.*, 1994, 1997; Franco *et al.*, 2006; Yuan *et al.*, 1996), though not in all cases (O'Neal *et al.*, 2000). Humoral antibodies might be neutralizing when directed towards the type-specifying antigens VP7 and VP4, but may also be directed towards the inner capsid proteins VP2 and particularly VP6 (Burns *et al.*, 1996; Feng *et al.*, 1997; Herrmann *et al.*, 1996), possibly interacting with DLPs intracellularly and preventing transcription or maturation ('intracellular neutralization') (Burns *et al.*, 1996; Cortes *et al.*, 2006). There is a rotavirus-specific cytotoxic T-cell response, but its exact role in overcoming an

infection, or in protection against subsequent infections, is not known (Franco and Greenberg, 1995; Franco *et al.*, 2006; Offit, 1994). Natural infection and appropriate vaccination (see below) seem to protect from severe disease in subsequent infections (Coulson *et al.*, 1992; Velasquez *et al.*, 1996), even if the serotype of the challenging virus differs from that of the previous infection or those contained in a vaccine.

ILLNESS, DIAGNOSIS AND TREATMENT

After a short incubation period of one to two days the onset of the illness is sudden, with watery diarrhoea lasting four to seven days, vomiting and rapid dehydration. All degrees of severity of disease are seen, including in apparent infections by so-called 'nursery strains' (frequent) (Gorziglia *et al.*, 1988), central nervous system infections (rare) (Iturriza-Gómara *et al.*, 2002a) and chronic infections and hepatitis in children with immunodeficiencies (rare) (Gilger *et al.*, 1992). In SCID mice, rhesus rotavirus (RRV) causes hepatitis (Riepenhoff-Talty *et al.*, 1987). Respiratory symptoms are not infrequently seen during rotavirus disease, however the question whether rotavirus is actively replicated in the lung and transmitted from there is still unresolved (Ramig, 2007).

Diagnosis of a rotavirus infection is relatively easy as such large numbers of virus particles (up to 10^{11} particles/ml of faeces) are shed. The main techniques are enzyme-linked immunoassays (ELISAs), passive particle agglutination tests and, when searching comprehensively for diarrhoeogenic viruses, EM (Figure 15.1). Serological assays have been used to establish G and P types

of rotavirus isolates, but these depend on the presence of triple-layered virus particles in the clinical specimen, which are often largely outnumbered by bilayered particles. Molecular techniques are therefore increasingly being applied for the purpose of typing, and also of detection. Rotavirus-specific oligonucleotide primers complementary to common and type-specific regions of the VP6, VP7 and VP4 genes allow sensitive detection, subgroup determination and typing for both G and P types, respectively, by RT-PCR (Gentsch *et al.*, 1992; Gouvea *et al.*, 1990; Iturriza-Gómara *et al.*, 2002c, 2003; Simpson *et al.*, 2003).

Treatment of rotavirus disease is by oral, subcutaneous or intravenous rehydration and pain relief as indicated. The formulae of oral rehydration solution (ORS) and reduced osmolarity 'light ORS', recommended by the World Health Organization (WHO), are widely used (Desselberger, 1999) (Table 14.2). Oral immunoglobulins seem to have an effect on the duration of diarrhoea and virus

shedding but are not routinely used (Bass, 2003; Desselberger, 1999; Guarino *et al.*, 1994). Enkephalinase inhibitors have been used to dampen the symptoms of severe diarrhoea caused by rotaviruses and other microorganisms (Salazar-Lindo *et al.*, 2000). For immunocompromized children, passive immunization with rotavirus antibodies obtained from eggs or milk has been found to be helpful (Davidson, 1996; Yolken *et al.*, 1985, 1988), but their wider use is not practicable.

EPIDEMIOLOGY

Rotaviruses are spread mainly via the faeco-oral route. Water, fomites and occasionally food may act as vehicles. Rotavirus particles are very resistant to environmental conditions in temperate climates. The high particle number in the faeces of children with acute rotavirus disease and the very small minimum diarrhea-causing dose

Table 14.2 Oral rehydration solutions (ORS) for the treatment of rotavirus-related diarrhoea

Component	Osmolarity (mmol l ⁻¹)	
	WHO ORS	'ORS light'
Sodium	90	60
Potassium	20	20
Chloride	80	50
Citrate	10	10
Glucose	111	84
Total osmolarity	311	224

Modified from Desselberger (1999). Both solutions were compared by an International Study Group on Reduced Osmolarity ORS Solutions (1995) and are equally in use.

Table 14.3 Cases of intussusception in double-blind, placebo-controlled phase III clinical rotavirus vaccine trials

Trial	Vaccinees		Placebos	
	N total	N IS	N total	N IS
Ruiz-Palacios <i>et al.</i> (2006) Monovalent rotavirus vaccine Two doses at two and four months of age <i>Observation period:</i> 32 days after any dose	31 673		31 552	
Vesikari <i>et al.</i> (2006) Pentavalent rotavirus vaccine Three doses at two, three and four months of age <i>Observation period:</i> 1. 42 days after any dose 2. 1 year after first dose	34 035	6 ^a	34 003	7 ^a
		6 ^a		5 ^a
		12 ^a		15 ^a

^aCases of intussusception (IS) evenly spread over observation periods.

($1DD_{50} = 10$ plaque-forming units (pfu); Ward *et al.*, 1986) ensure wide and efficient spreading to any susceptible host. Whilst there are marked seasonal peaks (winter/spring) in temperate climates, in tropical regions rotavirus infections and disease occur throughout the year.

The epidemiology of group A rotavirus infections is complex, as at any one time within a geographical location rotaviruses of different G types cocirculate (Desselberger *et al.*, 2001; Gentsch *et al.*, 1996; Ramachandran *et al.*, 1998; Santos and Hoshino, 2005). The relative incidence of G types also changes over time in the same location. Approximately 95% of cocirculating strains are types G1–G4 in most regions of temperate climate, typically G1P1A[8], G2P1B[4], G3P1A[8] and G4P1A[8], but other G types may be represented at high frequencies, particularly in tropical areas (Desselberger *et al.*, 2001; Gentsch *et al.*, 1996; Iturriza-Gómara *et al.*, 2000). Recently, G9 rotaviruses have been isolated as the predominant outbreak strains in several locations in the United States (Ramachandran *et al.*, 1998) and in Europe (Bányai *et al.*, 2005; Iturriza-Gómara *et al.*, 2002b; Marques *et al.*, 2007; Rahman *et al.*, 2005; Reidy *et al.*, 2005; Sanchez-Fauquier *et al.*, 2006; Steyer *et al.*, 2005; Tcheremenskaia *et al.*, 2007; Van Damme *et al.*, 2007). Besides being acquired in the community (Waters *et al.*, 2000), rotavirus infections are increasingly recognized as making up a significant proportion of nosocomial infections (Gleizes *et al.*, 2006). Group B rotaviruses have caused outbreaks of diarrhoea in children and adults in China (Hung, 1988) and have been isolated from sporadic cases of gastroenteritis in Calcutta, India (Kobayashi *et al.*, 2001; Krishnan *et al.*, 1999). Group C rotaviruses are associated with small outbreaks in humans (Caul *et al.*, 1990; Jiang *et al.*, 1995). Besides the accumulation of point mutations (genomic drift) (Iturriza-Gómara *et al.*, 2003), gene reassortment (genomic shift) (Iturriza-Gómara *et al.*, 2001, 2003; Matthjinsens *et al.*, 2006; Maunula and von Bonsdorff, 2002) seems to play a major role in generating the high diversity of rotaviruses. Animals are increasingly recognized as a significant reservoir for human rotavirus infections as animal rotaviruses have been found to infect humans directly and to form reassortants with human rotaviruses (Alfieri *et al.*, 1996; Awachat and Kelkar, 2005; Das *et al.*, 1993; Iturriza-Gómara *et al.*, 2004; Matthjinsens *et al.*, 2006).

VACCINE DEVELOPMENT

Rotavirus infections are recognized as a major viral infection in children (Figure 14.4), and development of vaccines has been in progress since the early 1980s (Vesikari

et al., 1984). Results were mixed for some time, owing to the enormous genomic and antigenic diversity of rotaviruses (for reviews see Angel *et al.*, 2007; Glass *et al.*, 2004, 2006b; Kapikian, 1994a, 1994b). Initially, animal rotaviruses (of simian or bovine origin) were used as live attenuated vaccines for humans. In many cases protection from *infection and/or mild disease* was only modest (40–50%), but 70–80% protection was achieved from *severe disease including dehydration* when a cocktail of different viruses was applied (Joensuu *et al.*, 1997; Pérez-Schael *et al.*, 1997; Rennels *et al.*, 1996). A tetravalent RRV-based reassortant vaccine (developed by Wyeth) received Food and Drug Administration (FDA) approval as a universal vaccine in the United States in August 1998, and recommendations for its usage have been issued (CDC, 1999a). The tetravalent vaccine contained a rhesus monkey rotavirus (RRV) of G3 type and three mono-reassortants individually carrying the VP7 gene of human serotypes G1, G2 and G4 in the RRV genetic background. More than 1.5 million doses were administered in the following 10 months. During that time a number of cases of intussusception were observed in vaccinees, particularly within three to seven days after the first dose, with an odds ratio of 27.9 (95% confidence interval 10.8–72.1) in a case-control analysis of intussusception (Murphy *et al.*, 2001). These observations led the CDC and the American Academy of Pediatrics (CDC, 1999b, 1999c) to withdraw the recommendation for use in infants, and vaccine production ceased. There has been considerable controversy about the vaccine-attributable risk of the occurrence of intussusception, which also occurs spontaneously. Estimates between 1 case per every 4700 and 1 case per every 32000 vaccinated children have been considered (Chang *et al.*, 2001; Kramarz *et al.*, 2001; Murphy *et al.*, 2001; Simonsen *et al.*, 2001). Intussusception was not found to be epidemiologically linked with natural rotavirus infection (Chang *et al.*, 2002; Velazquez *et al.*, 2004), although abnormal gut anatomy has been observed in children with acute rotavirus disease (Robinson *et al.*, 2004). A recent reanalysis of the data indicated that the age of vaccinees is a critical factor for intussusception to occur, as a disproportionately high number (>80%) of children who developed intussusception after the first dose of rotavirus vaccine were older than 90 days (Simonsen *et al.*, 2005). Based on these observations, so-called ‘catch-up vaccinations’ of children older than three months are contraindicated, even when using the more recently developed vaccines (see below).

Despite this major setback, work on alternative live attenuated rotavirus vaccines has continued (Angel *et al.*, 2007; Glass and Parashar, 2006b; Glass *et al.*, 2004, (2006a,b)). In particular, two promising vaccine candidates have emerged. A monovalent vaccine, developed

by GlaxoSmithKline, is derived from an attenuated human rotavirus isolate, 89-12, of the G1P1A[8] type. It has been found to be highly effective in preventing severe rotavirus gastroenteritis and hospitalization (85%) (Bernstein *et al.*, 1998, 1999) and did not present a risk of intussusception above background in over 30 000 vaccinees when compared to the same number of matched, double-blinded placebo controls (Ruiz-Palacios *et al.*, 2006) (Table 14.3). The monovalent vaccine provided good heterologous protection against G3P[8], G4P[8] and G9P[8] strains and was only slightly less efficacious only against infection with G2P[4] strains. The vaccine has now been licensed in Mexico, Brazil, Venezuela and numerous European countries, and has been incorporated into Extended Programme of Immunization (EPI) schemes for universal vaccination of birth cohorts in Mexico, Brazil, Venezuela, Belgium, Luxembourg and Austria.

The other rotavirus vaccine, developed by Merck, is pentavalent and based on the attenuated bovine rotavirus WC3 strain (G6P7[5]) (Clark *et al.*, 1996; Treanor *et al.*, 1995). It consists of a mixture of five mono-reassortants, in which the VP7 gene is individually replaced by the VP7 genes of human G1, G2, G3 and G4 strains, and the VP4 gene by that of a human P[8] strain, all on the background of the other ten genomic RNA segments being contributed by the WC3 strain. This vaccine was also found to be highly efficacious in preventing severe rotavirus gastroenteritis (98%) caused by G1, G2, G3, G4 and G9 strains. No link with intussusception was found in >32 000 vaccinees when compared with the same number of matched, double-blinded placebo controls (Vesikari *et al.*, 2006). The vaccine was licensed for use in the United States, has been recommended for universal use by the Advisory Committee on Immunization Practices (ACIPs) (Parashar *et al.*, 2006a) and has now been licensed in various other countries.

The impact of the new rotavirus vaccines, particularly in countries in which the decision has been made to vaccinate universally on the annual birth cohorts, remains to be seen (Desselberger *et al.*, 2006a). Although one of the vaccines has been tested in some developing countries, the real test of vaccinating populations of infants in the poorest parts of the world is still ongoing (Glass *et al.*, 2006). The risk of undesirable effects of live attenuated vaccines (clinical complications, reversion to virulence, genetic interaction with cocirculating wild-type rotavirus strains, etc.) is considered to be low but is not zero and may only become apparent, or be excluded, once millions of children have been vaccinated. Cases of intussusception may occur in temporal association with vaccination, although the overall background level of intussusception may not be increased (Angel *et al.*, 2007). Thus, intensive post-marketing surveillance is required during the

introduction of the new vaccines in many countries, and is in fact in place. There is a good case for including surveillance of animal rotaviruses in these exercises as well.

A lamb rotavirus strain LLR (G10P[12]) has been licensed as a live attenuated vaccine for human use in China, but no clinical trial data have become available (Glass *et al.*, 2004, 2006a,b). A tetravalent vaccine, based on mono-reassortants of human VP7 genes with the genes of the attenuated bovine UK Compton rotavirus strain, has been proven to be effective and safe (Clements-Mann *et al.*, 2001) and has been expanded into a hexavalent vaccine candidate (Kapikian *et al.*, 2005). Asymptomatic neonatal human rotavirus strains have also been developed as live attenuated candidate vaccines (Glass *et al.*, 2005).

Other rotavirus vaccines under development are: virus-like particles (VLPs) originating from baculovirus recombinants expressing structural proteins (VP2, VP6, VP4 and VP7) (Conner *et al.*, 1996); vaccines with enhancement of rotavirus immunogenicity by microencapsulation (Offit *et al.*, 1994); and DNA-based vaccines (Chen *et al.*, 1997, 1998; Herrmann *et al.*, 1996).

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Viruses other than Rotaviruses Associated with Acute Diarrhoeal Disease

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INTRODUCTION

Gastroenteritis in humans can be caused by viruses, bacteria and parasites. The pathogenesis differs considerably depending on the infectious agent. Clinical symptoms, however, are similar and range from mainly upper-gastrointestinal symptoms including vomiting to acute watery or bloody diarrhoea without any vomiting. Globally, viral gastroenteritis is a life-threatening major disease in infants and young children. This chapter presents an overview of gastroenteritis viruses except for rotaviruses, which are reviewed in Chapter 14.

Viruses which are known to cause human gastroenteritis (Figure 15.1) belong to genera of different virus families (Blacklow and Greenberg, 1991; Fauquet *et al.*, 2005):

- rotaviruses (a genus of the *Reoviridae*); see Chapter 14
- noroviruses (NoVs) and sapoviruses (SaVs) (two genera of the *Caliciviridae*)
- astroviruses (*Astroviridae*)
- enteric adenoviruses (species F of the *Adenoviridae*).

In terms of relative frequency of incidence in children, rotaviruses were found to account for 30–60%, caliciviruses for 8–30%, astroviruses for 6–9% and enteric adenoviruses for 3–15% of all cases of viral gastroenteritides (Krajden *et al.*, 1990; Bon *et al.*, 1999; Iturriza Gomara *et al.*, 2008). In adults, caliciviruses are the most

frequent viral cause of diarrhoea. In the Netherlands, between 1994 and 2005, 6707 faecal samples collected from patients involved in 941 outbreaks of gastroenteritis were tested for the presence of NoV, SaV, rotavirus, astrovirus and adenovirus. Viruses were identified as the causative agent in 84.5% of outbreaks, with the aetiological agent of 15.5% still unidentified. NoVs were detected as the causative agent in 78.1% of outbreaks, with rotaviruses, adenoviruses and astroviruses being associated with 4.9, 1.0 and 0.5% of outbreaks, respectively (Svraka *et al.*, 2007). Thus, recently the significance of caliciviruses for gastroenteric disease has been increasingly recognized.

Other viruses that are found in the gastrointestinal tract but are not regularly associated with diarrhoea are: enteroviruses including parechoviruses (*Picornaviridae*), reoviruses (*Reoviridae*), toroviruses (*Coronaviridae*), coronaviruses (CoVs, *Coronaviridae*) and parvoviruses including bocavirus (*Parvoviridae*). Human immunodeficiency virus (HIV; a member of the *Retroviridae* family) can infect the gut directly (Heise *et al.*, 1991). Under conditions of immunosuppression, herpes simplex viruses (*Herpesviridae*), cytomegalovirus (CMV, *Herpesviridae*), and picobirnaviruses (*Picobirnaviridae*) are also found to infect the gut and cause disease.

Many of the obligatory human gastroenteritis viruses do not grow at all, or only very poorly, in cell cultures, and therefore virus isolation is not the diagnostic method of choice. By contrast, electron microscopy (EM)

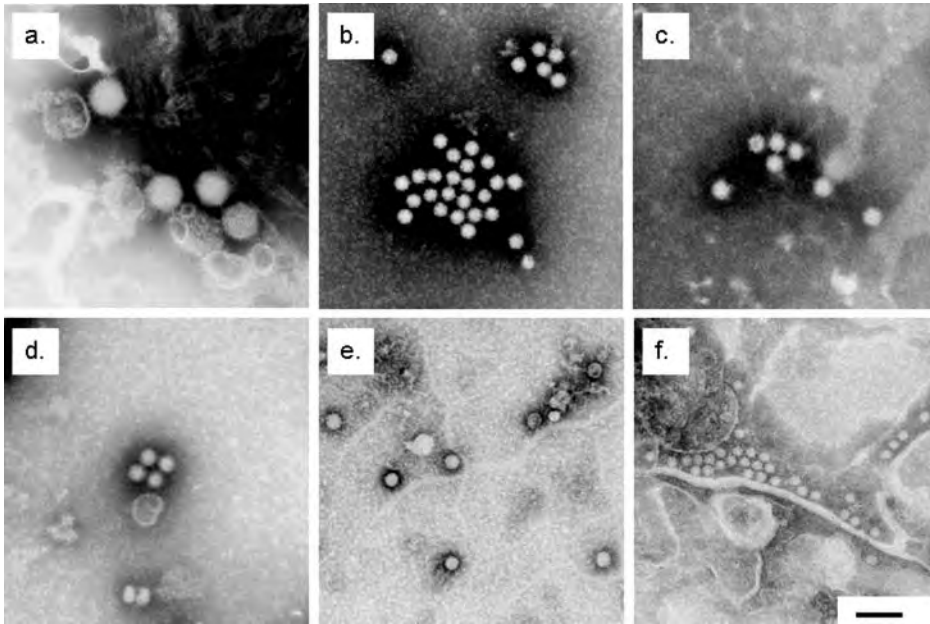


Figure 15.1 Electron micrographs of (a) adenovirus, (b) astrovirus, (c) sapovirus, (d) norovirus, (e) picornavirus and (f) parvovirus. (Negative staining with 1.5% phosphotungstate, pH 6.5; bar = 100 nm.) (Courtesy of Dr Hazel Appleton and Brian Megson, Virus Reference Department, Centre for Infections, HPA, London.)

permits the differentiation of viruses on the basis of their characteristic morphology (Figure 15.1). However, the sensitivity of detection (approximately 10^6 particles per ml) is low. In general, detection of rotaviruses by EM is easy as this virus is shed in very large numbers (up to 10^{11} particles per ml of faeces) during the peak of the illness. Astroviruses and enteric adenoviruses also occur in large numbers and are readily detected. Other viruses, particularly caliciviruses, are often only detectable by EM very early in clinically-apparent disease. The methods of polymerase chain reaction (PCR) and reverse transcription (RT)-PCR, which have much greater sensitivity (10–100 DNA or 20–100 RNA molecules, respectively, are detectable per reaction), are increasingly being used in diagnostic laboratories. RT-PCR and serological techniques using recombinant antigens have demonstrated the true extent of human infections with caliciviruses and astroviruses, and they are much commoner than previously thought.

Viral gastroenteritis infections follow two distinct epidemiological patterns. In early childhood, diarrhoea occurs as endemic disease, mainly caused by rotaviruses of group A, caliciviruses, astroviruses and adenoviruses of subgroup F. By the age of five years many children have been infected with all these agents, often without apparent symptoms. The main mode of infection is faeco-oral, but infection may possibly also happen by droplet or close

contact. The second pattern is as outbreaks and epidemics, mainly caused by caliciviruses, but sometimes by astroviruses and group B and C rotaviruses. These can affect all ages, and the viruses may be transmitted by infected food (e.g. shellfish) or water (Gray *et al.*, 1997; Hedberg and Osterholm, 1993).

In a community-based study NoV infection was detected by RT-PCR in 871/2422 (36%) cases of gastroenteritis and, remarkably, in 358/2205 (16%) of age-matched asymptomatic controls (Amar *et al.*, 2007). Symptomatic and asymptomatic NoV infections were detected in all age groups but were predominant in children, young adults and the elderly (Amar *et al.*, 2007) (Figure 15.2). Similarly, SaV was detected in 4% of cases and 2% of asymptomatic controls, but in contrast to NoV, symptomatic SaV infections were predominantly in children and asymptomatic infections were found more often in young adults (Figure 15.2). This highlights the lack of long-term immunity to gastroenteric viruses, with infections and re-infections occurring throughout life, particularly at times of frequent contact with children, as a child, as a parent of young children and as a grandparent (Gray *et al.*, 1993).

Treatment largely follows guidelines established since 1985, mainly using oral rehydration fluids containing electrolytes and sugar (Desselberger, 1999). Bismuth subsalicylate has been found to be beneficial in children with

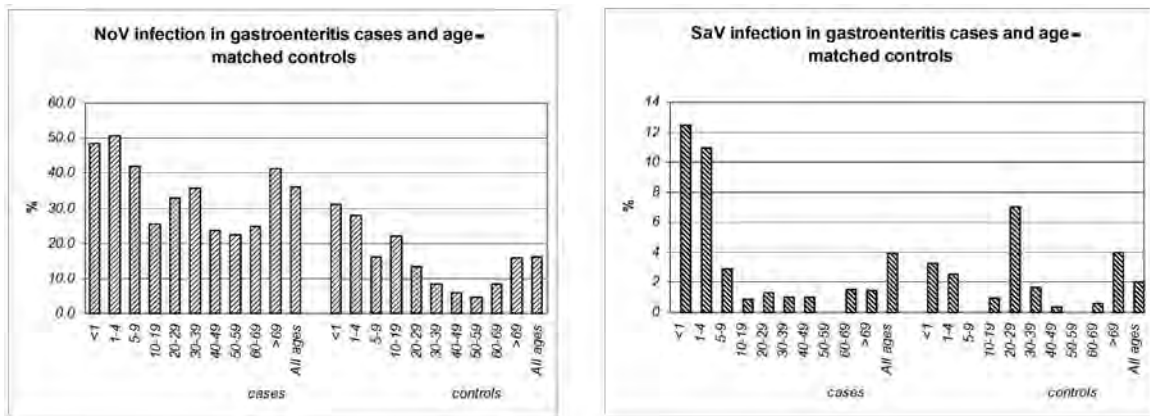


Figure 15.2 Incidence of symptomatic and asymptomatic norovirus and sapovirus infection by age. (Source: Amar *et al.*, 2007.)

acute watery diarrhoea (Figueroa-Quintanilla *et al.*, 1993). Agents used against abdominal cramping such as diphenoxylate or loperamide should be avoided as they can have serious side effects. In severe cases of diarrhoea, rapid fluid replacement by parenteral administration may be required. In developing countries where children are often malnourished, supplementary nutrition is an important component of the therapy. Public health measures to confine outbreaks include frequent hand washing, proper disinfection, removal of infected faeces and contaminated food or water, and exclusion of infected individuals from work. Outbreaks in clinical wards may require temporary closure to new admissions and restrictions on visiting and placements of staff.

Viruses regularly and irregularly causing acute gastroenteritis will be briefly described below (for concise review see Desselberger, 1998; for more extensive information see Chadwick and Goode, 2001; Cohen *et al.*, 2002; Desselberger and Gray, 2003). According to the relative degree of importance of different viruses for clinical human disease, caliciviruses are presented more extensively than the other viruses. Rotaviruses, associated with significant morbidity and mortality worldwide, are reviewed in Chapter 14.

ENTERIC ADENOVIRUSES

Genome and Structure

Adenoviruses are non-enveloped icosahedral viruses of 70–80 nm diameter, possessing a genome of linear ds-DNA of approximately 35 000 bp and a capsid with 240 hexons and 12 pentons at the vertices, which carry

projecting fibres (Berk, 2007). Their three-dimensional structure has been elucidated (Stewart *et al.*, 1991).

Classification

Human adenoviruses occur in 51 distinct serotypes ordered into six different species (A–F, previously called groups or subgroups) within the genus *Mastadenovirus* of the *Adenoviridae* family (Fauquet *et al.*, 2005). The classification is based on immunological, biochemical and biological differences. Within species, serotypes are differentiated by the reactivity of the two major capsid proteins, hexon and fibre. Within each species, DNA sequence homology is greater than 85%. Adenoviruses regularly associated with gastroenteritis are classified in species F, as serotypes 40 and 41 (Wold and Horwitz, 2007).

Replication and Pathogenesis

All adenoviruses grow well in human epithelial cells, with the exception of the fastidious or enteric adenoviruses types 40 and 41. These viruses grow poorly in Graham 293 cells (a human embryonic kidney cell line transformed by adenovirus type 5 DNA; Takiff *et al.*, 1981) or to higher titre in Graham 293 cells expressing the V-protein of the paramyxovirus SV5, which degrades the signal transducer and activation of transcription 1 (STAT1) and therefore prevents the STAT1-mediated interferon response (Sherwood *et al.*, 2007). Adenovirus replication has been studied in detail, mainly with types 2 and 5 of species C. Cell attachment is facilitated by the fibre protein, and uptake is via receptor-mediated endocytosis. Members of the immunoglobulin superfamily have been implicated as receptors, and integrins possibly as co-receptors, with CD46 identified as a receptor

for species B and species D adenoviruses (Gaggar *et al.*, 2005). Following uncoating, viral DNA moves to the nucleus, and a phased early and late gene expression is initiated. The early protein E1A acts as a transcriptional activator and is a potent blocker of apoptosis and interferon (IFN)- α and - β expression. Late gene expression is at the onset of viral DNA replication and is accompanied by blockage of cellular messenger ribonucleic acid (mRNA) expression. Virus assembly is in the cytoplasm. Virus particles are released after cell death, mediated by disruption of the cytoskeleton. Some adenovirus proteins seem to decrease the expression of major histocompatibility complex (MHC) class I antigens on the surface of infected cells, thus hindering susceptibility to adenovirus-specific cytotoxic T lymphocytes, and also to counteract tumour necrosis factor alpha (TNF- α) expression (for details see Berk, 2007).

Adenoviruses replicate in the epithelia of the human respiratory and gastrointestinal tracts, as well as in the conjunctiva and in lymphocytes. Various viral factors contribute to the pathogenesis. Whilst the pentons are directly cytotoxic, early viral proteins counteract TNF and apoptosis, and down-regulate the expression of MHC Class I molecules, thus preventing recognition by cytotoxic T cells. There is persistence of adenovirus-infection in lymphoid cells, the mechanism of which is poorly understood (Berk, 2007).

Diagnosis

Detection of enteric adenoviruses in faecal specimens is mainly by enzyme-linked immunosorbent assays (ELISAs) using species F-specific monoclonal antibodies. EM followed by immune electron microscopy (IEM) can also be used to identify these agents. Adenoviruses are detected in 4–15% of stools from children with gastroenteritis in many hospitals, outpatients clinics and day-care centres (Krajden *et al.*, 1990). PCR techniques have also been applied to the diagnosis of adenovirus infection (Allard *et al.*, 1990, 2001; Avellón *et al.*, 2001). However, of all the adenoviruses detected in faeces, only 30–50% belong to species F (Krajden *et al.*, 1990; Lew *et al.*, 1991), the others being mainly members of species B and D, infecting primarily the respiratory tract. By the age of two years, more than 40% of children possess neutralizing antibodies to adenoviruses types 40 and 41, suggesting that there are likely to be many inapparent infections (Shinozaki *et al.*, 1987).

Clinical Symptoms and Treatment

Clinically, adenovirus-associated diarrhoea does not differ from that caused by other viruses, although the duration of

symptoms may last slightly longer (3–11 days). The stools are watery and non-bloody (in contrast to some bacterial diarrhoeas). Fever and vomiting are common. Usually adenovirus gastroenteritis is a mild disease, and treatment is symptomatic. Cidofovir is active against adenovirus *in vitro* (De Clercq, 1996) and has been used in the treatment of disseminated adenovirus infections in bone marrow transplant recipients (Legrand *et al.*, 2001; Slatter *et al.*, 2005).

Epidemiology

Adenovirus infections occur worldwide as epidemic, endemic or sporadic infections. Enteric adenoviruses are mainly endemic, but outbreaks in hospitals and boarding schools have been reported. Most infections are in infancy and early childhood. The incidence of enteric adenovirus infections is between 4 and 7 per 100 person years in small children (Mistchenko *et al.*, 1992), without a seasonal preference. In immunocompromised persons, adenoviruses can cause severe systemic disease. Different genomic subtypes within types 40 and 41 have been observed (de Jong *et al.*, 1993).

Prevention and Control

At present there is no vaccine candidate for enteric adenoviruses. Control of hospital outbreaks is by cohort nursing of patients, use of gloves, gowns and goggles, and disinfection with sodium hypochlorite.

NOROVIRUSES AND SAPOVIRUSES (HUMAN CALICIVIRUSES)

Introduction

This group of viruses was first recognized from an outbreak of gastroenteritis in an elementary school in Norwalk, Ohio, USA in 1968, affecting half of the students and teachers (for reviews see Cubitt, 1994; Kapikian *et al.*, 1996). The outbreak was not due to a bacterial pathogen, and, finally, using IEM, the causative agent Norwalk virus (NV) was visualized as a 27–35 nm virus particle. Upon cloning and sequencing of the genome (Jiang *et al.*, 1993; Lambden *et al.*, 1993), NV was unequivocally classified as a member of the *Caliciviridae* family. With the advent of molecular diagnostic techniques it has become apparent that NoVs and SaVs are major causes of outbreaks of diarrhoea and vomiting in various population groups, and they are now recognized as the second most important cause of viral gastroenteritis (Fankhauser *et al.*, 1998; Koopmans *et al.*, 2001, 2003).

Structure and Genome

Typically, the surface of the particle carries cup-shaped depressions, which have given the name to this viral family (Latin 'calix' = 'goblet', 'cup'). The morphology of caliciviruses was analysed in more detail using cryoelectron microscopy and image reconstruction, once it became possible to produce recombinants and NoV particles from insect cells which were infected with a baculovirus recombinant expressing the capsid protein (Prasad *et al.*, 1994a, 1994b, 1999). The capsid consists of 90 dimers of a single capsid protein of 58 K molecular weight (monomer), which are arranged in such a way that large hollows are seen at the fivefold and threefold axes. These give the appearance of cup-like structures by EM. Each dimer forms a basal shell (S) domain. This is joined by a flexible hinge to a protruding (P) domain, which is further subdivided into subunits P1 and P2. NoVs recognize human histo-bloodgroup antigens (HBGAs) as receptors (Huang *et al.*, 2005). Human HBGAs are complex glycans found on the surface of red blood cells, on gastrointestinal and respiratory epithelia, and in saliva, milk and intestinal contents. The receptor binding site of the GII-4 strains of NoVs has been mapped to the outermost end of the P domain and forms an extensive hydrogen-bonding network with the saccharide ligand (Cao *et al.*, 2007).

The calicivirus genome consists of single-stranded RNA of positive polarity and approximately 7.7 kb size. The single-stranded RNAs are polyadenylated and have a length of between 7.3 and 7.7 kb (Figure 15.3). The genome composition of the SaVs bears a greater similarity to those of several animal caliciviruses than to human NoVs (Liu *et al.*, 1995; Matson *et al.*, 1995). This justifies their classification as a separate genus. All calicivirus genomes have three open reading frames (ORFs); ORF1 encoding nonstructural proteins, P48, NTPase, P22, VPg, 3CL^{pro} and an RNA-dependent RNA polymerase (RdRp); ORF2 encoding the viral capsid protein (VP1); and ORF3 at the 3' end of the genome encoding a small protein (VP2) of as yet unknown function (Hardy, 2005) (Figure 15.3; Table 15.1). There is a major difference between the genomes of NoVs and SaV; for SaV the ORF2 is in the same reading frame as ORF1 and is thus contiguous with the RdRP gene. For NoV, ORF1 and 2 overlap by a few nucleotides, with a -2 frameshift for ORF2 (Clarke and Lambden, 2001; Liu *et al.*, 1995).

Classification

NoVs and SaVs constitute two of the four genera of the *Caliciviridae* family (the other two being *Vesivirus* and *Lagovirus*, infecting animals). Sequence comparison has allowed the division of human NoVs into two, and

possibly three, genogroups, each containing multiple genetic clusters (Wang *et al.*, 2005; Zheng *et al.*, 2006). A bovine calicivirus (Jena virus) is closely related to genogroup I NoV (Liu *et al.*, 1999), whilst a porcine enteric calicivirus (PEC) is related to SaVs (Vinjé *et al.*, 2000a). Human SaVs are divided into five genogroups, each containing between one and four genotypes (Galimore *et al.*, 2006a). The classification of the *Caliciviridae* family is continually being updated as a result of the mutability of the calicivirus genome, which results from the accumulation of point mutations and genetic recombination.

Replication

As there is no *in vitro* monolayer cell-culture system available for human caliciviruses, their replication has been deduced from that of animal caliciviruses which have a similar genome organization and can be propagated in cell cultures. Feline calicivirus grows in CRFK cells, and an infectious cDNA clone has been constructed (Sosnovtsev and Green, 1995). From this it appears that caliciviruses interact with species-specific receptors. Proteins deduced from ORF1 may arise from appropriate co- and post-translational cleavage of a polyprotein precursor, in a way similar to the cleavage cascade identified for picornaviruses (Estes *et al.*, 1997). Murine NoV type 1 infects macrophage-like cells *in vivo* and replicates in cultured primary dendritic cells and macrophages, and therefore provides important tools, both *in vivo* and *in vitro*, for the investigation of NoV pathogenesis, replication and immunity (Wobus *et al.*, 2004). An infectious cDNA clone of the murine NoV has recently been obtained, allowing a detailed characterization of the viral replication cycle (Ward *et al.*, 2007). Straub *et al.* (2007) reported the culture of human NoV in a highly differentiated 3D cell-culture model of human small-intestinal epithelium. Guix *et al.* (2007) demonstrated that transfection of NoV RNA into human hepatoma cells led to the expression of viral antigens and viral replication, with release of viral particles into the cell-culture medium. Prior treatment of the RNA with proteinase K abolished protein expression after transfection, suggesting that there may be an important role, in infectivity and NoV genome expression, for the covalent linkage of RNA to a virus protein, similar to the role of VPg as a primer for RNA polymerase in picornavirus replication. Transfection of NoV RNA into cultured human hepatoma cells, bypassing receptor binding, internalization and uncoating, results in replication of the viral RNA and the release of virus particles. This contrasts with an inability to infect these cells conventionally, even in the presence of the glycans suspected to be involved in virus attachment. This suggests that attachment is not sufficient to lead to a productive

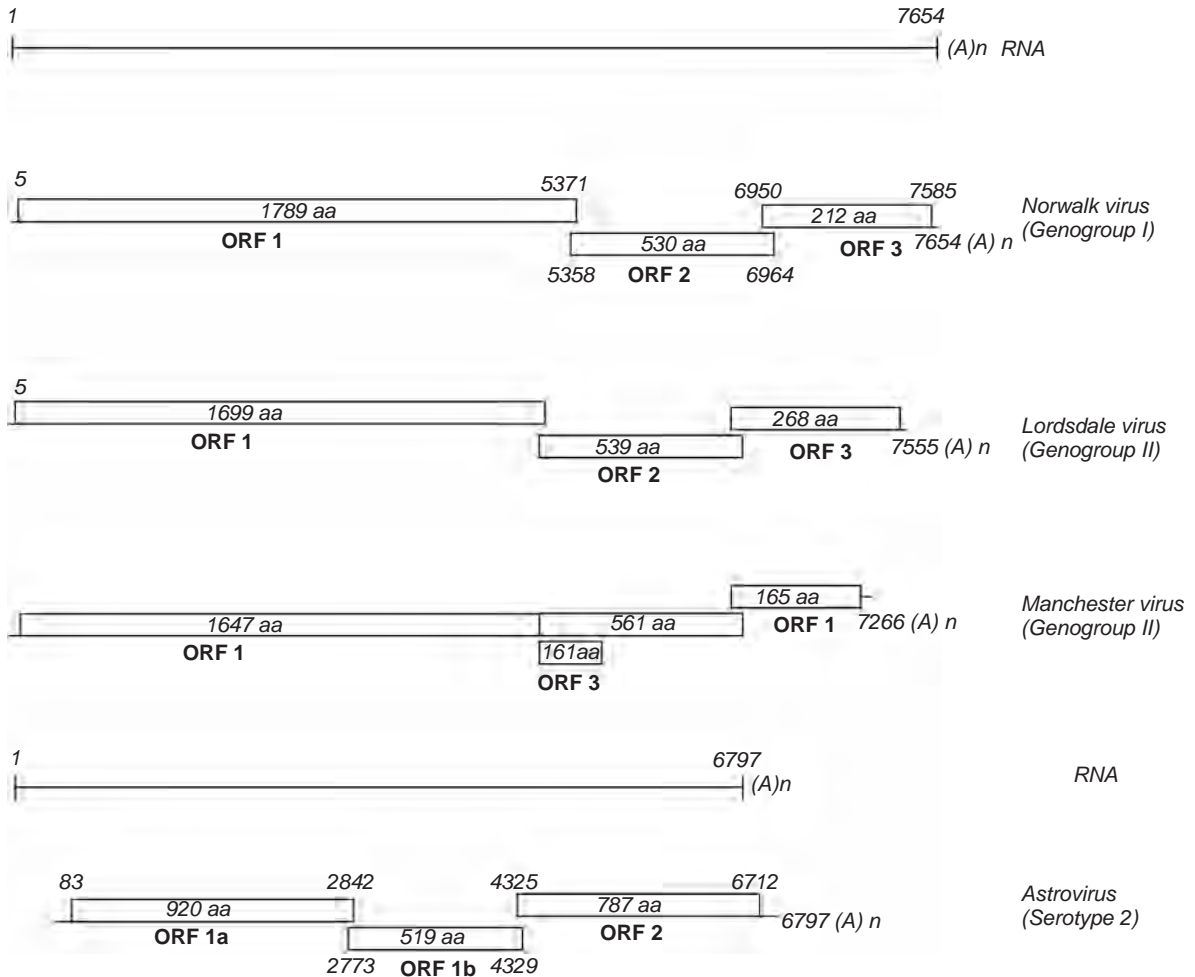


Figure 15.3 Genome organization of Norwalk virus (a norovirus of genogroup I), Lordsdale virus (a norovirus of genogroup II), Manchester virus (a sapovirus) and astrovirus (serotype 2). For nomenclature of noroviruses see Table 15.2. ORF, open reading frame; aa, amino acids; (A)_n, poly(A) tail of RNA. (Source: Estes *et al.*, 1997, slightly modified.)

infection and that other factors are required for entry and uncoating (Guix *et al.*, 2007).

Pathogenesis

The incubation period ranges from 10 to 50 hours, and diarrhoea lasts 24–48 hours. Ileum biopsies from ill volunteers showed that a symptomatic illness correlated with blunting of intestinal villi, crypt hyperplasia, cytoplasmic vacuolization and lymphocytic infiltration of the lamina propria. Small-intestinal brush border enzymes are decreased, and malabsorption and diarrhoea with abdominal cramps, nausea and vomiting result (Green, 2007).

Laboratory Diagnosis

This is mainly by direct EM or IEM, by ELISA (Gray *et al.*, 2007) or by RT-PCR assays (Ando *et al.*, 1995; Moe *et al.*, 1994). For the latter to be successful, broadly reactive primers must be available in order to detect most of the human caliciviruses. Initial use of such primers looked promising (Green *et al.*, 1995). Vinjé *et al.* (2000b) have developed primers with which 90% of outbreaks initially diagnosed by EM could be detected. NoV nucleotide sequence data, accumulated over time, indicate that the most conserved region of the NoV genome is the ORF1/ORF2 junction. Broadly reactive and highly sensitive genogroup-specific real-time quantitative

Table 15.1 Norovirus protein structure and function

Protein	Structural or nonstructural	Function
P48	Nonstructural	May disrupt intracellular protein trafficking
NTPase	Nonstructural	NTPase activity
P22	Nonstructural	May localize replication complexes
VPg	Nonstructural	May recruit translation machinery
3CL ^{pro}	Nonstructural	Protease activity
RdRp	Nonstructural	RNA-dependent RNA polymerase
VP1	Structural	Capsid protein
VP2	Structural	May play a role in viral genome packaging

RT-PCRs have been developed and are used extensively for the detection of NoVs in clinical samples (Kageyama *et al.*, 2003). These techniques have also been applied to detecting caliciviruses in shellfish and other foodstuffs (Atmar *et al.*, 1996; Lees *et al.*, 1995), and on environmental surfaces (Gallimore *et al.*, 2006) from which human infections may originate.

Immune Responses

The immune responses against human calicivirus infections have been examined in adult volunteer studies, using IEM and more recently ELISAs with recombinant antigens. However, volunteers are generally young adults who will have been infected with caliciviruses in childhood and have measurable pre-existing antibodies before challenge. It has not been determined if antibodies are neutralizing, as animal- and cell-culture models do not exist. Interestingly, more than 50% of adult volunteers appear to be susceptible to infection upon challenge (Graham *et al.*, 1994; Gray *et al.*, 1994; Johnson *et al.*, 1990; Parrino *et al.*, 1977).

Volunteer studies have shown that pre-existing NoV-reactive antibodies do not protect from re-infection in the longer run; on the contrary, higher pre-existing antibody levels seem to condition for more severe illness upon re-infection (Gray *et al.*, 1994; Parrino *et al.*, 1977). Some volunteers who fell ill after initial NoV challenge had developed immunity when rechallenged 6–14 weeks later (Parrino *et al.*, 1977). Cellular and humoral immune responses and infection were determined by examining pre- and post-challenge responses in volunteers aged

between 21 and 54 years of age who were challenged with a GII-2 (Snow Mountain) strain of NoV (Lindesmith *et al.*, 2005). Nine of fifteen volunteers were infected, and immune responses were characterized by a ≥ 4 -fold increase in serum immunoglobulin and by significant increases, on day 2 post-challenge, of serum IFN- γ and interleukin-2 (IL-2). No increases in concentrations of IL-6 or IL-10 were detected. PBMCs stimulated with NoV VLPs secreted IFN- γ . Antibody and cellular immune responses were cross-reactive within genogroup but not between genogroups (Lindesmith *et al.*, 2005).

Clinical Course

The clinical symptoms were documented in a study of 50 volunteers and were as follows: 82% (41) became infected, 68% symptomatically and 32% asymptotically. Most common symptoms were nausea, headache and abdominal cramps followed by diarrhoea and vomiting. Severe symptoms lasted for only 12–48 hours (Graham *et al.*, 1994). Persistent infection with caliciviruses and other viruses associated with chronic diarrhoea has been found in children with severe combined immunodeficiency (SCID) (Chrystie *et al.*, 1982) and in patients with acquired immune deficiency syndrome (AIDS) (Grohmann *et al.*, 1993).

Epidemiology

The availability of recombinant proteins expressed from cloned cDNAs has allowed the study of age-dependent antibody prevalence in both developed and developing countries. Epidemic gastroenteritis produced by caliciviruses is relatively mild. There is likely to be a large number of inapparent infections in early childhood, as it has been shown that 50% of three-year-old children already have NoV-specific antibodies, a number that increases to 80% in early adulthood (Gray *et al.*, 1993) (Figure 15.4). In less-developed countries, NoV-specific antibodies are produced after primary infection even earlier in life. In general, children appear to be infected with caliciviruses much more frequently than was recognized previously (Gray *et al.*, 1993; Numata *et al.*, 1994). Two distinct seroepidemiological patterns have been described for SaVs. The first pattern is characterized by high antibody prevalence at birth, reflecting the presence of maternal antibody, followed by a fall in prevalence during the first four months of age, and then a steady increase reaching a prevalence of 100% by the age of four years (Nakata *et al.*, 1988). The second pattern is one of low antibody prevalence in the first three months after birth, followed by a steady increase reaching a prevalence of 80–90% by the age of two to five years. The antibody

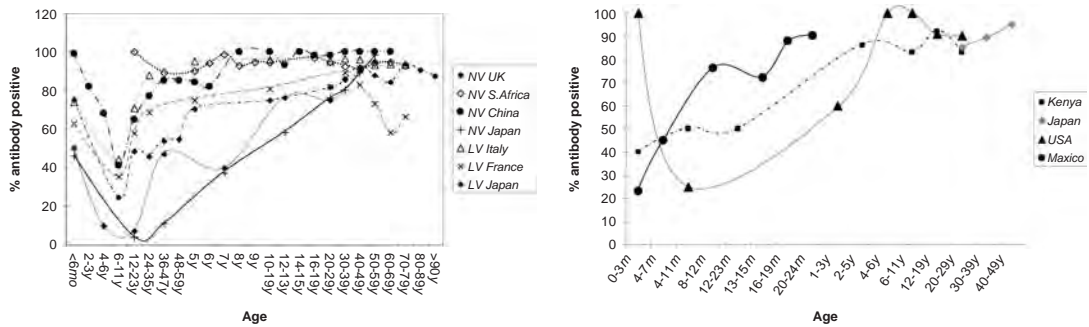


Figure 15.4 Age-related seroprevalence of antibody to (a) norovirus and (b) sapovirus in different countries. NV, Norwalk virus (GI-1); LV, Lordsdale virus (GII-4); MV, Mexico virus (GII-3) (Farkas *et al.*, 2006; Honma *et al.*, 1998; Jing *et al.*, 2000; Nakata *et al.*, 1985, 1988, 1998; Nicollier-Jamot *et al.*, 2003; Numata *et al.*, 1994; Pelosi *et al.*, 1999; Smit *et al.*, 1999).

prevalence in women of childbearing age in these populations is ~90%, but this is apparently not transferred as maternal antibody to offspring in their early months of life (Farkas *et al.*, 2006; Nakata *et al.*, 1998); this phenomenon requires further study.

Outbreaks of acute gastroenteritis that can be related to food or waterborne sources occur frequently in recreational camps, hospitals, schools, cruise ships, nursing homes and so on. (Hedlund *et al.*, 2000; Inouye *et al.*, 2000; Nakata *et al.*, 2000) and are associated with the ingestion of contaminated drinking or recreational water, uncooked shellfish, eggs, salads and cold foods. Mixed infections with caliciviruses of different genogroups have been observed (Gray *et al.*, 1997) and, as a consequence of this, genome recombination was found to occur (Jiang *et al.*, 1999; Phan *et al.*, 2007; Vinjé *et al.*, 2000b). Worldwide, outbreaks occur year round, and nosocomial infections with caliciviruses are common. The viruses are highly infectious and have been found to spread rapidly in volunteer studies. The primary and secondary attack rates are high (above 50%). Transmission is by the faeco-oral route and by projectile vomiting producing an aerosol which scatters these viruses in the environment (Caul, 1994). Viral shedding does not normally persist beyond 100 hours after the initial infection, but can be prolonged for up to two weeks. Shedding has also been detected by RT-PCR in samples collected from volunteers who remained well (Okhuysen *et al.*, 1995).

Diversity and Evolution

Diversity among NoVs is maintained through the accumulation of point mutations associated with the error-prone nature of viral RNA replication and genetic recombination upon dual infection of a single cell (Phan *et al.*, 2007).

The genomic diversity among human NoVs is characterized by three genogroups (GI, GII and GIV), and multiple genetic clusters within genogroups have been described. Genetic lineages made up of variants form the clusters. NoV variants may be associated with epidemics. Between 2002 and 2006, at least six variants (v1–v6) of the GII-4 NoV strain circulated worldwide, and v2 and v4 were responsible for global epidemics of gastroenteritis in 2002 and 2006, respectively (Gallimore *et al.*, 2007).

Host Genetic Resistance to NoV Infection

Carbohydrates of membrane glycoproteins or glycolipids are often associated with virus attachment to cell membranes. The H types 1, 2 or 3 and Lewis^b (Le^b) carbohydrate structures are HBGAs. These antigens are synthesized by the sequential addition of monosaccharides to precursor oligosaccharides that constitute the peripheral region of the glycolipids, as well as of O- and N-linked glycans of glycoproteins. The expression of α 1,2-linked fructose residues on surface epithelial cells of the gut and in body fluids is dependent upon the presence of a wild-type FUT2 allele (secretor gene). The trisaccharide Fuc α 2Gal β 3GlcNAc (H type 1) is recognized by NoV VLPs derived from GI-1 (NV), as are H type 3 and Le^b (Huang *et al.*, 2005) (see above). These oligosaccharides have an α 1, 2-linked fructose in common and are present in the gut on the surface of epithelial cells (Le Pendu *et al.*, 2006).

Although nonsecretors (FUT2^{-/-}), who represent 20% of the European population, are resistant to infection with the majority of NoV strains, and individuals of the B blood group (4–20%) are partly resistant, they are likely to be susceptible to strains that recognize the B blood group antigen or target the Lewis antigens under the control of the FUT3 gene (Tan and Jiang, 2005).

Prevention

The most efficient methods of prevention are measures of good hygiene (hand washing, disinfection and disposal of contaminated faeces and material, hygienic processing of food, withdrawal from work of ill food handlers, etc.) and sometimes closure of facilities (hospitals, nurseries, etc.). A vaccine seems possible, for example derived from VLPs (Jiang *et al.*, 1992) or from transgenic plants ('edible vaccines'; Mason *et al.*, 1996, 2002), but a better understanding of the significance of antibody responses for protective immunity, the duration of protection and the impact of short-term herd immunity is required. Prophylactic surveillance of key personnel in hospitals or food production (including keeping people with vomiting and loose stools off work) and of water sources helps to reduce calicivirus-induced outbreaks.

ASTROVIRUSES

Astroviruses are members of the family *Astroviridae* (Monroe *et al.*, 1993; Fauquet *et al.*, 2005). Virions possess a genome of single-stranded RNA of positive polarity and no envelope, like the *Picornaviridae* and *Caliciviridae* (Mendez and Arias, 2007).

Structure and Genome

Morphologically, astroviruses are 28–30 nm particles with typical five- or six-pointed star patterns on their surface, and sometimes also knob-like structures (Figure 15.1).

The astrovirus genome of approximately 6.8–7.2 kb comprises three ORFs (1a, 1b and 2), coding for a viral protease (ORF1a), an RNA-dependent RNA polymerase (ORF1b) and structural proteins (ORF2) (Willcocks *et al.*, 1994) (Figure 15.3). There is a 70 base overlap in ORFs 1a and 1b, containing sequences directing ribosomal frameshifting (to a –1 frame) for the reading of ORF1b (Brierley and Vidakovic, 2003; Lewis and Matsui, 1994, 1996; Marczinke *et al.*, 1994).

Classification

By 1995, seven serotypes had been distinguished by solid-phase IEM and had been confirmed as different genotypes (Noel *et al.*, 1995). Recently, a genotype 8, which may have arisen through intragenotypic astrovirus recombination, has been identified. Although rare overall, it has had relatively high incidences in Egypt and Spain (Belliot, *et al.*, 1997; Guix *et al.*, 2002; Naficy *et al.*, 2000).

Replication

Astroviruses grow well in human embryonic kidney, monkey kidney or human colon carcinoma cell cultures in the presence of trypsin (Lee and Kurtz, 1981). In all human astroviruses an RGD integrin recognition motif is found in the ORF2, and although experimental data is still lacking, it is postulated that integrin recognition plays a key role in astrovirus cell attachment (van Hemert *et al.*, 2007). After adsorption to and uncoating in cells, astroviruses produce full-length and subgenomic RNAs, the former leading to translation of ORF1a and ORF1a–1b fusion proteins, and the latter directing the production of the ORF2 protein precursor (Geigenmüller *et al.*, 2002, 2003). As mentioned above, the viral RNA polymerase is translated from ORF1b by –1 ribosomal frameshifting at an efficiency of approximately 5%.

Transfection of naked full-length genome RNA of human astrovirus type 1 was shown to lead to the production of infectious virus, allowing a reverse genetics system to be set up from cDNA (Geigenmüller *et al.*, 1997).

Pathogenesis

This is mainly deduced from studies of *in vitro* infected cells and of experimental animals. Adsorption occurs through a cellular receptor, and replication takes place in the cytoplasm. Animals infected with species-specific astroviruses (lambs, calves) show infection of mature enterocytes (at the tip of the villi) of the small intestine at 14–38 hours post infection, and diarrhoea is observed on days 2–4 (Mendez and Arias, 2007).

Clinical Course, Diagnosis, Treatment and Prevention

Clinically, astrovirus disease is similar to that caused by rotaviruses, although often milder.

Diagnosis is by EM, IEM (typing), astrovirus-specific ELISA (detecting all seven types; Herrmann *et al.*, 1990) and generic and type-specific RT-PCR (Noel *et al.*, 1995).

Treatment is symptomatic, and prevention in outbreaks consists of measures to interrupt person-to-person spread (good hygiene, disinfection, surveillance of food sources, etc.). At present, there is no specific antiviral treatment and no vaccine.

Epidemiology

Astroviruses are found in humans, lambs, calves, deer, piglets, mice, dogs and ducks, causing diarrhoea in most cases except for ducks, in which they cause acute hepatitis. Astroviruses are very species-specific. Human astrovirus infections are seen in infants and the elderly as endemic infections and occasionally as the cause of foodborne outbreaks of diarrhoea. The seasonal incidence

Table 15.2 Incidence of astrovirus serotypes in Oxford, UK, 1976–1992, and prevalence of astrovirus type-specific neutralizing antibodies in the Netherlands, 1994–1996

Astrovirus type	Astrovirus isolates Oxford, UK ^a (n = 291) (% type)	Astrovirus-neutralizing antibody Utrecht, the Netherlands ^b (n = 242) (% type-specific antibody)
1	64.9	91
2	11.3	31
3	9.3	69
4	11.3	56
5	2.1	36
6	0.3	16
7	0.7	10

^aFrom Lee and Kurtz (1994).

^bFrom Koopmans *et al.* (1998). For astrovirus type 8 see text.

peak is in the winter. Transmission is via the faeco-oral route, person-to-person contact and possibly fomites. The incidence of astrovirus infections in hospital-based and community studies has mostly been $\leq 5\%$ and 1–9% of viral causes of diarrhoea, respectively, but much higher incidence figures ($>10\%$) were recorded in Guatemala and Thailand. The incidence of different serotypes was determined from a collection of isolates in Oxford, UK (Lee and Kurtz, 1994) and has been complemented by an age-stratified study of the prevalence of neutralizing astrovirus antibodies in Utrecht, the Netherlands (Koopmans *et al.*, 1998) (Table 15.2). According to this, serotype 1 is most frequently found, followed by serotypes 2–4 at medium and serotypes 5–7 at low frequencies. The seroprevalence figures suggest that individuals can become infected by more than one serotype. Astrovirus infection is frequent in childhood (often inapparent), and 75% of 10-year-old children have astrovirus antibodies (Koopmans *et al.*, 1998). A high percentage (15%) of cases of diarrhoea in HIV-infected individuals are due to astrovirus infection (Grohmann *et al.*, 1993). Large foodborne outbreaks of astrovirus gastroenteritis have been recorded (Matsui *et al.*, 1994; Mitchell *et al.*, 1995; Oishi *et al.*, 1994).

Cocirculation of multiple astrovirus serotypes was shown by several groups in different locations (Cunliffe *et al.*, 1998; Monroe *et al.*, 2001; Mustafa *et al.*, 2000; Naficy *et al.*, 2000; Noel *et al.*, 1995). The different types cluster in two genogroups (A: types 1–5; B: types 6 and 7; Belliot *et al.*, 1997).

Although enteric astroviruses have been isolated from animals (Koci *et al.*, 2000) there is so far no firm indication that human astroviruses may have arisen from an animal reservoir.

GASTROINTESTINAL VIRUSES NOT REGULARLY ASSOCIATED WITH ACUTE DIARRHOEAL DISEASE

Details of the virology, immunology, diagnosis, clinical symptoms and epidemiology of these viruses are found in other chapters.

Enteroviruses and Parechoviruses

Enteroviruses (polioviruses, coxsackie viruses and echoviruses) and human parechoviruses (HPeVs 1 and 2)—all members of a genus of the *Picornaviridae*—infect humans via the alimentary tract, where they have their first site of replication (lymphoid tissues of the pharynx and the gut). Virus is usually excreted in the stool for several to many weeks and can also be isolated with ease from the pharynx during the first two weeks of infection. Most infections are asymptomatic. The main disease foci are the central nervous system (CNS) (spinal cord, brain, meninges), heart (myocardium and pericardium), skeletal muscles and skin. These are reached after a viraemic phase originating from the gut (Racaniello, 2007).

Diarrhoea is not a regular symptom of primary infection. Some echoviruses (notably types 4, 11, 14, 18, 19 and 22) as well as Coxsackie virus A1 have been documented as associated with outbreaks of diarrhoea (Patel *et al.*, 1985; Townsend *et al.*, 1982), but a consistent association has not been established. The clinical symptoms associated with HPeV1 and 2 infection are predominantly gastrointestinal and respiratory, with fewer CNS symptoms (Ehrnst and Eriksson, 1993).

Aichi virus, a member of the genus *Kobuvirus* of the family *Picornaviridae* (Yamashita *et al.*, 1998), was first isolated in BSC1 (African green monkey kidney) cells from a patient with oyster-associated gastroenteritis in Aichi, Japan. The name *Kobuvirus* is derived from the characteristic morphology of the ~ 30 nm diameter virus particle ('*kobu*' meaning 'bump' in Japanese). The virus has been found to be a consistent cause of sporadic cases and outbreaks of human gastroenteritis in Japan (Yamashita *et al.*, 1991, 1993). It has been a cause of traveller's diarrhoea in South East Asia (Yamashita *et al.*, 1995) and more recently also in Europe and South America (Oh *et al.*, 2006). Symptoms include diarrhoea, abdominal pain, nausea, vomiting and fever with abdominal pain, nausea and fever predominating (Yamashita *et al.*, 2001). The prevalence of neutralizing antibodies to Aichi virus increases significantly with age, from 7.2% in children under 5 years of age to 83.3–87.0% in adults of more than 35 years of age (Yamashita *et al.*, 1993).

Reoviruses

Reovirus, like *Rotavirus* a genus of the *Reoviridae* family, also primarily infects the gut via M cells and has its first round of replication in the Peyer's patches. From there reoviruses are carried in a viraemic step (or by retrograde transport along autonomous nerves) to the CNS, where they have their main disease targets in neuronal or ependymal cells. Reovirus pathogenesis has been studied in detail in mice, and particular pathogenetic factors have been associated with particular gene products (Tyler and Fields, 1996). Reovirus distribution is worldwide and can infect a variety of species, including baboons, bats, birds, cattle, humans, monkeys, sheep, snakes and swine.

At the end of human childhood, antibodies against all three serotypes of reovirus are found, suggesting past infection. No firm association of reovirus infection with any distinct human disease has so far been established, although reoviruses are not infrequently seen in the gut; an association with mild diarrhoea has been suggested (Tyler and Fields, 1996).

Coronaviruses and Toroviruses

These two genera of the *Coronaviridae* (Enjuanes *et al.*, 2000) cause respiratory and gastrointestinal infections in humans. Toroviruses, a well-established cause of diarrhoea in calves (Koopmans and Horzinek, 1994) and of asymptomatic infection in horses, were found to be associated with acute and possibly persistent diarrhoea in children (Koopmans *et al.*, 1997; Waters *et al.*, 2000).

CoVs are a recognized major cause of the common cold in man (Lai *et al.*, 2007). They cause gastroenteritis in animals (Kim *et al.*, 2001) and were found to be associated with cases of human diarrhoea (Gerna *et al.*, 1985). CoV-like particles were also seen in symptomless healthy subjects (Marshall *et al.*, 1989). The true significance of CoV infection for human enteric disease remains to be established (Lai *et al.*, 2007; Siddell *et al.*, 2007; Zhang *et al.*, 1994).

Disease associated with SARS-CoV is biphasic, including a prodromal influenza-like period characterized by myalgia, malaise, chills and fever, followed by the onset of respiratory and gastrointestinal symptoms. The epithelial cells of the mucosa of both the small and large intestines have been shown to be infected with the SARS-CoV, which is consistent with reports of viral shedding in stool samples (Guo *et al.*, 2008). The ability of the SARS-CoV to target cells of the immune system results in targeting of Peyer's patches, with a resulting decrease in both CD20-positive B cells and CD3-positive T cells and excessive atrophy of submucosal lymphoid tissue (Guo *et al.*, 2008).

Parvoviruses

These viruses are well-known pathogens associated with diarrhoea in animals. However, in humans no firm association with diarrhoea has been made, although parvovirus-like particles are not infrequently observed by EM as 'small round viruses' in human faeces.

Human bocavirus (HBoV), a recently-described new virus species belonging to the *Parvoviridae* family, was identified through molecular screening of respiratory tract samples (Allander *et al.*, 2005) and reported to be associated with upper- and lower-respiratory-tract infections (Bastien *et al.*, 2007). More recently, the spectrum of disease associated with HBoV infection has been widened to include gastroenteritis (Lee *et al.*, 2007). HBoV was detected in the stools of 9.1% of subjects in a study of Spanish children with acute gastroenteritis (Vicente *et al.*, 2007). Similarly, HBoV was detected in 2.1% of 1435 samples of faeces collected from children with fever and watery diarrhoea in Hong Kong (Lau *et al.*, 2007). In the absence of case-control studies it has yet to be determined to what extent HBoV is a respiratory and/or enteric pathogen.

Human Immunodeficiency Virus

The clinical virology of this member of the *Retroviridae* family (subfamily *Lentivirinae*) is extensively discussed elsewhere in this book. Here it should be mentioned that there is evidence for primary HIV infection in gut-associated lymphoid tissue and in enterocytes, which may contribute to an enteropathy with chronic diarrhoea in AIDS patients (Aziz *et al.*, 2005; Heise *et al.*, 1991; Kotler *et al.*, 1991; Levinson and Bennets, 1985; Nelson *et al.*, 1988; Rabeneck, 1994).

Cytomegalovirus and Herpes Simplex Virus

These two members of the *Herpesviridae* family are frequent co-infectants/superinfectants in the gut of HIV-infected individuals, causing mainly chronic colitis (Dieterich and Rahmin, 1991; Levinson and Bennets, 1985), but also oesophagitis, gastritis and cholangitis (Theise *et al.*, 1991). There may be interaction between HIV and CMV infections in causing gut disease (Skolnik *et al.*, 1988). With the introduction of highly active antiretroviral therapy (HAART) in 1996, it has become less urgent to initiate or maintain a specific anti-CMV therapy with ganciclovir (Pollok, 2001; Whitcup *et al.*, 1999).

Picobirnaviruses

These bisegmented dsRNA viruses of approximately 35–41 nm in diameter and icosahedral structure were

found in the gut of rats, guinea pigs, pigs and calves, and to be associated with cases of diarrhoea, although not regularly. Recently, besides astroviruses, caliciviruses and adenoviruses, picobirnaviruses were found to be significantly associated with diarrhoea in AIDS patients in case-control studies (Giordano *et al.*, 1999; Grohmann *et al.*, 1993). Similar dsRNA viruses have been demonstrated to infect *Cryptosporidium* spp. but there is little sequence identity between them and the typical picobirnaviruses (Wang and Wang, 1991; Xiao *et al.*, 2001). Reagents derived from the genomic sequence (Rosen *et al.*, 2000) will allow us to unravel the epidemiology of human and related picobirnaviruses. As the picobirnaviruses are much smaller than birnaviruses (infecting exclusively animals) and differ largely from birnaviruses in their nucleotide sequence (Wakuda *et al.*, 2005) and particle structure (F. Rey, personal communication), the proposal to classify them in a separate family, *Picobirnaviridae*, seems to be justified.

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Influenza

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INTRODUCTION

Although occasionally occurring as sporadic infections, influenza is more commonly and dramatically seen as local outbreaks or widespread epidemics of respiratory illness; these occur in most parts of the world, in most years (Figure 16.1). Epidemics can be traced anecdotally from the fifth century BC, the Hundred Years War and the court of Mary Tudor, to the more exact records of the last century. Unfortunately, knowledge gained in the last 70 years since isolation and characterization of the influenza virus has done little to prevent epidemics; it is not surprising, therefore, that influenza has been referred to as the 'last plague of mankind'. Epidemics can arise at any time but are usually concentrated in months of high relative humidity; outbreaks occur explosively, often with little or no warning, and the number of people infected can vary from a few hundreds to many hundreds of thousands, causing societal disruption. In addition, history records some nine occasions since 1700 AD when influenza has caused pandemics, and at these times millions were infected. In many years epidemics are short-lived, lasting days or weeks; however, pandemics can continue in successive waves for several months. It is the large number of persons infected during an outbreak of influenza, together with our inability to predict outbreaks, which has focused so much attention on this disease.

Influenza is a short-lived but relatively severe respiratory infection in healthy adults. Individual infection can be life-threatening to the elderly and to those with pre-existing heart, chest or metabolic disease, and infection may result in increased susceptibility to other infectious diseases. Depression and behavioural complications occasionally continue for months after the acute phase

of illness has passed. The seasonal or epidemic nature of influenza is less marked in tropical countries, and the burden of illness much less well defined.

Since the first isolation of an influenza virus in ferrets in 1933, there has been intensive research into the nature and control of the disease. Commissioning of an international network of communicating laboratories, the Global Influenza Surveillance Network (GISN), by the World Health Organization (WHO) to monitor the gradual changes in circulating viruses, occurred in recognition that coordinated global effort is needed to sustain virus-tracking activities (Kitler *et al.*, 2000). Nevertheless, despite intensive scientific and surveillance efforts, many fundamental questions concerning the nature of virus evolution and pathogenicity remain unanswered. Influenza A viruses have a wide host range; this together with a high rate of replication and a segmented viral genome, which allows for genetic reassortment and a mobile global animal reservoir, means that there is a continuing threat of the emergence and adaptation of a new influenza A virus to the human host.

Virus Structure

The influenza A, B and C viruses belong to the genus *Influenzavirus* in the family *Orthomyxoviridae*, within the negative-sense RNA virus order *Mononegavirales*. Influenza viruses grow on embryonated eggs or mammalian cell culture, and when examined in the electron microscope they are seen as approximately spherical particles with a diameter of 80–120 nm. After serial passage in the laboratory, some strains produce filamentous particles, and pleomorphic forms are not uncommon (Stuart-Harris *et al.*, 1985). The electron-microscopic appearance of

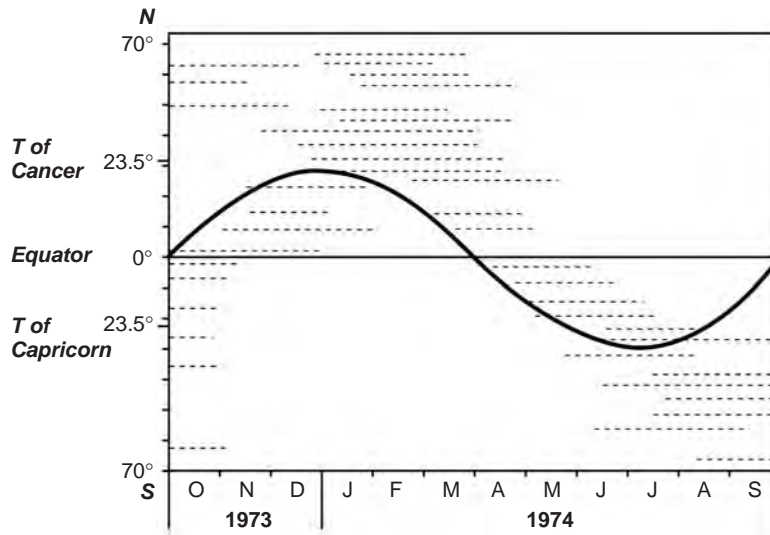


Figure 16.1 The annual timing of influenza epidemics by latitude. The timing of global influenza epidemics reported to WHO Geneva, Oct 1973 to September 1974, shown by latitude (broken lines). The curve shows the annual shift of midwinter around the globe. (Drawing based on Hope Simpson, 1981.)

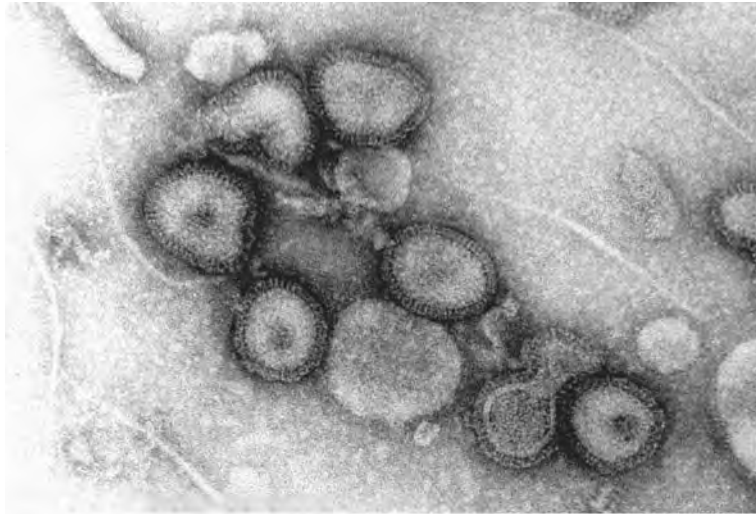
influenza virus particles is shown in Figure 16.2a, and a schematic diagram of a viral particle is shown in Figure 16.2b.

Each virus particle is composed of approximately 1% RNA, 70–75% protein, 20–24% lipid and 5–8% carbohydrate. Structurally, the virus consists of a single-strand RNA of negative polarity (Lamb and Krug, 1996) and relative molecular mass (M_r) $4 \times 10^6 - 6 \times 10^6$ segmented into fragments; the M_r s of the fragments range from 3×10^5 to 1×10^6 . The viral RNA is closely associated with the nucleoprotein (NP) to form the ribonucleoprotein (RNP), a helical structure termed the nucleocapsid; the NP has an M_r of approximately 60×10^6 , and there are approximately 1000 molecules in each virus particle. The NP is a type-specific antigen. It occurs in one of three antigenic forms, which provide the basis for the classification of influenza viruses into types A, B and C.

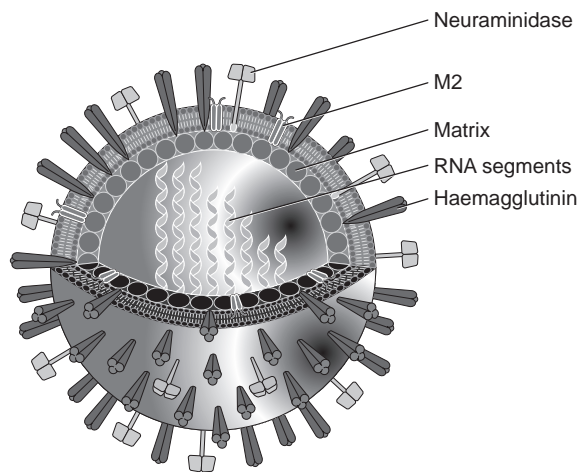
Surrounding the nucleocapsid of influenza A is a second protein referred to as the matrix protein (M1) which contributes 35–45% of the mass of the virus particle. The M_r of the M1 protein is 23×10^6 , and there are approximately 3000 molecules in each virus particle. A second M protein, termed M2 and coded by the same segment as the M1 protein, is important in virus replication; there are relatively few copies of M2 present in each virus particle, 14–68 molecules, which are inserted into the viral membrane external to the M1 protein; this is a lipid bilayer derived from the host cell which constitutes approximately 20–24% of the virus.

Two virus glycoproteins are inserted into the membrane; these are rod-shaped structures projecting out from the virus particle to give the spiky appearance of the surface (Figure 16.2a and b). The first of these glycoproteins is the haemagglutinin (HA), which is composed of two separate fragments, termed HA1 and HA2, joined together by a disulphide bond (Skehel *et al.*, 1984). The complete HA molecule is composed of a trimer of these subunits, each of M_r $75-80 \times 10^6$, and 20% carbohydrate, to give a total M_r of approximately 225×10^6 . There are approximately 1000 HA molecules on each virus particle; each HA particle is 14–16 nm in length and 4 nm in diameter, and is inserted in the lipid membrane by a hydrophobic tip. The HA forms 25–30% of the protein of the virus. The function of the HA is the attachment of the virus to the surface of host cells during the initial stages of virus infection; the HA also attaches to receptors on erythrocytes, causing haemagglutination, a property which forms the basis of several *in vitro* tests for the detection of virus and virus-induced antibody. The subtype classification of influenza A viruses is based on the different antigenic forms of the HA molecule, H1–H16.

The second glycoprotein extending from the surface of the virus particle is the neuraminidase (NA). There are 100–200 NA molecules on the surface of each virus particle; they are 10 nm long and 4 nm in diameter, and composed of four identical subunits, each of M_r approximately 60×10^6 , and an unknown amount of carbohydrate; these structures account for some 7% of the total viral protein. The NA molecules are attached to the viral membrane by a



(a)



(b)

Figure 16.2 (a) The electron microscopic appearance of influenza A/Taiwan/1/86 (H1N1) particles grown in embryonated eggs. Magnification $\times 200\,000$. Negatively stained with phosphotungstic acid. (Micrograph courtesy of Hazel Appleton HPA.) (b) Schematic diagram of an influenza virus particle.

stalk terminating in a hydrophobic tip; the complete structure has a mushroom appearance (Laver *et al.*, 1984) and a total M_r of approximately $200\text{--}250 \times 10^6$. Suggested functions of the NA include the removal of sialic acid residues from cell surfaces to promote virus absorption, and the removal of virus receptors on the infected cell surface, to facilitate release of newly-formed virus particles (Colman, 1998). The NA glycoprotein is antigenically variable, and differences are used in the subtype classification of the viruses: N1–N9.

Influenza virus particles also contain an RNA-dependent RNA polymerase complex; this consists of three proteins, termed polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase acid (PA) protein; the M_r s vary (range $80\text{--}90 \times 10^6$) and there are approximately 50 molecules of each species in each virus particle, which are closely associated with the nucleocapsid. These proteins have a complex role in virus-specific RNA synthesis during virus replication. A recent finding is the presence of a second small

nonstructural protein in infected cells synthesized from the PB1 gene, termed PB1-F2, which appears to have a mitochondrial localization and a role in the induction of cell death (Chen *et al.*, 2001). Viral RNA codes for two further nonstructural proteins, termed NS1 and NS2. These are expressed in infected cells; NS1 is synthesized early after infection, while NS2, now termed nuclear export protein (NEP), appears late and is found in virus particles (Paragas *et al.*, 2001). These viral proteins are multifunctional and have roles in RNA binding and interaction with host-cell responses (reviewed by Palese, 2005).

Most studies on the structure of influenza virus particles have been carried out on influenza A viruses; however, sufficient work has been done with influenza B viruses to suggest general similarity in size, composition and structure. Some differences in the genetic organization of influenza B have been found, most notably in the coding arrangements for the accessory protein BM2, and the presence of NB protein coded on gene 6, which does not have a homologue in influenza A viruses. The significance of these differences for virus natural history and epidemiology is not known. The influenza B virus also differs from influenza A viruses by the presence of an antigenically-distinct NP, and this, together with the antigenic specificity of other virus proteins, underlies differences in epidemiological behaviour. Antigenic variants occur less frequently, there are no antigenic subtypes or animal reservoirs for influenza B, and epidemics tend to occur less frequently than do influenza A epidemics. As a result, children tend to be older when experiencing their first influenza B infection.

The size, composition and structure of influenza C viruses are similar to those of influenza A; the main difference is an antigenically-distinct NP, and the presence of a Haemagglutinin-Esterase protein, which combines the function of the HA and NA of influenza A and B, with a different substrate specificity of 9-*O*-acetyl-*N*-acetylneuraminic acid (Rogers *et al.*, 1986). These differences characterize a virus which has clinical and epidemiological properties distinct from those of both influenza viruses A and B, since it causes a much milder illness.

Host Range

Influenza B and C are viruses of humans, with very few reports of sporadic infections occurring in nonhuman mammalian hosts (seals, pigs and dogs). In contrast, influenza A viruses are primarily viruses of avian water-based birds, with all subtypes H1–H16 and N1–N9 being found in this natural reservoir in a variety of H and N combinations (Figure 16.3). Restricted subtype combinations have established and adapted to mammalian hosts (humans: H1N1, H3N2; pigs: H1N1, H3N2; and horses: H3N8, H7N7). However, there is enormous potential for zoonotic infection of humans with different subtypes, either from pigs or avians, and for cross-species transmission from water birds to aquatic mammals which share an ecological niche (seals and whales); from water birds to gallinaceous birds (domestic poultry), which may in turn infect mammalian hosts such as felines (cats and tigers), canines (mink) or humans; from horses to dogs (Figure 16.4); and from humans to susceptible animals,

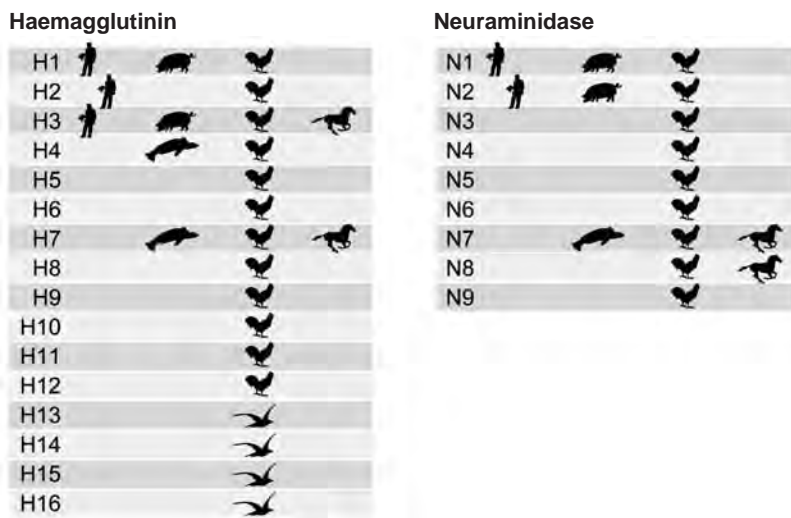


Figure 16.3 HA and NA subtype distribution in different animal species.

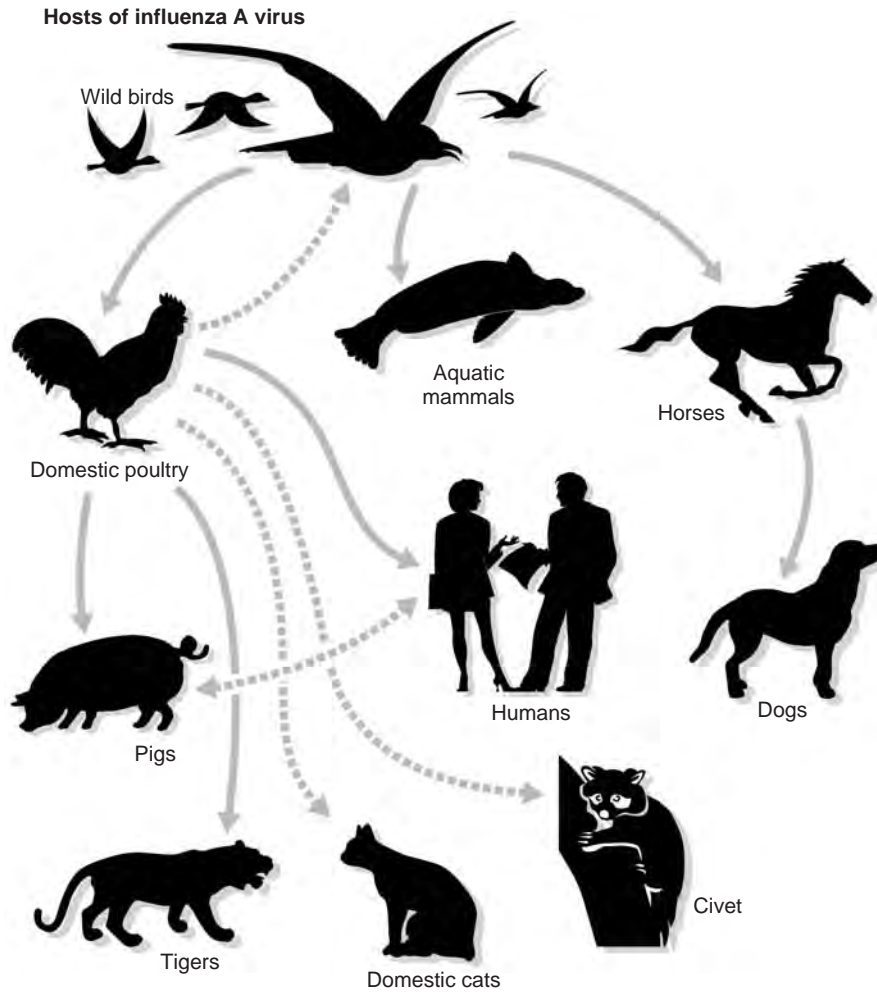


Figure 16.4 The ecology of influenza A. Dotted lines represent routes of transmission which are less well documented.

as in the original identification of influenza virus from a ferret infected by a human.

Replication

Most studies on the replication of influenza viruses have been carried out using influenza A strains, but the limited studies with influenza B virus have not indicated major differences in the mechanism of replication of this virus type. When an influenza A virus is inoculated on to cell cultures, there are three possible outcomes. First, the virus may fail to initiate infection, commonly because the cells do not possess the receptor essential for virus binding to cells. Second, the virus may undergo an incomplete growth cycle, known as abortive infection; this occurs in a variety of cell lines, including HeLa cells, L cells

and human diploid fibroblast cells. There is no accepted explanation for abortive replication, but because of a block at some stage of the normal replication cycle large numbers of virus particles are produced which are deficient in RNA content and are non-infective. Abortive infection is also seen when excessive amounts of virus are inoculated on to permissive cells, such as amniotic cells of the embryonated hen's egg, where the effect is described as the Von Magnus phenomenon. Third, infection may be permissive and result in the production of infectious virus; the various stages of replication are shown in Figure 16.5.

Receptor Binding

Permissive infection by influenza virus occurs in a range of cultured mammalian cells; typically dog, baboon and

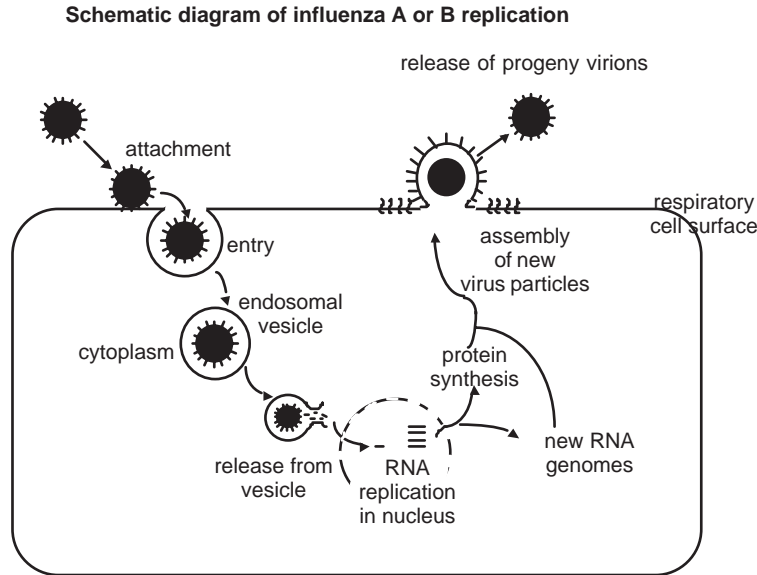


Figure 16.5 Schematic diagram of influenza A or B replication.

monkey kidney are preferred cell lines, as well as other continuous cell lines of human origin. These generally require that trypsin is added to the medium, since proteolytic cleavage of the HA into HA1 and HA2 by host enzymes is essential for virus infectivity and multicycle replication. Cells lining the amniotic and allantoic cavities of the embryonated hen's egg also support viral replication. Infection is initiated by adsorption of the virus to the cell surface. Adsorption requires the interaction of two molecules; these are the sialic acid-containing receptors on the surface of the cell, and the virus HA. Some 28 species of sialic acid receptors, together with sugar chains, are known (Suzuki *et al.*, 2001), and the specificity is important as there is selective binding of both avian and human strains to different sialic acid residues. Avian strains preferentially bind to $\alpha 2,3$ sialic acid, and human strains preferentially bind to $\alpha 2,6$ linked sialic acid (Rogers *et al.*, 1983) (Figure 16.6); neither preference is absolute, although receptor characteristics are important in determining virus–host range (Matrosovich *et al.*, 1997). Pathogenicity is also affected by genetic determinants. Some viruses can cross species barriers via other receptors (Stray *et al.*, 2000). The role of HA in virus binding is demonstrated in studies which show inhibition of virus binding with antibody to HA. Viral adsorption to cells provokes receptor-mediated endocytosis of virus particles via clathrin-coated pits, which can be seen by electron microscopy to appear approximately 20 minutes after infection. Virus internalization is followed by fusion of the viral envelope with endosomal membranes, a procedure

which requires the acidic environment at pH 5.0–6.0 in the endosomes, and the separate availability of HA1 and HA2 fragments of HA to allow insertion of the viral membrane into that of the host membranes. An acidic environment within the virus particle is achieved via the M2 protein in the virus membrane, which acts as an ion channel to allow hydrogen (H^+) ions into the virion. The low pH also frees the M1 protein from RNP, thus allowing the 'uncoated' RNA complex to migrate to the cell nucleus (Wang *et al.*, 1997).

Nucleic Acid Replication

The events of virus replication in the cell nucleus are complex and remain to be fully understood (Lamb and Krug, 1996). Information to date suggests that transcription of the messenger ribonucleic acid (mRNA) from all RNA fragments takes place immediately after entry into the cell nucleus. Initially, similar amounts of each mRNA are produced; however, this is followed by a regulatory phase in which the synthesis of mRNA coding for the NP and the NS1 predominate; it is clear that mRNA synthesis is regulated at all stages, and that this regulation is controlled by virus proteins, particularly P proteins NP and NS1 (Portela *et al.*, 1999). Synthesis of mRNA involves, sequentially, cap recognition and binding, endonuclease activity, initiation and then elongation, to produce an mRNA species which is a shorter and polyadenylated complementary copy of virion(v) RNA. First, the polymerase complex binds to a methylated-cap structure present on heterologous, cellular RNA; this cap recognition is a function

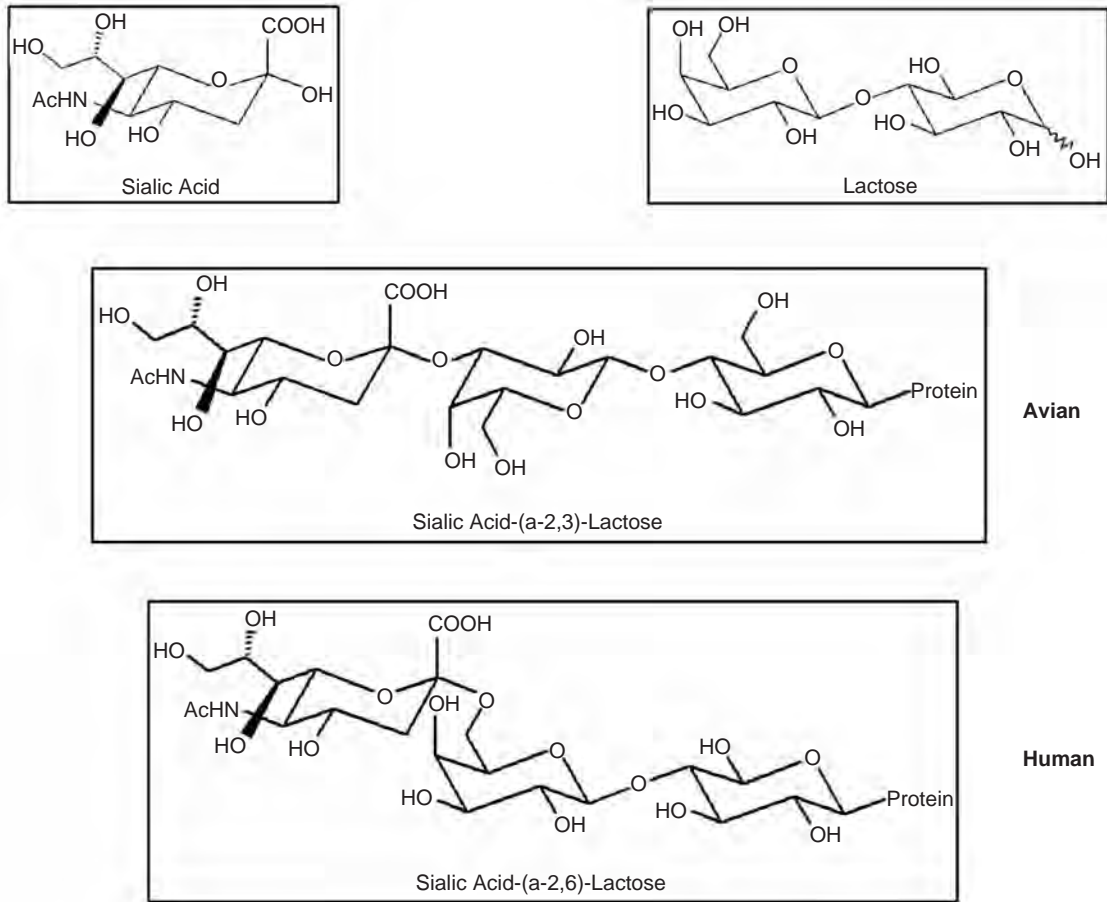


Figure 16.6 Linkage of sialic acid receptors for avian and human influenza viruses.

of PB2 protein. Second, a nucleotide sequence of 10–30 bases is cleaved from the cap structure by endonuclease activity, termed ‘cap snatching’. This structure forms the starter molecule for viral mRNA synthesis; it has been suggested that this cleavage is one of the functions of PB1 or PB2 proteins (Blok *et al.*, 1996). The cap sequence incorporates a G complementary to the penultimate C of the vRNA to complete the initiation step, and this is a function of the PB1 protein. Finally, elongation takes place to produce complete mRNAs; this step requires binding to NP, and elongation is thought to be controlled by the PA protein. Cap snatching from the host RNA requires active host RNA synthesis, and virus replication is dependent on this process; for this reason, inhibitors such as α -amanitin inhibit influenza virus replication.

Although interaction occurs between PB1 and PB2, and PB1 and PA, no direct interaction between PB2 and PA has been demonstrated to date; the exact role of these interactions in viral replication remains to be determined.

The mRNAs leave the cell nucleus and bind ribosomes, and translation of viral proteins is initiated.

The synthesis of a complementary, full-length, positive-sense RNA replication intermediate (A^- RNA), which is neither capped nor polyadenylated and does not require primer initiation and then subsequent production of negative-sense progeny virion(v) RNA, occurs after the time of peak production of mRNA and protein synthesis. Full-length vRNAs are produced in approximately equimolar amounts throughout infection, and are synthesized in the infected cell nucleus. The process requires the RNA polymerase to switch to a replicase mode and requires NP function, although how this is carried out is poorly understood. The time sequence for the appearance of the various viral proteins has been studied extensively: the presence of NP can be detected approximately two hours after virus infection, and the concentration rises to a maximum at five to six hours; the NP1 protein appears five hours after infection,

and has been identified in the cytoplasm and, to a lesser extent, the nucleus of infected cells; the HA and NA appear approximately four hours after infection.

The processes described above interfere with normal host-cell functions; specifically, there is a preferential synthesis of viral components at the expense of host-cell components. One of the chief mediators of the above is the NS1 protein, which is one of the most abundantly produced proteins synthesized in infected cells (Liu *et al.*, 1997). The NS1 protein inhibits the processing of cellular RNA by a number of mechanisms; the result of this is the cessation of cellular gene expression, and in particular their nuclear export. An important consequence of the activity of NS1 is to antagonize interferon production and thus interfere with the cellular response to virus infection (García-Sastre, 2001); indeed, this may be the most important role of NS1, since it is not essential for viral replication, as seen in studies in which NS1 mutants replicate in interferon-negative cells. Alteration of NS1 may be an important means of attenuating viruses for vaccine purposes

Virus Assembly

Assembly of new virions within infected cells begins with the binding of NP to newly-synthesized vRNAs to form RNP (Elton *et al.*, 1999). RNPs exit the nucleus in association with M1 and NEP proteins, and at the cell membrane they are enclosed by an envelope which contains HA, NA and M2 proteins; it is proposed that the M1 protein is the major driving force in this process (Gómez-Puertas *et al.*, 2000). Virus escapes by budding from lipid rafts in the plasma membrane; the process of virus release is detectable as early as five to six hours after infection, and is maximal seven to eight hours after infection. An important element in this process is believed

to be the NA, which prevents aggregation of virus at the host membrane by hydrolysis of sialic acid residues from the viral envelope at the cell membrane.

VIRAL VARIATION

Historical Aspects

Annual epidemics of influenza are associated with attack rates of 5–30% of the population, a significant increase in rates of hospitalization and death, predominantly among elderly and the very young. Nine pandemics have been recorded since 1700, which began at a focal point and spread rapidly through the world to infect hundreds of millions of individuals (Figure 16.7). Accurate documentation of pandemics was first seen in the 1889–1892 pandemic, which spread from Russia into Europe and the United States; infection presented as an upper-respiratory-tract infection of sudden onset and short duration; the total number of cases was high; and deaths were most numerous amongst infants and the elderly, but occurred in a relatively small percentage (0.05%) of the 25–30% of the world population infected. The pandemic 1918–1920 is known as the Spanish influenza. This pandemic spread worldwide for some six to eight months, and gave rise to an infection of unusual virulence which commonly caused a severe pneumonia; some 40–50 million deaths occurred, principally among young adults (Potter, 1998). The effects of the pandemic caused international panic. The number of deaths recorded is probably only a fraction of the true number: many countries did not record figures. India was said to have lost a generation, and 1.5 million deaths occurred in sub-Saharan Africa. The propaganda surrounding the World War of

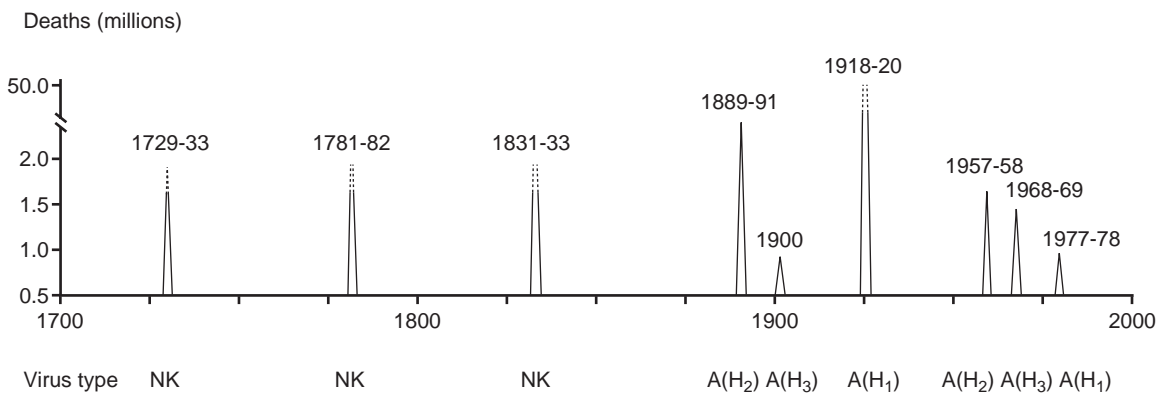


Figure 16.7 Schematic of influenza pandemic, based on historical accounts from 1700. Gives years of pandemics, estimated numbers of deaths in each pandemic and causal virus. NK, not known.

1914–1918 undermined the accuracy of data from Europe, and the Bolshevik revolution in Russia resulted in ignorance of the effects of the pandemic in that country (Crosby, 1976).

The definition of a pandemic of influenza is an outbreak of infection arising in a specific geographical region, spreading worldwide and infecting a high percentage of people. A pandemic is caused by a strain of influenza virus which could not have arisen by mutation from strains previously circulating. The influenza outbreaks of 1932–1933 and 1947–1948 were caused by viruses related to each other and to the virus that caused the pandemic of 1918–1920; thus, by the above definition, the widespread outbreaks of 1932 and 1947 were not pandemics. However, a pandemic did occur in 1957, which originated in China and spread throughout the world, infecting 40–50% of people and causing over 1 million deaths, mainly in the elderly population; however, fatalities among the younger victims were more conspicuous in the early months of the epidemic than later, and this is of note in other pandemics (Simonsen *et al.*, 1998). Pandemic infection occurred again in 1968, and the event was similar to that of 1957 (Figure 16.7). Pandemic infection broke out in 1976, due to an influenza virus very closely related to a virus strain which disappeared in 1947. This pandemic was therefore limited to the young,

since many persons born before 1947 had retained immunity to infection. Prior to 1976, the appearance of a new pandemic virus had been accompanied by the disappearance of the virus subtype previously causing infections in man; however, from 1976 the old and new subtypes co-circulated, and this continues to the present day (Figure 16.8).

No discernible pattern for predicting pandemics can be seen; however, the largest gap between pandemics over the last 150 years has been 40 years. In conclusion, the historical record of influenza indicates almost annual occurrences of epidemics, punctuated at intervals of 10–40 years by pandemics: there is little evidence to suggest that this situation will not continue into the future (Potter, 2001).

Antigenic Shift

Since 1933, the viruses which cause pandemic and epidemic outbreaks have been isolated and compared. Viruses isolated from patients infected in the epidemic years 1933–1946 and 1947–1956 show wide variations, but antiserum from ferrets experimentally infected with these viruses show serological cross-reactivity. Sequence data confirmed that the two groups of virus, represented by A/PR/8/34 and A/FM/1/47, were related and belonged

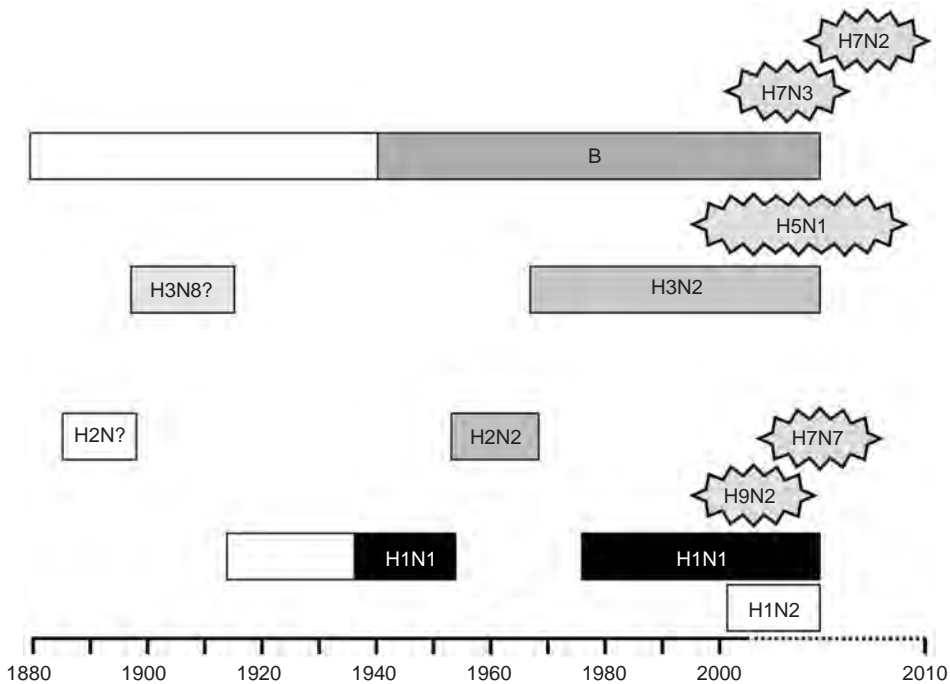


Figure 16.8 Circulation of influenza types and subtypes in humans since 1900. The jagged symbols represent limited transmission of avian viruses to humans.

to the H1 subtype. RNA sequences recovered and amplified by the polymerase chain reaction (PCR) from post-mortem lung tissue from two US soldiers who died in 1918 also confirmed that the Spanish influenza pandemic was caused by an influenza A (H1N1) virus (Taubenberger *et al.*, 1997). In contrast, the virus subtypes which emerged to cause the pandemics of 1957 and 1968 were unrelated to each other or to the H1N1 subtype, and could not have arisen by mutation from the preceding subtypes. This demonstrates that when pandemic infections are recorded, new influenza virus subtypes have emerged in a population that has no immunity; a phenomenon known as antigenic shift. The historical pattern for pandemic influenza is for a new virus subtype to emerge and spread rapidly, causing explosive outbreaks in many countries. Population immunity to the new subtype is built up over a period of years, and further outbreaks are more limited. However, after 10–40 years a new virus subtype emerges, with an HA which does not cross-react with antibody generated to the HA of the previous virus subtype, and to which the population is therefore largely susceptible. This initiates a new cycle of influenza pandemic and subsequent epidemics. Sudden changes in antigenicity can also occur in the virus NA molecule, although the impact is not as great as a change in the HA.

Although the HA antigens of different influenza A virus subtypes show no cross-reactivity in serological haemagglutination inhibition (HI) tests (Table 16.1), a degree of heterosubtypic immunity may exist, as evidenced in the phenomenon known as ‘original antigenic sin’, first reported by Francis *et al.* (1953). This term describes the observation that infection by an influenza virus induces antibody to the other virus subtypes that the individual has been infected with previously; indeed, the titre of antibody to the earlier-infected subtype can be several-fold greater than to the current infecting virus. This is probably due to B memory cells, which persist after infection, being stimulated by new virus serotype,

and suggests a relatedness in the HA antigens of different virus serotypes not detected in HI and other serological tests.

The importance of antigenic shift in producing new antigenic subtypes which spread to cause pandemic infection is evident, but despite some 100 years of intensive research, the origin of pandemic viruses is uncertain. The most widely held theory for the origin of human pandemic influenza viruses is that new virus subtypes are reassortant viruses arising during double infection of a single host. As influenza A viruses have a wide host range, dual infection with mammalian-adapted and animal virus in a species permissive for replication of both could result in a reassortant virus with surface antigen(s) from the nonhuman virus and other genes from a mammalian-adapted virus. Reassortant viruses can be produced experimentally from human and animal parents and may occur naturally. Viruses with segments derived from humans, pigs and avians have emerged to cause disease in pigs in North America since 2000 (Webby *et al.*, 2004). This, together with the emergence and spread of H1N2 derived from circulating human H1N1 and H3N2 (Gregory *et al.*, 2002), indicates that reassortment does occur both between species and within species. Close studies of avian species in South East Asia confirms the enormous genetic diversity which can be generated through segment exchanges within aquatic and land-based birds (Webby *et al.*, 2007). It has been suggested that reassortment of the whole HA molecule from an avian influenza virus into a new pandemic strain is not essential, and that only some of the sequences from avian virus may be necessary to produce an HA of radically different antigenic form, although there is no evidence to support this so far. Influenza B viruses do not occur in animals and do not exhibit antigenic shift; this is also indirect evidence for reassortment as the mechanism for the emergence of new influenza A virus subtypes.

Table 16.1 Antigenic shift in the haemagglutinin of influenza A viruses

Virus subtype	Virus strain	Serum HI antibody titre to influenza virus ^a			
		A/PR/8/34	A/FM/1/47	A/Sing/1/57	A/HK/1/68
H1N1	A/South Carolina/1/18	NK	NK	NK	NK
	A/PR/8/34	1280	<10	<10	<10
	A/FM/1/57	<10	640	<10	<10
H2N2	A/Sing/1/57	<10	<10	1280	<10
H3N2	A/HK/1/68	<10	<10	<10	1280

NK, not known; <10, limit of detection of HI antibody.

^aInfluenza A viruses grown in eggs were tested in HI tests with antisera from ferret infected intranasally with live virus and bled three to four weeks later. The serum HI antibody titre against eight HA units of virus is listed. The titres in bold represent reactivity of a virus with homologous antisera.

The pandemics in the years 1889, 1900, 1918, 1957, 1968 and 1976 were due to influenza A virus subtypes H2, H3, H1, H2, H3 and H1, respectively (Figure 16.7). Thus of the 16 HA types identified in nature, only influenza viruses A (H1, H2 and H3) have caused human pandemics; and over the observation period of approximately 100 years, they have occurred sequentially. From these observations it has been suggested that only these subtypes can cause pandemics and that they exist cryptically in nature, and can emerge when the antibody state of the population has fallen to levels which allow pandemics to occur. Although serological studies have detected the presence of antibodies to influenza virus A (H3), which was first seen in 1968 in serum from aged persons collected before the pandemic, no reservoir for the virus in humans before this date has been found. The evidence supporting this theory remains fragile.

Concern about the emergence of the next pandemic has been demonstrated by the epizootic of influenza A (H5N1) virus infection in chickens in South East Asia, which first came to attention in 1997. Zoonotic infection of 18 humans was associated with 6 case fatalities (de Jong *et al.*, 1997). Although stringent control measures involving chicken slaughter and improved hygiene suppressed further human infections for a few years, Hong Kong experienced further human cases in 2003, with H5N1 viruses which had evolved significantly from those seen in 1997 (Shinya *et al.*, 2005). Since this time there has been infection of humans with H5N1 in at least 10 countries in South East Asia, Europe and the Middle East through direct exposure to poultry or wild birds, with a case fatality of approximately 50%. Zoonotic infections with influenza H7, H9 and H10 emphasize the necessity for concern with respect to the continuing pandemic threat posed by viruses in an animal reservoir with close proximity to humans, leading to intensified surveillance in humans and animals, and heightened preparedness for a potential pandemic (WHO, www.who.int/csr/disease/avian_influenza/ai_timeline/en/index.html).

Antigenic Drift

When the viruses of the same subtype from various epidemic seasons between pandemics are compared in cross-HI tests, they exhibit strain differences. Although all viruses belong to the same subtype they are not identical; these changes are termed 'antigenic drift'. Table 16.2 shows the major HA variants found in viruses isolated in 1968–1979. All belong to the H3N2 subtype associated with the epidemic of 1968. A/HK/1/68 virus reacts most strongly with homologous antiserum, and cross-reactions are seen with other viruses in the series; the degree of cross-reactivity decreases as the time difference increases. Similar drift can be seen in the strains isolated from 1979 to the present day (not shown). The practical effects of antigenic drift are that infection by one strain may induce some cross-reactive antibody and partial immunity to viruses of the same subtype, but as time passes and virus diversity increases, this immunity is likely to be less and less effective. In this way viruses alter from year to year, giving rise to new strains that cause fresh epidemics.

It is considered that virus variants occurring naturally by mutation are selected by antibody pressure in an immune or partially-immune population. New viruses are not neutralized completely by antibody to pre-existing virus strains, and are therefore capable of causing new epidemics. Virus strains with antigenic changes detected by serology and biochemical techniques can be recovered by growth in low concentration of antibody. Antigenic drift also occurs in the NA antigen. Epidemics due to new virus strains exhibiting antigenic drift do not have such a great clinical impact as those due to viruses showing antigenic shifts, since partial immunity is present due to previous infection. Antigenic drift has been shown for influenza subtypes H1, H2 and H3, and also for influenza B, and is widely accepted to account for the inter-pandemic seasonal outbreaks. Recent work on the global circulation of H3N2 viruses examined the emergence of new antigenic drift strains, and indicated

Table 16.2 Antigenic drift in the haemagglutinin of influenza A (H3N2) in the years 1968–1979

Virus subtype	Virus strain	Serum HI antibody titre to influenza virus ^a				
		A/HK/1/68	A/Eng/42/72	A/PC/1/73	A/Vic/3/75	A/Bang/79
H3N2	A/Hong Kong/1/68	1280	320	40	40	<10
	A/England/42/72	240	2560	80	120	40
	A/Port Chalmers/1/73	40	120	5120	320	160
	A/Victoria/3/75	<10	40	640	1280	160
	A/Bangkok/1/79	<10	<10	80	160	640

^aThe titres in bold represent reactivity of a virus with homologous antisera. <10, limit of detection of HI antibody.

the importance of particular global regions in South East Asia as the source of new antigenic-drift variants (Russell *et al.*, 2008)

VIRUS CLASSIFICATION

The antigenic differences of the HA and NA antigens of influenza A viruses provide the basis of their classification into subtypes. This classification is of practical importance as the antigenic differences are critical for the understanding of virus epidemiology and for vaccine production. At the present time the agreed classification is shown in Table 16.3. Previously, the results of serology studies suggested that the HA of the influenza virus strain which affects swine, termed H_{sw}, was similar to that which caused the pandemic of 1918–1920, and this has been confirmed by sequence data from the two viruses. Strains occurring in the years 1933–1948 were classified as a subtype termed HO, and those occurring in 1947–1957 were termed H1. Recent studies have shown that the HA of all these viruses are related, and the classification shown in Table 16.3 groups these viruses into a single subtype, termed H1. The HA of human subtype H3 cross-reacts with the HA of equine influenza virus Heq2 and avian influenza virus Hav7, and these three are now grouped together into a single subtype, H3. Further subtype classification shown in Table 16.3 gives the new and old designations. Recent structural studies on the virus HA have refined the understanding of subtype classification and indicated some structural features distinguishing subtypes (Ha *et al.*, 2002).

Antigenic differences also occur in the NA antigen of the influenza A viruses. The antigenic form designated N1 is found in all human influenza A viruses isolated prior to 1957, and the form designated N2 has been found in all human isolates recovered since that time. Strains having the N1 NA recurred in 1976, and have persisted since that date. The other antigenic variants of the NA occur in viruses isolated from birds and horses (Figure 16.3).

Using the above classification it is possible to describe any influenza virus in terms of its subtype specification. Every influenza virus is referred to as A or B, followed consecutively by the place of isolation, the laboratory number and the year of isolation. Following this designation, the subtype designation is given in parenthesis. Thus, influenza virus A/Hong Kong/1/68 (H3N2) signifies an influenza A virus isolated from a patient in Hong Kong in 1968, and of subtype H3N2. Within any subtype as described above, a number of strains can occur as a result of antigenic drift, and these are important in epidemiology and in vaccine design, since antibody against one strain does not completely neutralize viruses of other strains; thus, minor variations need to be identified and, where necessary, alterations to vaccine composition made accordingly. Virus strains of different subtype can be distinguished by a variety of tests, including serological tests using homologous and heterologous polyclonal and monoclonal sera, and genetic analysis focussing on the genes for viral HA and NA. In this way, large numbers of differing strains are identified and distinguished within any one influenza A subtype. Tracking the antigenic drift which gives rise to strain variation is of crucial importance in influenza research and surveillance.

Table 16.3 Nomenclature of influenza A viruses

Haemagglutinin		Neuraminidase	
Subtype	(Previous designation)	Subtype	(Previous designation)
H1	(HO, H1, H _{sw})	N1	(N1)
H2	(H2)	N2	(N2)
H3	(H3, Heq2, Hav7)	N3	(Nav2, Nav3)
H4	(Hav4)	N4	(Nav4)
H5	(Hav5)	N5	(Nav5)
H6	(Hav6)	N6	(Nav6)
H7	(Heq1, Hav1)	N7	(Neq1)
H8	(Hav8)	N8	(Neq2)
H9	(Hav9)	N9	(Nav6)
H10	(Hav2)		
H11	(Hav3)		
H12	(Hav10)		
H13	(–)		
H14	(–)		
H15	(–)		
H16	(–)		

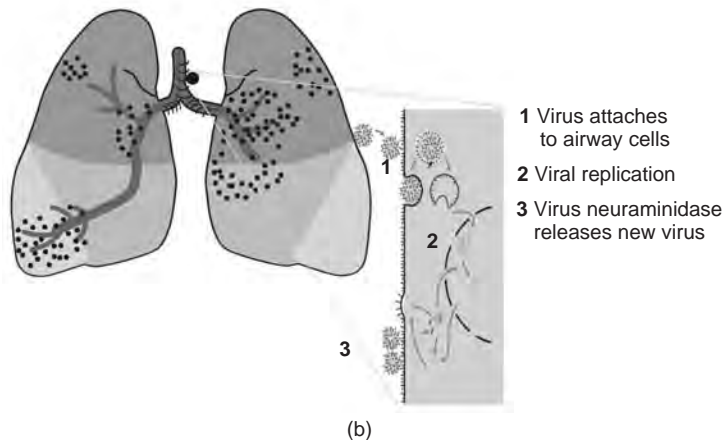
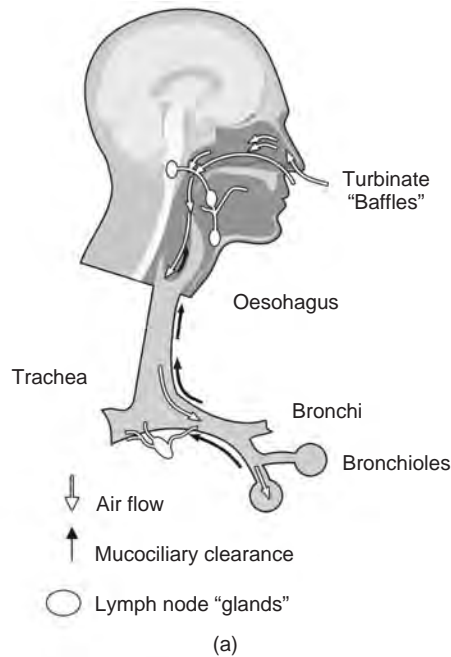


Figure 16.9 (a) Anatomy of human airways. White arrows indicate direction of airflow, black arrows indicate direction of mucociliary clearance. (b) Preferred sites of human influenza virus replication in the tracheobronchial tree.

PATHOGENESIS

Evaluation of disease spectrum and case fatality in different pandemics and epidemics demonstrates that there are significant differences in disease severity caused by different influenza virus strains, most notably the severity of 1918 influenza, and more recently the high case fatality caused by H5N1 zoonotic infection in man. Unusual disease syndromes may also occur in certain populations, for example the high rates of influenza-associated

encephalopathy seen in Japan, which indicate the potential for contribution of host factors in infection outcome.

There is no agreed explanation for the pathogenesis of human influenza. However, historical and virological investigations have broadly defined the effects of virus infection. Infection is the result of inhaling respiratory droplets from infected persons; these droplets containing virus are deposited on the mucus blanket lining the respiratory tract (Figure 16.9a and b). Much virus is destroyed by nonspecific immune barriers, such as mucus binding,

which is functional at this site, or inactivated by natural inhibitors containing sialic acid present in serum or mucosal fluids, which can bind virus HA and competitively inhibit virus binding to cells (Matrosovich and Klenk, 2002). However, some virus escapes these inhibitors, release from mucus being assisted by the action of virus NA allowing attachment by the virus HA to receptors on the cells of the respiratory epithelium; epithelial cells of both the upper and lower respiratory airways are rich in sialic acid receptors. Following viral attachment, replication proceeds; virus can be isolated following acute infection from 1 to 7 days, with peak titres usually occurring at 48–72 hours after the onset of symptoms. Histological studies on nasal exudate cells and tracheal biopsies have indicated that the major site of virus infection is the ciliated columnar epithelial cells. Following infection, these cells become progressively rounded and swollen, and the nucleus appears shrunken and pyknotic; the cytoplasm becomes vacuolated, the nucleus degenerates and ciliation is lost, and eventually cells slough off their basement membranes.

The progression of changes in the cells of the respiratory epithelium suggests the tracheal bronchial epithelium is an early site of infection. Thus, early lesions in the tracheobronchial mucosa have been described in uncomplicated influenza, clinically evident as bronchitis and tracheitis; the tissues show increased permeability of vascular capillary walls, oedema, polymorphonuclear infiltration and phagocytosis of degenerate epithelial cells. The basement membrane is not affected.

Because of the generalized symptoms present in uncomplicated seasonal human influenza, viraemic spread from the respiratory tract has been suggested. Influenza has been associated with electrocardiogram (ECG) and electroencephalogram (EEG) changes; some unconfirmed reports of virus antigen in brain and heart have been recorded; and influenza has been temporally associated with cases of virus encephalitis, particularly among children. However, demonstration of viraemia has not been conclusive; blood samples from children infected with influenza were not found to contain virus when tested by PCR (Mori *et al.*, 1997). In addition, the failure to prove viraemia at least in some cases leaves unexplained the myalgia and malaise which are commonly seen in influenza, and the sudden and marked temperature rise which follows infection. Clearer understanding of the relationship of innate immune response to infection, and of viral gene expression, are likely to provide explanations of these host responses to infection.

Zoonotic Infections

Zoonotic influenza infections with nonhuman subtypes of influenza A demonstrate a different disease spectrum and

pathogenicity profile (Table 16.4). H5N1 infections are associated with a disseminated viral infection, with virus being recovered from a number of anatomical sites, including brain and gastrointestinal (GI) tract, with evidence of viraemia (Beigel *et al.*, 2005; de Jong *et al.*, 2005). Infection is thought to initiate lower in the respiratory tract, as a consequence of the receptor preference of avian influenza strains for α 2,3 sialic acid receptors, which occur much more frequently in cells of the lower airway, giving rise to an initial lower-respiratory-tract infection or pneumonia (Shinya *et al.*, 2006). Limited autopsy evidence from cases of H5N1 is consistent with respiratory failure and interstitial pneumonia, but also evidence of multisystem failure. Whilst there is some evidence of extra-respiratory spread of virus in a few cases, this may not account for all the pathogenic features. Immune dysregulation is considered an important contributor to pathogenesis as there is autopsy evidence for haemophagocytic syndrome (reviewed by Gambotto *et al.*, 2008; To *et al.*, 2001). H7 infections are particularly associated with unilateral or bilateral conjunctivitis, which is not commonly seen in seasonal influenza or H5N1 infections

Animal Models

One of the key differences between highly-pathogenic and low-pathogenic strains in an avian host is the restriction of replication to cells of the avian GI tract in a low-pathogenic strain. Disseminated infection is a hallmark of high-pathogenicity avian influenza infection, with a wide range of tissues supporting viral replication. Pathogenesis studies of avian influenza viruses in avian hosts clearly identify molecular determinants of virulence being directly associated with the cleavability of the HA into HA1 and HA2. H5 and H7 subtypes of influenza exist in highly-pathogenic and low-pathogenic forms, the difference between these strains being the amino acids present at the cleavage site between HA1 and HA2. The highly-pathogenic forms of avian influenza contain a series of basic amino acids, usually generated through insertional mutation at the carboxyl terminal of HA1. These basic amino acids increase the size of a protein loop in the stalk region of the HA protein, making the site more accessible to a wider range of host proteolytic enzymes, enhancing the ability of the virus to replicate in a wider range of tissues (Steinhauer, 1999) (Figure 16.10).

Molecular determinants of pathogenicity in an avian host may play a role in mammalian hosts, but may not be necessary or sufficient to account for high pathogenicity in a mammalian host. The 1918 influenza is known to have high virulence for humans. This virus lacks a polybasic HA cleavage site, yet retains virulence in ferret and monkey models. Additional virulence determinants in viral

Table 16.4 Zoonotic infections with influenza A

Subtype and country	Spectrum of illness	Case no.	Fatality
H5N1	Respiratory and multisystem	>300	~60%
H7N7	Conjunctivitis and respiratory	2–3	—
United States 1980s		1	—
England 1995		83	1
Netherlands 2003	Conjunctivitis and respiratory	5–6	0
H7N3		1	—
Canada 2004		2	—
England 2006		3	0
H7N2 United States 2004		2	0
H9N2 Hong Kong 1999–2003	Respiratory	2	0
H10N7 Egypt	Respiratory		

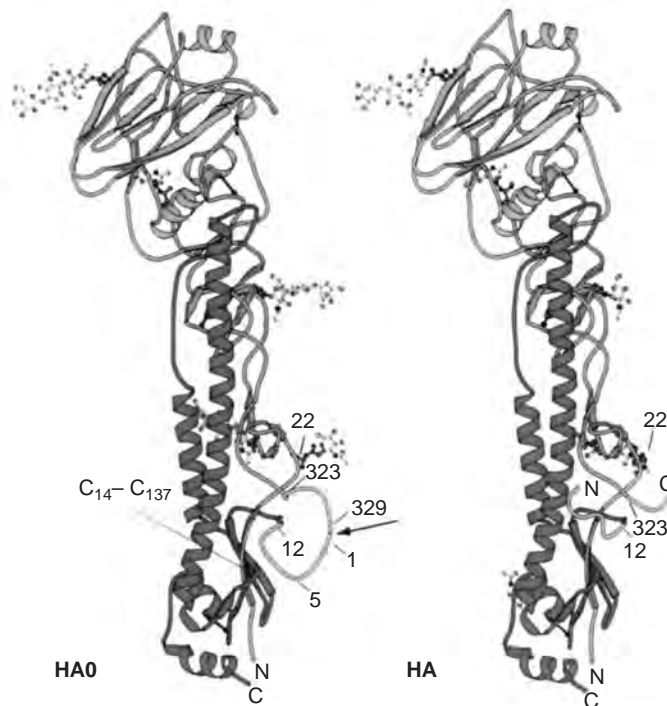


Figure 16.10 Representation of HA proteolytic cleavage site. HA0, uncleaved HA; HA, cleaved HA. Arrow indicates accessibility of cleavage site for proteolytic enzymes. (Source: Steinhauser, 1999, with permission.)

genes are likely to include mutations in viral nonstructural genes and genes associated with viral replication, implicated in adaptation to mammalian hosts. The entire sequence of influenza viruses has been known since the late 1970s, but the molecular basis of virulence in mammalian hosts is still not understood. It is not known why encephalitis syndromes occur at high frequency following influenza infection in children in Japan (Morishima *et al.*, 2002), why H5N1 viruses are associated with such lethal

infection in humans and animals, or why unusual clusters of fatalities accompany occasional drift variants of human influenza (Bhat *et al.*, 2005). Linking viral gene analysis with analysis of host response to infection should be a final step towards understanding influenza pathogenesis in humans and eventually determining predictive markers for clinical outcome and therapeutic intervention. Consideration of zoonotic influenza infection requires a broader diagnostic approach, looking at different body fluids

including blood and faeces, and samples from lower in the respiratory tract. The ability to analyse viruses recovered from different compartments offers a tantalizing prospect of looking in detail at 'within host' variation, and what clues this may yield for better understanding of pathogenesis.

CLINICAL FEATURES

Uncomplicated Infection

Influenza has been described as an unchanging disease due to a changing virus, and this description underlines the relative constancy of the clinical presentation of the infection. Detailed analysis of the symptoms seen in the groups of patients studied in the years 1937–1941, 1947 and 1957 indicate some differences in the relative incidence of some symptoms; however, these are probably due to the variable recording of different observers. Although the clinical presentation of uncomplicated influenza in any one age group is generally agreed (Figure 16.11), variation in the incidence of certain symptoms does occur for different ages; vomiting and convulsions are rarely seen except in infants; croup is more a feature of infection in young children; sore throats and myalgia are seen more commonly in adults.

Following droplet infection from infected individuals, the incubation period is approximately 48 hours, but may vary from 24 to 96 hours; the variation may depend on the size of the infecting dose. The onset of the illness is

usually abrupt, and many patients can pinpoint the hour of onset. The symptoms in adults commonly include a marked fever, headache, photophobia, shivering, a dry cough, malaise, aching muscles and a dry tickling throat or other features of laryngeal irritation, which can lead to the voice becoming husky and even lost. The fever is usually continuous and classically lasts three days, at which point the temperature falls and the symptoms abate (Gentile *et al.*, 1998; Hayden *et al.*, 1998) (Figure 16.12). In a percentage of cases, a second fever spike may occur after this time; it will be smaller than the first one but will give the common biphasic fever curve. Of the acute symptoms listed, the cough may persist for several days; the eyes are often watery, burning and painful in movement; the nose can be blocked or may have a purulent discharge; cervical adenopathy is unusual but has been described; and myalgia is more severe in the leg muscles, but also may involve the extremities. Although the infection usually resolves within seven days, listlessness and depression are common residual complaints.

Studies of the clinical illness resulting from infection by influenza B virus show close similarity to that caused by influenza A virus. Some authors have suggested that influenza B infections are milder than those caused by influenza A, with less myalgia and higher incidence of nasal symptoms, and differences have been reported in the incidence of sweating and other symptoms; however, these differences are small. In contrast, influenza C infection is generally accepted as a relatively mild respiratory infection of young children and a mild upper-respiratory-tract infection of adults that is rarely diagnosed.

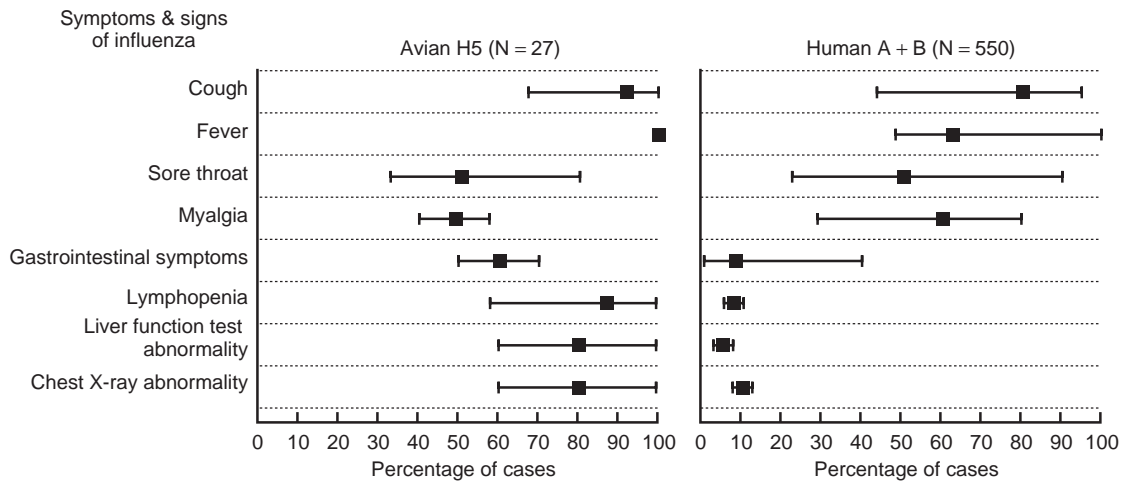


Figure 16.11 Symptoms and signs of seasonal and avian influenza based on Fig 19, Nicholson (1998), and Beigel *et al.* (2005). Overall incidence and highest and lowest incidence of symptoms recorded during several studies of individuals with virologically-confirmed seasonal influenza A or H5 avian influenza.

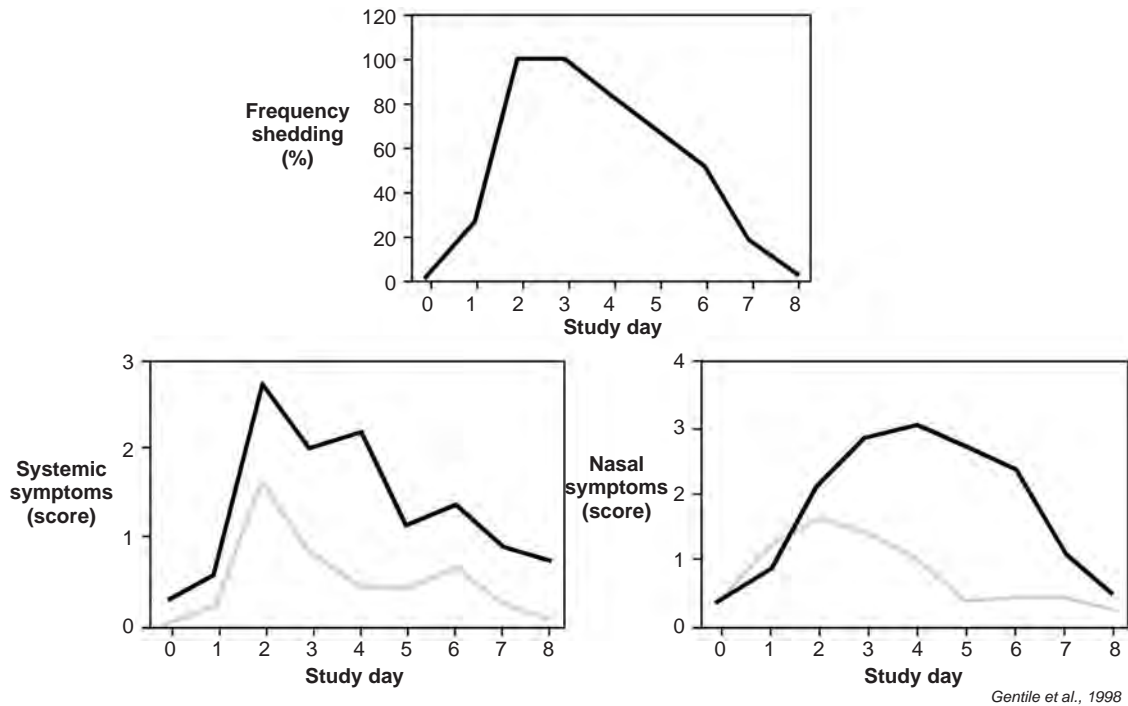
Gentile *et al.*, 1998

Figure 16.12 Relationship between virus shedding and systemic and nasal symptoms of influenza A H1N1. (Source: Gentile *et al.*, 1998, with permission.) Dark line represents virus shedding.

Tracheobronchitis and Bronchitis

All series of patients studied have included a small proportion in which the respiratory symptoms were more severe. These patients have a productive cough, chest tightness and substernal soreness. Rales and rhonchi are commonly heard but the lungs are radiologically clear. These symptoms are most commonly seen in patients with chronic obstructive bronchitis and in older persons, and it is evident that age and chronic pulmonary disease predispose to severe bronchitis, which can result in death.

Pneumonia

Pneumonia in patients with influenza virus infection can be primary viral pneumonia or secondary bacterial infection. In viral pneumonia, patients develop persistent fever with leukocytosis, dyspnoea, hypoxia and cyanosis following the acute symptoms described above. Sputum specimens will show no clear bacteriological cause, and a proportion of these patients will die of diffuse haemorrhagic pneumonia. Autopsies show congested, dark-red lungs, and the mucosa of the trachea and bronchi will be hyperaemic. Microscopic examination of lungs section has consistently revealed tracheitis and bronchiolitis with

haemorrhage, hyperaemia, a small cellular infiltrate and loss of ciliated epithelium. An alveolar exudate containing both neutrophils and mononuclear cells in haemorrhage is common. Cases of influenza viral pneumonia are relatively uncommon but are demonstrated in most influenza epidemics; pneumonia can occur in previously young and healthy persons, but it is more commonly associated with patients with pre-existing cardiovascular disease, such as rheumatic heart disease. The pathology of the viral pneumonia which killed many relatively young and previously healthy people during 1918–1920 was unique to that epidemic, and contained pathological features not described before or since (Crosby, 1976). Thus, following the acute symptoms of influenza described above, some patients showed increasing tracheobronchitis and bronchiolitis, shortage of breath and the appearance of mahogany spots around the mouth, which would extend and coalesce into a violaceous cyanosis until ‘a white man could not be distinguished from a coloured’. With increasing cyanosis, blood-stained fluid would froth from the mouth and death would follow from suffocation. The time from onset of infection to death varied from a few hours to 2–3 days. Post-mortem examination would not show the signs of secondary bacterial pneumonia; rather, the lungs con-

tained up to a litre of blood-stained, frothy and fibrin-free fluid; petechial and confluent haemorrhages were seen in the lining of the trachea and bronchi; and the lung tissue exhibited intense inflammatory changes with marked adenopathy.

Secondary bacterial pneumonia may be more common than primary viral pneumonia, and usually occurs late in the course of the disease. It usually ensues after a period of improvement from the acute symptoms of infection. The symptoms and signs are those of typical bacterial pneumonia and of the organisms involved, commonly *Staphylococcus aureus* and *Haemophilus influenzae*, although other bacteria may also be found. The association of *S. aureus* with secondary bacterial pneumonia following influenza is much more common than might be anticipated, and there appears to be a good reason for this: cleavage of the virus HA by bacterial proteases (Tashiro *et al.*, 1987). The incidence of secondary bacterial pneumonia is most common in the elderly and those with underlying disease, such as congestive heart failure and chronic bronchitis; in addition, patients with diabetes mellitus, renal disease, alcoholism and those who are pregnant may also have increased susceptibility to secondary bacterial infection.

Myositis and Myoglobinuria

In addition to myalgia, which is a characteristic feature of acute influenza infection, clinical myositis and myoglobinuria can occur. Symptoms usually develop soon after subsidence of the acute upper-respiratory-tract symptoms; the muscles are painful and tender to touch, but neurological symptoms are not evident. Changes in serum transaminases and creatinine phosphokinase levels accompany these symptoms, and histological examination of muscle biopsies has revealed necrosis of the muscle fibres and a mononuclear cell infiltration.

Reye's Syndrome

A syndrome characterized by encephalopathy and fatty liver degeneration was originally described in 1929, and more fully characterized by Reye *et al.* (1963). Later observers noted an association of recent viral infection with this syndrome, now termed Reye's syndrome. Typically, a previously normal child has a virus-type prodromal illness, followed in a few days by vomiting, altered consciousness and occasionally convulsions; the liver may be enlarged, and there is evidence of hepatic dysfunction, with raised transaminases and blood ammonia levels. Forty to fifty percent of patients admitted to hospital for Reye's syndrome die. At autopsy, an enlarged, pale and fatty liver is usually seen, and histological examination shows diffuse panlobar microvesicular fatty infiltration. The brain shows evidence of encephalitis with

cerebral oedema. Outbreaks of Reye's syndrome have been recorded in conjunction with influenza A and influenza B epidemics, and there is clustering of cases in the winter months.

An experimental disease similar to Reye's syndrome can be induced in mice by intravenous inoculation with influenza B virus; other studies have suggested that acute viral infection in conjunction with cofactors may be responsible for initiating the pathology of the disease. In particular, the use of high concentrations of aspirin during an acute virus infection has been suggested as a possible precondition for development of Reye's syndrome. There is epidemiological data to support this association, because Reye's syndrome is now rare, following widespread advice to give paracetamol, not aspirin, to febrile children.

Otitis Media

Although considered by many to be a bacterial infection, or a bacterial infection secondary to viral disease, it has been increasingly recognized that otitis media could be the result of influenza infection. Diagnosis of this in young children is associated with influenza epidemics, and in many cases bacterial causes of infection cannot be demonstrated. Association of influenza with otitis media is now recognized epidemiologically, but there is a need for detailed virological examination of patients presenting with otitis media, particularly in the age group zero to four years (Fleming, 2000).

Influenza in Pregnancy

The literature contains a number of reports of an increased incidence of congenital malformations (Conover and Rossmann, 1990) and neural-tube defects (Lynberg *et al.*, 1994) following influenza virus infection during pregnancy. In contrast, transplacental passage of the virus has not been demonstrated satisfactorily (Irvine *et al.*, 2000), and prospective studies of congenital abnormalities following epidemics of influenza have failed to establish a relationship between influenza-virus infection and these abnormalities. Reports of the association of maternal influenza with schizophrenia and Parkinson's disease in the offspring exist, but evidence of association is both fragile and contradictory. Earlier retrospective studies of small groups of patients infected during influenza epidemics suggested an increased risk of schizophrenia where infection took place in the second trimester of pregnancy (O'Callaghan, 1991); however, other similar studies have failed to confirm these results. There are methodological difficulties, particularly in the early studies, which relied on small numbers of patients asked to recall events 20 years or more in the past and on questionable evidence for past influenza infection. Three

larger studies published in 1999 found no evidence of an association between influenza-virus infection in pregnancy and later schizophrenia in the offspring (Bradbury and Miller, 2000). Follow-up studies of influenza during pregnancy have indicated increased severity of influenza illness among pregnant women, but no evidence of increased medical problems among the offspring. The recommendation for immunization of pregnant women is a recognition of the severity of this infection in this group, particularly from the pandemics of 1898 and 1918, when the death rates in pregnancy were considerable.

Other Complications

Although influenza in healthy adults is normally severe but of short duration, resolving in three to five days, complications can occur, particularly in elderly patients and in those with predisposing conditions, as outlined above. In addition, virus infection can result in a number of other less well understood complications. Influenza can cause ketoacidosis in diabetic patients, even in relatively mild cases of infection. Acute viral encephalopathy can occur and Guillian–Barré syndrome is a rare but recognized complication; both can lead to death. Histological examination of brain tissue has shown no gross abnormalities, but small changes consistent with virus encephalitis have been shown. The pathogenesis of the neurological complications is unknown, since virus recovery from the brain has rarely been documented.

Epidemiological studies have associated influenza A with sudden unexplained death and sudden infant death syndrome (SIDS), sometimes called ‘cot death syndrome’ (Zink *et al.*, 1987). Firm population data associating acute influenza infection with SIDS have been sought by many but are difficult to obtain, and the association is made on circumstantial evidence. Studies evaluating the burden of mortality due to influenza in young children have demonstrated that the importance of influenza as a serious pathogen may be significantly underestimated in this age group. The emergence of new antigenic-drift variants may be associated with unusual mortality in children experiencing a primary influenza infection, as occurred in the winter of 2003/2004 with A H3N2 Fujian-like antigenic variants (Bhat *et al.*, 2005). Factors underlying the severity of infection in young children and host predisposition to fatal outcome remain to be determined.

Zoonotic Infections

There is a spectrum of illness associated with the presentation of zoonotic avian influenza infection, which is associated with the infecting subtype (Table 16.4). However, in the early stages of infection it may be difficult to

distinguish zoonotic influenza infection from seasonal influenza infection as many presenting features are shared. H5N1 infections are associated with illness onset up to seven days post exposure, with predominant clinical features of high temperature, cough and shortness of breath (Figure 16.11). A high proportion of cases experience either transient or lasting respiratory failure during the course of a severe illness, which often involves multi-system failure. Although the majority of cases have been respiratory in presentation, a handful have been documented with atypical presentation involving fever, GI disturbance and diarrhoea, without obvious disease of the respiratory tract. Early clinical signs include disturbances in liver-function tests, lymphopenia and the presence of interstitial pneumonia on X-ray, which are not typically seen in seasonal influenza (Figure 16.11). Progression of disease typically involves respiratory failure and acute respiratory-distress syndrome, with death most frequently being due to respiratory failure. Recovery will begin to occur 7–10 days after illness onset, and tends to coincide with development of neutralizing antibodies. H7 infection is most likely to lead to unilateral or bilateral conjunctivitis. Of 83 people infected with H7N7, approximately 40% had evidence of virus in the upper respiratory tract, with 10% having a mild respiratory illness (Koopmans *et al.*, 2004). A single case fatality due to acute respiratory failure was recorded in an adult male.

DIAGNOSIS OF INFECTION

During seasonal epidemics of influenza, large numbers of patients are seen with similar influenza symptoms, increasing the probability of a correct clinical diagnosis. Concordance between clinical diagnosis and laboratory-proven cases of influenza are good, with correlations of 70% at the peak of the epidemic (Zambon *et al.*, 2001). However, influenza A and B can co-circulate, and mixed infections of influenza and other viruses have been reported; under these circumstances clinical diagnosis can be misleading and a diagnosis on clinical grounds cannot be confidently made. It may be considered that clinical diagnoses at the time of an epidemic are relatively easy and possibly of limited value. In contrast, laboratory diagnosis of isolated sporadic cases of suspected influenza should be carried out wherever possible, since the predictive value of clinical assessment is much lower, and infection may represent the first case of an impending epidemic or infection by a new virus strain, as has been seen with zoonotic H5, H7 and H9 infections of humans.

Laboratory diagnosis of influenza infection during the acute phase of illness in humans relies on isolation or

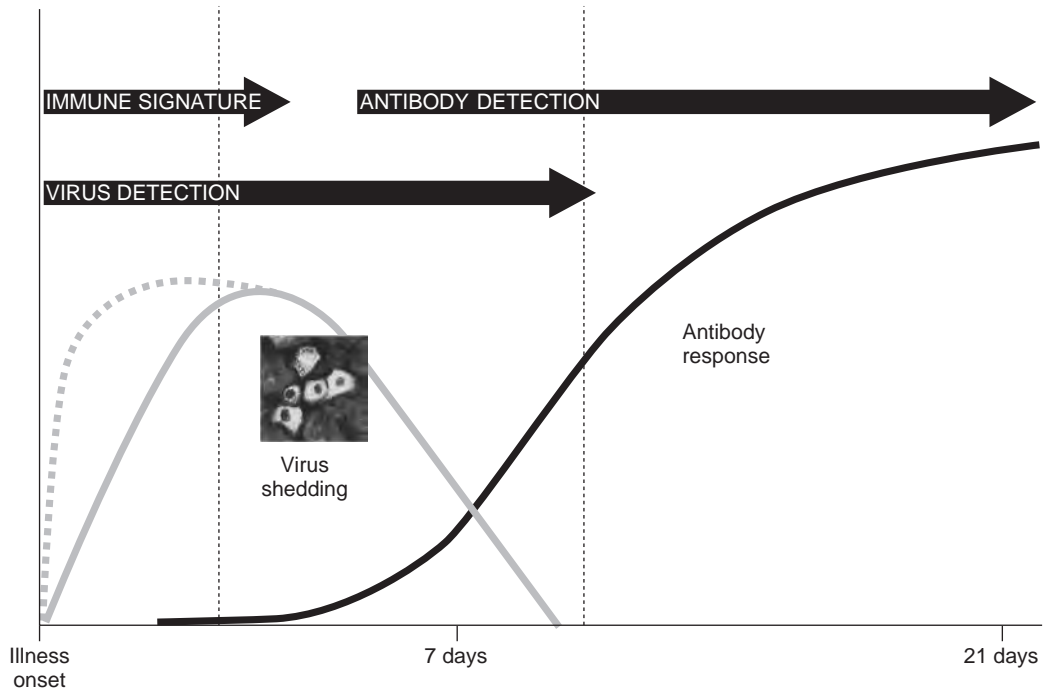


Figure 16.13 Diagnosis of influenza infection. Broken line indicates seasonal influenza virus shedding, compared with delayed shedding peak of avian H5 influenza.

detection of viral components during the period of viral replication, using a variety of techniques (reviewed by Zambon, 1998) (Figure 16.13). For seasonal human influenza A (H1N1, H3N2 and B), viral shedding peaks within several days of the onset of illness, and is readily detectable in the upper respiratory tract for up to five to seven days. In contrast, the peak of virus shedding following zoonotic infection may occur longer after illness onset and may be prolonged. Throat or nasopharyngeal swabs can be taken into a suitable transport medium, or nasal washing can be collected; comparative studies have shown that virus can more commonly be isolated from nasal washing than from other specimens. In all cases, the rapid transfer of specimens, or proper maintenance of specimens where delay may occur, is important for virus isolation.

Influenza viruses A and B, present in pathological specimens and collected into transport medium, can be cultured by inoculation on to mammalian cells or by amniotic inoculation of embryonated hens' eggs. Continuous cultures of kidney cells from dogs, rhesus monkeys, baboons or chicks are the preferred cell substrate. The addition of trypsin to ensure the cleavage of virus HA and HA1 and HA2 is usually necessary for virus infectivity in cell culture. After adsorption and incubation of virus-infected

cells, newly-produced virus can be detected by a number of methods. Free virus released into the maintenance medium of the cell culture can be detected by haemagglutination with erythrocytes, as described for amniotic fluid (Figure 16.14). Virus HA is inserted into the membranes of infected cells during replication and erythrocytes will adhere directly to these infected cells; this phenomenon is termed haemadsorption and can be observed under the microscope. Finally, viral protein can be detected in infected cells by fixation and staining by immunofluorescence (IF) (Figure 16.15). If specimens are inoculated into embryonated eggs, the virus is adsorbed from the fluid of the amniotic cavity on to cells of the amniotic membrane, where multiplication occurs, releasing newly-formed virus back into the amniotic fluids. After two to three days of incubation, virus can be present in high titres in the amniotic fluid and can be detected by adding aliquots of harvested amniotic fluid to chick, turkey, guinea pig or human erythrocytes and observing haemagglutination. Egg fluids negative for virus can be passed to further embryonated eggs and retested; specimens which do not reveal virus after two passages are recorded as negative in eggs. In recent years, there has been alteration in the receptor of circulating seasonal influenza viruses, which makes the use of embryonated eggs less favourable for the primary

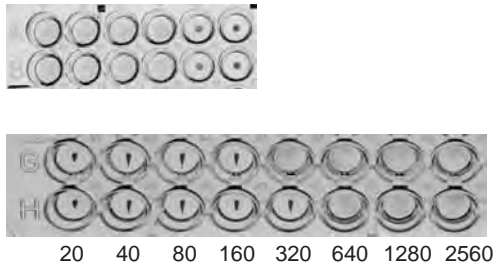


Figure 16.14 Haemagglutination and haemagglutination inhibition. (a) Demonstrates haemagglutination in tissue-culture supernatant in the presence of virus. As virus is diluted out, erythrocytes settle out to form a button. (b) Demonstrates haemagglutination inhibition (HI). Row G demonstrates serum with an HI antibody titre of 160, and row H serum with an antibody titre of 320 (HI titre is measured as the last well in which there is complete inhibition of haemagglutination reaction).

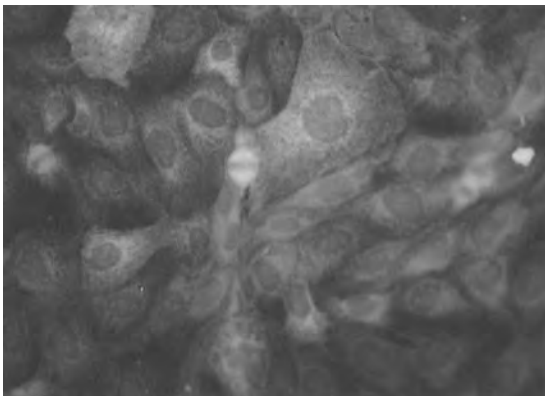


Figure 16.15 Immunofluorescence of influenza B-infected cells. Magnification $\times 400$.

isolation of human strains of influenza (Thompson *et al.*, 2006), although this is the culture medium of choice if there is a suspected avian influenza strain in a zoonotic infection.

Virus Recognition

Influenza viruses isolated from embryonated hens' eggs or cell culture can be identified by serological methods. First, influenza virus can be recognized as A, B or C by complement fixation (CF) tests using extracts of infected cells or embryonic membranes, which contain high titres of NP antigen, and standard antisera against influenza A, B or C viruses.

Further classification of influenza isolates into subtypes and strains is a highly specialized activity. Each virus

isolated is tested by HI test in comparison with a range of virus subtypes and strains with appropriate reference antisera. Virus strains are standardized to contain a fixed amount of HA activity by titration against erythrocytes, and then reacted against a range of dilutions of each antiserum. By observing the patterns of HI against the various antisera, the virus subtype and strain is identified. Should the virus isolate not be inhibited by any of the sera to the same titre known for the reference virus, the clinical isolate may be a new subtype or strain; homologous serum against the strain is then prepared in animals, and more extensive cross-HI tests are performed. These tests require the addition of new antisera to update the battery of sera used. In addition, the NA of the virus strain may also be typed serologically. This is done by identifying which antisera prepared against the various influenza NAs inhibit the NA of unknown virus to the same titre as against homologous virus; the indicator system in this test is an NA substrate, such as fetuin.

Virus Shedding

Viral replication outside the respiratory tract or viraemia is not a feature of human seasonal influenza, and conjunctival infection is rare. In contrast, in zoonotic influenza H5N1 infection virus can be detected in respiratory-tract samples, blood and faeces, indicating a systemic illness during the acute illness phase (Beigel *et al.*, 2005). The highest viral shedding occurs lower in the respiratory tract, reflecting the cell tropism of H5N1 virus and the distribution of appropriate receptors in the lower respiratory tract (Shinya *et al.*, 2006; van Riel *et al.*, 2007). Although H5N1 viral shedding may be prolonged and viral replication may be higher than in seasonal human influenza infection, the concentration of viral protein or nucleic acid in accessible upper-respiratory-tract secretions is lower. Zoonotic influenza A H7 infection is associated with virus shedding in conjunctival fluid, but only in a minority of cases is virus recovered from elsewhere in the respiratory tract (Koopmans *et al.*, 2004). Virus-shedding patterns in zoonotic influenza A virus infection therefore bring additional diagnostic opportunities to detect virus in different body compartments, but they also bring significant challenges. The experiences of avian influenza A infection in humans in the last few years (H5N1, H7N7, H9N2, H7N3), with serious illness presenting unexpectedly, underlines the difficulties of rapid influenza diagnosis. Initially, H5N1 illness may be indistinguishable from seasonal influenza, or worse still, in rare cases, it may bear no resemblance to human influenza, as has occurred in Thailand and Vietnam (de Jong and Hien, 2006). Detection of highly-conserved viral internal protein or genes (NP, M or NS) in respiratory secretions is sufficient to determine whether infection is due to influenza

A rather than influenza B or C (type-specific diagnosis), but does not inform which influenza A subtype (H1–H16) is present. Subtype-specific diagnosis requires analysis of the surface proteins or their gene segments. The HA and NA genes are subject to rapid genetic drift, leading to amino acid substitution on the surface of these proteins. Detection diagnostics based on these genes or proteins require frequent updating to take account of rapid evolution. Hitherto, the precise diagnosis of influenza A subtype (H1–H16; N1–N9) has not been considered relevant for clinical management of human influenza, although it is important for surveillance purposes (Zambon, 1998).

There are important clinical and public-health advantages that accrue from more rapid laboratory confirmation of subtype-specific diagnosis during the acute phase of illness. There is now a clear shift towards nucleic acid amplification (NAA) techniques for clinical virology diagnosis generally. Increasing use of automation facilitates high-throughput, real-time PCR to provide sensitive, specific, quantitative detection of virus nucleic acids, including influenza A, in different body fluids within hours of sample taking. Reverse-transcription polymerase chain reaction (RT-PCR) shows a high concordance with results obtained from slower culture and serological methods (Ellis and Zambon, 2002; Weinberg *et al.*, 2004; Zambon, 1998). Ensuring that influenza diagnostic techniques based on NAA techniques such as PCR are both sensitive and specific requires accurate knowledge of sequence diversity. Portions of gene segments which are highly conserved are chosen as detection targets, for example NP, MP (matrix protein and NS1 for type-specific diagnosis (A, B or C). Subtype-specific diagnosis (H1–16; N1–9) requires targeting of the highly-variable viral HA and NA genes. All strategies based on detection of HA of any subtype require frequent verification that single-base mutations found in newly-emerging drift variants do not affect test performance. It is essential to regularly update reagents to take account of sequence diversity and different genetic lineages detected in circulating viruses. Thus, efficient and effective subtype-HA-specific nucleic-acid-based diagnosis, either for human or zoonotic influenza infection, requires coordination between surveillance laboratories generating HA or NA sequence data from newly-emerging strains, and diagnostic end users, if subtype-specific diagnostics are to be applied in clinical laboratories.

Pinpointing the emergence of antigenic variants of human influenza requires growth of influenza viruses from clinical material. It takes a few days to obtain virus particles in adequate concentrations (approximately 10^7 – 10^8 particles per ml of culture fluid) to provide sufficient HA protein for analysis. Innovations, including the use of engineered mammalian cell lines such as MDCK cells

expressing enhanced levels of 2,6 sialic acid virus receptor (Matrosovich *et al.*, 2003), may enhance sensitivity for detection of human viruses, but have not reduced the time taken for virus to grow. The rapidity of viral evolution, the difficulty of working with highly-pathogenic viruses, the diversity of circulating strains, the limited access to original clinical material containing influenza virus and the delay in availability of sequence data from contemporaneous strains all make it difficult for the medical-devices industry to validate tests designed for human diagnosis. The predictive value of any diagnostic test, even one with 99% sensitivity and 99% specificity, is less than 70% when disease frequency is low, indicating the necessity for sensitive tests and confirmation strategies when trying to diagnose rare infections such as H5N1. Viral detection microarrays on a bite-sized chip could be one way forward for specific subtype diagnosis, but it may be a decade or more before this technology can be deployed in a low-skill format.

Rapid point-of-care tests (PoCTs) for influenza were developed alongside drugs for treatment of human influenza in the 1990s, to assist individual patient management ‘Test and Treat’. These tests require limited skill and equipment to provide a result in 10–30 minutes, in time to influence prescription in a physician’s office or at the bedside. Current PoCT formats use antibodies to detect conserved type-specific viral NP or M protein present in respiratory secretions. This allows detection of influenza A or B, but it may not always distinguish between them and does not indicate which subtype of influenza A is involved. A diversity of technologies can be applied to this task (reviewed in Nicholson *et al.*, 2003), including lateral-flow immunochromatography, solid-phase capture ELISA with optical or colorimetric read-outs, and activation of viral NA enzyme activity with substrate detection. Although influenza PoCT devices are widely used in some countries, for example Japan, in conjunction with antiviral drug prescription for seasonal influenza, the global uptake has been limited due to the costs of such tests, although they are useful in outbreak settings. Early data from clinical cases of H5N1 indicate that existing PoCT tests perform poorly in comparison to H5 virus-culture investigation (Beigel *et al.*, 2005), partly because the concentration of H5 virus protein in respiratory secretions from the upper airways is limited and partly because of the inherent sensitivity limitations of the tests.

Direct PCR sequencing of clinical material containing influenza virus does not require virus cultivation. This approach has been successfully applied to fresh, frozen and archived human tissue, and has provided important diagnostic information, for example the viroarchaeology of 1918 influenza in exhumed cadavers and frozen sections (Taubenberger *et al.*, 1997). Although the influenza virus

genome is relatively small, consisting of approximately 15,000 nucleotides, the technologies for whole-genome sequencing have not been applied to any great extent to investigate variation in influenza virus strains. Recently, however, whole-genome sequencing of a library of animal viruses (Obenauer *et al.*, 2006) has indicated the wealth of information that can be obtained from this approach. Similar analysis of human influenza A strains collected from a single locality over several years has also revealed a greater degree of diversity and reassortment than had been expected (Holmes *et al.*, 2005). Exploring the relationship between sequence diversity, antigenic variation and virus evolution allows the development of mathematical models for virus evolution (Smith *et al.*, 2004). Useful mathematical models would increase the ability to anticipate the emergence of significant antigenic variants and prediction of virus variation, rather than the reactive observational analysis of circulating strains currently undertaken.

Diagnostic challenges may ultimately become research opportunities, if the technical solution to providing better diagnosis at the point of patient care can be married to the technical achievements of recovery of nucleic acid, sequence reconstruction of viral genomes and information capture about outcomes of infection.

Immunological Diagnosis of Influenza Infection

Infection with influenza viruses creates a symphony of innate immune responses in the individual, rapid analysis of which could be used to differentiate H5 infection from human influenza infections or other viral or bacterial infections. The concept of using detection of a host immune signature based on innate immune responses to diagnose a specific viral infection is a very attractive area for microarray development, but these efforts have yet to translate into simple, cheap devices for patient diagnosis or disease-severity prediction. This approach has been used to try to tease apart the immune-response events occurring following infection with highly-pathogenic viruses in animal models (Kobasa *et al.*, 2007) and is likely to be a very active area for development in the next decade. Results in humans with H5N1 point to events early post infection determining outcome of infection, in particular high pharyngeal viral load, viral RNA in blood and host response with high levels of immunocytes and cytokines (de Jong and Hien, 2006). However, available data do not provide any pragmatic markers that can be used to predict outcome or provide specific diagnosis. Immunological diagnosis of influenza will rest for the foreseeable future on the detection of specific antibody responses.

Infection with influenza virus results in the development of antibody to the viral envelope glycoproteins HA and NA, as well as to internal proteins M, M2 and NP.

Serum immunoglobulin M (IgM), IgA and IgG to HA appear within two weeks of infection. Peak antibody responses occur within two months and decline thereafter (Murphy and Clements, 1989). Serological tests provide the most sensitive and practical alternative for diagnosis in the absence of virus isolation or detection but, since they require a convalescent serum specimen, the diagnosis is retrospective. However, a diagnosis may be made on a single serum sample by demonstrating the presence of a virus-specific IgM response; such responses may be present for about eight weeks, occasionally longer, following influenza infection, although this is not a widely used technique as it lacks specificity in adults.

The demonstration of a fourfold or greater increase in antibody titre in the convalescent serum as compared to the acute serum is considered diagnostic of infection. A traditional method of measuring antibody titre is by CF: soluble antigen is extracted from embryonic membranes of infected cells and is reacted against a range of dilutions of the acute and convalescent sera. A more specific test for antibody to influenza virus is the HI test. For this, paired sera are treated to remove nonspecific inhibitors, a series of dilutions are added to a standard quantity of intact virus and, after incubation, erythrocytes are added. The presence of antibody is indicated by inhibition of haemagglutination, and the highest serum dilution that inhibits haemagglutination is recorded as the titre of HI antibody in the serum specimen (Figure 16.14).

Infection by influenza virus results in a rise of serum antibody titre, but the requirements for an equal to or greater than fourfold rise in titre of HI or CF antibody reflects the inaccuracy of these tests for detecting increases in antibody. A more precise method of measuring antibody is by the single radial haemolysis (SRH) technique. Here, influenza virus is coated onto sheep red cells, and after suspension in melted agar with complement the agar is poured into dishes or on to glass slides. After setting, wells are cut in the agar and inoculated with dilutions of test sera. The presence of antibody in the sera is detected by lysis of the red cells, as antibody combines with complement and antigen on the red-cell surface. This lysis can be seen with the naked eye, and the amount of antibody present is directly related to the area of haemolysis. The procedure is more sensitive than CF or HI antibody and has a greater degree of precision: a 50% increase in zone area of haemolysis reflects a rise in antibody and is evidence of recent infection. Sera do not require pre-treatment to remove the nonspecific inhibitors which plague the HI test, although the difficulty in standardizing this test has precluded its widespread use.

Measurement of serum HI titres in convalescent sera has been used as a surrogate measure for neutralizing antibodies, and has been recognized as a correlate of immune

protection for many years. It remains the mainstay of serological diagnosis of influenza and assessment of an individual's exposure to different strains of influenza. ELISA assays using antibody/antigen sandwich and capture immobilization on sophisticated chemically-derivatized solid surfaces, microparticles and beads have substantially enhanced detection of specific antibodies in body fluids to nano- or picogram quantities, improving the sensitive, accurate detection of many viral infections. Unfortunately, ELISA antibody titres do not readily measure functionality of antibody and may not distinguish between recent and past infection. It is therefore difficult to relate ELISA detection of influenza IgG antibody to definitive diagnosis of recent infection or to any known serological correlates of protection.

Zoonotic infection with influenza A subtypes (H5 and H7) presents further serological challenges. The classical techniques of HI antibody detection have proved to be insensitive for detection of antibodies to these subtypes, either following natural infection or vaccination, which has necessitated the development of modified serological tests that are restricted to a handful of laboratories globally (Stephenson *et al.*, 2003a). The limited serological data from accumulated field studies investigating community transmission of H5 influenza do not suggest that there is appreciable subclinical H5 disease in parts of the world where H5 is endemic in poultry (Ortiz *et al.*, 2007). However, because of the severity and, often, the geographical remoteness of the human cases so far, relatively few serological investigations have been carried out, in part because there is a lack of easily-portable, low-technology serological tests. Recombinant protein expression and the capability arising from reverse-genetic techniques has not yet impacted on human influenza serological diagnosis to deliver serological techniques which give clues about early subtype-specific antibody response to infection, or to provide a substitute for assays which give information about correlates of protection but require the use of live virus. These gaps serve to emphasize the difficulty of translating technical innovations into practical diagnostics for a rapidly-evolving virus that causes poorly-differentiated clinical disease.

TREATMENT AND PREVENTION

The clinical severity of influenza, with high temperatures, respiratory symptoms, myalgia and severe prostration, requires most patients to seek bed rest during the acute phase of illness; the exhaustion and depression which follow may require further convalescence. It is clearly desirable that adequate means of treatment and prevention should be developed. Progress has been relatively slow,

although significant leaps forward in antiviral therapy and vaccine effectiveness have been achieved in the last 10 years.

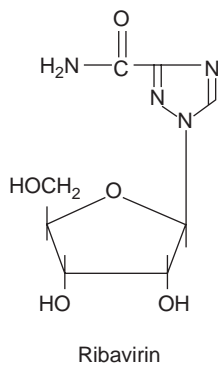
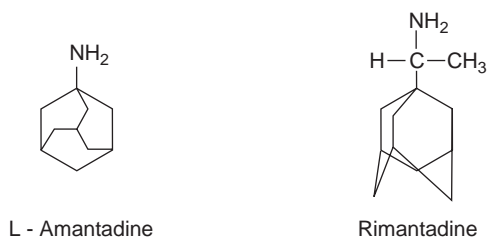
Treatment

At the present time, the treatment of seasonal influenza is usually symptomatic. Most adult patients are advised to rest for two to three days until the acute symptoms have subsided. In view of the evidence that a combination of salicylates and acute influenza infection underlies the pathogenesis of Reye's syndrome in children, paracetamol is given in place of salicylates to treat common symptoms of headache and malaise. Codeine linctus may relieve cough and antibiotics are indicated where chest complications are present or suspected. The use of prophylactic antibiotics in patients with chronic chest disease who thus have a higher risk of developing post-influenzal pneumonia is contentious; some advocate this practice, but the incidence of secondary bacterial pneumonia is not reduced and infection may be by antibiotic-resistant organisms.

Since the earliest conception of antiviral chemotherapy, influenza has been one of the target diseases against which suitable antiviral compounds should be developed. The search for such compounds has used three methods. First there is a rational approach, which predicts a substance that would interfere with virus replication; this requires an exact knowledge of the molecular events of virus infection and multiplication, and such information is rarely complete; however, this approach identified ribavirin. The second method involves serendipity, and requires the random screening of chemical compounds in the hope that an active compound will be found by chance; this method identified amantadine. Lastly, there is the structural approach: the three-dimensional structures of the HA and NA of influenza virus have been elucidated and the identification of the active sites in these structures of virus replication predicts the structure of compounds which may interfere with their function; this strategy led to the development of the neuraminidase inhibitor (NI) drugs (reviewed by Hayden and Pavia, 2006) (Figure 16.16).

Amantadine, Rimantadine

Amantadine and rimantadine are synthetic, water-soluble primary amines with a symmetrical structure; they consist of a stable base with an active amino group (Figure 16.16). In experimental studies, the compounds inhibit the growth of influenza virus in cell culture and limit virus replication in mice, ferrets and other experimental animals. They also reduce tissue damage by influenza virus in infant rats and protect hamsters from infection from virus-infected animals placed in the same cage (Potter and Oxford,



Neuraminidase Inhibitors

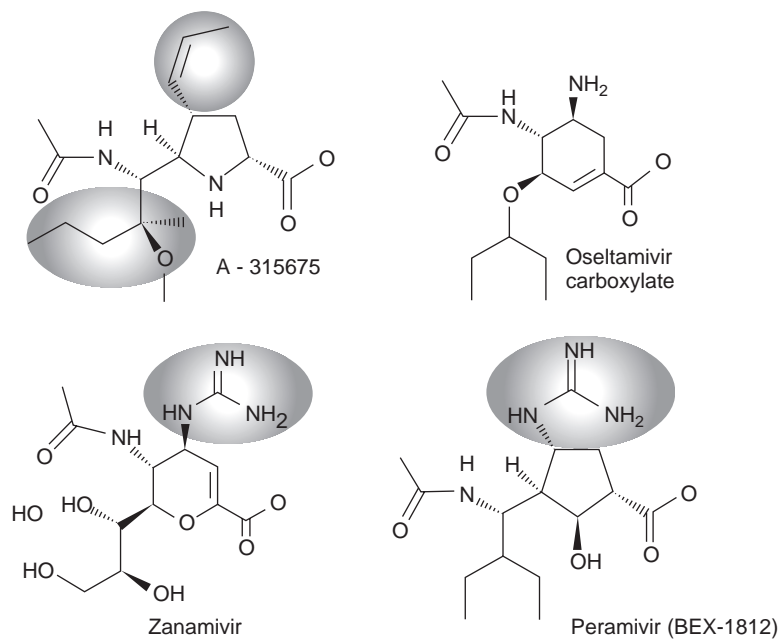


Figure 16.16 Antiviral drugs used against influenza A and B. Shaded areas of neuraminidase-inhibitor molecules represent the areas in contact with substrate binding site.

1977). On the other hand, the compounds are not equally active against all influenza A viruses and do not work against influenza B. Studies on the mode of action of amantadine have indicated two features of the antiviral activity. First, the compound acts at the level of virus uncoating by lysosomal enzymes; this process requires a functional ion channel formed by the M2 protein and a fall in pH within the virus particle for optimal fusion of the viral membrane with host membrane, but amantadine blocks the viral ion channel and thus uncoating and subsequent virus replication is limited. Second, the action of amantadine on the M2 ion channel actively inhibits the transport of HA and other viral subunits to the cell surface and actively inhibits the viral assembly process (Lin *et al.*, 1997).

Clinical studies with amantadine have shown that the compound can occasionally induce mild neurological symptoms; these include insomnia, loss of concentration and mental disorientation. The symptoms are quickly shown by susceptible individuals, and cease when treatment is stopped. If these symptoms do not occur within 24 hours of initiation of amantadine treatment, they are unlikely to occur at all. Both prophylactic and therapeutic studies have been carried out. Prophylactic studies in schools or universities have demonstrated approximately 60% protection for those taking amantadine prophylactically. In therapy trials, amantadine significantly reduced the duration of fever (51 hours as opposed to 74 hours in the placebo group), and illness (2.5 days as opposed to 3.5 days). Rimantadine is the preferred compound, since, although not as effective as amantadine, it is less toxic (Arruda and Hayden, 1996), but it is not licensed in many parts of the world, including Europe.

Resistance to Amantadine/Rimantadine

Resistant strains arise readily *in vitro* and during treatment and are transmitted without loss of fitness. Resistance to both drugs is conferred by mutations at key residues in the transmembrane region of the M2 protein, particularly at residues 26, 27, 30, 31 and 34. The most commonly occurring mutation is S31N. In the last five years it has become apparent that the majority of circulating seasonal influenza A H3N2 strains are naturally resistant to amantadine (Bright *et al.*, 2005), as are avian H5N1 influenza strains, which limits the usefulness of these drugs when applied without knowledge of viral subtype.

Ribavirin

The structure of the synthetic nucleoside analogue 1B-D-ribofuranosyl-1,2,4-triazole carboxymide (ribavirin) is shown in Figure 16.16. The compound inhibits the replication of a wide range of RNA and DNA viruses *in*

vitro, including both influenza A and influenza B, and limits influenza virus replication in mice and ferrets. The compound probably acts by inhibition of virus nucleic acid synthesis, through alteration to cell metabolism. Antiviral compounds which act through alteration to cell metabolism have broad antiviral activity and do not generate resistant viruses; these two properties are true for ribavirin. Ribavirin is well tolerated in concentration 200-fold greater than that necessary to inhibit virus replication; however, the compound has been reported to have immunosuppressive effects, although these are completely reversible once treatment is stopped. Clinical studies have demonstrated a significant therapeutic effect on symptoms of both influenza A and B (Stein *et al.*, 1987), and the compound is very effective in animal studies when given by aerosol combined with rimantadine or amantadine (Hayden, 1996).

Neuraminidase Inhibitors

The activity of the NA enzyme, situated on the outer surface of the influenza virus particle, is important in the replication of both influenza A and B viruses. The three-dimensional crystalline structure of this glycoprotein has been established (Laver *et al.*, 1984), and although the structure can vary between influenza strains, X-ray crystallography and site-directed mutagenesis indicate that the active site is conserved (Laver *et al.*, 1999). It is apparent therefore that drugs that combine with the active site would have antiviral activity. The first active antineuraminidase compound, 4-guanidino-Neu5Ac2en (zanamivir, Relenza), was shown to have good antiviral activity against influenza A and B *in vitro* and, given as an aerosol, was safe and effective in animal models and in clinical studies (Monto *et al.*, 1999). The structure of this compound is shown in Figure 16.16. The second compound, with similar structure and properties (3*R*, 4*R*, 4*4*)-4 acetoamido-5-amino-3-(1-ethyl[*rp*][*pxy*])-1 cyclohexane-1 carboxylic acid (oseltamivir, Tamiflu), given orally as a prodrug, shows similar antiviral activity in volunteer studies (Nicholson *et al.*, 2000) and is also licensed for treatment. Several other similar compounds are in development, for example peramivir (Sidwell and Smee, 2002).

Treatment of adults with seasonal influenza with these compounds is demonstrably effective: the incidence, severity and duration of illness can be reduced. The effect of the drugs is maximized when given early in infection, in the first 48 hours of illness onset, but they may still be effective when given later in infection when illness is severe (McGeer *et al.*, 2007). NIs can be given prophylactically to family members of an index case, and as part of seasonal prophylaxis in institutional settings so as to interrupt transmission of influenza. The

two licensed drugs oseltamivir and zanamivir both act on the enzyme active site; the former is an oral drug, given twice a day; the latter is a topical drug, given by inhalation because of poor oral bioavailability.

The WHO advises the use of oseltamivir for treatment of human H5N1 cases, and oseltamivir or zanamivir for prophylaxis (Schunemann *et al.*, 2007). Early treatment with drug, within five days, substantially improves case fatality (Gambotto *et al.*, 2008) compared with individuals receiving drug late into illness presentation, a situation reminiscent of data obtained with human seasonal influenza, where early treatment substantially reduces viral replication and provides a better clinical outcome. However, several factors may limit the efficacy of oseltamivir in treatment of H5N1. These include late presentation of illness, substantial irreversible pathological tissue damage at the time of treatment initiation, high replication capacity of the H5N1 viruses and emergence of resistant viruses. Together these factors may affect the efficacy of treatment of H5N1 infections, but in view of the severity of infection, antiviral treatment should not be withheld even in those presenting late.

Resistance to Neuraminidase Inhibitors

Resistance to NI drugs has been difficult to generate *in vitro*, requiring multiple passages, and resistant viruses are generally compromised in fitness, because mutations conferring resistance reduce the stability of the protein and/or enzymatic activity. Surveillance of natural isolates prior to the licensure of NI drugs in 1999 did not demonstrate the existence of naturally-occurring resistance (McKimm-Breschkin *et al.*, 2003). Since the time of licensure, the usage of oseltamivir has far exceeded that of zanamivir, probably for reason of ease of application. Surveillance studies since the introduction of NI drugs indicate that the level of resistance to oseltamivir in unselected viral isolates is detectable, with approximately 0.1–1% even in regions with high drug use, such as Japan. Studies of viral isolates recovered from individuals post treatment indicate that approximately 4% of adults and approximately 18% of children may have resistant viruses, although these resistant viruses may not necessarily be transmitted to others. Taken together, the data are suggestive of low-level spread of drug-resistant viruses in the community following the introduction of this class of drugs (Monto *et al.*, 2006; NISN WER, 2007). The genetic mutability of influenza viruses is clearly demonstrated by the emergence of drug-resistant H1N1 influenza in the winter of 2007/2008, as a consequence of spontaneous mutation at position 274 (Histidine to Tyrosine H274Y) in the absence of drug pressure (Lackenby *et al.*, 2008). Such viruses are clearly transmissible and able to compete with other circulating H1N1 strains, although

their long-term persistence is not yet demonstrated. The development of NI drug resistance is to a certain extent subtype dependent, as the exact conformation of each NA subtype active site varies in detail, which affects the nature of drug binding and the potential for generating virus that is drug resistant (McKimm-Breschkin *et al.*, 2003; Russell *et al.*, 2006) (Table 16.5). The landscape of resistance in the NI drugs has altered substantially following the emergence of drug-resistant viruses in the absence of drug pressure. The impact of antiviral resistance on the usefulness of these drugs will require close monitoring in the coming years, particularly in light of the emergence of drug resistance in H5N1 infections and of natural variation in susceptibility found amongst these viruses (de Jong *et al.*, 2005; McKimm-Breschkin *et al.*, 2007).

Clinical experiences of the use of the existing antiviral drugs for treatment of influenza indicate that a search for new targets for antiviral therapy or for immune modulation is urgently required. Although immune modulation is an attractive approach to severe influenza infection, particularly avian influenza H5N1 infections, a much clearer rationale is required for understanding the nature of immediate/innate immune responses. Sixteen of nineteen patients with H5N1 who received corticosteroids did not survive, indicating lack of benefit from empirical adjunct steroid treatment (Gambotto *et al.*, 2008).

Prevention

Vaccines are the mainstay of control and prevention of seasonal influenza, and have been used since the 1940s, with gradual incremental improvement in the accuracy of strain composition, formulation for vaccine potency and reduction of side effects. The majority of vaccines used globally are inactivated killed-virus vaccines, which are administered annually to ‘at-risk populations’ at the beginning of the winter season in developed countries in the Northern or Southern Hemisphere. There has been a long-standing tradition of the use of live-attenuated vaccines in Russia, and since 2000 the use of live-attenuated vaccine using well-characterized cold-adapted attenuated influenza strains has been recommended and introduced for children in the United States. Despite these developments, the search for improved vaccines continues. Recent innovations include the shift towards the use of mammalian cell culture as the substrate for growth of vaccine viruses. This substrate reduces the dependency of vaccine production on the availability of embryonated eggs and improves the flexibility and scaleability of bulk production. The gradual introduction of reverse-genetics technology (Fodor *et al.*, 1999; Neumann *et al.*, 1999) will serve to improve the yield of vaccine strains and the speed at which vaccine candidate strains can be made available to manufacturers.

Table 16.5 Neuraminidase inhibitor resistance profile

NA mutation	NA type/subtype	Fold decrease in neuraminidase enzyme susceptibility			
		Oseltamivir	Zanamivir	Peramivir	A-315675
E119V	A/N2	R (>50)	S (1)	S (1)	S (1)
R292K	A/N2	R (>1000)	S (4–25)	R (40–80)	S (8)
H274Y	A/N1	R (>700)	S (1)	R (40–100)	S (3)
R152K	B	R (>30–750)	R (10–100)	R (>400)	R (150)

R, resistant; S, sensitive.

Correlates of Immunity to Influenza

The antigens of the influenza virus particle which stimulate immunity to subsequent infections have been identified. The results of challenge studies indicate that immunity is induced by the host immune responses to the virus HA and, to a lesser extent, to the NA. Some evidence suggests that the immune response to the M2 and NP proteins may also contribute to immunity.

Studies to determine which immune responses correlate with protection against infection indicate that the serum antibody titre against the viral HA is the most important; thus susceptibility to influenza virus infection is inversely related to the titre of serum HI antibody. This is true for both experimental challenge with attenuated virus and natural infection with virulent virus, and a serum HI antibody titre of approximately 30–40 and 20–30 represents a 50% protective level of antibody against infection by homologous influenza A and B virus, respectively (Potter and Oxford, 1979). Further, the degree of cross-immunity for viruses of different subtypes of strain is directly related to the degree of cross-reactivity of the HA antigens: immunization with an influenza-virus vaccine confers no protection against challenge with virus of different subtype, since there is no cross-reactivity of the HA of the two viruses. In addition to conferring relative protection against infection, the serum HI antibody is reported to both reduce the severity of infection and decrease virus spreading from infected persons. Similar studies have shown that serum NI antibody also confers protection against influenza virus infection; this has been shown in studies with experimental animals and in observations of natural infection in humans. Thus, anti-NA antibody has been shown to confer protection against the clinical effects of influenza virus infection, not infection *per se*, and in the presence of an NI antibody, infection will induce protective HI antibody, whilst clinical symptoms are considerably reduced and may not occur. A generally-held view is that the serum HI antibody is more important in determining immunity than the serum NI antibody, as indicated

by several studies. The importance of cellular immune responses in immunity to infection remains to be established (McMichael *et al.*, 1983), although the role of cellular immunity in assisting clearance of infection is well established; however, cellular immunity is an important prerequisite for humoral antibody response in clinical disease.

From the above it is clear that an influenza vaccine must contain the surface HA and NA antigens of the virus in a form which will stimulate serum HI and NI antibody, local IgA antibody and possibly cellular immunity. It is essential that the vaccine contains the antigens of recently-isolated virus strains; although some cross-reactivity and corresponding cross-immunity are seen between viruses of the same subtype, the most solid immunity is found following immunization with virus homologous to the infection strain. Thus, whatever the type of vaccine used, it is necessary to review virus-strain composition and ensure regular updating to match new antigenic drift variants as they occur.

Inactivated-virus Vaccines

At the present time and for the last two decades, strains of influenza A (H3N2) and (H1N1) and influenza B have been circulating in any one year (Figure 16.8); thus, the currently-infecting strains of each of the three viruses are grown in parallel for vaccine production. Inactivated virus vaccines are prepared by inoculating the currently-circulating strain of influenza virus into embryonated hens' eggs or mammalian cell culture: culture fluids are harvested after two to three days of incubation and centrifuged by zonal centrifugation in order to concentrate and purify the virus particles, which are inactivated with formalin or β -propiolactone. Vaccines are standardized by HA content for intramuscular inoculation. The three viruses are mixed to contain 15 μ g HA of each virus in each dose, to constitute a trivalent vaccine. Inactivated vaccines may be either whole-virus vaccines, prepared as described here, or split-product or subunit vaccines, where further purification steps are undertaken after virus inactivation (Figure 16.17).

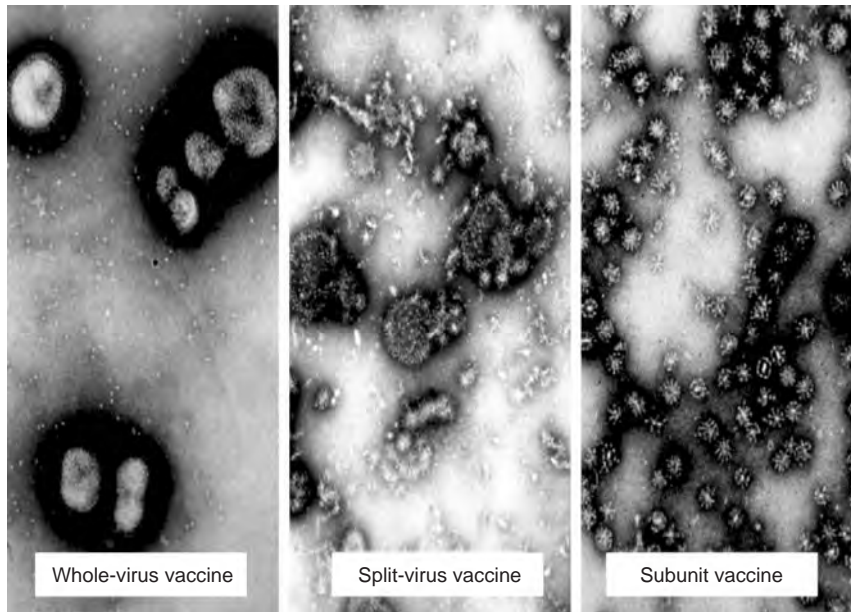


Figure 16.17 Electron micrographs of different types of inactivated influenza vaccines. (Source: Wood, 1987.)

Whole-virus Vaccines

Clinical studies with whole vaccines prepared in this manner have recorded some local pain in approximately 20–30% of vaccinees, and symptomatic reactions, such as fever, headache and muscle pain, in about 5% of persons; however, these responses are usually mild and ephemeral. The vaccine induces serum HI and NI antibody responses, which confer protection on 60–90% of volunteers against challenge virus infection and significant prevention of hospitalization and deaths in the elderly (Nichol and Treanor, 2006). The vaccine may be given annually to the young or elderly, and higher doses may be given to the elderly, who give relatively poor immune responses to conventional vaccine (Keitel *et al.*, 1996). The serum antibody response persists at a protective level for one to five years, depending on the vaccine virus strain and the age of the vaccinees; however, subsequent infecting virus strains may show antigenic drift and the vaccine-induced antibody will be less effective in protecting against these new strains. In contrast, the antibody response to a vaccine containing virus of the new subtype against which the vaccinees have had no past experience is relatively short-lived, and 60–80% of the antibody may have disappeared by 12 months after immunization.

Split-virus Vaccines

Because of the relatively high incidence of reactions seen in vaccines given as inactivated whole-virus vaccine, at-

tempts have been made to reduce reactogenicity while preserving the ability to induce satisfactory titres of serum antibody (immunogenicity). For this, virus pools grown, purified and inactivated as described above are treated with detergents to disrupt the virus particles; inoculation of virus produced in this way induces fewer reactions in volunteers than whole-virus vaccines and the serum antibody responses and protection afforded against subsequent challenge are similar. For these reasons, there has been a gradual shift to split vaccines from whole-virus vaccines, to the extent that probably over 80% of inactivated vaccines given from 2000 have been either split or subunit vaccines.

Subunit-virus Vaccines

Since only virus HA and NA antigens are required to induce immunity, vaccines containing purified surface antigens and free of virus RNA and core proteins have been investigated; these are conventionally given as aqueous suspension, but may be administered with carrier compounds to enhance the immune response (O'Hagan *et al.*, 2007). Volunteers given aqueous subunit vaccines intramuscularly experience fewer reactions than those given whole-virus vaccines. In years of antigenic drift, when the population is primed, serum HI antibody responses to whole and subunit saline vaccines are similar, and since aqueous vaccines are less reactogenic, these are preferred.

However, when vaccinees are not primed by previous exposure to viruses of the same subtype, the serum HI antibody response to whole vaccine is significantly greater than for subunit vaccine; in order to achieve protective levels of antibody, two doses of subunit vaccine may be necessary, and the search for an acceptable adjuvant to overcome this need is an important research activity. The choice of vaccine formulation may therefore be dependent on the immune status of the population being vaccinated. In the event of antigenic shift and the emergence of a novel virus subtype to which the population has no prior exposure, the choice of vaccine may depend on which is most immunogenic at lowest antigen dose in different age groups. In general, in this situation it is accepted that two doses of vaccine may be needed to generate a sufficiently protective immune response.

Vaccine Adjuvants

Subunit vaccines, though demonstrating improved reactogenicity profiles, remain short of ideal in protection against influenza, and experimental studies to find a suitable carrier/adjuvant to enhance antibody reproduction have been pursued vigorously for some years. A large number of adjuvants are known but few are suitable for intramuscular injection: ISCOMS and MF59 enhance serum antibody responses, but there is a modest increase in the incidence of side-effect reactions; and immune modulators such as interleukin 2 (IL-2), which enhance antibody responses in mice, have not been investigated in human volunteers. Subunit trivalent influenza vaccines adjuvanted with MF59 have now been licensed for seasonal influenza and are beginning to be used; however, the real advantage of adjuvants may be demonstrated in populations naïve to a particular influenza subtype, as has been demonstrated in clinical vaccine trials of H5N3 and H5N1 influenza vaccines (Leroux-Roels *et al.*, 2007; Nicholson *et al.*, 2001; Stephenson *et al.*, 2003b). As the threat of pandemic influenza remains, the field of pandemic vaccine development is actively investigating the use of different adjuvants to enhance the immunogenicity of vaccines and produce dose-sparing vaccines.

Live-virus Vaccines

Inactivated influenza vaccines induce serum HI and NI antibodies to protective levels in the majority of vaccinees; however, the local IgA antibody and cellular immune responses are disappointing. In contrast, there is evidence that immunization with live-attenuated influenza vaccine induces a full range of immune responses and a more solid immunity than inactivated vaccines. The preference for vaccine delivered noninvasively and the

reactogenicity profile of inactivated vaccine have continued to encourage the development of live vaccines against influenza. Influenza viruses can be attenuated by serial passage in embryonated hens' eggs, by chemical mutation or by laboratory passage at reduced temperatures (cold adaptation). Using these techniques, viruses can be produced which infect and immunize volunteers without producing appreciable clinical illness. Attenuated viruses produced by one of the above methods have been mixed with wild-type viruses causing current infections to produce reassortants, which contain the RNA fragments coding for the wild-type HA and NA surface antigens and all the other genetic material (internal genes) from the attenuated strain. These reassortants can be produced relatively quickly in the laboratory and, when inoculated intranasally into volunteers, produce few and mild symptoms, and induce both serum and local HI and NI antibody against the wild-type virus and immunity to challenge virus infection (Beare, 1982). Two strains, A/Ann Arbor/6/60 (H2N2) and B/Ann Arbor/1/66, have been used to produce attenuated influenza A and B vaccine reassortants for over three decades (Maassab and DeBorde, 1985); in all studies, the selected reassortants have been shown to be safe and effective, easily administered and suitable for all ages.

The development of a suitable influenza-virus vaccine initially requires the production and purification of a suitable reassortant from an established, suitable and attenuated virus and the wild-type virus. Vaccine virus must be attenuated for various groups of volunteers of different ages and susceptibility, must induce antibody responses and must protect against challenge virus infection. This has been demonstrated for the Ann Arbor cold-adapted vaccine strains, in a series of large randomized controlled trials over a number of years, resulting in licensure in the United States for use in children older than five years (Belshe *et al.*, 2007). The superiority of these vaccines in an elderly population has not yet been demonstrated, and remains in some doubt, since the existence of antibody in elderly adults as a result of lifetime exposure to influenza may preclude sufficient replication of an attenuated influenza to achieve a boosting of immunity.

Other Approaches to Vaccine Development

In addition to the above influenza vaccines, several other approaches are possible and under investigation. Large quantities of influenza-virus antigens can be made by cloning the relevant genes into a variety of vehicles for expression; this would make vaccines more available, since quantity would not be limited by the availability of embryonated hens' eggs. An example of such an approach is the use of recombinant protein HA vaccine produced by baculovirus expression, which produces an extremely

pure product but requires large quantities of protein to establish immunogenicity (Treanor *et al.*, 2007). Reverse genetics has been developed, which allows virus to be produced from susceptible cells transfected with plasmids coding for each of the eight gene segments of the virus RNA (Fodor *et al.*, 1999; Neumann *et al.*, 1999); this opens the possibility of laboratory-designed live and inactivated influenza vaccines. Clinical vaccine studies with H5N1 have shown that vaccines developed in this way are both safe and immunogenic (Bresson *et al.*, 2006; Treanor *et al.*, 2006), and this technology, together with the use of mammalian cell culture, is likely to revolutionize the field of influenza vaccines in the years to come.

One of the limitations of influenza-vaccine usage is the requirement to inoculate intramuscularly; to obviate this, inactivated vaccines have been tested intranasally. Inactivated vaccines given intranasally are generally poor immunogens: these vaccines need to be combined with adjuvant/carrier to be effectively immunogenic. Licensure in Switzerland of inactivated virosomal vaccine adjuvanted with a powerful mucosal adjuvant, LTK, was unexpectedly followed by an increased incidence of Bell's palsy (Mutsch *et al.*, 2004), which has reinforced caution in the development of intranasal delivery of inactivated vaccines. DNA vaccines coding for virus HA have been used successfully in animal models (Wong *et al.*, 2001)

and have demonstrated immunogenicity in human clinical studies (Drape *et al.*, 2006), and represent an exciting new development for influenza vaccines.

Vaccine Recommendations

At the present time, live-attenuated influenza-virus vaccine is available for use in the United States and Russia (although different strains are used), but not in Europe, and the novel methods of producing vaccine outlined above remain in their infancy. The majority of currently-available vaccines are inactivated split or subunit vaccines mainly produced from virus grown in hens' eggs with a clearly-defined annual production schedule (Figure 16.18), although this is likely to shift to mammalian-cell production over the next 10 years, improving the scalability and flexibility of production of inactivated vaccines. Of these, the most popular are the aqueous subunit vaccines, which have replaced the earlier whole- and split-virus vaccines in many countries. These vaccines produce relatively few reactions, and such reactions are usually mild and of relatively short duration; they produce serum antibody in the large majority of subjects, and immunity to infection in 60–90% of vaccinees. Early fears that these vaccines may be unsuitable in certain patient groups, such as those with multiple sclerosis, acquired immunodeficiency syndrome

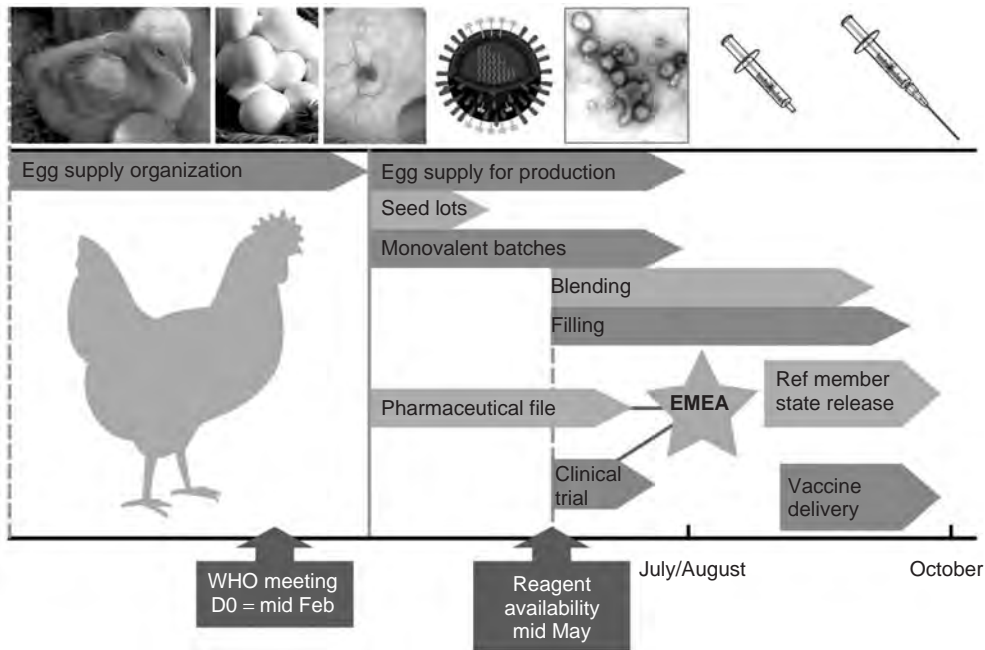


Figure 16.18 Annual production schedule of inactivated influenza vaccine produced from embryonated eggs. EMEA, European Medicines Agency.

(AIDS) or diabetes, have been largely discounted by more recent and larger studies.

A substantial literature assembled over the past three decades attests to the safety and the efficacy of inactivated influenza vaccine; these studies were carried out in children, in healthy adults and in the elderly. Meta-analysis of collected studies from the literature in both healthy young and elderly people indicates that immunization is both safe and effective in producing immunity, and in the elderly can prevent approximately one in five cases of influenza illness and one in four deaths, where there is a good match between the influenza strains in the vaccine and those in circulation (Nichol and Treanor, 2006; Ohmit *et al.*, 2006; Vu *et al.*, 2002). With this information it is difficult to understand the reluctance of some health-care workers to receive vaccine themselves; immunization rates for health-care workers in many Western countries are less than 20%. Finally, although inactivated influenza vaccines are effective in the elderly population, they are less effective than in younger people, since the immune response is impaired by age; however, recent clinical studies suggest that a combination of inactivated vaccine given intramuscularly and live vaccine given intranasally can be more effective than killed vaccine on its own. The use of live vaccines could result in this strategy being used to further protect the vulnerable and aged population.

Vaccine is recommended in most Western countries for all aged over 65 years, for patients with chronic lung or heart disease, for patients with metabolic disorders such as diabetes, renal disease or immunodeficiencies, including children, for whom influenza is life-threatening, and for residents of institutions for the elderly or disabled; other groups, such as household contacts of high-risk persons and health-care workers, should be considered for immunization, together with pregnant women after the first trimester. Recently there has been considerable impetus to extend use of vaccine to young children, for whom influenza is a more serious clinical disease and in whom the virus replicates to higher titres, thus identifying them as a focus of infection for others (Neuzil, 2004). For the remainder of the population, immunization against influenza is a debatable subject; some authorities hold that vaccines should be available for all and used according to the wishes of doctors and patients, while other protest that the further widespread use of vaccine is not justified (Abramson *et al.*, 2006).

A particularly active field of vaccine research currently is that of pandemic vaccine development (vaccines for nonhuman subtypes of influenza), which is serving to highlight the limitations of existing vaccines and also pioneering new approaches to vaccination, including new vaccines, methods of formulation and antigen-sparing strategies (Stephenson *et al.*, 2004). Vaccines will be

in very short supply in the event of a new influenza pandemic, and continuing effort is urgently needed to ensure effective use of both vaccines and drugs should one emerge.

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Parainfluenza Viruses

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INTRODUCTION

Human parainfluenza viruses (HPIVs) are a leading cause of hospitalization for acute respiratory-tract disease (ARD) in young children. Although the characteristic HPIV-mediated illness is laryngotracheobronchitis (croup), their repertoire in the paediatric population also includes upper-respiratory-tract infection, as well as bronchiolitis, pneumonia and lower-respiratory-tract pathology. Parainfluenza viruses are also able to trigger community-acquired ARD, requiring hospitalization in adults, as well as morbidity and mortality in immunodeficient patients and transplant recipients.

Despite the considerable progress that has been made during the past five years, vaccination against HPIV is not yet available. However, vaccines based on cold-adapted viruses have been tested in phase II trials and recently-established reverse-genetics systems manipulating infectious HPIV cDNAs allow for the development of promising vaccine candidates. In addition, results from reverse-genetics-based genome-modification studies, as well as detailed analysis regarding the binding of those viruses to cellular receptors and the concomitant regulation of fusion between viral envelope and cellular membranes leading to viral internalization and release, shed light on the complex pattern of infection and replication of, and immunity against, HPIVs. Large epidemiological studies continuously underline the important contribution of HPIV infections, including the less well studied HPIV type 4 serotypes, in paediatric and adult hospitalizations, which is often underestimated, partly due to

the attention on other viral pathogens. Unravelling of the molecular, cellular and epidemiological aspects of HPIV infection, aided also by efficient nucleic-acid-based detection methods, was of great benefit in the battle against these pathogens. For instance, elucidation of mechanisms used by HPIVs to infect and replicate within hosts, and in addition to escape interferon (IFN)-driven host antiviral immune surveillance has already led to the application of effective intervening strategies. Agents targeting particular events of the viral replication cycle (e.g. attachment to and replication in target cells) are promising. Comprehensive reviews regarding epidemiological studies and molecular mechanisms regulating HPIV infection, propagation and treatment continuously appear, and the reader is encouraged to refer to them for further information (Henrickson, 2003; Moscona, 2005).

TAXONOMY

Parainfluenza viruses are single-stranded negative-sense enveloped RNA viruses belonging to the order *Mononegavirales*. Four major HPIVs have been genetically and antigenically identified (HPIV1–4). Furthermore, there are two subtypes of HPIV4 (A and B), as well as some subgroups/genotypes of HPIV1 and HPIV3. All HPIVs are members of the *Paramyxovirinae* subfamily in the large *Paramyxoviridae* family, and classified into different genera (Figure 17.1). Thus, human and animal PIV1s and PIV3s belong to the *Respirovirus* (former *Paramyxovirus*) genus, whereas PIV2s and PIV4s are members of the

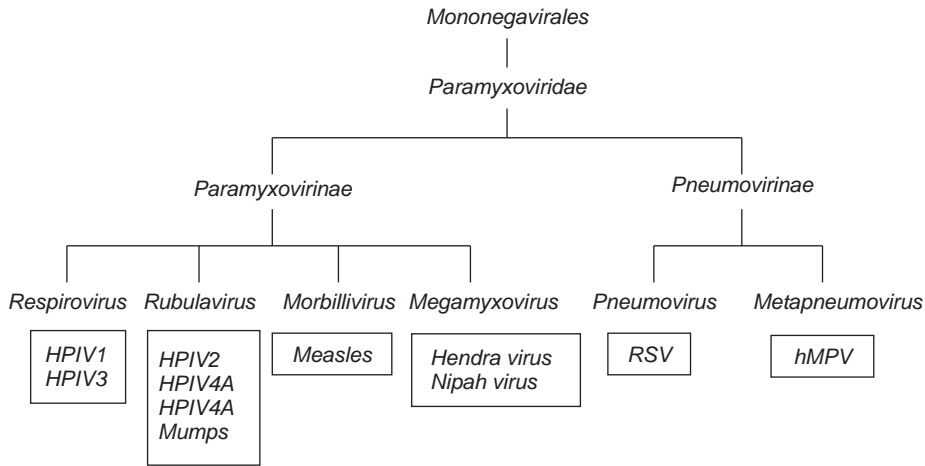


Figure 17.1 Classification of human parainfluenza and related viruses. From top to bottom: order, family, subfamily and genus—the viruses are listed in blue. For abbreviations, see text.

Rubulavirus genus. The human pathogen mumps virus is also a member of the *Rubulavirus* genus. These two genera, together with the *Megamyxovirus* genus (to which the recently-identified Hendra and Nipah viruses belong) (Mounts *et al.*, 2001; Nichol *et al.*, 2000) and the *Morbillivirus* genus (which includes measles virus), form part of the *Paramyxovirinae* subfamily (Figure 17.1). The *Pneumovirinae* subfamily contains the *Pneumovirus* genus, the most important member being human respiratory syncytial virus (RSV), as well as the *Metapneumovirus* genus, to which human metapneumoviruses (hMPV) belong (van den Hoogen *et al.*, 2002).

Sendai virus (SeV), simian virus 5 (SV5) and bovine PIV3 (BPIV3) are well-studied animal counterparts of HPIV1, HPIV2 and HPIV3, respectively. In particular, SeV was originally isolated from the lung of a fatal case of newborn pneumonitis in Sendai, Japan in 1953, but is considered a murine virus (referred to also as murine PIV1, MPIV1) since it readily infects and causes disease in mice, and there have been no reported further outbreaks of the human disease (see ‘Receptors, Virus Entry and Host Range’). Notably, SV5s isolated from different species, including humans, show impressive similarities in sequences and infection properties, suggesting that SV5 may be able to persistently infect humans. Accordingly, its designation as HPIV5 has been proposed (Chatziandreou *et al.*, 2004). The related avian Newcastle disease virus (NDV) belongs to the *Avulavirus* genus of the *Paramyxovirinae* subfamily. Several additional PIVs able to infect and propagate in various animal species have been identified (listed in Henrickson, 2003).

STRUCTURE AND PHYSICAL PROPERTIES

The nonsegmented, negative-stranded RNA species of PIV form genomes approximately 15–16 kb long, encoding at least six mRNAs. They also contain short (less than 100 nt long) untranslated regions at their 3′ and 5′ ends, named leader and trailer respectively, which contain important replication and transcription promoter elements (see ‘Replication’). The gene order starting from the 3′ end is: N-P-M-F-HN-L. These combine to form the nucleocapsid and the envelope of the virion (Figure 17.2). Whereas HN and F proteins mediate binding of HPIVs to target cells and subsequent fusion to cellular membranes, the matrix protein is necessary for viral assembly and budding. Shifts in reading frame, as well as read-through and RNA-editing events, allow the encoding of additional protein species by the P, M and F parts of the genome (see ‘Replication’). These species, designated V, C and D, are characteristic of each HPIV subtype, playing important roles in replication, assembly, maturation and virulence. Some of them may be expressed only in infected cells and are involved in modulation of host immune responses.

With the exception of phosphoprotein P, each of those proteins has a characteristic molecular weight (MW) that does not essentially differ among the different HPIVs (N: 66–70 kDa; M: 28–40 kDa; F: 60–66 kDa; HN: 69–82 kDa; L: 175–251 kDa) (Henrickson, 2003). In contrast, P proteins from HPIV1 and HPIV3 are smaller (49–53 kDa) than those of HPIV2 and HPIV4 (83–90 kDa). MW patterns of the HPIV proteins when run in electrophoresis gels may differ from those predicted,

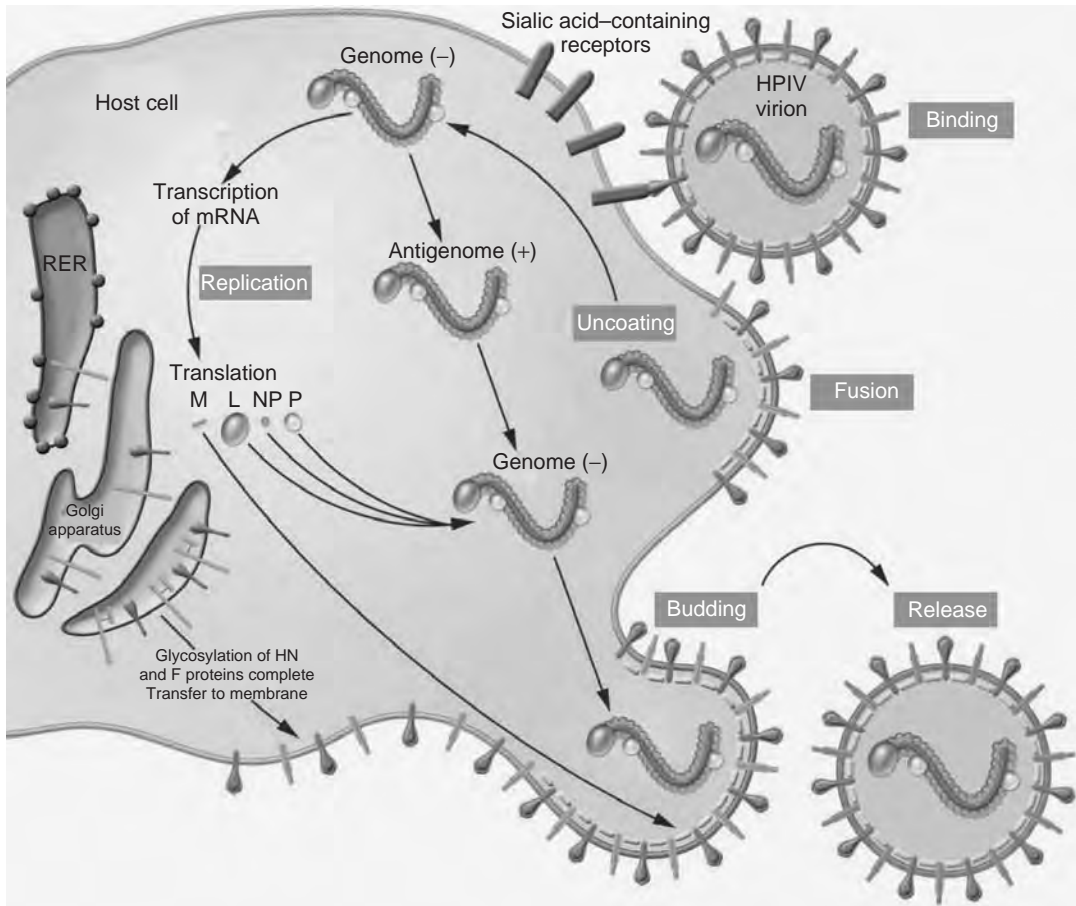


Figure 17.2 The parainfluenza life cycle. Upon binding to sialic acid receptors, viral envelope fuses to plasma membranes, PIV enters the target cell and (–)RNA genome is released into the cytoplasm. Following transcription and replication, L, NP and P proteins are produced and form the newly-produced genomes. M protein is transferred to the plasma membrane, where together with glycosylated HN and F produced through cellular organelles (rough endoplasmic reticulum (RER) and Golgi apparatus), it enriches the plasma membrane regions that allow budding of newly-formed infectious virions. (Source: Moscona, 2005, with permission.)

due to post-translational modifications (glycosylation or phosphorylation).

Viral RNA is encapsidated within multiple copies of the N protein, resulting in helical nucleocapsids. These are further associated with P and L proteins to form the ribonucleoprotein (RNP) complex (Figure 17.2). Accordingly, N is present in abundance in the RNP of the virion to perform its encapsidation function (2600 molecules), whereas the proportions of the L and P proteins are lower (30 and 300 molecules, respectively).

The resulting virions are pleomorphic, roughly-spherical, lipid-containing enveloped particles, 120–300 nm in total diameter (Figure 17.3). The nucleocapsid strands are 12–17 nm in diameter, with cross-striations at intervals of 4 nm. Their total MW is at least 500×10^6 Da and

their density in sucrose is $1.18\text{--}1.20 \text{ g ml}^{-1}$. HPIV virions can be morphologically distinguished from influenza viruses by their thicker and nonsegmented nucleocapsids and from other paramyxoviruses by either morphological (e.g. thinner nucleocapsid than that of *Pneumovirinae* RSV and hMPV) or enzymatic criteria (e.g. absence of neuraminidase activity by morbilliviruses) (Henrickson, 2003).

RECEPTORS, VIRUS ENTRY AND HOST RANGE

Infection of target cells by PIVs is initiated by attachment of virus through interaction of the HN glycoprotein with a cell-surface receptor containing sialic acid (*N*-acetyl

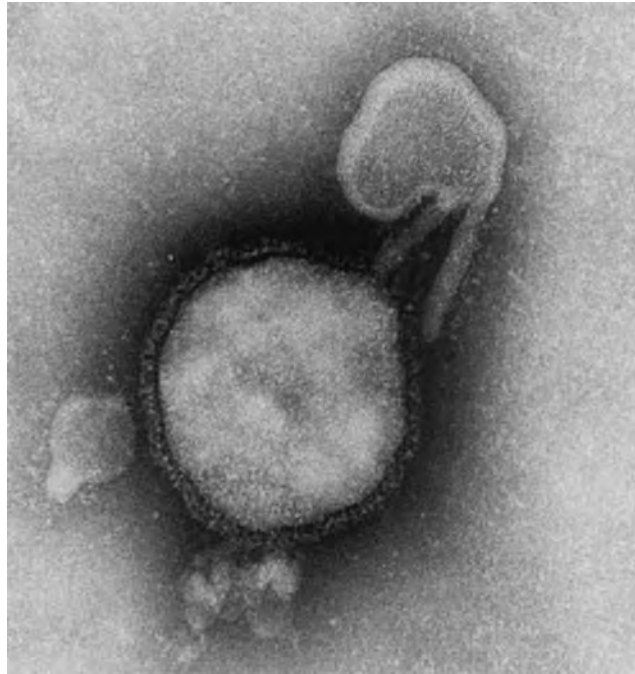


Figure 17.3 The parainfluenza virion. Electron microscopy appearance showing surface glycoproteins. Magnification $\times 275\,000$. (Source: Henrickson, 2003, with permission.)

neuraminic acid, Neu5Ac). F and HN envelope proteins then regulate the process that allows the fusion of the virus envelope with the plasma membrane. Following penetration, the virion gets uncoated and the viral nucleocapsid material is released into the cytoplasm, serving as template for both transcription and replication. Next the replica genomic RNA is packaged into progeny virions that are generated through budding out of plasma membrane areas rich in HN, F and M proteins (Figure 17.2). The M protein triggers budding by interacting with the nucleocapsid and the cytoplasmic components of HN and F proteins, thus mediating the alignment of the former with viral-protein-rich areas of plasma membrane.

HPIV3 infection of airway epithelial cells is polarized and restricted to ciliated cells, as also shown by the use of recombinant HPIV3 engineered to express enhanced green fluorescent protein (EGFP) (Zhang *et al.*, 2005). Characteristically, PIV3 enters and can be released by type II alveolar polarized epithelial cells, preferentially from the apical surface (Bose *et al.*, 2001) with microtubules actively regulating the release process. F-protein trafficking also seems to be restricted to the apical surface of ciliated cells, suggesting that budding may occur from cilia (Zhang *et al.*, 2005).

Both sialoglycoproteins (e.g. glucophorin) (Suzuki *et al.*, 1984; Wybenga *et al.*, 1996) and gangliosides can

serve as specific viral receptors for PIVs (Holmgren *et al.*, 1980; Suzuki *et al.*, 1985; Umeda *et al.*, 1984). It has been shown that SeV is able to bind to both ganglio-series and neolacto-series gangliosides, with a terminal *N*-acetylneuraminic acid (NeuAc) linked to galactose (Gal) by an $\alpha 2-3$ linkage (NeuAc $\alpha 2-3$ Gal) as isoreceptor. HPIV1 and HPIV3 subtypes bind to neolacto-series gangliosides of $\alpha 2-3$ linkage with different binding specificity (Suzuki *et al.*, 2001). Glycoarray-based analysis comprising 285 oligosaccharides demonstrated that HPIV1 and HPIV3 bind only to a subset of glycans that contain $\alpha 2-3$ -linked sialic acids (Amonsén *et al.*, 2007), in contrast to influenza viruses, which use the $\alpha 2-6$ linkage. Differences among HPIV serotypes do exist: HPIV1 binding requires a minimal motif (Neu5Ac $\alpha 2-3$ Gal $\beta 1-4$ GlcNAc), while a longer oligosaccharide is normally needed for HPIV3. Moreover, additional molecules such as heparin sulphate may regulate binding and entry of HPIV3 (Bose and Banerjee, 2002). Finally, cell-surface-expressed nucleolin seems to be required for virus internalization during infection of human lung epithelial cells (Bose *et al.*, 2004).

HN not only mediates HPIV binding to sialic acid containing cellular receptors, but has two additional important properties: it uses its neuraminidase activity

to cleave sialic-acid-containing receptors and undergoes conformational changes which activate the fusion activity of the HPIV F protein. The crystal structure of HPIV3 HN was resolved, indicating that its tertiary structure is a six-sheeted β -propeller (Figure 17.4) and that the sialic acid binding and cleaving functions contained in the globular head of the molecule are not separated (Lawrence *et al.*, 2004). Accordingly, both functions are inhibited by zanamivir (4-GU-DANA, Relenza), a sialic acid analogue. On NDV HN two different active sites appear to exist: site I, showing both receptor binding and neuraminidase activities, and site II, involved only in receptor binding; the latter has a higher avidity for sialic acid receptors than site I and is resistant to zanamivir inhibition (Murrell *et al.*, 2003).

Upon binding to sialic acid receptors HN undergoes a conformational change, which in turn triggers a conformational change in F protein that makes it assume its fusogenic function. Recently, a second zanamivir-resistant active site was also found in HPIV3 HN, which mediates both binding to sialic acid and triggering of the fusion function of F protein (Porotto *et al.*, 2007). N-terminal heptad repeats and C-terminal heptad repeats (N-HR and C-HR) contained within the latter confer a trimeric core coiled-coil formation, and proteolytic cleavage by endoproteases, such as furin and Kex2, into two subunits (membrane distal F₂ and membrane proximal F₁) is required for the exposure of the hydrophobic fusion peptide contained in the new N-terminus of the F₁ subunit. The fusion peptide contains 25 hydrophobic residues highly conserved among the paramyxovirus species. Upon F-protein activation it inserts itself into the target membrane in a transient intermediate, anchoring to both viral

and cell membranes. Then N-HR and C-HR associate into a tight coiled-coil-containing complex, and subsequent re-folding brings together the viral and cell membranes and leads to their fusion, using the free energy generated by the coiled-coil-formation step (Moscona, 2005).

By cleaving sialic acid HN can promote the release of newly-formed virions from the cell surface, allowing them to penetrate additional cells (Huberman *et al.*, 1995). Surprisingly, inhibition of HPIV HN activity by 4-GU-DANA while blocking receptor binding and thus fusion and viral entry, actually aids in the release of newly-formed virions from the infected cells by blocking the interaction between them and their receptors (Porotto *et al.*, 2001). However, this is not the only mode of PIV propagation. PIV envelope proteins accumulating in the cell surface of the infected cells can fuse with neighbouring cells, leading to syncytium formation. Again, neuraminidase activity of HN controls the process by modulating the number of available receptors on the adjacent cells (Moscona and Peluso, 1992). In particular, it has been reported that syncytium formation by HPIV3 is reduced when the target cells are infected at high multiplicity of infection (MOI). The absence of cytopathic effect (CPE) from these ‘persistently-infected’ cells is due to sialic acid cleavage by the HN neuraminidase in a manner proportional to viral loading (Moscona and Peluso, 1993). This phenomenon can be reproduced at low MOI by addition of exogenous (bacterial or viral) neuraminidase. On the other hand, infection with HPIV1 and 2 at high MOI fails to inhibit syncytial formation *in vitro*, as appears to be the case also for infection of ciliated epithelial cells by recombinant HPIV3 (Zhang *et al.*, 2005). Interestingly, the observed CPE cannot be



Figure 17.4 Structure of HN dimer of HPIV3. The N- and C-termini of each monomer are shown in dark and lighter grey, respectively, with the rest of the polypeptide in other shades. Interaction with sialic acid (ball and stick representation) is also depicted. (Source: Adapted from Lawrence *et al.*, 2004, with permission.)

blocked by bacterial or NDV neuraminidase treatment, but it is significantly reduced by HPIV3 neuraminidase, suggesting different cleavage specificities of PIVs' neuraminidases (Ah-Tye *et al.*, 1999). Nevertheless, these data show that in addition to the differences in binding specificities, CPE formation is also differentially regulated by the individual HPIV serotypes, a fact that might have particular impact on their pathogenesis pattern.

Viral interference, a state whereby cells infected by a specific virus are refractory to subsequent infections by the challenge virus, is induced by several mechanisms, including attachment interference. For example, expression of NDV HN inhibits infection of the target cells by NDV (Morrison and McGinnes, 1989). Furthermore, it seems that the neuraminidase activity of HPIV3 depletes the cells of appropriate receptors and protects them from re-infection not only by HPIV3 but also by HPIV2 (Horga *et al.*, 2000). This heterologous interference pattern suggests additional regulatory mechanisms of PIV infection and propagation.

It is plausible that the binding specificities of parainfluenza viruses would determine host range of the particular subtypes. Indeed, sialoglycoproteins localized on erythrocyte membranes mediate the well-studied PIV-mediated agglutination of these cells (haemadsorption activity). In addition, blood-group-I-type gangliosides able to bind HPIVs have been detected in the cell surface from human and bovine, but not equine, erythrocytes. Accordingly, HPIV1 and HPIV3 are unable to induce agglutination of horse erythrocytes, but succeed in agglutinating human- and bovine-derived ones (Suzuki *et al.*, 2001).

However, receptor specificity is not the only determinant of PIVs' host range. Cell-specific replication patterns, as well as cell-specific escape from immune-regulatory mechanisms of the virus, seem to play more important roles in this issue, as described in the following sections.

Finally, it should be noted here that the host range for some PIV species has not been conclusively defined. For instance, SV5, originally isolated from monkey tissue, seems to be a canine virus, but it has also been proposed to be a human pathogen (Chatziandreou *et al.*, 2004). Similarly, SeV, considered to be mainly a murine virus, has been reported to propagate equally well as its homologue HPIV1 in the respiratory tract of primates (Skiadopoulos *et al.*, 2002a).

REPLICATION

Following viral entry and release of the viral RNP into the cytoplasm, transcription is the first event initiating the viral replication cycle. Transcription is thought to start at the 3' end of viral RNA and generates the six canonical

mRNAs by a sequential start–stop mechanism. Conserved sequences flanking the 3' and 5' termini of each PIV gene, termed gene start (GS) and gene end (GE), mediate transcription initiation and termination/polyadenylation, respectively.

Following this initial transcription phase, sequences located in the leader of the 3' end of PIV RNA promote the replication of the nascent RNA, so that the entire genome is copied into a positive-sense replica called the antigenome. A prerequisite for this synthesis is the ability for read-through transcription ignoring the GS and GE signals of each particular mRNA. Subsequently, promoter sequences found in the 3' end (trailer) of the PIV antigenome drive the synthesis of genomic RNA, leading to the replication of the entire PIV genetic material (Figure 17.5).

The whole transcription and replication procedure is thought to be mediated by the RNA-dependent RNA polymerase complex of P and L proteins, and the nucleocapsid N protein. Indeed, efficient HPIV3 transcription and replication has been demonstrated by a minireplicon (i.e. an otherwise 'empty' recombinant viral genome containing only the promoter elements for transcription and replication), when these three proteins were provided in trans by cotransfection (Durbin *et al.*, 1997). Using a similar approach, HPIV2 infection could be achieved using the full-length HPIV2 cDNA (Kawano *et al.*, 2001). It seems that P acts as transactivator of L, which is the catalytic subunit of the polymerase complex. Moreover, the L protein is not able to bind by itself to the N-RNA complex to initiate RNA synthesis, but requires the formation of the L–P complex. Interestingly, N–P and P–P complexes have also been observed in the infected cells. The N–P complex seems to play an important role in encapsidation of the nascent RNA chains during genome replication. The N-terminus of the P protein is responsible for the formation of the soluble N–P complex, whereas the C-terminus mediates P binding to the N-RNA template, as well as the formation of the stable L–P complex (De *et al.*, 2000). According to a recently-proposed model, the polymerase enters the template at approximately the location of the N GS but then scans the template bidirectionally to find a GS signal and initiate transcription (Hoffman *et al.*, 2006). In addition to the role of viral proteins, cellular actin microfilaments seem to play an important role in HPIV3 RNA synthesis and replication (Gupta *et al.*, 1998). Importantly, the actin-binding β -catenin interacts with HPIV3 RNP at the periphery of infected cells and can be packaged in HPIV3 virions, and functions as transcriptional activator of HPIV3 (Bose and Banerjee, 2004).

The promoter elements for SeV and HPIV3 replication have been mapped (Hoffman and Banerjee, 2000;

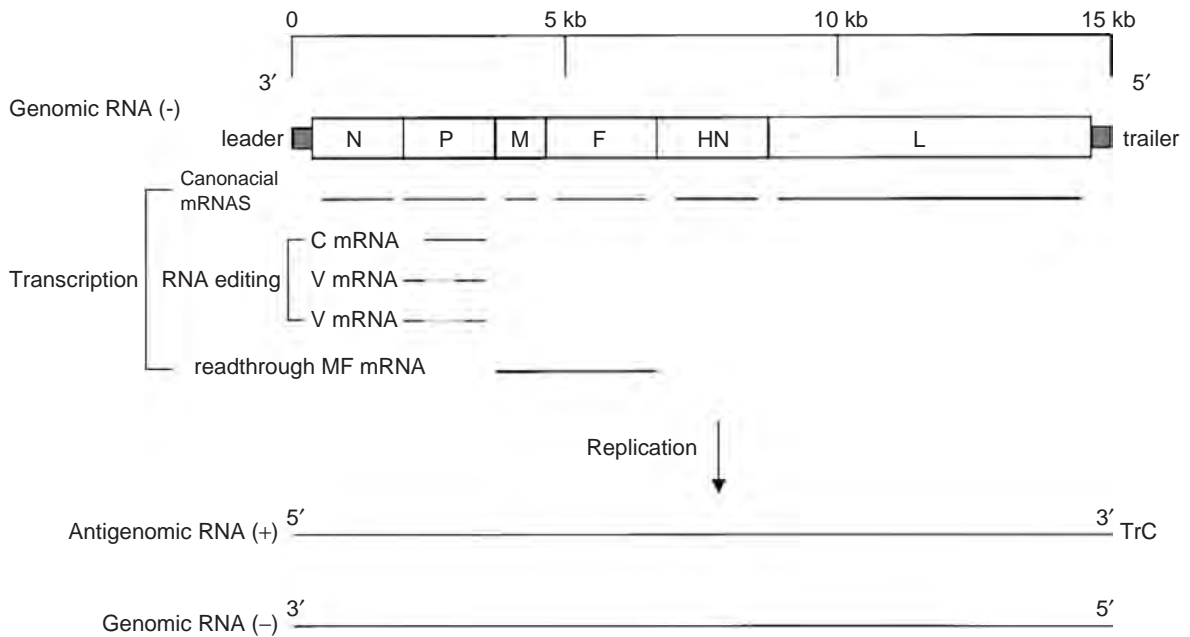


Figure 17.5 The genomic organization and main transcriptional and replication events of parainfluenza viruses. The genomic organization of the PIV genome (3' to 5' direction) as found in the RNP complex of the virion is shown in the upper part of the scheme. Cistron sizes were designed to roughly correspond to the original sequence length, depicting thus relative sizes (in thousands of RNA bases, kb). Upon RNP release into the cytoplasm of a target cell, canonical, as well as alternative, transcriptional events produce the six main mRNAs and additional species (for their role in virus life, see text). Dashed lines within V and D products represent sequences not included in the fusion mRNAs and indicate inclusion of the second ORF. Promoter sequences found in the 3' (leader) of the genome drive not only the transcription but also a round of replication ignoring gene initiation and stop signals, thus producing the antigenomic RNA (depicted in 5' to 3' direction). Promoter elements (TrC) found in the 3' of the latter trigger a second round of replication, generating negative-sense original PIV RNA.

Tapparel and Roux, 1996). The first 12 nucleotides of the 3' leader are essential for HPIV3 replication driven by the leader (Le) promoter. Similarly, the respective region 1–12 of the 3' terminus of the antigenome is critical for the synthesis of genomic DNA driven by the so-called trailer complementary (TrC) promoter (Hoffman and Banerjee, 2000). In addition, cis-elements found within regions 13–28 and 79–96 promote replication, whereas the intergenic region and the start sequence of N gene (49–61) promote transcription (Hoffman *et al.*, 2006).

Replication of most paramyxoviruses, with the remarkable exception of RSV (Samal and Collins, 1996), obeys the so-called 'rule of six' (Calain and Roux, 1993): it is efficient only if the nucleotide length of the genome is a multiple of six. This special feature, thought to reflect an association of the NP protein strictly with six nucleotides in the nucleocapsid, is an absolute prerequisite in SeV, but can be partially circumvented in HPIV2 or HPIV3 (Durbin *et al.*, 1997; Kawano *et al.*, 2001).

Apart from the 'canonical' N, P, M, F, HN and L proteins, a number of alternative transcriptional and translational events have generated additional gene products by PIVs (Figure 17.5). Many of these proteins modulate the transcription and/or replication processes of these viruses.

In particular, an overlapping open reading frame (ORF) located within the P region encodes the nonstructural C protein found in HPIV1, 2 and 3. The C protein of SeV has been reported to inhibit RNA replication by down-regulating the promoter of genomic RNA (Cadd *et al.*, 1996). It also contributes to the SeV pathogenicity for mice (Kurotani *et al.*, 1998). Similarly, the C protein of HPIV3 inhibits viral transcription, and the elements responsible for this inhibition have been localized within a coiled-coil region of the molecule (Malur *et al.*, 2004).

A polymerase slippage on a cis-acting sequence motif in the template P gene leads to the addition of nontemplate nucleotides during mRNA synthesis. Transcription of these elements leads to reading-frame shifts

and internal ORFs are expressed as chimeras fused to the amino-terminus of the P protein. This RNA editing leads to the translation of an additional V protein by the HPIV3 genome. In HPIV2, however, which lacks the above-mentioned C ORF, it is actually the insertion of two G residues that produces the mRNA encoding the P protein, whereas the unedited mRNA (which is actually the exact copy of the P gene) encodes the V protein (Kawano *et al.*, 2001). The differences between the PIV subtypes do not stop here: the V protein is a structural protein detected in the HPIV2 virion (Ohgimoto *et al.*, 2001), but is a non-structural component of SeV (Lamb and Choppin, 1977), whereas the HPIV1 genome does not encode V protein at all (Matsuoka *et al.*, 1991). Recovery of infectious virions from an HPIV2 cDNA modified to lack V-protein expression [HPIV 2V(-)] was successful using the usual reverse-genetics protocols, but the titres obtained were 10–100 times lower than those of unmodified HPIV2, although RNA synthesis was not impaired in the former. Furthermore, HPIV2V(-) virions were anomalous in size, consisting of heterogenous populations and being of larger mean diameter (220 nm vs. 165 nm) (Kawano *et al.*, 2001). The regulation of virus growth by V protein seems to be mediated by a complex formed between V protein, the viral NP complex and proteins provided by the host (i.e. AIP (ALG-2 interacting protein)/Alix). Inhibition of the latter by the small-interference RNA (siRNA) methodology is able to suppress virus growth in cell cultures (Nishio *et al.*, 2007). In addition, the V protein is involved in HPIV-mediated STAT (signal transducers and activators of transcription) degradation, an event essential for HPIV propagation through evasion from the host's immune response (see sections below).

Finally, RNA editing of the P region in HPIV3 and BPIV3 results in the generation of a unique protein named D, not encountered in other paramyxoviruses, which is the fusion product of the N-terminus of P protein and a second internal ORF (Galinski *et al.*, 1992; Pelet *et al.*, 1991).

In addition to synthesizing monocistronic mRNA, the viral polymerase in some cases ignores the termination GE and initiation GS signals. Accordingly, bicistronic M–F read-through transcripts are abundant in cells infected by HPIV1 or HPIV3, but not by SeV (Bousse *et al.*, 1997; Spriggs and Collins, 1986). This capacity to read through was due to cis-elements contained in the long noncoding region of the HPIV1 F gene, and when these sequences were introduced in the SeV M–F boundary they conferred M–F read-through transcription in the modified *Respirovirus* (Bousse *et al.*, 2002). This modification was further shown to reduce the levels of F protein and virus propagation in the infected cells, resulting in reduced pathogenicity of the virus for mice. Thus, failure of transcriptional termination in specific gene boundaries

of the PIV genome may form an additional regulatory mechanism of replication and pathogenesis associated with the parainfluenza viruses.

The mechanisms regulating PIV replication may also contribute to determination of tissue tropism by these viruses. For instance, although Madin–Darby bovine kidney (MDBK) epithelial line cells are refractory to HPIV1, the virus is able to infect them and its genetic material is transcriptionally active in this environment. However, replication seems to be severely impaired, since only trace amounts of genomic RNA and no nucleocapsid formation are detectable following infection, rendering these cells nonpermissive for HPIV1 infection (Tao and Ryan, 1996). Amino acid differences observed at given positions in the otherwise highly homologous BPIV3 and HPIV3 proteins are host-range specific and consistent with the ability of each strain to preferentially propagate in either bovine or primate tissues (Bailly *et al.*, 2000).

During PIV replication the nascent RNA associates with the N–P complex to form the helical nucleocapsid, with matrix protein M playing a central role in this encapsidation procedure (Coronel *et al.*, 2001). Lastly, the nucleocapsids associate with the viral-envelope proteins (M, HN and F) at the plasma membrane and new infectious virions are released from the cell surface, further propagating infection (Figure 17.2).

VIRAL TRANSMISSION, INCUBATION AND SHEDDING

Although transmission has not been extensively studied, it is believed that close-contact transmission and surface contamination may be more important than small-particle aerosol spread or direct hand contact in HPIV transmission (Henrickson, 2003). Similar to RSV, HPIVs live for long periods on skin and cloth (e.g. survive for up to 4 hours on porous surfaces and 10 hours on nonporous surfaces) (Brady *et al.*, 1990). Thus, prevention is particularly important within hospitals and clinics. Common detergents, antiseptics and disinfectants readily remove HPIVs from such surfaces.

Mucous membranes of the nose and throat are the primary sites of infection. After an incubation period of two to eight days, viral replication occurs in the nasopharyngeal epithelium, and one to three days later it is spread throughout the tracheobronchial tree, including the lower respiratory tract. The virus is normally not propagated beyond the respiratory tract, but HPIV has been isolated from cerebrospinal fluid, pericardial and myocardial material, white blood cells and liver in several cases of immunocompromised as well as immunocompetent patients (Reviewed in Henrickson, 2003).

Recurrent infections are a common event and seem to occur more than once, even during adulthood (Marx *et al.*, 1999). However, symptoms become progressively more restricted to the upper respiratory tract.

Shedding of HPIV3 at four to six weeks after onset of respiratory-tract infection (RTI) was observed in a paediatric population (Frank *et al.*, 1981). Increased shedding has also been correlated with more severe RTI symptoms in HPIV1-infected children (Hall *et al.*, 1977). In a remote community model (an isolated population in an Antarctic station), HPIVs could be isolated throughout a period of complete social isolation of greater than eight months (Muchmore *et al.*, 1981). Persistent infection has been demonstrated *in vitro*, where the levels of viral neuraminidase activity and sialic acid receptors or genetic alterations of host cells seem to modulate the process; HPIV2 may use different mechanisms than the other serotypes to achieve this (Ah-Tye *et al.*, 1999). Altered or compromised immunity seems to be responsible for cases of persistent HPIV infection in patients (Henrickson, 2003).

PATHOGENESIS

HPIV infection and illness is associated with both upper and lower-respiratory-tract symptoms. Furthermore, HPIVs have been clearly associated with the development of croup, pneumonia and bronchiolitis in susceptible individuals, mainly children (see 'Epidemiology' and 'Clinical Features'). Pathologic features of these conditions, such as airway inflammation, necrosis and sloughing of the epithelium, oedema, excessive mucus production, alveolar filling and interstitial infiltration of the lungs, should therefore be considered consequences of HPIV-associated airway lesions (Marx *et al.*, 1999).

HPIV infections are also associated with the accumulation of ions and fluid in the respiratory tract. This may at least partly be due to a direct effect on ion transport by the respiratory epithelium (activation of Cl^- secretion and inhibition of Na^+ absorption), as shown in a model of SeV infection of tracheal epithelium (Kunzelmann *et al.*, 2004). The effect seems to be mediated by binding of SeV to a HN-resistant glycolipid, possibly asialo-GM1.

CPEs, mainly syncytium formation, are thought to be of crucial importance for HPIV pathogenesis. Thus, HPIV1 and HPIV2 have been shown to readily infect and propagate in cultured human tracheal epithelial cells (Stark *et al.*, 1991). CPE including early syncytium formation has also been demonstrated in this system. The role of HN-receptor interaction in the process has been demonstrated in experiments using variants of HPIV3

that contain single amino acid changes in HN. These altered HN molecules show increased avidity for sialic acid receptors, and as a result the respective variants are highly fusogenic and destroy a cell monolayer more rapidly than wild-type (wt) HPIV3. More importantly, they cause alveolitis and more severe interstitial pneumonitis than wt HPIV3 in a reliable cotton-rat model for PIV infection (Prince *et al.*, 2001). The enhanced inflammatory response caused by HN variants is dissociated from viral replication or infectivity and is thought to be caused by HN-directed alterations in chemokine expression of the host (Moscona, 2005).

The main body of evidence concerning HPIV-mediated airway inflammation comes from animal studies, since early experimental infection protocols of human volunteers (Kapikian *et al.*, 1961; Lefkowitz and Jackson, 1966; Smith *et al.*, 1967; Tremonti *et al.*, 1968) have not been continued and HPIV inoculation was poorly infectious (Clements *et al.*, 1991).

Rats and mice develop bronchiolitis and pneumonia following PIV infection, with characteristics similar to the human pathology (Mo *et al.*, 1995; Porter *et al.*, 1991; Sorden and Castleman, 1991). Although the respiratory epithelium is the major site of infection in such models, olfactory infection with SeV has been demonstrated in mice. Respiratory inflammation (IFN- γ , IL-2, IL-6, TNF- α (tumour necrosis factor- α) secretion) is evident in the bronchoalveolar (BAL) fluid of C57BL/6J mice inoculated with nonfatal doses of SeV, peaking between day 7 and day 10, the time point at which the virus is cleared from the lungs (Mo *et al.*, 1995). Infiltration of BAL fluid and bronchioles by inflammatory cells (neutrophils, lymphocytes, macrophages and mast cells) has been reported in an SeV-infected rat (Sorden and Castleman, 1991). Eosinophils, macrophages and neutrophils accumulated in the BAL fluid of guinea pigs four days post-PIV3 infection and this was associated with airway hyperreactivity (Toward *et al.*, 2005). In addition, the expression of chemokines such as MCP-1 (monocyte chemoattractant protein-1), RANTES (regulated-on-activation normal-T-cell-expressed-and-secreted protein), GRO- α (growth-related oncogene- α) and IL-8 is induced by SeV in human cells (Hua *et al.*, 1996; Le Goffic *et al.*, 2002). Infection of epithelial cells by HPIV4 enhanced epithelial IL-8 and IL-6 production by both NF- κB - and AP (activator protein)-1-mediated transcriptional up-regulation, as well as by inhibition of their mRNA degradation (Roger *et al.*, 2004). Moreover, the chemokines RANTES and MIP-1 α (macrophage inflammatory protein-1 α) have been also found elevated in nasal secretions of paediatric patients with acute upper-respiratory illness attributed to a number of respiratory viruses, including HPIV (Bonville *et al.*, 1999).

Airway inflammation and hyperresponsiveness are also induced by HPIV3 in guinea pigs, characterized by increased release of and response to histamine, and BAL enrichment with eosinophils, neutrophils and monocytes (Folkerts *et al.*, 1993; Graziano *et al.*, 1989; van Oosterhout *et al.*, 1995). Eosinophil accumulation is well correlated with the degree of airway responsiveness, as well as with the significantly-elevated eotaxin levels following HPIV3 inoculation (Scheerens *et al.*, 1999). Interestingly, the viral content detected in the lungs of inoculated animals presensitized with ovalbumin (i.e. eosinophil enriched) was considerably reduced (80%) compared to the nonsensitized ones (Adamko *et al.*, 1999). Treatment with an antibody to IL-5, a known eosinophil chemoattractant, reversed the ovalbumin effect and inhibited viral clearance. Thus, similarly to what has been previously reported for RSV (Domachowske *et al.*, 1998), eosinophils, when activated by the HPIV virus, apart from mediating inflammatory activity, may also exert antiviral action.

In accordance with the increased airway hyperresponsiveness and the HPIV-mediated eosinophilia established in the animal models, naturally-occurring HPIV3 infection has been associated with mild exacerbation of asthma (Matsuse *et al.*, 2005). However, no correlation could be found between HPIV3 and sputum cytokine (IL-5, IL-10 and IL-12) and eosinophil marker (eosinophilic cationic protein) levels. In contrast, cysteinyl leukotrienes were significantly increased during HPIV3-associated mild asthma exacerbation (Matsuse *et al.*, 2005).

Finally, both HPIV2 and HPIV3 have been reported to induce expression of intercellular adhesion molecule-1 (ICAM-1) in tracheal and other human epithelial cells (Gao *et al.*, 2000; Tosi *et al.*, 1992a). Notably, ICAM-1 also serves as receptor for the majority of rhinoviruses, another important group of respiratory pathogens. This increase may induce adhesion of inflammatory cells in airway epithelium, further augmenting local inflammation, as has been shown for neutrophils in *in vitro* systems (Tosi *et al.*, 1992a, 1992b). Indeed, from the limited human studies available, HPIV3 infection has been associated with BAL neutrophilia in a patient experiencing unilateral bronchiolitis obliterans (Peramaki *et al.*, 1991), whereas bronchial neutrophils and eosinophils were found significantly increased in otherwise healthy individuals during common colds caused by respiratory viruses including HPIV (Trigg *et al.*, 1996).

The above data clearly indicate an inflammatory impact of PIV infection, consistent with an early report showing increased nasal secretion of IFN in children infected by PIVs (Hall *et al.*, 1978); however, further human studies would be required to establish its clinical impact.

ANTIGENICITY AND IMMUNITY

Almost all children by the age of three years demonstrate serologic evidence of HPIV infection (Henrickson, 2003). Sera obtained from subjects one to two weeks following HPIV infection contain antibodies able to inhibit HPIV-induced adsorption of erythrocytes to cultured cells. These so-called haemagglutination inhibition (HI) antibodies persist in the host for several years, whereas complement-fixing antibodies against the whole virion (V-CF) appear one to two weeks later and tend to persist only for approximately one year. Neutralizing antibodies are detected at the same time as HI antibodies. Although IgG1 constitutes the majority of the antibody response against HPIV, one third of adults show increased IgG3, IgG4, serum IgA and IgM levels.

Antibodies can be elicited against the HN, N, P, F and M proteins. However, HN seems to possess the greater antigenicity. Twenty-one epitopes have been mapped in HPIV1 HN (Henrickson and Savatski, 1997). They are organized in five non-overlapping antigenic sites (I–V) and a sixth site connecting sites I, II and III. By examining clinical isolates collected during a period of 35 years and from different geographic regions, evidence was provided showing that only 33% of these epitopes were conserved among isolates. Two sites were found in all isolates, but another one was missing from isolates collected in the last 15 years. Similarly, some monoclonal antibodies against HN and N of HPIV1 strains generated in the 1990s did not react with isolates obtained in the 1970s and 1980s (Komada *et al.*, 1992), in contrast to an earlier report suggesting HN homogeneity by finding a single change in the HPIV1 HN antigenic pattern in a limited number of isolates obtained between 1981 and 1989 (Hetherington *et al.*, 1994). Monoclonal antibodies raised against HPIV3 allowed the mapping of 5, 2, 6 and 6 epitopes in HN, F, N and M proteins, respectively (Rydbeck *et al.*, 1987); this analysis also revealed antigenic variation among isolates obtained throughout a six-year period. Similarly, considerable antigenic variation has been found among clinical HPIV2 isolates, as well as HPIV4A and HPIV4B. In general, analyses of all HPIV serotypes indicate higher antigenic and genetic heterogeneity than initially believed, suggestive of antigenic changes that, although slow, are reminiscent of the evolutionary pattern of influenza B (Henrickson, 2003). Accordingly, some of the standard sera raised against HPIVs in the previous decades fail to detect the virus in serologic tests imposing the use of more recent materials as antigenic sources.

At least 20 epitopes have been located in the F protein of HPIV3 (van Wyke Coelingh and Tierney, 1989). From

those, only 14, organized in three non-overlapping antigenic sites, induce the production of neutralizing antibodies. In addition, HPIV3 frequently accumulates mutations producing F epitopes that efficiently bind to antibodies but are resistant to neutralization. Similarly, antibodies against HPIV4 (of both subtypes) can be divided into three groups: those showing high neutralizing, HI and hemolysis inhibition activities, those neutralizing the virus but unable to inhibit haemagglutination, and those exhibiting low neutralizing and HI activity (Komada *et al.*, 1989). The F protein seems to possess neutralizing-related epitopes in this case.

The antigenic capacity of HPIV proteins is complex and often unpredictable. The HN glycoproteins of related PIV subtypes for instance may be antigenically dissimilar even if they share common epitopes, as is the case for HPIV3 (Ray and Compans, 1986). However, even when the amino acid identity between HN proteins of closely-related viruses is high (e.g. between HPIV1 and SeV) there may be a limited conservation of epitopes and high-antigenic diversity (Komada *et al.*, 1992). In the case of the HPIV4 subtypes (4A and 4B), antigenicity of the N protein is highly conserved between them, but monoclonal antibodies raised against F or HN proteins show low cross-reactivity with the heterologous subtype viruses, although there is a high degree of amino acid identity between the respective glycoproteins (Komada *et al.*, 1989). By constructing HPIV4B cDNAs with putative N-glycosylation sites identical to those of 4A, it could be shown that the mutant 4B subtype was antigenically closer to 4A than to the wt HPIV4B (Komada *et al.*, 2000), suggesting that N-glycosylation pattern determines, at least partially, the limited cross-reactivity observed between PIV species.

The severity of respiratory illness and the duration of virus shedding seem to be reduced in the presence of high titres of neutralizing antibodies (Chanock *et al.*, 1961). However, the fact that HPIV3, for instance, can re-infect an individual within a short time interval, and even in the presence of neutralizing antibodies (Bloom *et al.*, 1961; Glezen *et al.*, 1984; Welliver *et al.*, 1982), and may cause infection of a persistent nature (Gross *et al.*, 1973; Muchmore *et al.*, 1981) suggests that the virus fails to induce a state of long-lasting immunity. These events cannot be explained by the relatively stable antigenic determinants. On the other hand, the fact that the most severe symptoms develop in subjects with defects in cell-mediated immunity or severe immunodeficiency syndromes (Dorman *et al.*, 1999; Taylor *et al.*, 1998) indicates that the interaction of PIVs with the T-cellular compartment of the host's immune response may be of crucial importance for the fate of both the virus and the infected host.

Cytotoxic responses preferentially mediated by $\alpha\beta$ T cells (although $\gamma\delta$ T cells and natural killer (NK) cells are also involved) are elicited against HN, P and NP proteins, and regulate clearance of the HPIVs from the lower airways. Cytokine induction in response to HPIV3 has been shown to inhibit both T-cell proliferation and cytotoxicity *in vitro* (Sieg *et al.*, 1994, 1995). In particular, HPIV3 induced IL-10-inhibited proliferation of CD3⁺ peripheral blood mononuclear cells (PBMCs) derived from healthy human donors and infected *in vitro* by the virus (Sieg *et al.*, 1996). In addition, the HPIV3-infected lymphocytes failed to respond to IL-2, suggesting that IL-10-inhibition of T-cell function may support enhanced virus survival. HPIV3 infects and replicates poorly in human monocytes, simultaneously increasing their survival by induction of granulocyte macrophage colony forming factor (GM-CSF) expression (Plotnicky-Gilquin *et al.*, 2001). In addition, the virus replicates extensively in human dendritic cells (DCs), accelerating their apoptosis. The surviving DCs seem to undergo maturation via increased production of IL-12 following HPIV3 treatment, but mature DCs were poor stimulators of allogeneic T-cell proliferation (Plotnicky-Gilquin *et al.*, 2001). Recent evidence suggests that the neuraminidase activity of HN of HPIV3 may be responsible for the induction of DC maturation and the production of a characteristic pattern of cytokines (Horga *et al.*, 2005). Thus, HPIV3 infects and differentially regulates the growth and response of these two professional antigen-presenting cell populations. Infected DCs may actually contribute to the active dissemination of the virus, whereas only small numbers of surviving pulmonary DCs should be able to reach the draining lymph nodes; however, these cells would have low stimulatory properties, thus aiding survival of the virus. The infected monocytes might also constitute a reservoir for HPIV3.

HPIV3 induces the expression of major histocompatibility complex (MHC) class I and class II molecules in human alveolar epithelial cells (Gao *et al.*, 1999). Although IFN- β is also induced and acts as an intermediate in this process, viral antigens also contribute to MHC induction. MHC class I induction is virus-replication-independent, whereas infectious virions are required for induced expression of MHC class II molecules. Such an induction pattern could contribute to HPIV3 infection immunopathology by increasing cytotoxic T-lymphocyte (CTL)-mediated lysis of respiratory epithelium.

PIVs are able to escape IFN-mediated antiviral immune responses. Type I IFN (IFN- α and IFN- β) responses are mediated by both STAT1 and STAT2 transcription factors, whereas type II (IFN- γ) uses only STAT1. It has been shown that the V protein of SV5 is responsible for blocking IFN antiviral responses in human cells by

targeting STAT1 for degradation by the proteasome (Didcock *et al.*, 1999). Degradation is independent of IFN signalling and targets both phosphorylated and unphosphorylated STAT1. Although practically all PIVs seem to resist IFN responses (Young *et al.*, 2000), there are differences in the mechanisms used by the individual members of the subfamily to achieve this. In particular, HPIV3 and SeV have been shown to block both type I and type II IFN pathways, whereas HPIV2 is able to block only type I, by down-regulating STAT2 (Young *et al.*, 2000). Accordingly, HPIV2 replication is severely impaired in IFN- γ pretreated cells (Andrejeva *et al.*, 2002). The inability of HPIV2 to block IFN- γ -mediated defense could suggest why HPIV2 infections are generally less severe than HPIV3-mediated ones (see 'Clinical Features'). In addition, human STAT2 transfected into mouse cells confers on SV5 the ability to mediate STAT1 degradation and to escape from type I response in an otherwise refractory environment (Parisien *et al.*, 2002), suggesting that STATs may serve as intracellular determinants of PIV host range by affecting each other's degradation. There is also species specificity since, besides STAT2 degradation, HPIV2 V protein can also abolish STAT1 in an *in vitro* system, depending on whether the host cells are of mouse or human origin (Precious *et al.*, 2005). Interaction of V protein with partners such as the UV damaged DNA binding protein (DDB) and Cullin4A (Cul4 α), which associate with the E3 ubiquitin ligase complex, is crucial for this process. Although HPIV4 is able to associate with STAT1, STAT2, DDB and Cul4 α , it is unable to induce STAT degradation and thus escapes antiviral IFN responses (Nishio *et al.*, 2005), a fact that may explain the more modest symptoms observed with HPIV4 infection. Recent *in vitro* studies indicate that in addition to V protein, the C protein of HPIV3 may also mediate suppression of antiviral responses using a somewhat different mechanism, namely by inhibiting STAT1 phosphorylation (Malur *et al.*, 2005). On the other hand, the C protein of HPIV1 inhibits both activation of IFN regulatory factor 3 and IFN production in A549 airway epithelial cells (Van Cleve *et al.*, 2006). Nevertheless, examination of V and C proteins from several PIV species suggests that V proteins may use different mechanisms than C proteins to suppress double RNA-induced IFN- β production targeting particular signalling molecules, such as melanoma differentiation-associated protein 5 (MDA-5) (Komatsu *et al.*, 2007). In addition, the highly homologous HPIV1 and SeV (murine PIV1) or their C proteins both block the IFN Jak/STAT pathway in human cells. However, when human cells are pretreated with IFN, although the replication of both viruses is initially inhibited, it is subsequently recovered only for the human PIV1. Conversely, SeV overcomes suppression in IFN-pretreated murine cells,

again emphasising that PIV-mediated anti-IFN activity is species-specific (Bousse *et al.*, 2006).

Finally, HPIV3 has been shown to induce type I IFN expression in human epithelial cells (Gao *et al.*, 2001), consistent with the reported elevated IFN levels following parainfluenza infection in children (Hall *et al.*, 1978). HPIV3-induced IFN- α/β inhibits signals downstream of class II, major histocompatibility complex, transactivator (CIITA) mRNA accumulation, a transcription factor regulating IFN- γ -induced MHC class II expression. On the other hand, HPIV3 antigens directly inhibit CIITA mRNA accumulation (Gao *et al.*, 2001), the overall effect being an inhibition of IFN- γ -induced MHC class II expression in epithelial cells; that is, the primary target of respiratory virus replication (Gao *et al.*, 2001). This may result in regulation of CD4⁺ cell activation, in addition to PIV-mediated regulation of macrophages, B cells and DCs.

EPIDEMIOLOGY

HPIVs are one of the leading causes of hospitalization for ARD in young children. It has been estimated that they can account for 9–30% of hospitalizations for paediatric acute respiratory illnesses (Henrickson, 2003). Studies with paediatric populations across the United States revealed that during 1970s–1990s HPIV infections were responsible for approximately 65% of croup, 20–40% of upper-respiratory-tract infections (URTIs) and 20% of lower-respiratory-tract infections (LRTIs) (Weinberg, 2006). However, the high prevalence of HPIV infections may have been underestimated (at least 1.5 times), since most large cohort studies were based on less sensitive detection methods (cell culture, serology) than the conventional and real-time reverse transcriptase PCRs (RT-PCRs) that are currently being applied. Real-time RT-PCR-based diagnosis of respiratory viruses including HPIVs increased the calculated incidence of infection by a viral respiratory pathogen from 24 to 43% in infants and from 3.6 to 36% in adults hospitalized for respiratory illness (van de Pol *et al.*, 2007).

Similarly to RSV, HPIV transmission is likely to take place by aerosolization of large droplets rather than by aerosol (small-droplet) spread (Hall, 2001; Henrickson, 2003). Virus-laden droplets expelled from lower-respiratory or nasal secretions of infected individuals primarily infect mucous membranes of the nose and throat, where, after a two-to-eight-day incubation period, HPIVs are replicated and eventually spread into the lower respiratory tract. Viral pneumonia, otitis media, bronchiolitis, fever of unknown etiology and nonspecific URTI are often encountered among HPIV patients (Weinberg, 2006). Among serotypes, HPIV1 and HPIV2 are

generally more associated with croup, URTI and pharyngitis (Knott *et al.*, 1994), whereas HPIV3 is in addition a major cause of infant bronchiolitis and is also associated with the development of pneumonia in susceptible subjects (Hall, 2001). HPIV3 seems to infect younger children than HPIV1 and HPIV2 (50–67% are infected by one year of age (Weinberg, 2006)). HPIV4 subtypes cause a rather mild illness; however, lower-respiratory-tract symptoms have been documented (Lindquist *et al.*, 1997). In an HPIV4 outbreak in a developmental-disabilities unit a portion of the paediatric patients infected by HPIV4 showed LTRIs, including respiratory failure requiring mechanical ventilation (Lau *et al.*, 2005). Although less studied, HPIV4 may cause more severe pathology than previously believed; HPIV1 and HPIV3 are generally thought to confer the more symptomatic phenotypes, followed by HPIV2 and the less symptomatic HPIV4s.

Serological evidence suggests that almost 50% of infants have been infected by HPIV3 by the time they reach their first birthday (Parrot *et al.*, 1962). In contrast, HPIV1 and 2 are encountered more frequently in toddlers and preschool children. Although HPIVs mainly affect the paediatric population, adult infection is not uncommon, and is usually associated with mild URTI and common cold (Makela *et al.*, 1998). Nevertheless, a portion of community-acquired pneumonia in adults may be associated with HPIV infection (Marx *et al.*, 1999). By collecting data from England and Wales for the period 1975–1997, Laurichesse *et al.* (1999) were able to illustrate the different age-distribution patterns of all four HPIV subtypes (Figure 17.6). In contrast, they could not find any significant gender-dependent differences.

The relative circulation rates differ among the individual HPIV serotypes. A US national prospective study analysed over 40 000 reported positive test results covering a 15-year period (1990–2004) (Fry *et al.*, 2006) and found that more than half of HPIV infections were due to HPIV3 (52%), followed by HPIV1 (26%), HPIV2 (12%) and HPIV4 (2%). These results were in accordance with a previous study that followed successive cohorts formed over a period of 20 years (1974–1993) in Nashville by 1429 otherwise healthy infants and young children of less than five years of age; that study identified HPIV3 as the most common HPIV pathogen isolated from LRTI specimens (59%), followed by HPIV1 (29%) and HPIV2 (12%) (Reed *et al.*, 1997). In all, HPIV isolates represented 17.4% of all viral cultures that yielded a virus in that study, with 10% of the children having a symptomatic HPIV3 LRT infection. The higher incidence of HPIV3 infections was consistent with the annual epidemic pattern of HPIV3 isolation during this period, in contrast to the biennial outbreaks of HPIV1 and HPIV2. In addition, Laurichesse *et al.* (1999) reported finding HPIV3 more frequently isolated from children aged 3–12 months, whereas the other two species were more common in the second year of life.

HPIV1 outbreaks are correlated with well-defined biennial rises in croup outbreaks usually recorded in the autumn (Hall, 2001). HPIV1 outbreaks occur biennially between late September and December, with an outbreak duration of 13–15 weeks (Fry *et al.*, 2006). HPIV2 outbreaks have been thought to follow those of type 1, being generally less predictable, though simultaneous incidence has also been recorded (Hall, 2001). More-recent data, however, suggest an annual occurrence from October to

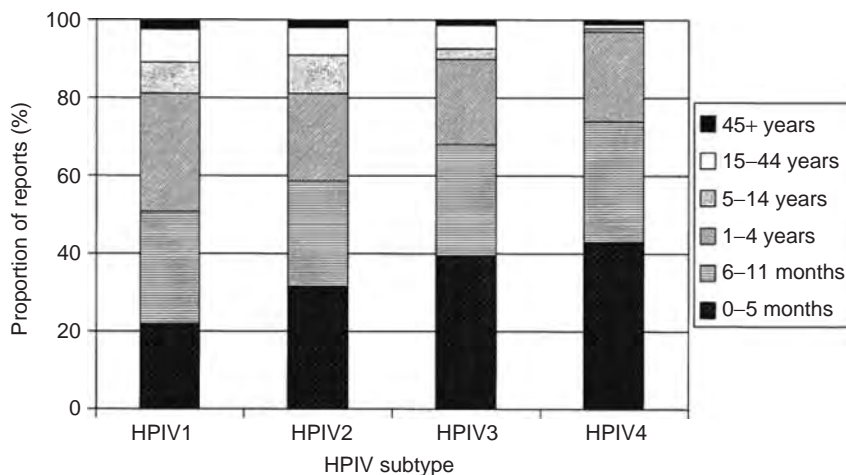


Figure 17.6 Age distribution of HPIV laboratory reports in England and Wales, 1985–1987. (Source: adapted from Laurichesse *et al.*, 1999.)

December (Figure 17.7), with outbreak duration varying between 1 and 18 weeks (Fry *et al.*, 2006).

As mentioned, HPIV3 infections seem to be considerably more frequent (Fry *et al.*, 2006; Laurichesse *et al.*, 1999) and to occur annually, with peaks recorded during spring and summer. Outbreaks of this virus last longer than those of the other members of the family (Hall, 2001). Importantly, Fry *et al.* demonstrated recently that in the years with no HPIV1 activity, HPIV3 incidence (occurring annually between April and June; Figure 17.7) seemed to be significantly more prevalent, characterized by either an elongated spring outbreak or a second smaller outbreak in the fall (Fry *et al.*, 2006). The study used the cumulative-sum method, which determines periods of peak viral incidence, relying on the presence of significantly more positive tests than those observed in the preceding weeks. The emerging interaction between HPIV1 and HPIV3 may be of particular importance for designing preventive strategies.

HPIV4 isolates are rare (Figure 17.7) and accordingly their seasonal pattern is less well characterized (Fry *et al.*, 2006); however, HPIV4 diagnosis is probably underestimated and previously-undetected cases can be identified with more sensitive methods, such as real-time RT-PCR (Templeton *et al.*, 2005). Although the majority of HPIV4 clinical isolates are of subtype B, as many as 95% of adults have antibodies against HPIV4A, compared to 75% that have antibodies against HPIV4B.

Croup incidence is associated more with HPIV1 epidemiology than with the other HPIV subtypes. In a study conducted between winter and spring of three consecutive years in Canadian patients with croup, HPIV1 was isolated in 30.3% of cases and HPIV3 in 3.5%. Peak monthly HPIV1 isolation occurred in November in this study and in January in another study of similar design (McLean, 2000). By analyzing croup hospitalization collective data from the United States, Marx *et al.* (1997) confirmed the unique pattern of HPIV1 epidemics: HPIV1 has produced national epidemics of acute RTI in the United States in the odd-numbered years between 1979 and 1993, whereas it has rarely been isolated in between the peaks. Notably, HPIV1 was calculated to be responsible for ~18% of all cases of croup recorded and ~37% of all cases with determined aetiology, indicating the leading role of this HPIV subtype in the disease. While the major peaks of croup occurred biennially during October in odd-numbered years, minor peaks were produced annually around February (Figure 17.8). Interestingly, these trends were also age-dependent, with epidemics of children under five years closely following the biennial pattern, whereas for older children the annual winter peak was equally as high as that of the autumn, and moreover there were no apparent differences between odd- and even-numbered years in the magnitude of the autumn peak. The pattern of the autumn peaks coincided well with

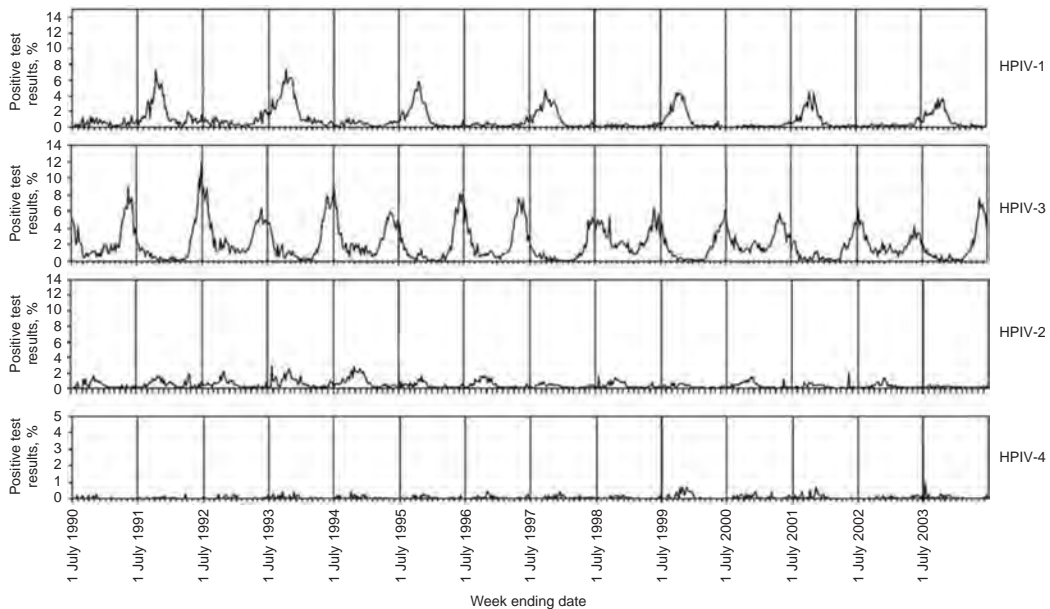


Figure 17.7 Seasonal trends in HPIV incidence. Distribution of positive tests for all HPIVs in the United States during 1990–2004, based on weekly reports to the National Respiratory and Enteric Viruses Surveillance System (NREVSS). (Source: adapted from Fry *et al.*, 2006.)

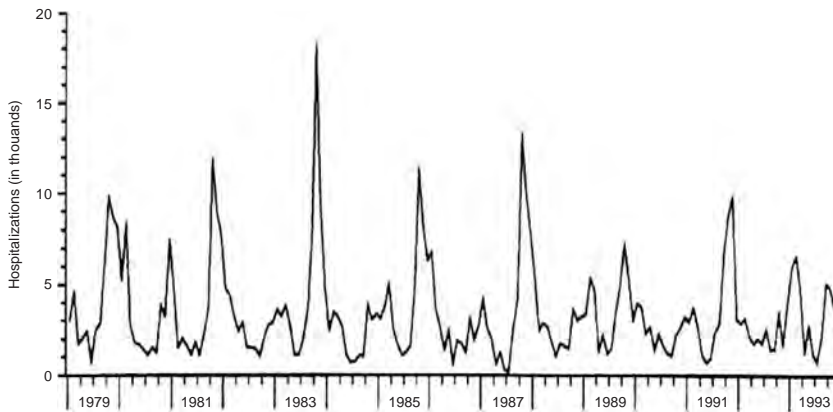


Figure 17.8 Collective data from croup hospitalizations (children <15 years of age) in the United States, 1979–1993. 22.5% of all croup hospitalizations per biennium, and 36.7% in odd-numbered years, can be attributed to HPIV1. (Source: Marx *et al.*, 1997, with permission.)

peaks of HPIV1 isolation across the United States (Marx *et al.*, 1997).

Another population-based prospective study suggested that during their respective epidemic seasons, HPIV1 and HPIV3, but not HPIV2, were among the most commonly detected infectious agents in adult patients hospitalized for LRTI (together with RSV and influenza A and B) (Marx *et al.*, 1999). Importantly, no HPIV1 and very few HPIV3 infections could be documented in between the epidemic seasons. Although similar rates of incidence were observed for HPIV1 and HPIV3 in this adult population in the epidemic seasons (2.5% and 3.2% of hospitalized patients, respectively), the likelihood of clinically-serious disease was greater for HPIV3 infection. In contrast, the role of HPIV2 was emphasized in a study among hospitalized children with LRTI, where it was suggested that HPIV2 predominantly associates with inspiratory difficulties, as compared with HPIV1 and HPIV3 (Korppi *et al.*, 1988).

CLINICAL FEATURES

Acute laryngotracheobronchitis (croup) is the major clinical manifestation, and the hallmark of HPIV (especially HPIV1) infection in children. The syndrome is characterized by croupy cough, inspiratory stridor, cry or hoarse voice, difficulty during inspiration and in-drawing of the chest wall in the subcostal, intercostal or supraclavicular areas (McLean, 2000). There are frequent rhonchi in the lungs and air entry into the chest is inhibited. Accordingly, diminished inflation of the lungs is revealed in some cases by radiology. In some cases there is also severe airway obstruction due to swelling of the epiglottis or the aryepiglottic folds of the larynx, which may

require emergency tracheotomy to reestablish inspiration. Upon hospitalization (usually 3–24 hours after the onset of the clinical syndrome) the symptoms usually subside relatively quickly (one to two days). Influenza, measles and chickenpox may also induce croup in paediatric patients.

Parainfluenza viruses have also been involved in LRTI and other complications of upper URTI. About 30–50% of patients developing URTI due to parainfluenza infection may experience otitis media (Knott *et al.*, 1994). About 15% of HPIV infections also involve the lower respiratory tract, with some 2.8% of patients requiring hospitalization (Reed *et al.*, 1997). A study analyzing the prevalence of HPIV1 and HPIV2 in US children under six years of age receiving emergency treatment and/or requiring admission due to LRTI during the fall of 1991 reported that PIVs were associated with croup (48%), bronchiolitis (10%), pneumonia (12%), cough with fever (29%) and wheeze with fever (19%) (Henrickson *et al.*, 1994). HPIV association with bronchiolitis has also been clearly documented (Hall, 2001; Welliver *et al.*, 1986). Pneumonia and bronchiolitis due to HPIV3 infection occur mainly in the first six months of life, as is also the case for RSV (Reed *et al.*, 1997). HPIV3 infection has also been associated with mild exacerbation of asthma (Matsuse *et al.*, 2005).

A report examining data collected over a period of 20 years concerning immunocompetent children aged under five years with a clinically significant ARD revealed the absence of significant differences among clinical signs and symptoms between the three main HPIV serotypes. The most common symptoms comprised coryza and cough, with fewer than 10% of cases experiencing high body temperatures (Reed *et al.*, 1997). There were no significant differences between upper and lower RTI among the subtypes, with LRTI being more rare than

expected. In contrast, HPIV3 was more often associated with acute otitis than HPIV1 and HPIV2 combined (Reed *et al.*, 1997). In a recent outbreak of the less well studied HPIV4 in a developmental disabilities unit, affecting 38 children 3 three staff members, URIs were the major symptoms, but LRTIs occurred in 7% of the children. A case of respiratory failure requiring ventilation support was also reported in the same outbreak. Genetic analysis suggested that the outbreak was probably of the HPIV4A serotype (Lau *et al.*, 2005).

Although HPIV infection is thought to concern mainly paediatric patients, it has also been associated with LRTI in adults. Association of HPIV3 with adult croup has recently been documented, providing evidence suggesting that the disease may be severe in adults as well as in the paediatric population (Woo *et al.*, 2000). Furthermore, in a prospective study among adults hospitalized during 1991–1992, HPIV1-mediated symptoms and disease included wheezing, rhonchi and one case of lobar pneumonia (Marx *et al.*, 1999). Wheezing and rhonchi were also recorded among HPIV3-infected patients, at rates significantly higher than those reported among patients experiencing a bacterial pneumonia (*C. pneumoniae*, *M. pneumoniae*, etc). 27% of HPIV3-infected hospitalized patients developed lobar pneumonia.

Nosocomial spread of community-acquired parainfluenza viruses transported into transplantation units by employees or visitors may cause severe infection of patients receiving immunosuppressive treatment following bone marrow transplantation. The severity depends on the degree of immunosuppression, the virus subtype, the presence of other infections and the timing of the infection (before or after engraftment) (Wendt and Hertz, 1995). A high mortality rate due to pneumonia, reaching even 37%, has been reported for HPIV infections in cases of children and adults receiving bone marrow transplantation, as well as of infants with combined immunodeficiency (Frank *et al.*, 1983; Wendt *et al.*, 1992); however, lower mortality rates have also been documented (Dignan *et al.*, 2006). In a recent outbreak within a haematology unit, nine deaths occurred among haematopoietic stem cell transplant recipients after community-acquired HPIV3 was introduced into the clinic by a patient. Another patient became chronically infected by the same strain of HPIV3 and passed the virus over to other patients, which in combination with a later RSV outbreak ended up with the disastrous outcome of 9 deaths among 27 infected patients (Jalal *et al.*, 2007). In patients with severe combined immunodeficiency syndrome or acute myelomonocytic leukemia, HPIV infection may be associated with the development of persisting, severe and sometimes fatal LRTI, including giant-cell pneumonia (reviewed in Henrickson,

2003). IFN- γ -deficient patients are also more susceptible to HPIV3 infection (Dorman *et al.*, 1999). Moreover, patients undergoing lung transplantation are significantly susceptible to HPIV infection (Vilchez *et al.*, 2003). Infections occurred in 1.6–11.9% of the recipients among several studies, most probably almost a year after transplantation, and were highly associated with allograft rejection. As many as 32% of HPIV-infected recipients developed active bronchiolitis obliterans six months after infection, severely impairing long-term survival of lung transplants. Notably, HPIV3 has been associated with rejection of renal and liver transplants. On the other hand, HPIV infection may be asymptomatic in patients undergoing hemopoietic cell transplantation, in contrast to RSV and influenza infections (Peck *et al.*, 2007).

In immunocompromised patients, infection of HPIV is not restricted to the respiratory tract, but may affect diverse tissues, including myocardium. Characteristically, in a retrospective study samples obtained from a small group of patients with congenital immunodeficiency who died following HPIV infection were examined for signs of HPIV presence (immunohistochemistry) and related cytopathology (e.g. presence of giant cells). As well as lung and bronchi, in most of the cases HPIV infection and cytopathic events were documented in additional tissues, including pancreas, kidneys, bladder, gastrointestinal tract, spleen, thymus and/or lymph nodes and small blood vessels in various organs (Madden *et al.*, 2004). Furthermore, *in vitro* studies suggested that HPIV3 is able to infect epithelial cells of pancreatic, bladder, colon and thymus origin, suggesting that HPIV infection may be spread to other organs, at least under immunocompromised conditions, thus increasing its morbidity and mortality.

Rarely, parainfluenza infection has been also shown to affect the central nervous system and brain, even in immunocompetent subjects associated with acute and chronic neurological disease, suggesting a potential neurotropism in certain hosts. Thus, PIV has been isolated from children and adults with meningitis (Arguedas *et al.*, 1990; Arisoy *et al.*, 1993; Vreede *et al.*, 1992). Furthermore, the presence of PIV has also been documented in syndromes such as Guillain–Barre and Reye's (Powell *et al.*, 1973; Roman *et al.*, 1978). A particular problem has been the development of febrile seizures in children hospitalized for HPIV infection: in that case HPIV4B seems to be more aggressive (up to 62% association) than the other serotypes (17% for HPIV3 and 7% for HPIV1). Hendra and Nipah viruses have been reported to cause severe encephalitis, and are associated with mortality in children and adults (Mounts *et al.*, 2001; Nichol *et al.*, 2000). In addition, HPIV1, HPIV2 and HPIV3 have been identified in the airways of children with acute encephalitis.

Finally, HPIV infections have been documented in several syndromes in the absence of other pathogens, including acute respiratory-distress syndrome in children, parotitis, hepatitis, gastroenteritis, as well cardiovascular (myocarditis and pericarditis) and muscular disorders (rhabdomyolysis) (reviewed in Henrickson, 2003).

DIAGNOSIS

As for all respiratory viruses, identification of parainfluenza viruses in clinical specimens can be achieved by detecting whole virus, viral proteins and viral RNA in clinical samples, as well as by investigating host antibody responses. Methods include: identification of the virion or viral proteins by electron microscopy or immunofluorescence, respectively; virus culture and identification based on CPEs in cultured cells; a variety of serology techniques to detect antibody responses; and detection of PIV genetic material with the aid of the RT-PCR method. The latter has proved to be a very reliable, rapid and sensitive method in a number of applications (either in its conventional or in the real-time format), allowing the simultaneous detection of other respiratory viruses in the same specimen, and it is being used in current large epidemiological cohort studies.

Nasopharyngeal secretions or throat swabs constitute the preferred specimens for all PIV detection methods, with the latter in general giving less satisfactory results (McLean, 2000).

Whole-virus Detection

Viral-particle identification can be achieved by electron microscopy either in nasopharyngeal aspirates and lavages or in lung biopsies. Microdrops of secretion or garglings are placed on electron-microscope grids (McLean, 2000). Negative-staining electron-microscopic examination can be performed immediately, and virions morphologically identified as paramyxoviruses (~140 nm total diameter, roughly spherical, see 'Structure'). Concentration by centrifugation or use of antisera may be necessary in cases of very low levels of viral particles within particular specimens. In addition, discrimination between orthomyxoviruses and paramyxoviruses is difficult since they have similar electron-microscopic appearance. Although quick, this method would additionally require immunofluorescence for HPIV subtyping.

Protein-based Methods

Protocols for immunofluorescent-based subtyping require the centrifugation of nasopharyngeal secretions. Throat swabs can also be directly smeared on to slides,

which are then dried and fixed in acetone. HPIV-specific antibodies can then be applied for direct or indirect immunofluorescence-based detection. This can be also conducted in combination with antibodies against other respiratory viruses. In a relevant protocol the RSV-specific antibody used was rhodamine-labelled, whereas antibodies against HPIV1–3, influenza A and B and adenovirus were labelled with fluorescein. Following RSV test in one spot, a second reaction in another spot determines which of the other viruses is present. The sensitivity of this very rapid method was ranked as higher than enzyme-linked immunoassay (EIA), but lower than cell culture (Landry and Ferguson, 2003).

Whole-virus intranasal inoculation in hamsters or guinea pigs produces hyperimmune sera used in HPIV serologic tests. Monoclonal antibodies to all four serotypes of HPIV have also been produced, which show minimal cross-reactivity, in contrast to commercially-available antisera (Henrickson, 2003). Immunofluorescent foci in the cytoplasm of cells exposed to an antibody specific for a given subtype, which are absent when the specimen is incubated with antisera against another subtype (or virus), document serotype-specific HPIV infection of the patient. This method, when used in combination with electron microscopy, allows same-day confirmation of HPIV infection. It is also the most rapid and sensitive method available for HPIV detection and subtyping in tissue culture (Henrickson, 2003). However, the timing of sampling is of crucial importance since acute infection, during which viral shedding is greatest, may have occurred before hospitalization. Moreover, the detection of antigen in nasal specimens of immunocompromised and elderly patients has been claimed to be a rather insensitive method (Englund *et al.*, 1997; Sable and Hayden, 1995; Whimbley and Ghosh, 2000). Finally, direct immunofluorescence staining of HPIVs in clinical material shows considerable variability in sensitivity, although specificity is universally high (reviewed in Henrickson, 2003). The best results were obtained with HPIV3.

In addition to conventional immunofluorescence microscopy, flow-cytometry analysis with or without PCR can also be used for the detection of PIVs (Lee *et al.*, 2007). EIA, radioimmunoassay (RIA) and fluoroimmunoassay (FIA) techniques have been applied to PIV detection, showing sensitivities between 75 and 95% with polyclonal sera (Henrickson, 2003).

Virus Culture

For the virus to be recovered by tissue-culture techniques, the nasopharyngeal secretions or throat swabs are typically resuspended in 2 ml cell-culture medium containing 10% fetal bovine serum (FBS). Viral suspensions retain viability in this medium and can be kept, if necessary, for

24 hours at 4°C before being transferred to tissue culture. For longer delays, freezing at -70°C is recommended. Susceptible cultures are primary human fibroblast or primary monkey kidney (PMK) cell cultures and several continuous cell lines (e.g. the monkey-kidney-derived LLC-MK₂, CV-1, Vero, HMV-II, HEp-2, MDCK, L929, HeLa cells, etc.) (Henrickson, 2003). The infection protocol should be performed both at 37°C and at 33°C, since some strains seem to prefer the lower temperature conditions prevailing in the upper respiratory tract. Most HPIV clinical isolates can be detected 10 days after initial inoculation. However, this period may be unacceptably slow in emergency cases in the clinic. Recently, mixtures of cell lines have shown improved viral detection abilities: co-cultures of the influenza-susceptible mink-lung-derived Mv1Lu cells and the human adenocarcinoma A549 cell line (R-mix) have been shown to synergistically detect respiratory viruses with greater sensitivity than single cultures. A relevant protocol consists of the inoculation of R-mix monolayers with a clinical sample on coverslips; 24 hours later a coverslip is stained with a panel of antisera against different respiratory viruses, and, given a positive result, cells from parallel coverslips are further examined for the presence of individual viruses with monoclonal antibodies. HPIV, influenza and RSV have been detected with this method up to four days earlier than the conventional cell-culture technique and with equal sensitivity (Barenfanger *et al.*, 2001).

Upon propagation in cell culture, haemagglutinin-containing viruses such as HPIVs can be identified by their ability to induce erythrocyte haemadsorption by infected cells. For this the supernatants should be removed and the cell monolayer exposed to 0.1% suspension of guinea pig or avian erythrocytes in saline. Haemadsorption is then observed under a light microscope. By adding 0.5% suspensions of guinea pig or avian erythrocytes to cells plated in 96-well plates and inoculated by twofold serial dilution of viral preparations, the titre of specimens can be determined with precision (McLean, 2000). Since the same effect can be mediated by all HPIVs, as well as by the influenza viruses, serotyping (haemadsorption inhibition test, HA_I) of the viral isolate is then necessary for confirmation of the patient's virus type or subtype. To that end, 250 µl suspensions containing 10 antibody units of antisera raised against each PIV and influenza serotype are mixed with the fresh, titrated viral isolate, diluted so that the latter contains four haemagglutinating doses. Since even in normal sera activities inhibiting PIV-mediated haemagglutination are present, an inactivation step should be performed before the haemadsorption test. Such nonspecific activities are sensitive to heat or periodate treatment or incubation with receptor-destroying enzyme (RDE); the latter

binds to and inactivates serum-derived HPIV-binding activities (Canchola *et al.*, 1965; Smith *et al.*, 1975). After 10 minutes incubation at room temperature, 0.5% suspensions of erythrocytes are added and the serotype against which the specific antiserum inhibits haemadsorption will coincide with the serotype of the viral isolate. Alternatively, cells inoculated with specimen and showing haemadsorption can be placed on slides and incubated with anti-PIV antibodies for immunofluorescent detection, as described above. Although both procedures can be completed within 2–3 hours, the rate-limiting step is the previously-conducted tissue-culture isolation, rendering the method a complex and time-consuming process.

Serological Techniques

The prerequisite for serological identification of PIV infection is the collection of both acute-phase and convalescent blood samples. The former should be collected as soon as possible after onset of the symptoms, whereas the latter is obtained later, usually after two to six weeks. The paired sera should then be treated with heat and periodate or receptor-destroying enzyme to inactivate inhibitory substances and should be applied as serial twofold dilutions in HI tests against four haemagglutinating doses of each parainfluenza serotype (McLean, 2000). Virus infection is considered to be confirmed if antibody conversion from negative to positive can be documented. Alternatively, if both sera are positive, a fourfold difference in antibody titre is considered confirmation of serological evidence of infection with the respective PIV serotype. Again, although the final step of this method is quick (two hours), the requirement of the specimen obtained during convalescence limits its usefulness to retrospective diagnosis of acute illness. Furthermore, this method is not that sensitive for young infants, where antibody may be detected only after recurrent infections in some cases (McLean, 2000).

Apart from HI antibodies, neutralizing and complement-fixing antibodies can also be detected in patients' paired sera, although the latter method is thought not such a useful test for clinical diagnosis (McLean, 2000). For neutralization tests, cultured cells should be inoculated with 100 tissue culture infective doses of PIV subtypes mixed with twofold serial dilutions of patients' sera. Three days later haemadsorption assays should be inhibited by positive sera. RIA and enzyme-linked immunosorbent enzyme (ELISA) tests are also available for detection of antibody responses to HPIV infection, though they are not widely used.

An inherent problem in serologic diagnosis of acute HPIV is that ELISA and HI tests are not able to discriminate between HPIV1 and HPIV3, whereas mumps

antibodies can cross-react (Henrickson, 2003). Complement fixation may serve as an alternative method since it is characterized by minimal heterologous cross-reactivity; however, it is the least sensitive of the serologic assays.

RNA-based Diagnosis

RT-PCR protocols allow the specific detection of HPIV genetic material (RNA) in clinical specimens (Karron *et al.*, 1994). Total RNA is extracted from nasopharyngeal secretions or throat swabs previously kept at -70°C in appropriate virus transport medium (VTM) (e.g. Hanks balanced salt solution enriched with 0.5% bovine serum albumin (Corne *et al.*, 1999)) to avoid deterioration of genetic material. Throat swabs can be broken directly into a denaturing solution to inactivate all RNase activity and then stored or transported at room temperature (Carman *et al.*, 2000). Extracted RNA is reverse transcribed into cDNA and the HPIV cDNA is then subjected to conventional PCR (single-round or nested), multiplex PCR (simultaneously detecting more than one pathogens) or real-time PCR (can be also multiplex) with HPIV-specific primers. In such protocols, serotype-specific primers have been designed, usually against the HN or P coding regions of HPIV1–4, exploiting the differences in nucleotide sequence occurring between these species (Aguilar *et al.*, 2000; Corne *et al.*, 1999). Nucleotide diversity observed within each serotype (e.g. Henrickson and Savatski, 1992; van Wyke Coelingh *et al.*, 1988) should also be taken into account and areas of low homology should be selected for serotype-specific primer-pair design (Corne *et al.*, 1999). This method can be readily accomplished within one working day, a fact of obvious importance for the effective treatment of patients. PCR products (amplicons) can also be sequenced and the diversity of individual serotypes within characteristic genes such as HN can be studied, providing valuable epidemiological information concerning particular HPIV outbreaks (Echevarria *et al.*, 2000; Jalal *et al.*, 2007).

Conventional, nested and multiplex RT-PCRs are available for simultaneous determination of HPIV serotypes alone (Aguilar *et al.*, 2000; Corne *et al.*, 1999; Echevarria *et al.*, 1998) or other respiratory viruses (Fan *et al.*, 1998; Freymuth *et al.*, 1997; Grondahl *et al.*, 1999; Hindiyeh *et al.*, 2001; Kehl *et al.*, 2001; Liolios *et al.*, 2001) from clinical samples.

The well-known Hexplex (Prodesse, Waukesha, WI) formulation is a multiplex method allowing the simultaneous detection of HPIV1–3 and other respiratory viruses (e.g. influenzas A and B, as well as RSV A and B) that has achieved high sensitivity and specificity scores in clinical samples (Kehl *et al.*, 2001). Such highly-sensitive protocols allow the detection of HPIVs in samples with low virus copy numbers, or of viral RNA without presence of

live virus, both of which may be missed by conventional cell-culture techniques (Fan *et al.*, 1998). The detection limits of multiplex RT-PCR protocols can go down to 1 tissue culture infectious dose (TCID₅₀) for cultured PIV viruses (Corne *et al.*, 1999) or 100–140 copies per ml, depending on the serotype (Fan *et al.*, 1998). The sensitivity of this highly-specific and fast method is generally at least as high as combined tissue culture and immunofluorescence (Corne *et al.*, 1999), and sometimes even more sensitive (Aguilar *et al.*, 2000; Fan *et al.*, 1998). Multiplex real-time PCR methods for HPIV detection are also available (Hu *et al.*, 2005). In a relevant study a two-tube multiplex reaction in combination with molecular beacons was used to discriminate all four HPIVs, RSV and influenza viruses (Templeton *et al.*, 2004). The minimal amount of detectable HPIV1, HPIV2, HPIV3 and HPIV4 RNA was 0.01, 0.001, 0.1 and 0.0001 of the TCID₅₀, respectively. When applied to clinical samples this proved more sensitive than cell culture and, importantly, considerably less time-consuming (it can provide results within six hours). In addition, real-time PCR protocols applied for the detection of panels of respiratory viruses including HPIVs show increased diagnostic efficiency in comparison to cell culture and direct immunofluorescence-based diagnosis (van de Pol *et al.*, 2007).

A combination of RT-PCR with enzyme immunoassay techniques (RT-PCR-EIA), consisting of hybridization of the HPIV amplicon with biotinylated RNA probes generated against amplicon internal sequences, has also been used (Karron *et al.*, 1994). In addition, new emerging techniques achieve high sensitivity scores by probe hybridization and electroluminescence, or by molecular beacons (Hibbitts *et al.*, 2003). Finally, methods combining multiplex RT-PCR and microsphere flow cytometry (MultiCode-PLx system; Eragen Biosciences) have been reported to rapidly and efficiently detect multiple respiratory pathogens, including PIVs (Lee *et al.*, 2007; Nolte *et al.*, 2007).

The fast-developing array technology has been also employed to detect viral pathogens. Microarrays containing 1600 oligonucleotides targeting viral genomes were used to identify up to 140 distinct viral prototypes including HPIVs (Wang *et al.*, 2002). A recently-developed commercial custom-designed resequencing respiratory pathogen microarray by Affymetrix needs further validation, but initial promising results have been reported (Lin *et al.*, 2006). Provided it is able to achieve high sensitivity scores, such methodology may replace current detection protocols in the future, mainly due to its ability to handle large amounts of data and to provide more composite information concerning the pathogens than the other methods.

PREVENTION

There are no established anti-HPIV vaccines available for clinical use. Early attempts with formalin-inactivated whole HPIV1, 2 or 3 viruses failed, probably due to inadequate neutralizing activity against the native HPIVs and/or failure to induce mucosal immunity against HPIV HN and F proteins. Currently, vaccination strategies include immunization against particular proteins of HPIVs (i.e. HN and F), the use of attenuated strains (e.g. animal strains or strains obtained *in vitro* by cold-adaptation) and engineered approaches (i.e. recombinant animal/human viruses or constructs containing genetic material of multiple viruses generated by reverse genetics).

BPIV3 is a promising vaccine candidate against HPIV3, with which it shares sequence and antigenic similarities (Clements *et al.*, 1991). The bovine subtype is characterized by attenuated replication in the respiratory tract of primates; however, it is immunogenic and protective against HPIV3 challenge. The antibody responses to BPIV3 vaccination have been evaluated in phase 2 clinical trials in infants (Lee *et al.*, 2001). Bovine-attenuated PIV3 induced antibody production in seronegative children (Karron *et al.*, 1995a). The safety characteristics of BPIV3 vaccine were evaluated in a phase II trial, where vaccinees of two groups received doses of either 1×10^5 or 1×10^6 TCID₅₀ (Greenberg *et al.*, 2005). Both groups showed similarly good seroconversion rates and minimal BPIV3 isolation after the fourth dose, suggesting the vaccine efficiently immunizes and is being well tolerated by children.

Another attenuated, serially-passaged (passage 45), cold-adapted human PIV3 (Hall *et al.*, 1992) gave promising results in both seronegative and seropositive children and infants aged from 6 months to 10 years (Karron *et al.*, 1995b). In addition, the cold-adapted HPIV3 cp45 was stable and did not revert to wild-type as reported with previous early-passage HPIV3 approaches, whereas it is more immunogenic than BPIV3. A phase II trial examined the properties of HPIV3 cp45 on a vaccination protocol in 380 children (226 seronegative for HPIV3) aged 6–18 months (Belshe *et al.*, 2004b). No adverse effects were reported for the 15-day period following immunization and the vaccine appeared both safe and efficient for seronegative children, 84% of which developed more than fourfold increase in antibody titres. Furthermore, the HPIV3 cp45 has a low risk of transmission, as revealed by a protocol following the infection characteristics of seronegative children who were in close contact (play-groups) with infected vaccinees (Madhi *et al.*, 2006). In addition, this HPIV3 cp45 was used in combination with an RSV-attenuated strain in a phase I double-vaccination protocol (Belshe *et al.*, 2004a) and promising results were

obtained, showing that the combination did not affect the vaccine properties of the monovalent formulations.

In contrast to attenuated type 3 viruses, the type 1 SeV, although considered as a murine subtype, has been shown to replicate equally as well as human PIV1 in the upper and lower respiratory tract of primates (Skiadopoulos *et al.*, 2002a). This, together with the fact that this serotype was originally isolated from humans, has so far excluded its use as a vaccine in humans.

Recently, by using powerful reverse-genetics methodology, a recombinant infectious PIV virus was produced expressing the HN and F proteins of HPIV1 on the cold-adapted HPIV3 backbone mentioned above (Skiadopoulos *et al.*, 1999). This construct was proven to protect against HPIV1 challenge in primates (Skiadopoulos *et al.*, 2002b), while retaining its attenuated phenotype. In addition, by replacing the HPIV3 HN and F sequences with chimeric ORFs, encoding the HPIV2 ectodomains fused with the transmembrane and cytoplasmic domains of HPIV2, Skiadopoulos *et al.* (2002b) succeeded in generating a vaccine candidate against HPIV2.

This methodology allows inclusion of more than one antigenic viral glycoprotein in the attenuated backbone. Thus, a recombinant HPIV3 was generated that additionally expressed HN of HPIV1 and HPIV2; the emerging infectious virions conferred immunity against all three HPIVs in hamsters, while retaining the attenuated pattern of infection (Skiadopoulos *et al.*, 2002b). Further extension of the repertoire could include vaccination against other respiratory pathogens, such as RSV types A and B (Schmidt *et al.*, 2002). Conversely, reverse genetics was also used for the development of an attenuated influenza virus that, instead of HN of influenza, expresses that of HPIV. Mice immunized with this vaccine developed immunity against both viruses, which allowed them to survive lethal doses (Maeda *et al.*, 2005).

Additional strategies include the insertion of point mutations associated with temperature-sensitive or non-temperature-sensitive attenuation of HPIV strains. Using a combination of such mutations comprising the P/C, L and HN genes of HPIV1, Skiadopoulos and his group were able to identify a combination of the five examined genetic alterations that rendered the HPIV1 strain attenuated and stable (Bartlett *et al.*, 2005). The resulting recombinant HPIV1 rC(R84G/F170S)L(Y942A/L992C) was efficient as vaccine in African green monkeys. However, single mutations have been shown to allow reversion to wt HPIVs and larger deletions of two to six nucleotides have been employed to confer attenuation properties that are stable, while retaining immunogenicity. The attenuated phenotype seems to be due to disruption of C-mediated inhibition of IFN production and signalling (Van Cleve *et al.*, 2006). Individual deletions confer attenuation in

hamsters but not in African green monkeys, and vice versa, whereas by examining combinations, researchers end up with HPIV mutants that are stable, attenuated in both animal systems of experimental infection, and able to provide high immunogenicity and protection against native HPIV challenge. One such combination of mutations and deletions in C and HN proteins showed good characteristics and will be evaluated clinically as a live intranasal HPIV1 vaccine (Bartlett *et al.*, 2006), suggesting that this technique may be valuable for the establishment of candidate vaccines for clinical trials. Finally, insertion of mutations from heterologous paramyxoviruses into the L gene of HPIV2 generated mutants with attenuated phenotype when used in hamsters, which are candidates for HPIV2 vaccine development (Nolan *et al.*, 2005).

Additional viruses may be also used in such combinations, for instance RNA replicons based on an attenuated alphavirus backbone. Such constructs were engineered to express PIV3 HN and were able to elicit HN-directed immunogenicity in mice and hamsters, as well as to protect hamsters against PIV3 challenge (Greer *et al.*, 2007). Clinical studies evaluating the clinical efficacy of such elegant recombinant vaccine candidates in humans are awaited with particular interest.

TREATMENT

A major problem limiting development and use of efficient therapeutic treatments is that many clinicians do not count HPIV infections among the common pathogens in adult respiratory infections (Hall, 2001). The major clinical need is in young children with croup. Current therapy includes supportive care and steroid therapy if needed.

Studies investigating the action of steroids in mild to severe croup have been reviewed and analyzed (Moss *et al.*, 2002). Although smaller studies may bias results showing larger effects, it has been confirmed that nebulized steroids are effective in significantly reducing the severity of croup (e.g. improvement of croup scores by five hours) in children attending the emergency departments of hospitals. Notably, administration of immune-response stimulants or cytokines improved host defense to PIV challenge in *in vitro* systems and animal models (reviewed in Henrickson, 2003).

Croup is not the only manifestation of HPIV-mediated disease. Patients may develop tracheobronchitis, bronchiolitis, pneumonia or otitis media, each one requiring specific treatment. In extreme cases, excessive secretions may require emergency removal by bronchoscopic aspiration, whereas bacterially-complicated HPIV-triggered pneumonia requires antibiotics.

HPIV replication and pathology are currently targeted for the development of inhibitor drugs, synthetically

generated or derived from natural sources (Jiang *et al.*, 2001). The broad-spectrum antiviral agent ribavirin and its analogues are known to bind and inhibit HPIVs (Gabrielsen *et al.*, 1992; Ghose *et al.*, 1989). Aerosolized ribavirin has been used in immunocompromised patients for parainfluenza virus infections where the use of corticosteroids may lead to HPIV infection and pneumonia, both associated with mortality (Elizaga *et al.*, 2001; Englund *et al.*, 1997; Nichols *et al.*, 2001); however, the results were rather disappointing since ribavirin did not affect either the duration of viral shedding or mortality rates associated with HPIV3 infection (Nichols *et al.*, 2001). Nevertheless, it is still considered a potential therapeutic intervention in cases of immune deficiency where alternatives are not obvious (Stankova *et al.*, 2007).

Stages in the viral replicative cycle that are targeted for antiviral drugs include virus entry (HN binding to sialic receptors) and HN-mediated activation of F protein (reviewed in Moscona, 2005). A hydrophobic pocket in the C-terminus of the N-HR trimer, one of the heptad-repeat-containing peptides that are essential for fusion by F protein, may interfere with the stability of a hairpin intermediate. Accordingly, small molecules that bind to the hydrophobic pocket probably impair the fusion of both RSV and SV5. Sialic acid analogues, such as the 4-Guanidino-Neu5Ac2en (GU-DANA; zanamivir) originally designed to inhibit influenza infections (Greengard *et al.*, 2000), inhibit HN neuraminidase action and thus indirectly inhibit the triggering of fusion activity of F protein and syncytial formation by HPIV3 (Porotto *et al.*, 2001). These could therefore be potential candidates as inhibitory agents of viral entry and cytopathology. However, PIV variants (HN mutants) with increased receptor-binding avidity can escape the inhibitory effect of this agent (Murrell *et al.*, 2003), partly due to reduced affinity of the HN receptor-binding site for this compound, in parallel with increased avidity of the HN mutant for the original receptor (Porotto *et al.*, 2004). In addition, analogues designed to block neuraminidase function of NDV *in vitro* failed to block virus binding and entry, indicating that the differences among PIV members require that treatment of each one should be considered individually (Porotto *et al.*, 2004). Moreover, by interfering with HN-receptor interaction 4-GU-DANA enhances the release of newly-formed virions (Porotto *et al.*, 2001). Oseltamivir, another inhibitor of influenza neuraminidase, is less similar to the natural substrate than zanamivir but triggers more drug-resistant viral strains to be developed, in accordance with the notion that the less modifications the analogue contains in comparison to HN, the more efficient for clinical use it may be (Moscona, 2005). Several relevant analogues are currently being tested in members of the HPIV family (e.g. Ikeda *et al.*, 2004, 2006)), as

well as in combinations of HPIV and bacterial infections (Alymova *et al.*, 2005).

Synthetically-modified pyrimidine bases of uracil have been shown to specifically inhibit SeV replication (Saladino *et al.*, 2001). Further approaches include benzothiazole derivatives, carbocyclic 3-deazaadenosine (Cc3Ado), calcium elenolate, ascorbic acid, protein-synthesis inhibitors (puromycin) and nucleic-acid-synthesis inhibitors (reviewed in Henrickson, 2003). Endoplasmic reticulum α -glucosidase inhibitors (castanospermine and 1-deoxynojirimycin) known to block the trimming of N-linked glycosylation have recently been used and were shown to reduce the infectivity of newly-released HPIV3 particles, probably by inhibiting the first steps of HPIV3 envelope glycoprotein processing (Tanaka *et al.*, 2006). It is hoped that some of these innovative approaches may lead to effective strategies against HPIV infections in the future.

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Respiratory Syncytial Virus

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INTRODUCTION

*On the barely born
the Gods bestow
the most potential,
the greatest woe.*

C.B.H.

Respiratory syncytial virus (RSV) infections are a common and concerning conundrum. This ubiquitous agent is the major respiratory pathogen of young children, with life-threatening illness occurring most frequently in the first few months of life, and is commonly followed by the sequelae of recurrent wheezing. Worldwide acute respiratory disease is the leading cause of morbidity and mortality in young children, causing an estimated 4 million deaths each year in children within the first five years of life (Selwyn, 1990). Viruses are the most frequent cause, and RSV is the leading viral agent. Much about this virus, however, we do not understand—its immunity, its ‘inner soul’ and its control.

RSV was first isolated in 1956 (Morris *et al.*, 1956) from a colony of chimpanzees suffering from the common cold, and thus was initially called chimpanzee coryza agent (CCA). The human source of the pathogen was subsequently recognized by an identical agent being recovered from the respiratory secretions of an infant with pneumonia (long strain) and from a child with laryngotracheobronchitis (Chanock *et al.*, 1957).

The virus has now been recognized throughout the world as causing annual outbreaks of pneumonia and bronchiolitis in infants, and both upper- and lower-respiratory-tract infections in older children and adults.

In temperate climates peak periods of RSV activity generally occur in the winter and spring (Stensballe *et al.*, 2003). The predictability of its pattern of activity is singular among respiratory viruses.

THE VIRUS

RSV belongs to the order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Pneumovirinae* and in the genus *Pneumovirus*. In size, RSV lies between the larger paramyxoviruses and the smaller influenza viruses. The genome of the A2 strain of RSV is composed of 15 222 nucleotides, and the complete sequence of the A2 strain gene has now been delineated. RSV is an enveloped RNA virus with nonsegmented, single-stranded, negative-sense RNA genome, which encodes for 11 proteins. Each messenger RNA (mRNA) encodes for one major protein, except for M2 mRNA which encodes for two different proteins, M2 and M1. Eight are structural proteins (L, G, F, N, P, M, M2-1 and SH), two are nonstructural (NS1 and NS2) and M2-2 is a regulatory protein (Collins *et al.*, 2006). Of the structural proteins, three compose the viral capsid, N (nucleoprotein), P (phosphoprotein) and L (polymerase), and two are associated with the matrix membrane, the nonglycosylated M and M2 proteins. The other three structural proteins, the glycosylated F (fusion), the G (attachment) proteins and the small nonglycosylated hydrophobic protein, SH, are transmembrane surface proteins.

These two large glycoproteins, F and G, projecting from the surface give the envelope the appearance of a thistle (Figure 18.1) and make them integral to the immunity and pathogenicity of the virus. The G protein

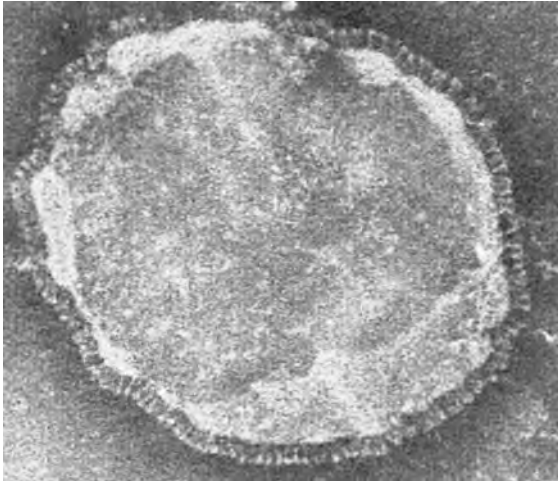


Figure 18.1 Negative contrast electron micrograph of RSV. The fringed envelope is variable in shape. The surface projections are about 15 nm long and the envelope contains a helical nucleocapsid which may occasionally be visible (negative contrast, 3% potassium phosphotungstate, pH 7.0; 6200 000). (Courtesy of Professor C.R. Madeley.)

is involved in initial attachment of the virus to the host cell and the F protein mediates penetration into the host cell by fusing the viral and host cell membranes. The G and SH proteins, however, augment this fusion process.

Neutralizing antibodies are produced to the F and G proteins, and fusion-inhibiting antibodies to the F protein. Antibody to the F protein also provides heterologous immunity to both strain groups, whereas antibody to G affords little protection against heterologous strains. Infants are able to produce both F and G antibody after initial RSV infection, but the response to the G protein is more variable. In young infants, the heavily glycosylated G protein is a poor immunogen, and pre-existing maternal antibody results in a greater degree of dampening of the infant's own antibody response.

Antigenic variation among strains of RSV has resulted in the two major strain groups, designated A and B, which have been delineated on the basis of their reactions with monoclonal and polyclonal antisera. The major antigenic differences between these two strain groups have been linked to the G protein (Collins *et al.*, 2006). The amino acid sequence of the G protein differs by about 50% between the two strain groups, and the antigenic relatedness is only 3–7%. The amino acid sequence of the F protein, on the other hand, is relatively conserved, allowing neutralizing antibody to the F protein to be cross-reactive. The F proteins of the prototype A and B strains exhibit greater than 90% amino acid homology and high antigenic relatedness. Intragroup variations also occur, with

an amino acid diversity for the G protein within the group varying from about 12% for group B to about 20% for group A (Cane, 2001; Cane and Pringle, 1995). In tissue culture, RSV produces a characteristic syncytial appearance with eosinophilic cytoplasmic inclusions. RSV has a growth cycle which, after inoculation, consists of a period of adsorption, with about 50% of the inoculum being absorbed in 2 hours, followed by an eclipse period of 12 hours. The subsequent log phase of replication of the new virus occurs over about 10 hours. At the time when maximal quantities of virus are obtained, about 50–90% of the virus remains cell-associated on the surface of the cell. Non-infectious incomplete virions comprise most of the cell-associated virus. Peak titres with the laboratory prototype group A virus (long strain) usually occur in 48 hours. Generally 10 plaque-forming units are produced for each infected cell. Under certain conditions, such as repeated high passage, continuous infection may develop, which is associated with a diminished amount of cell-free virus and a loss of the characteristic cytopathic effect.

EPIDEMIOLOGY

RSV is characteristically epidemic and global in distribution. Its seasonal pattern (Figure 18.2) is distinctive in that it predictably produces a sizeable outbreak of infections each year (Hall, 2001a; Kim *et al.*, 1973a). However, the timing and length of the outbreaks of RSV vary according to the geography (Mullins *et al.*, 2003; Stensballe *et al.*, 2003). Areas with warm or tropical climates tend to have more prolonged outbreaks, and some may detect the presence of RSV infections throughout the year. Nevertheless, certain months tend to have more pronounced swells of RSV activity. In the United Kingdom and the temperate parts of the United States, the peak period of RSV activity generally occurs from January through March, and in the USA the overall RSV activity is usually 20 or more weeks, stretching from the end of October to May.

The role of strain variation in the epidemiology, severity and clinical impact of an RSV outbreak remains unclear (Brandenburg *et al.*, 2000; Gilca *et al.*, 2006; Moore and Peebles, 2006; Papadopoulos *et al.*, 2004). Strains from both groups appear to circulate concurrently, although the proportion from each group may vary by year and locale (Cane, 2001; Hall *et al.*, 1990; Kim *et al.*, 2007b). In most areas in which this has been examined, A strains tend to dominate, and outbreaks composed almost entirely of B strains are uncommon. Studies of strains from cities across the United States have shown substantial annual differences, suggesting that the more influencing factors may be local rather than national (Anderson *et al.*, 1991). Homotypic immunity to previous strains

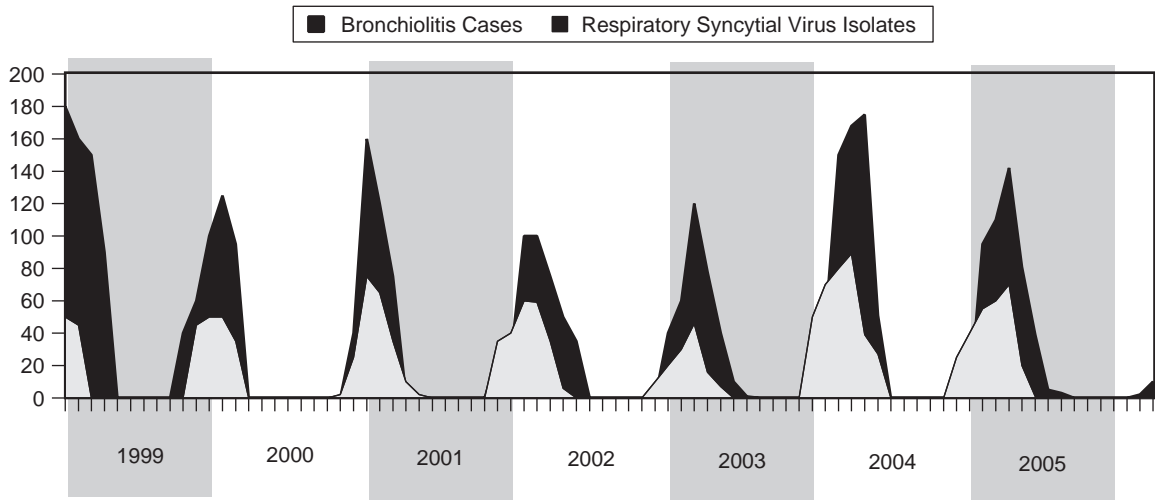


Figure 18.2 The number of cases of bronchiolitis and the number of isolates of respiratory syncytial virus from children under five years of age evaluated in private offices and outpatient facilities from 1998 to 2005, identified by a community surveillance programme in Monroe County, NY.

may also play a role, as several distinct, but varying, genotypes within a strain group appear to predominate each year in a community (Cane, 2001; Peret *et al.*, 1998). Some studies have suggested that the magnitude of an outbreak and the severity of clinical illness are greater with A-strain infections, while others have not. This indicates the difficulty in separating out the roles of strain variation from the collage of other environmental and host factors that contribute to the severity associated with RSV infections (Hall *et al.*, 1990; McConnochie *et al.*, 1990; Papadopoulos *et al.*, 2004; Toms, 1990).

In most places the size of the annual RSV outbreak will fluctuate. Nevertheless, over 11 consecutive years the number of hospital admissions for children with RSV lower-respiratory-tract disease in Washington, DC, did not vary more than 2.7-fold, demonstrating the consistent and persistent impact of this virus (Kim *et al.*, 1973b). During the period of peak activity, RSV is the dominant cause of lower respiratory illness in young children, despite the overlapping seasons of the other viral respiratory pathogens of young children, such as influenza, the parainfluenza viruses and human metapneumovirus (hMPV) (Hall *et al.*, 2005; Iwane *et al.*, 2004; Nicholson *et al.*, 2006; Wolf *et al.*, 2006).

The striking characteristics of RSV's epidemiology from the clinical aspects are first, it is highly contagious. Second, lower-respiratory-tract disease associated with primary infection is almost entirely confined to the child less than three years of age, and is most common and severe during the first six months of life. Third, re-infections occur throughout life and often within short

intervals (Glezen *et al.*, 1986; Hall *et al.*, 1991; Henderson *et al.*, 1979). Fifty per cent or more of infants have been estimated to acquire RSV infection during their first year of exposure to RSV, and approximately 40% of these infections will result in lower-respiratory-tract disease.

Essentially all children have been infected with RSV during the first three years of life. In a prospective longitudinal study of families in Houston, TX, Glezen *et al.* (1986) showed that 69% of infants in the first year of life were infected with RSV, and one third of those infections were associated with lower-respiratory-tract disease. In the second year of life, 83% of the infants acquired RSV infection, and 16% had lower-respiratory-tract disease. Even during the third and fourth years of life, one third to one half of the children were infected with RSV, and about one quarter had lower respiratory tract involvement (Glezen *et al.*, 1986). The attack rate in day-care centres is even higher, with 65–98% of the children becoming infected during each of the first three years of life and up to one half of the infections being manifest as lower-respiratory-tract disease (Henderson *et al.*, 1979). Recurrent infections, therefore, are not only common but may occur within a single season.

Transmission

Nosocomial Infection

RSV appears to spread with equal facility and alacrity on hospital wards housing young children (Berner *et al.*, 2001; Goldmann, 2001; Halasa *et al.*, 2005; Hall, 2000a; Hall *et al.*, 1979). The epidemiologic, immunologic and

clinical characteristics of RSV explain its propensity for becoming a major nosocomial hazard on paediatric wards. Epidemics occur yearly, resulting in the admission of a large number of young infants with primary infection, who tend to shed virus in high titres and sometimes for prolonged periods. Individuals of any age in contact with these infants compose a large susceptible population, as immunity is not durable. Most of these cross-infections result in symptomatic illness, and about half will involve the lower respiratory tract. The morbidity and cost are considerable, and the mortality may be high among young children with underlying prematurity, cardiopulmonary and immunodeficiency diseases (Berner *et al.*, 2001; Hall, 2000a; Hall *et al.*, 1979, 1986; Meissner, 2003; Weisman, 2003). Early recognition that RSV activity is present in the community and institution of appropriate infection control measures have been effective in reducing nosocomial infection rates as high as 40–45% among contact infants (Goldmann, 2001; Greenough, 2001; Hall, 1983).

RSV nosocomial infections may commonly involve both staff and adult patients. Among immunocompromised patients, especially transplant recipients, RSV infection tends to be serious and difficult to control (Champlin and Whimbey, 2001; Goldmann, 2001; Kim *et al.*, 2007a; Whimbey and Ghosh, 2000). Visiting children with mild colds are often the source. In compromised patients, nosocomially-acquired RSV infection frequently is not recognized as being caused by RSV but is thought to be one of the opportunistic infections to which such patients are prone, which may be clinically similar. The outcome, however, may be as devastating and even fatal.

Hospital personnel may also have a high rate of RSV infection, acquired nosocomially or in the community. This may appear insignificant since cases are frequently mild upper-respiratory-tract illnesses not requiring absence from work (Hall, 2000a, 2001b). These unnoticed illnesses of hospital personnel, nevertheless, may initiate and be integral to the continued spread of RSV on wards housing children or adults.

Modes of RSV Transmission

The spread of RSV appears to require close contact with infectious secretions, either by large particle aerosols or by fomites (Hall, 2000a; Hall and Douglas, 1981). Small particle aerosols, which can traverse greater distances, appear to be an infrequent mode of transmission for RSV. Hospital staff are apt to spread the virus by touching contaminated secretions or objects while caring for an infected patient. Self-inoculation may then occur by the inadvertent rubbing of their eyes or nose, the major portals of entry for RSV.

How long RSV remains infectious on inanimate objects varies according to the environmental conditions,

especially the dew point, and the type of contaminated surface. Generally, hard, nonporous surfaces allow longer duration of viable virus. During the winter months of the usual RSV outbreak, RSV in freshly-obtained secretions from hospitalized infants was recovered from countertops for up to 6 hours, from contaminated gloves for 1.5 hours, from cloth gowns and paper tissue for 30–45 minutes, and from the hands of hospital personnel for 20 minutes. Furthermore, infectious virus could be transferred to the hands of hospital personnel touching these contaminated surfaces and could be recovered from their hands for up to 25 minutes, thus allowing self-inoculation to occur and also transmission to other individuals via contaminated hands (Hall *et al.*, 1980). This underscores the observation that good hand washing is the single most effective means of controlling the spread of RSV (Hall, 2007a). Effective hand washing may be accomplished by traditional methods with soap or with other cleaners and water, or by the more recently preferred alcohol-based hand sanitizers, which provide extended protection (Centers for Disease Control and Prevention, 2002).

PATHOGENESIS

The incubation period for RSV is usually three to six days, but can range from two to eight days. Inoculation of the virus occurs through the upper respiratory tract, primarily the eyes or nose (Hall *et al.*, 1981). The virus subsequently spreads along the epithelium of the respiratory tract, mostly by cell-to-cell transfer along intracytoplasmic bridges. As the virus spreads to the lower respiratory tract it may produce bronchiolitis and/or pneumonia.

Pathology

Early in bronchiolitis a peribronchiolar inflammation with lymphocytes occurs, and the walls, submucosa and adventitial tissue appear oedematous (Hall, 2000b; Openshaw, 2005) (Figure 18.3). This progresses to the characteristic necrosis and sloughing of the bronchiolar epithelium, which may also be associated with a proliferative response of the epithelium. Plugging of these small airways results from the sloughed, necrotic material leading to obstruction of the flow of air, the hallmark of bronchiolitis. The tiny lumina of an infant's airways are particularly prone to obstruction from such inflammatory exudate and increased secretion of mucus.

If the bronchiolar lumina are incompletely obstructed, air trapping will occur distal to the sites of partial occlusion. Similar to a ball-and-valve mechanism, airflow is less impeded during the negative pressure of the infant's inspiration, but with the increased pressure of expiration,

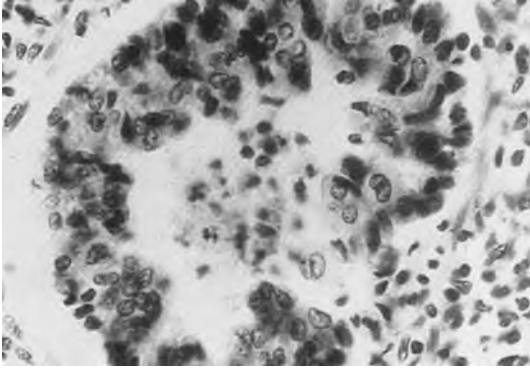


Figure 18.3 Pathological findings of an infant dying with RSV bronchiolitis. The wall of the bronchiole appears inflamed and oedematous, and sloughed, necrotic material fills the lumen in the small airway.

the airway lumen narrows, causing greater or complete obstruction to the flow of air. The trapped air causes the characteristic hyperinflation of bronchiolitis, which, when adsorbed, results in multiple areas of focal atelectasis.

Resolution of these pathologic changes may take weeks. Although the bronchiolar epithelium may show signs of regeneration in three to four days, ciliated cells rarely are present within the first two weeks. Other changes in the submucosal glands and types of cell in the airway may persist for even longer periods.

The pathologic findings of bronchiolitis may progress or coexist with those of pneumonia during RSV infection. In the latter, infiltration of the interstitial tissue with mononuclear filling and collapse of the alveoli are observed.

IMMUNITY

Immunity to RSV continues to be a major area of interest and research. Much progress has been achieved recently, but many of the complexities and conundrums remain unresolved. Multiple factors contribute to the variability of the immune response among different individuals. These include the host's epidemiologic and clinical experience, their genetic background and the characteristics of the virus (Openshaw, 2005; Openshaw and Tregoning, 2005; Moore and Peebles, 2006).

Innate Immunity

Innate immunity is evoked when RSV first infects the respiratory epithelial tissue and antigen-processing cells. The innate response is characterized by the synthesis of chemokines and pro-inflammatory cytokines and the

presence of inflammatory cells. The release of chemoattractant chemokines results in the trafficking of polymorphonuclear cells, eosinophils and interferon- γ (IFN- γ) secreting natural killer (NK) cells to the respiratory tract.

Toll-like receptors (TLRs) on alveolar and mononuclear cells appear to be important in mediating and characterizing the initial response. The viral-TLR interaction produces a cascade of immune-modifying and antiviral mediators. In animal-model experiments the components of TLR-mediated immune responses vary according to the TLRs involved. The effectiveness of the infant's innate immune response in modifying the initial and subsequent infection is unclear. However, this is likely affected by RSV's ability to evade certain antiviral components of the immune response, such as the IFNs (Johnson *et al.*, 2005).

Humoral Immunity

Specific maternal antibody is present in the cord blood of essentially all infants. Although the presence of this passive antibody has been suggested as playing a role in the pathogenesis of immune-mediated disease among infants with RSV, evidence has accumulated over the past two decades, indicating that maternally-derived antibody provides early protection for the infant. The level of passive neutralizing antibody in the cord blood has been correlated with an older age of first infection (Glezen *et al.*, 1986). This has been corroborated by experiments in animal models showing protection against RSV infection by passive administration of high-titred neutralizing antibody to RSV. The subsequent successful use of intravenous immunoglobulin with enhanced levels of RSV-neutralizing antibody for protecting high-risk infants against serious disease confirmed the beneficial effect of specific humoral antibody. This led to the current use of prophylaxis against RSV infection with monoclonal antibody for premature and other high-risk infants (American Academy of Pediatrics (AAP), 2006).

Primary RSV infection produces a specific, but transitory, serum IgM antibody response, followed by detection of IgG antibody during the second week after infection, which declines after one to two months. The neutralizing antibody response of young infants is generally blunted, with diminished responses to the F and G proteins. These proteins are glycosylated, especially the G protein, and thus are poor immunogens in infants. Maternal antibody also interferes with the antibody response, especially to the G protein. An IgA serum antibody response in young infants may not be detectable. After repeated infection an anamnestic response generally occurs in all three immunoglobulin classes. The serum antibody response to the F protein is broadly cross-reactive between the two strain groups, as the neutralizing epitopes are well conserved

among RSV A and RSV B strains. In contrast, antibody to the G protein is poorly cross-reactive and specific according to the group and genotype.

During the first six months of life, when the serum antibody responses are generally suboptimal, the infant's innate immunity is bolstered primarily by mucosal IgA (Welliver, 2003). IgA, IgG, IgM and IgE RSV-specific antibodies bound to epithelial cells and free in nasal secretions have been identified during the course of RSV infection in infants.

Cellular Immunity

Systemic and local cell-mediated immune responses appear to be key to the severity and recovery from RSV infection. This is supported by the observation that children and adults who have deficient cell-mediated responses with or without concurrent deficits in humoral antibody production tend to have prolonged, severe and sometimes fatal infection with RSV (Champlin and Whimbey, 2001; Hall *et al.*, 1986; Jarvis *et al.*, 1983; Whimbey and Ghosh, 2000). Recognition that the immune response to RSV may be integral in the pathogenesis of disease, indeed more important than the cytopathology engendered by direct viral invasion, came initially from studies aimed at explaining the experience with the first RSV vaccine, the formalin-inactivated vaccine, developed in the 1960s. In the trials of this first vaccine for RSV, those infants who received the vaccine developed more severe disease when subsequently exposed to the wild virus than did those children who had not received it; 80% were hospitalized compared to 5% of the control children (Chin *et al.*, 1969; Fulginiti *et al.*, 1969; Kapikian *et al.*, 1969; Kim *et al.*, 1969, 1976). Serologic studies demonstrated that the high levels of circulating antibody to the F and G proteins in the vaccinees were deficient in neutralizing and fusion-inhibiting capacity. In the lungs of fatal vaccinee cases peribronchiolar infiltration and increased levels of eosinophils were observed (Chin *et al.*, 1969; Murphy *et al.*, 1986).

Subsequent studies have suggested that integral to the pathogenesis of disease associated with both the formalin-inactivated vaccine and with primary infection is the T-cell response (Graham *et al.*, 1991, 1993; Moore and Peebles, 2006; Openshaw, 2005; Openshaw and Tregoning, 2005). These studies, primarily those using the Balb/c mouse model, demonstrated that the immune response during RSV infection varied according to whether it was elicited predominately by Th1 cells or by Th2 cells. Th1-dominant responses have been associated with viral clearance and control of the severity of clinical disease, and are characterized by induction of IFN-secreting CD4⁺ and CD8⁺ cytotoxic lymphocytes with production of IL-2, IFN- γ and IFN- α . In contrast, Th2-dominant responses are

primarily characterized by CD4⁺ cells producing IL-4, IL-5 and IL-13, pulmonary eosinophilic infiltration and IgE antibody, and are associated with the clinical manifestations of hyper-reactive airways and increased severity of disease (Openshaw and Tregoning, 2005; Openshaw *et al.*, 2003; Welliver, 2003;).

A fine balance of both Th1 and Th2 responses appears necessary for optimal clinical outcome. The multiple factors that affect this balance are only partially delineated, but include the infant's age, genetic background and the type of antigen provoking the immune response. Live virus and the F protein induce Th1-dominant responses, whereas inactivated virus, as in the formalin-inactivated RSV vaccine, and the G protein result in Th2-dominant responses. Although the experimental data have generally indicated a central role of Th1 versus Th2 cellular responses in determining the type and severity of disease associated with RSV, other studies have not confirmed this (Anderson *et al.*, 1994; Welliver *et al.*, 2002).

Information concerning the immune response remains incomplete, confusing and at times contradictory. However, several conclusions appear warranted: (i) the immune response to RSV is affected by multiple host and viral factors which currently prohibit predicting the clinical outcome of a child infected with RSV. (ii) The importance of the pathogenic role of the immune response in producing RSV disease is supported by the observation that *in vivo* the direct cytopathology produced by replicating RSV is only moderate and not necessarily concordant with the clinical manifestations. (iii) Innate immunity is important in the initial protection of the infant against primary infection, but also may quickly be altered by acquired immunity from T- and B-cell responses elicited during the characteristically high levels of viral replication which occur during primary infection (Openshaw, 2005). (iv) The acquired cellular immune response is integral in the clinical expression of RSV infection. Animal-model experiments suggest that the balance between the Th1 and the Th2 responses is critical in determining the immunologic and clinical outcome. These responses may be of prime importance during the first several months of life, when RSV infection most frequently results in hospitalization. During this period, the relatively weak Th1 responses may unfavourably tip the precarious balance of immunity and result in more severe disease in some infants.

CLINICAL FEATURES

Infection in Infants and Young Children

The importance of RSV as a cause of lower-respiratory-tract disease in the young is illustrated by the observation that the periods of peak occurrence of pneumonia

and bronchiolitis in young children signal the presence of RSV in the community. RSV has been reported in various studies as causing up to 10% of croup cases, 5–40% of the pneumonias and bronchitis in young children and 50–90% of the cases of bronchiolitis in infants. In the United States bronchiolitis has been shown to be the leading cause of all infant hospitalizations (Leader and Kohlhasse, 2003). The annual estimated number of hospitalizations for bronchiolitis attributed to RSV from 1994 to 1996 was 51 000–82 000 children within the first year of life and 62 000–100 000 children within the first five years of life (Shay *et al.*, 1999). RSV pneumonia was estimated to contribute another 11 000–44 000 hospitalizations for children under one year of age. In emergency departments, 22 visits attributed to RSV per 1000 infants have been estimated to occur each year (Leader and Kohlhasse, 2003). Worldwide, RSV is estimated to cause 600 000–1 million deaths among children less than five years of age (Simoes, 1999). In the United Kingdom approximately 3% of each year's birth cohort, approximately 20 000 infants, are admitted for bronchiolitis every winter, and 3% of these children need assisted ventilation (Sharland and Bedford-Russell, 2001). Similar admission rates for bronchiolitis have been estimated for Europe, Australia and North America. However, children with high-risk conditions may have substantially greater rates of admission (Carbonell-Estrany, 2003; Meissner, 2003; Nicholson *et al.*, 2006; Weisman, 2003). In the United Kingdom and North America, readmission rates for RSV bronchiolitis of premature infants (<32 weeks gestation) have been reported as about 6–8%, and 12–17% for infants with chronic lung disease. The mortality from RSV bronchiolitis, nevertheless, has been significantly reduced in recent years to an estimated 0.13% (PREVENT-Study-Group, 1997; Sharland and Bedford-Russell, 2001).

Primary RSV Infection

The first RSV infection is almost always symptomatic, but may be as mild as a common cold or as severe as a life-threatening lower-respiratory-tract infection. The manifestations of RSV infection initially observed in infants are usually those of a febrile upper-respiratory-tract infection. Lower-respiratory-tract involvement commonly becomes evident within several days. Although fever is common during the early phase of the illness, the infant may be afebrile by the time the lower-respiratory-tract disease becomes prominent and hospitalization is undertaken.

The harbinger of the lower-respiratory-tract disease is often a worsening cough. As the disease progresses, tachypnoea and dyspnoea develop, overtly marked by retractions of the chest wall. The hallmarks of bronchiolitis are wheezing and hyperinflation, often associated with a

strikingly elevated respiratory rate and sometimes with diffuse crackles. In pneumonia, the crackles may be localized or diffuse and may be accompanied intermittently by wheezes. Indeed, bronchiolitis and pneumonia often appear to represent a continuum and may be difficult to differentiate clinically. The variability in auscultatory findings and in the respiratory rate within short periods of time is common enough in infants with lower-respiratory-tract infection due to RSV to be considered characteristic. The course of the illness similarly may be variable, lasting from one to several weeks, but most infants will show clinical improvement within three to four days of the onset of the lower-respiratory-tract disease, and most children hospitalized with RSV are discharged within three to seven days, with an average of three to four days (Iwane *et al.*, 2004; Purcell and Fergie, 2004).

Evaluation of the severity of the lower-respiratory-tract disease in these young infants is often problematic. Multiple factors present during the initial evaluation have been associated with an increased risk of severe disease (American Academy of Pediatrics Subcommittee on Diagnosis and Management of Bronchiolitis, 2006). Among these are atelectasis on chest roentgenogram, an oxygen saturation by pulse oximetry of <95%, an appearance of clinical toxicity, and tachypnoea with a respiratory rate of ≥ 70 breaths per minute. An increased respiratory rate may result not only from RSV-induced compromised pulmonary function, but also from the concurrent presence of fever. Fever and the localization or extent of the wheezing or crackles generally do not correlate with the severity of illness.

The clinical assessment of hypoxaemia is difficult as clinically-important degrees of hypoxaemia may exist without the presence of cyanosis. The hypoxaemia results from diffuse involvement of the lung parenchyma, which may not be evident on chest roentgenogram, but causes an abnormally low ratio of ventilation to perfusion. Alveolar hypoventilation and progressive hypercarbia may develop but are rare in infants given good supportive care and supplemental oxygen if required.

The roentgenographic picture of infants with lower-respiratory-tract disease due to RSV may vary from a virtually normal appearance to one that mimics bacterial pneumonia. The severity of the infant's illness generally is not mirrored by the roentgenographic changes. Infants with the typical findings of bronchiolitis, hyperinflation and minimal peribronchial increased markings nevertheless may be severely ill and hypoxaemic. The most characteristic findings on the chest radiograph with RSV lower-respiratory-tract disease are hyperinflation, interstitial infiltrates—which are often perihilar, involving more than one lobe—and atelectasis, especially of the right-middle or right-upper lobes (Friis *et al.*, 1990;

Simpson *et al.*, 1974; Wildin *et al.*, 1988). This latter finding is commonly mistaken as resulting from bacterial pneumonia.

In the Newcastle upon Tyne (UK) studies of infants hospitalized with RSV lower-respiratory-tract disease, hyperinflation was present in over half the children and increased peribronchial markings in 39% (Simpson *et al.*, 1974). Hyperlucency, which occurred as the sole abnormality in 15% of the cases, was particularly associated with RSV infection. Consolidation was present in approximately one quarter of the cases and was usually subsegmental in distribution. The roentgenographic abnormalities, and sometimes the hypoxaemia, tend to persist beyond the time of clinical improvement.

RSV infection during the newborn period occurs less frequently than during the subsequent several months, but is not rare (Mlinaric-Galinovic and Varda-Brkic, 2000; Heerens *et al.*, 2002). The manifestations of the disease in the newly-born, however, may be atypical and missed clinically (Hall *et al.*, 1979). Neonates, especially in the first two to three weeks of life, commonly do not have the typical clinical findings of lower-respiratory-tract disease. More often RSV is initially manifested by such nonspecific signs as lethargy and poor feeding. Upper-respiratory-tract signs may be present in only about half. Premature infants appear to be more susceptible to infection, and the mortality may be high among those in neonatal intensive care units.

Primary infections may also be manifested only as upper-respiratory-tract disease or tracheobronchitis, especially in older infants and children. Ear infection associated with RSV infection is common, and 20–30% of infants hospitalized with RSV infection manifest otitis media. RSV may be recovered from 0 to 60% of middle ear aspirates of children with otitis media. It may be detected as the sole pathogen or in conjunction with bacterial agents. Epidemiologic and laboratory studies have indicated that RSV is a major cause of otitis media among infants and during the first years of life, and may have a more complicated course (Anderson, 2001; Chonmaitree *et al.*, 1992). In general, upper-respiratory-tract infections associated with RSV, whether primary or recurrent, tend to be more prolonged and severe than the usual cold (Hall, 2001b).

Acute Complications of RSV Infections

Apnoea is a frequent complication of RSV infection in young infants, occurring in up to 20% of hospitalized cases (Church *et al.*, 1984; Openshaw *et al.*, 2003). Apnoea may be the initial sign of RSV infection, preceding overt respiratory signs, and is non-obstructive. Apnoea is most likely to occur in premature infants with a gestation of 32 weeks or less and in those of young

postnatal age, less than 44 weeks postconceptional age. A history of apnoea of prematurity has also been identified as a significant risk factor for the development of apnoea with RSV infection. Although the prognosis for such infants has not been defined accurately, it does not appear to place the infant at increased risk of subsequent apnoea (Church *et al.*, 1984).

Infants with RSV bronchiolitis appear to be particularly predisposed to aspirate, which may be clinically manifest as airway hyper-reactivity (Hernandez *et al.*, 2002). One study conducted over a 12 month follow-up period showed that 83% of infants who had been hospitalized with RSV bronchiolitis developed reactive airway disease if given no ribavirin nor therapy for aspiration. However, among the infants who were given thickened feedings along with early ribavirin therapy, the proportion with episodes of reactive-airway disease was reduced to 45% (Khoshoo *et al.*, 2001). The reduction was greater with both ribavirin and thickened feedings than with either therapy alone.

In developed countries, secondary pulmonary bacterial infection or sepsis is an unusual complication (<1% of cases) (Hall *et al.*, 1988; Purcell and Fergie, 2004; Titus and Wright, 2003). In support of this are studies showing that antibiotic therapy did not result in an improved rate of recovery of infants with RSV lower-respiratory-tract disease (National Guideline Clearinghouse, 1998). The most frequent concurrent bacterial infection detected among children with bronchiolitis or RSV infection is urinary-tract infection (Levine *et al.*, 2004; Purcell and Fergie, 2004). The most likely reason for their coexistence is epidemiologic rather than RSV infection predisposing the infant to bacterial urinary-tract infection. RSV infections among hospitalized infants and the initial detection of urinary-tract abnormalities signalled by a urinary-tract infection commonly occur at the same age. In developing countries, however, concurrent pulmonary bacterial infection is more common and may be a major factor leading to the high mortality rate among infants in these countries.

More frequently co-infections which occur among young children with RSV infection are from other viruses (Glezen, 2004; Legg *et al.*, 2005). Usually these concurrent viral infections are from other respiratory viruses, which tend to circulate during the same season as RSV, such as influenza, parainfluenza viruses, hMPV, adenoviruses, rhinoviruses and bocaviruses. Whether children with these dual infections are more likely to have severe infection is unclear, but this may be affected by which virus is concurrently present and whether the virus is being detected during the acute phase of the infection or during a period of prolonged shedding from past illness.

Children at Increased Risk from RSV Infection

Certain groups of children appear to be at risk of developing complicated, severe or even fatal RSV infection (Meissner, 2003; Weisman, 2003). Among these the highest risk groups are those with chronic lung, cardiac and immunosuppressive conditions. In addition, acute or chronic conditions which affect a child's ability to handle respiratory secretions may augment the severity of the RSV infections. In children with nephrotic syndrome, cystic fibrosis and other chronic illnesses, RSV may also cause exacerbations and complications of their underlying disease. Among hospitalized infants with RSV infection the most frequent of the underlying conditions are those with pulmonary disease, especially chronic lung disease following prematurity. These children comprise about 10–15% of the infants hospitalized with RSV infection. Their risk of developing prolonged and complicated infection with RSV extends into the second year of life for those who continue to require medical therapy (Groothuis and Nishida, 2002; Meissner, 2003; Weisman, 2003). Infants of very low birth weight, <1500 g, and of gestational ages ≤ 28 weeks have the highest risk of severe and fatal illness (Holman *et al.*, 2003).

Although immunocompromised children are a smaller proportion of the high-risk children hospitalized with RSV infections, they are particularly important because of the severity of their infection and their susceptibility to nosocomial RSV infection. The degree of immunosuppression correlates with the risk of complicated infection. Thus, those with congenital immunodeficiency diseases and recipients of bone marrow or solid organ transplantation are at highest risk, and may develop lower-respiratory-tract disease at any age, which may be severe or even fatal (Whimbey and Ghosh, 2000; Champlin and Whimbey, 2001; Kim *et al.*, 2007a).

Young children with compromised cellular immune function during an acute or recurrent RSV infection may have higher viral loads than immunocompetent children of similar age, but not than young infants with primary infection. More notable is the prolonged and often intermittent shedding that frequently occurs in children with deficient cellular immunity. In the more compromised children, RSV may be detected for weeks to months in secretions from the lower respiratory tract, which may not be concurrently identified in the upper-respiratory-tract secretions. The viral loads and duration of shedding in these children is highly related to the diagnostic techniques. The rapid antigen tests applied to upper-respiratory-tract secretions are particularly variable in their sensitivity and reliability for diagnosis of these children. On the other hand, reverse transcriptase polymerase chain reaction (RT-PCR) assays are highly sensitive and specific, resulting in the

detection of more prolonged shedding among both immunocompromised and immunocompetent children. However, the clinical correlation with detection and quantitation of shedding by RT-PCR assays requires further study.

The risk of more complicated RSV illness among patients with human immunodeficiency virus (HIV) infection is less clear, but appears to vary according to the stage of their disease, the presence of concurrent viral and bacterial illnesses, and therapy. A limited number of children with RSV infection who are in different stages of HIV infection have been studied, but generally their illness appears less severe than among those who are severely immunosuppressed from transplantation and congenital immune-deficiency diseases (Chandwani *et al.*, 1990; King, 1997; Madhi *et al.*, 2000, 2001, 2002). In some studies the clinical outcome after viral respiratory infections, including influenza, has not differed appreciably between children with and without HIV infection (Madhi *et al.*, 2002).

Infants hospitalized in the first few months of life with uncorrected cyanotic congenital heart disease are potentially at particular peril (Meissner, 2003). Although all the factors or types of cardiac condition associated with a poor prognosis have not been defined, pulmonary hypertension accompanying congenital heart disease may increase the risk appreciably. Recent surgical and technical advances and early correction have substantially reduced the mortality and morbidity in infants with congenital heart disease. Nevertheless, their risk for fatal RSV disease still appears to be more than three- or fourfold greater than that estimated for other infants hospitalized with RSV infection (Fixler, 1996; MacDonald *et al.*, 1982; Meissner, 2003; Navas *et al.*, 1992).

Sequelae Associated with RSV Infections

The most frequent sequelae following RSV lower-respiratory-tract disease in early infancy is recurrent wheezing, sometimes associated with prolonged alterations in pulmonary function. The potential link between RSV bronchiolitis in early infancy and the later development of hyper-reactive-airway disease or asthma is an area of much interest, research and controversy (Douvillat *et al.*, 2007; Henderson *et al.*, 2005; Martinez, 2003, 2005; Openshaw *et al.*, 2003; Sigurs *et al.*, 2005; Stensballe *et al.*, 2006; Taussig *et al.*, 2003). Studies have reported rates of subsequent reactive-airway disease of approximately 30–50%, but most of these involved children who were hospitalized and thus had more severe bronchiolitis (Wennergren and Kristjansson, 2001). These episodes of recurrent wheezing tend to occur most frequently during the first couple of years after hospitalization and improve with age, lasting for several years or

up to adolescence. However, the link between reactive airway disease and RSV is not clearly defined. Whether RSV lower-respiratory-tract infection early in life is truly causal of these later sequelae is controversial.

Much of the current research has shown that the components of the cellular immune response elicited by RSV during bronchiolitis are those associated with asthma, predominately Th2, and the eosinophilic response observed in the lungs of animals with a Th2 response to RSV is similar to that seen in the secretions of children with an allergic response. Despite this, atopy does not appear to be the major factor in predicting which children will develop such long-term pulmonary abnormalities (Martinez, 2003). In a subgroup of children, however, atopy does appear to be a factor in increasing their risk of more severe RSV disease and the subsequent complication of recurrent wheezing (Welliver, 2000; Welliver and Duffy, 1993; Welliver *et al.*, 2002).

Multiple environmental, anatomic and other host factors appear to interact in both predisposing an infant to developing bronchiolitis with primary RSV infection and in augmenting the risk of recurrent wheezing. The importance of the genetic background in determining the variation in the clinical and immune response to RSV has been suggested by the increasing number of studies suggesting that certain gene variants (polymorphisms) correlate with a risk of more severe disease. Among these are certain variants of the genes encoding IL-4 and the IL-4 receptor α -chain, genes for IL-8, IL-10 and chemokine receptor (CCR) (Goetghebuer *et al.*, 2004; Hoebee *et al.*, 2003, 2004).

Infection in Older Children and Adults

Repeated RSV infections occur throughout life, and the interval between infections may be only weeks or months (Hall *et al.*, 1991). In older children and adults these repeated infections are usually manifest as upper-respiratory-tract infections or sometimes as tracheobronchitis. In a minority of adults, usually less than 15%, the infection may be asymptomatic (Hall, 2001a, 2001b). Even in young healthy working adults, and in military recruits, RSV infection causes considerable morbidity and work absenteeism, similar to that resulting from influenza (Hall, 2001b; O'Shea *et al.*, 2005). RSV infection has been shown to produce in young healthy adults hyper-reactivity of the airways to cholinergic stimulus, which may last for eight weeks after infection.

RSV infection in the elderly may be more severe and have some similarities to infection at the other end of the age spectrum (Falsey and Walsh, 2000; Falsey *et al.*, 2005; Nicholson *et al.*, 1997). In older individuals, RSV has been associated with pneumonia, exacerbations of chronic bronchitis and underlying cardiac disease, and can

produce a flu-like syndrome indistinguishable from that of influenza. RSV infection in older adults occurs more frequently than is generally recognized and has been shown to have an impact equal to that of nonpandemic influenza. Although the rates of RSV infection in this population vary from year to year and according to the general health of the individual, about 5–10% of those in assisted-living or long-term-care facilities will acquire RSV infection each year, and 2–5% will succumb. A large prospective study of viral respiratory infections among healthy elderly patients, high-risk adults and hospitalized adults showed that among those who were followed prospectively over four years, the incidence of RSV infection was 5.5% each year, which was nearly double that observed for influenza (Falsey *et al.*, 2005). Furthermore, the clinical manifestations and severity of the disease associated with RSV infection were similar to those from influenza. The morbidity from RSV infection was 8% and from influenza 7%.

DIAGNOSIS

The diagnosis of RSV lower-respiratory-tract illness is often made on the basis of typical clinical and epidemiologic findings (American Academy of Pediatrics Subcommittee on Diagnosis and Management of Bronchiolitis, 2006). Laboratory-confirmed diagnosis of RSV infection may be made by identification of the viral antigen by rapid diagnostic or molecular techniques, viral isolation or serology (Henrickson and Hall, 2007).

Viral isolation has been the standard upon which other diagnostic methods have been judged, but molecular techniques such as RT-PCR are increasingly becoming the gold standard. Viral isolation is dependent on the quality of the laboratory tissue-culture techniques, the specimen and the age of the patient. For infants, viral isolation can be sensitive and specific, but for older individuals tissue-culture diagnosis may be less sensitive, as individuals with a recurrent infection shed smaller quantities of virus and for shorter periods.

Nasal washes or tracheal secretions are generally the best specimens for isolation, but they are not as readily obtained as swabs of the upper respiratory tract. An acceptable alternative to nasal washes and lower-respiratory-tract aspirates is the combination of two swabs, one from the nasopharynx and one from the throat, placed together in one vial of viral transport media. The specimen should be inoculated on to tissue culture promptly and without subjecting it to major temperature changes. RSV is a relatively labile virus and withstands freezing and thawing poorly. Specific cytopathic changes usually appear within three to five days in sensitive continuous cell lines, such

as Hep-2. The use of shell vials allows earlier diagnosis (Figure 18.4).

The disadvantages of viral isolation include the required technical expertise, the number of days for identification (three to seven), the necessity to monitor consistently the sensitivity and quality of the cell lines used, and the expense. The major advantages of isolation techniques are the detection of concurrent viruses and having the virus available to freeze for future research.

Many rapid assays are commercially available for detecting RSV antigens, primarily immunosorbent assays (EIAs) and optical immunoassays (OIAs). They offer the advantages of ease of performance, rapidity and low cost. The sensitivity and specificity of these kits, however, may be highly variable and they should only be used during the peak periods of RSV activity. During periods of low prevalence, as at the beginning and end of the RSV season, the sensitivity and specificity fall markedly. These tests are also less sensitive in certain populations, such as the elderly and immunocompromised patients (Henrickson and Hall, 2007).

Both direct and indirect immunofluorescent reagents, utilizing either polyclonal or monoclonal antibodies, are available. They possess a high degree of specificity and sensitivity, about 95% if performed by experienced laboratory personnel (Figure 18.5). The reliability of these immunofluorescent techniques over the rapid EIA and

OIA assays is an advantage, but the required experience and time, along with the variable patterns produced by different antibodies utilized among vendors, are disadvantages.

Of the currently available methods, PCR assays are generally the most sensitive and specific and allow simultaneous detection of multiple viruses. The use of nested PCR assays augments the sensitivity, and RT-PCR assays offer additional benefits of a diminished number of procedural steps and, therefore, less time and chance for contamination. The major advantage these assays have offered for diagnosing RSV infection in children is illustrated by comparison of the use of RT-PCR to tissue culture. Of 983 nasal/pharyngeal specimens from children in Rochester, NY, 496 were determined to be positive for RSV, which was defined as the detection of RSV by RT-PCR two or more times and/or by isolation of RSV in tissue culture (Henrickson and Hall, 2007). Of these, 99% were positive by the RT-PCR assay, 51% by tissue culture and 49% by both methods. RSV was detected by viral isolation alone in only 1% of the specimens. The RT-PCR assays were equally sensitive for group A and B RSV strains. The advantages and continued improvements in these assays predict that they will become more widely available for clinical laboratories.

Multiple serologic tests are available, mostly in research laboratories. Neutralization assays and EIA, using

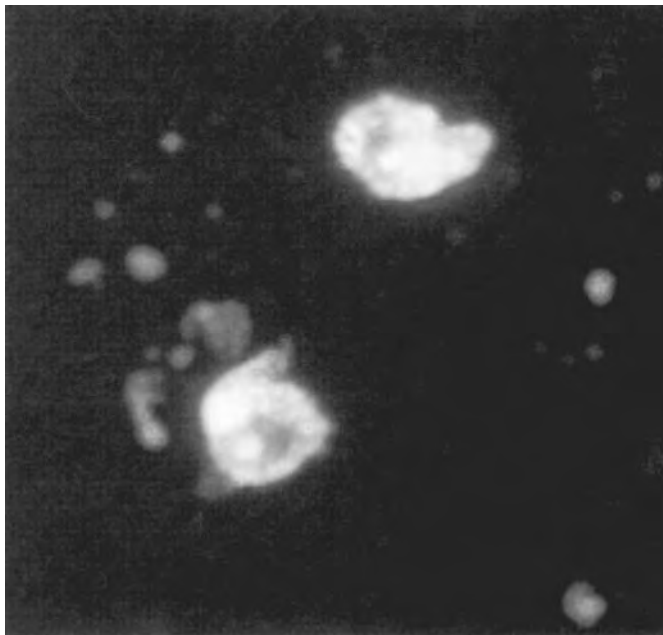


Figure 18.4 Positive indirect immunofluorescent antibody test on nasal secretions from an infant with lower-respiratory-tract disease due to RSV.

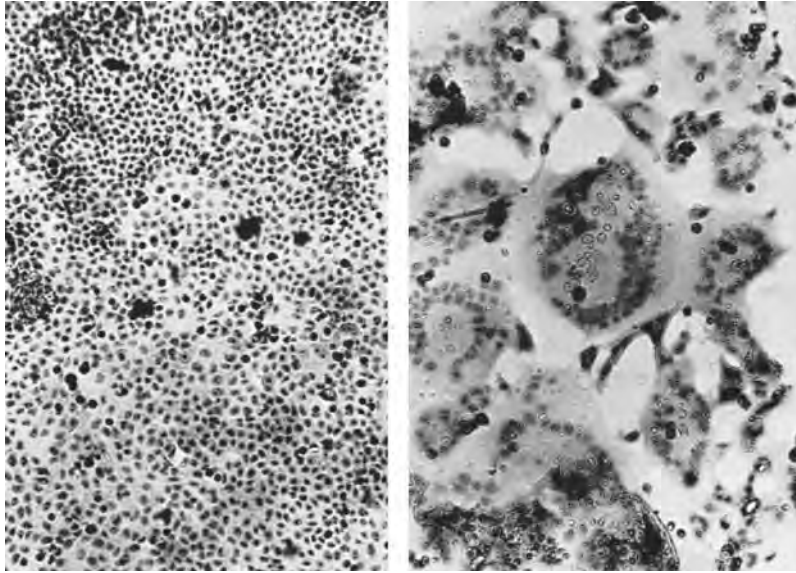


Figure 18.5 Stained preparations of uninfected HeLa cells (left) and cells infected with RSV (right). Syncytial formation is well illustrated by the infected monolayer.

the purified major RSV glycoproteins F and G, are most frequently used, and they offer the possibility of detecting specific antibody classes. Assays for IgM antibodies to RSV are of limited usefulness, as IgM antibodies are commonly not detectable in patients with proven infection and, if present, may appear anywhere from 6 to 40 days from the onset of the illness (Dowell *et al.*, 1996; Vikerfors *et al.*, 1988).

Serologic diagnosis by detecting antibody rises in acute and convalescent sera is rarely of help in the acute management of the patient since seroconversion usually does not occur for at least two weeks and may require four to six weeks. Furthermore, among 10–30% of patients some serologic assays may be falsely negative (Falsey *et al.*, 2005; Henrickson, 2004). Young infants in particular may have poor antibody responses which are not detectable by some antibody assays.

MANAGEMENT

Most infants do well with their RSV infection with just the usual care given for fever, maintenance of adequate hydration and clearing of the nasal passages by gentle bulb suction as necessary. For the more severely affected infants with lower-respiratory-tract disease, the quality of the supportive care is most important (Panitch, 2003). Documentation of the blood gases in the more severely ill hospitalized infant is essential to determine the need

for supplemental oxygen. Most infants respond well to relatively low concentrations of oxygen, since the major parenchymal abnormality is an unequal ratio of ventilation to perfusion. In the severely-ill infant, frequent assessment of the blood gases may be required to monitor for hypercarbia, and if progressive this may indicate the need for airway intervention. Hypercarbia and recurrent apnoea are the most frequent reasons for assisted ventilation. Hypoxaemia, unresponsive to oxygen therapy, is less common.

The antiviral agent, ribavirin (1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide), is the only agent currently approved for specific treatment of RSV infection (Jafri, 2003). Ribavirin is a synthetic nucleoside which appears to interfere with the expression of mRNA and has an unusually broad spectrum of antiviral activity, affecting a variety of both RNA and DNA viruses *in vivo* and *in vitro*. No toxicity has been shown with ribavirin therapy (Krilov, 2002) and development of resistance to ribavirin by RSV strains or other viruses has not been observed. Ribavirin is administered by small-particle aerosol, usually for 12 or more hours per day, but may be in shorter or intermittent periods until clinical improvement, which is usually in two to five days. Analysis of eleven randomized trials of the use of ribavirin showed seven demonstrated benefit, and four did not (American Academy of Pediatrics Subcommittee on Diagnosis and Management of Bronchiolitis, 2006; King *et al.*, 2004). Studies examining the effect of ribavirin therapy on the long-term pulmonary sequelae have given mixed results. Because of the

heterogeneity of the studies and the relatively low numbers of patients studied, the use of ribavirin is variable and controversial. It is expensive and its relative cost to benefit is unclear. For these reasons the drug is not recommended for routine management of infants with RSV infection. Its use may be considered for those patients at high risk for severe disease, and the decision made according to the individual circumstances (American Academy of Pediatrics (AAP), 2006; American Academy of Pediatrics Subcommittee on Diagnosis and Management of Bronchiolitis, 2006).

Bronchodilators, glucocorticosteroids and antibiotics are commonly used for the treatment of RSV bronchiolitis (Behrendt *et al.*, 1998). Among hospitalized infants, 75–80% are administered bronchodilating agents and 10–40% receive glucocorticoids, yet neither of these is routinely recommended (American Academy of Pediatrics Subcommittee on Diagnosis and Management of Bronchiolitis, 2006). The multiple trials that have evaluated α -adrenergic bronchodilators have been variable in design, agents used, patients included and measured outcomes. Systematic reviews of their use for children with bronchiolitis have concluded that insufficient evidence exists to recommend their use routinely in children with their first episode of wheezing (AHRQ, 2003; American Academy of Pediatrics Subcommittee on Diagnosis and Management of Bronchiolitis, 2006). However, these guidelines do allow the option for a carefully monitored trial of inhaled bronchodilating agents for some cases. They further stress that such a trial should be carefully and objectively evaluated, and that bronchodilator therapy should be continued only if benefit is demonstrated.

The studies examining the use of glucocorticosteroids for bronchiolitis also have been generally of varied design, making comparisons difficult. However, several reviews analysing the randomized and controlled trials concluded the evidence was not sufficient for a recommendation of routine use (AHRQ, 2003; American Academy of Pediatrics Subcommittee on Diagnosis and Management of Bronchiolitis, 2006). Although two controlled studies evaluating oral glucocorticosteroid therapy for bronchiolitis showed benefit, a more recent and larger study involving 20 emergency departments did not (Corneli *et al.*, 2007). This placebo-controlled trial examined the outcome of 608 children 2–12 months of age who presented with their first episode of bronchiolitis. No significant difference in the rate of hospitalization or in the assessment of the respiratory status existed between those children who were administered one dose of oral dexamethasone (1 mg kg^{-1}) and those who received placebos. Also of note was that no significant benefit was observed among the subgroup of children with asthma or an atopic family history. This large, well-designed study supports the

current recommendations against the use of glucocorticosteroids for this defined population of first-time wheezers (American Academy of Pediatrics Subcommittee on Diagnosis and Management of Bronchiolitis, 2006; Hall, 2007b).

Antibiotics are commonly administered to young infants with RSV lower-respiratory-tract disease, even among children with laboratory-confirmed RSV infection. In part, this may relate to the concern that infiltrates from atelectasis represent bacterial pneumonia or secondary pulmonary bacterial infection, both of which are rare. Prophylactic use of antibiotics has not been shown to be effective and is not warranted among children with RSV infection. Antibiotics should be used only when specific indications of a bacterial infection are present.

Chest physiotherapy, cool-mist therapy, aerosolized therapy with saline and chest percussion are not recommended for the routine management of children with bronchiolitis (National Guideline Clearinghouse, 1998; Perlstein *et al.*, 1999).

PREVENTION

For decades now, research has been directed towards the development of a safe and effective vaccine for RSV (Domachowske and Rosenberg, 2005; Greenberg and Piedra, 2004; Moore and Peebles, 2006; Piedra *et al.*, 2003). Immunization for RSV, however, poses singular problems. A vaccine for RSV would need to be administered within the first few weeks of life, when maternal antibody is consistently present and when the repertoire of both T-cell and B-cell responses is limited, especially among high-risk premature infants, for whom the vaccine is most needed. For these same reasons determining the correlates of the immunogenicity and effectiveness of a vaccine in this age group is particularly problematic. Furthermore, since repetitive infections are the mien of RSV, an RSV vaccine ideally should be able to produce more durable immunity than that observed after natural infection.

Following the experience with the initial formalin-inactivated vaccine, subunit and especially live-attenuated vaccines have been sought. The advantages of live-attenuated vaccines over subunit vaccines are the potential induction of both local mucosal and systemic humoral immunity, better and cellular responses and intranasal administration.

Live-vaccine candidates have been developed by cold-adaptation (cold-passage, cp) and chemical mutagenesis to produce temperature-sensitive (ts) mutants that will replicate at the low temperatures (32–35 °C) of the upper respiratory tract, but not at the higher temperatures in the lower respiratory tract (Collins and Murphy, 2005). The

initial 'cp' and 'ts' vaccine candidates proved to be too reactive or overly attenuated, and some were associated with prolonged shedding of virus. The mutations produced by these methods tended to be genetically unstable, variable and unpredictable.

The development of reverse genetics, which allows the recovery of infectious RSV from c-DNA clones, has now allowed the development of specifically-defined mutations, to be incorporated into candidate vaccines. Multiple 'cp/ts' mutations generated by reverse genetics have been introduced singularly or in combination into recombinant wild-type RSV, and their level of attenuation evaluated in animal models. Further 'designer gene' candidate vaccines have been generated by reverse genetics of RSV mutant strains with single or multiple gene delineations. These appear promising and are currently considered among the prime candidates for immunization (Karron *et al.*, 2005).

Multiple subunit RSV vaccines have been developed, and several have progressed into clinical trials in adults and children. These vaccines have primarily focused on the major RSV surface glycoproteins, F and/or G, which are the major targets of neutralizing antibody. Several generations of the F subunit vaccines have been evaluated in trials of healthy and elderly adults, pregnant women, and healthy and high-risk children beyond a year of age. Some have been administered with carriers and adjuvants which can augment and modulate the type of immune response, such as liposomes, biodegradable microspheres and vesicles of immunostimulating complexes. While these vaccines have appeared safe and well-tolerated, they are not sufficiently immunogenic in seronegative infants, and the neutralizing antibody response is frequently suboptimal. The RSV F subunit vaccines are able to reduce the overall occurrence of RSV infection, but not the incidence of lower-respiratory-tract disease if infection of the upper respiratory tract has already been established (Simoes *et al.*, 2001). Higher levels of neutralizing antibody have been induced in adults by a subunit vaccine containing purified F, G and M proteins from an RSV A strain, but the antibody levels obtained were not durable.

The hurdles currently existing for the development of an effective vaccine for RSV indicate that immunization for RSV in infants is unlikely to be available soon. Some of these candidate vaccines, however, could become available in the interim and provide some protection for groups of older individuals who are at high risk for RSV infection, such as the elderly and children with chronic underlying conditions, and could result in considerable progress toward the goal of controlling, if not eliminating, RSV infections.

Alternative means of protection against RSV infection have focused on diminishing the severity of RSV disease

in high-risk infants. The use of passively-administered humanized monoclonal antibodies became possible with the licensure of palivizumab, a humanized RSV monoclonal antibody, in 1998. Palivizumab was developed from a mouse monoclonal antibody that recognizes a protective epitope of the F protein. Two large randomized and placebo-controlled trials were integral in the licensure of palivizumab (Impact-RSV-Study-Group, 1998; PREVENT-Study-Group, 1997). These studies demonstrated that the rate of hospitalization for RSV illness was reduced over 50% among those infants who received prophylaxis, compared to those who received placebo. Among the different subgroups the hospitalization rates were reduced from 39 to 78%. Post-licensure studies have continued to show a significant reduction in the necessity for hospitalization among high-risk infants with RSV infection. The American Academy of Pediatrics currently recommends palivizumab prophylaxis for selected groups of children less than two years of age with chronic lung disease, prematurity and functionally-important congenital heart disease. Palivizumab is recommended in the United States to be administered in five-monthly doses to span the major months of RSV circulation. Because the potential benefit compared to the expense will vary according to the child's risk factors, such as gestational and postnatal age and presence of underlying conditions, the recommendations are subject to variable interpretation and often the decision must be based on individual circumstances.

Palivizumab is not currently recommended to diminish the risk of subsequent wheezing, as this use has not been well studied. However, a cohort study of premature infants who did and who did not receive palivizumab were prospectively followed for two years for the occurrence of recurrent wheezing. Those who had received prophylaxis had a significantly greater reduction in subsequent wheezing, suggesting palivizumab prophylaxis may have later beneficial effects in preterm infants (Simoes *et al.*, 2007).

Despite the proven efficacy of palivizumab in reducing hospitalization from RSV infection in high-risk infants, its use remains controversial. The economic analyses have varied, but most have not demonstrated an overall saving in health-care costs when the majority of high-risk children receive prophylaxis. Palivizumab has not been shown to be effective in the treatment of RSV disease, nor has it been demonstrated to diminish significantly the rate of mortality for infants with RSV. However, the mortality rate currently from RSV infections is extremely low. Further generations of humanized recombinant monoclonal antibodies derived from palivizumab have been developed. One of these, motavizumab, which has a binding affinity 70 times greater than that of palivizumab, was

Table 18.1 Infection control procedures, both standard precautions and contact precautions, for prevention of respiratory syncytial virus (RSV) infection. Recommended by Centers for Disease Control and Prevention

Recommendation category, procedure	Comment(s)
Category 1-B recommendations ^a	
Hand washing	Water with soap or antibacterial agent or waterless antiseptic hand rub
Wearing gloves	Combined with hand washing before and after each glove change; may diminish self-inoculation
Wearing gowns	When direct contact with patient or patient secretions is likely
Wearing masks plus eye protection	Eyes and nose are major sites for inoculation
Housing patient in private room or in a cohort isolated from other patients	Patients with documented infection can be grouped and isolated from other patients; beds should be separated by >0.9 m
Use of dedicated patient-care equipment. Sometimes recommended with less or no supporting evidence	Equipment, including toys, assigned to specific patients
Staff assigned according to patient's RSV status	Specific staff care only for patients with RSV infection
Visitor restrictions during RSV season	Some qualify by restricting young children only
Screening visitors for illness during RSV season	Visitor assessed by trained personnel and/or advised by use of an educational patient-information list

^aCenters for Disease Control and Prevention 1-B recommendations based on 'strong rationale and suggestive evidence', strongly recommended for all hospitals and 'reviewed as effective by experts in the field'. Reprinted from Hall, 2000a; see also Centers for Disease Control and Prevention, 2003; Gardner, 1996.

recently demonstrated in a large phase 3 trial to result in a significantly greater reduction in hospitalization for RSV infection than that following palvizumab prophylaxis.

The prevention of RSV infection in normal infants, however, currently is not possible because of the ubiquitous and contagious nature of the virus. Infection-control procedures may be effective in limiting nosocomial spread of RSV, but are of limited effectiveness and feasibility in the home. Of the infection-control procedures employed to limit the nosocomial spread of RSV, annual education of staff and good hand washing are most important (Hall, 2000a) (Table 18.1). The use of gloves may be of benefit when compliance with consistent and careful hand washing is less than optimal. The routine use of gowns and masks has not been shown to be of additional benefit. The use of gowns, however, may be advisable during periods of close contact, during which an infant's secretions are apt to contaminate clothing. Since RSV primarily infects via the eyes and nose, surgical masks which cover the nose and mouth are of limited value, but eye-nose goggles have been shown to be beneficial (Hall, 2000a).

Other procedures of potential, but less well proven, benefit include isolation or cohorting of RSV-infected infants and assignment of nursing personnel to care for either infected or uninfected infants, but not both simultaneously. During epidemic periods the number of patient contacts and visitors should be limited. Cleaning of objects and surfaces contaminated with infant secretions is

also advisable. RSV is a relatively labile virus and can be eradicated from surfaces by most any cleaning agent and certainly by those that are routinely used in hospitals, including soap and water, detergent and chemical disinfectants. Elective admissions of infants with high-risk conditions should be avoided during the epidemic periods of RSV. For those infants who must be admitted, particular care must be employed to prevent such cross-infections.

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Adenoviruses

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INTRODUCTION

Adenoviruses were discovered in the early 1950s from a military recruit with atypical pneumonia. Epidemiologic studies soon identified these viruses to be the major cause of acute respiratory disease (ARD) in military recruits. Later, these viruses were described as proven agents of infant gastroenteritis and conjunctivitis. Adenoviruses are also associated with pharyngitis, pharyngoconjunctival fever (PCF), pertussis-like syndrome, keratoconjunctivitis, bronchiolitis, pneumonia, acute haemorrhagic cystitis (HC), hepatitis, meningitis, encephalitis, myocarditis, pericarditis, genital infections, pancreatitis and disseminated disease. Clinical manifestations vary with the host, the site of pathology, and the serotype involved. In recent years, adenoviruses have been increasingly recognized as significant viral pathogens with high morbidity and mortality, especially among immunocompromised patients. In addition, increasing attention is being given to their utility as vectors for gene therapy.

Adenovirus infections are common, have worldwide distribution and occur throughout the whole year. These infections are frequent during childhood, where they tend to be self-limited and induce serotype-specific immunity. Adenoviruses are endemic in the paediatric population; epidemics and outbreaks with higher morbidity and mortality can occur in children and adults.

Human adenoviruses belong to the *Adenoviridae* family and the *Mastadenovirus* genus. They are divided into six species, designated A through F. To date, 52 adenovirus

serotypes have been described. The recently-described serotype 52 has a pending species classification into species G, as proposed by Jones *et al.* (2007). About half of the known serotypes have been recognized as causing illness. Most of the higher numbers for serotypes have been isolated from immunocompromised individuals but not necessarily associated with disease.

Adenoviruses are medium-sized, non-enveloped icosahedral viruses with a single linear, double-stranded DNA molecule. They are highly resistant to inactivation and readily spread in closed populations.

Adenovirus diagnosis is mostly carried out using direct methods, including virus isolation in cell culture, antigen detection by immunofluorescence (IF), immunohistochemistry, genome detection with or without amplification and, to a lesser extent, electron microscopy (EM). Molecular methods using DNA amplification by polymerase chain reaction (PCR) have increased the sensitivity and rapidity of diagnosis. More recently, the use of 'real-time' PCR assays has permitted even faster results and the quantification of the virus, allowing therapeutic intervention, prognosis and the follow-up of patients with severe disease.

Although no specific treatment for adenoviruses is available, there is evidence that some antiviral agents such as cidofovir and ribavirin are useful in treating some infections.

Sensitive, rapid and early diagnosis is important, given the disease severity and high mortality rates in some patient populations.

DESCRIPTION AND CHARACTERISTICS OF THE VIRUS

History

Hilleman and Werner (1954) described the recovery of a new virus from throat washings obtained from a military recruit with primary atypical pneumonia during a respiratory epidemic in 1952–1953 at Fort Wood. Rowe *et al.* (1953) described the spontaneous degeneration of cultures of adenoidal tissue obtained by routine adenoidectomies in children by an ‘adenoid-degeneration agent’. Tests performed by both groups gave evidence of an immunological relationship between the two agents. These viruses were initially called ‘adenoid-degeneration agent’ (Rowe *et al.*, 1953) and ‘RI-67’ (respiratory infection from recruit #67) (Hilleman and Werner, 1954) but in 1956 the term adenovirus was adopted as viruses were first recovered from adenoid tissues (Enders *et al.*, 1956). Retrospective serological procedures demonstrated that adenoviruses were present and associated with ARD in soldiers at Fort Bragg during World War II.

Taxonomy

Adenoviruses are widespread in nature and have been isolated from human and many animal species. The family *Adenoviridae* has been subdivided into four genera: *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus* and *Siadenovirus* (Mayo, 2002).

The *Aviadenovirus* genus, from the Latin ‘*avi*’, meaning ‘bird’, is limited to viruses of birds.

The *Mastadenovirus* genus, from the Greek ‘*mastos*’, meaning ‘breast’, infects mammals and includes human, simian, bovine, equine, porcine, ovine, canine and opossum viruses (Benko *et al.*, 2005).

The *Atadenovirus* genus, named in recognition of the high A+T content in the genome, has been recovered from reptiles, birds, marsupials and ruminants.

The *Siadenovirus* genus, from ‘*Si*’ for ‘sialidase’, was named in recognition of the presence of a putative sialidase homologue gene and infects amphibians and birds.

The regions E1A and E1B, E3 and E4 are present only in the *Mastadenovirus* genus. In the other genera, the location and organization of these three early regions is very different.

Basic Virology

Six of the approximately nineteen *Mastadenovirus* species (formerly called subgenera) infect humans and are designated A through F, with species B further subdivided into subspecies B1 and B2. Species G has been proposed as a new species. Species classification is defined by immunological, biological and biochemical characteristics, specifically on the basis of oncogenicity in rodents, the differential haemagglutination patterns with erythrocytes from different species, the lengths of their fibres and the percentage of G+C in the genome (Table 19.1). Transformation occurs primarily in rodent cells infected with species A serotypes, which integrate into the host genome and induce cellular replication without producing infectious virus. Although all adenoviruses can transform cells in tissue culture, there is no clear evidence that they cause cancer in humans.

Different adenovirus serotypes are described within one species. A serotype is defined on the basis of its immunological distinctiveness, as determined by quantitative neutralization with animal antisera. Initially only 24 antigenically-distinct adenoviruses were described. In 1960, serotypes 25–33 were recovered from stool samples and were not associated with known diseases. Types 40

Table 19.1 Classification of the 52 human adenovirus serotypes by species characteristics

Species	Serotypes	Oncogenic potential	% G+C	Haemagglutination		Fibre length (nm)
				Rhesus	Rat	
A	12, 18, 31	High	48–49	–	+ /–	28–31
B1	3, 7, 16, 21, 50	Weak	50–52	+	–	9–11
B2	11, 14, 34, 35	Weak	50–52	+	–	9–11
C	1, 2, 5, 6	None	57–59	–	+ /–	23–31
D	8–10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39, 42–49, 51	None	58	+ /–	+	12–13
E	4	None	57–61	–	+ /–	17
F	40, 41	None	57–59	–	+ /–	~29
G (proposed)	52	Nd	Nd	Nd	Nd	Nd

Nd: not determined yet.

and 41 were associated with gastroenteritis, and the last nine serotypes (43–51) were isolated from AIDS patients (de Jong *et al.*, 1999). In 2007, the new strain postulated as adenovirus 52 was identified in an outbreak of gastroenteritis (Jones *et al.*, 2007). Within one serotype there may be different genotypes, which suggest changes of the genomic DNA not associated with serological changes. Genome typing can be done by restriction endonuclease analysis of full-length viral DNA, by PCR or by sequencing. Forty genotypes for adenovirus 2, twelve for adenoviruses 7 and eleven for adenovirus 11 are some of the examples obtained by restriction enzyme analysis (Adrian *et al.*, 1986). The prototype strain is named with the letter 'p', and the subsequent genotypes with letters 'a', 'b', 'c', 'd' and so on. Most of the prototype strains are not currently circulating, probably due to a continuous

evolutionary process. Although adenoviruses are DNA viruses with a DNA polymerase, giving high fidelity due to proofreading capability, the emergence of new serotypes and genotypes is well known, especially among species D. The appearance of multiple serotypes or genotypes among adenoviruses and papilloma viruses (another DNA virus) suggests an evolutionary mechanism. Beside homologous recombination as a primary mechanism for evolution, it has been proposed that illegitimate recombination can occur among adenoviruses (Crawford-Miksza and Schnurr, 1996).

Structure

Adenoviruses are medium-sized, non-enveloped icosahedral viruses 70–90 nm in diameter (Figure 19.1). Each particle contains a single linear, double-stranded DNA

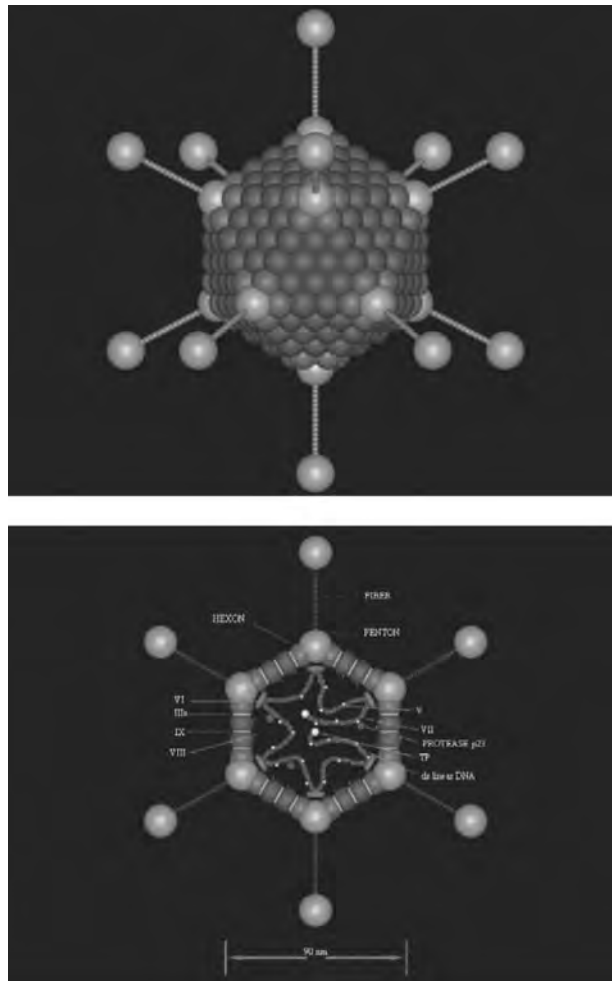


Figure 19.1 Schematic graphic of adenovirus particle. Location of proteins and genome.

molecule of about 36kb, encoding approximately 40 genes (Davison *et al.*, 2003). Major transcription units include early genes E1A, E1B, E2, E3 and E4, genes expressed later in the early phase (IX and IVa2), and late genes (L1–L5.) One or more (depending on the serotype) virus-associated (VA) non-translated genes are also described.

The protein capsid is formed by 252 capsomeres. The 240 hexons are grouped into 20 triangular faces, with 1 penton at each of the 12 vertices. Each penton consists of a base and the fibre, a rod-like projection of variable length with a terminal knob that interacts with cellular receptors.

The polypeptides are named with Roman numerals (I–X) and some of their functions are still unknown (Table 19.2). The hexon, penton and fibre (polypeptides II, III and IV, respectively) are the major components of the capsid and the principal antigenic determinants of the virus (Figure 19.1).

The hexon has antigenic sites common to all human adenoviruses. Neutralizing antibodies are directed against the ϵ epitope of the hexon. Hexons also contains type-specific sites which induce neutralizing antibodies. Fibres have type-specific and some species-specific antigenic determinants, and are responsible for haemagglutination *in vitro*.

Adenoviruses have a linear, nonsegmented, double-stranded DNA of about 36kb. The terminal nucleotide sequences are inverted repetitions and there is a protein of 55 kDa covalently attached to the 5' end of each strand. Both of these features are related to viral DNA replication, which contains two identical origins.

Resistance to Physico-chemical Agents

As non-enveloped viruses, adenoviruses are highly resistant to physical and chemical agents and are resistant

to inactivation at room temperature. They remain infectious at room temperature for prolonged periods in certain fomites, giving them a high potential for spread. Some serotypes, especially those associated with epidemic keratoconjunctivitis (EKC) have the ability to survive on nonporous surfaces for 35 days (Nauheim *et al.*, 1990). Transmission is person-to-person but they can also spread through water, fomites and instruments. Nosocomial infections and severe outbreaks have been reported.

They are stable at low pH and resistant to gastric and biliary secretions, thus allowing the virus to replicate and achieve a high viral load in the gut. They are resistant to ether and isopropyl alcohol. Sodium hypochlorite (500 ppm) for 10 minutes, or immersion in water bath at 75°C for 30 seconds, at 60°C for 2 minutes or at 56°C for 30 minutes can be used to inactivate. Effective surface disinfections include ~1900 ppm available free-chlorine, 70% ethanol, 65% ethanol with 0.63% quaternary ammonium compound, and 79.6% ethanol with 0.1% quaternary ammonium compound. These disinfectants should be allowed to contact all environmental surfaces for at least a minute (Rutala *et al.*, 2006). Hand hygiene with an antimicrobial soap and water should be used when adenoviral contamination may have occurred. Adenoviruses survive freezing with minimal loss of infectivity.

Replication

Replication begins by attachment of the fibre knobs to cell-surface receptors. Most human adenoviruses species A, C, D, E and F use the coxsackie adenovirus receptor (CAR) protein, which is a member of the immunoglobulin superfamily, as the initial receptor (Roelvink *et al.*, 1998).

The species B attachment receptor has not been identified, although recent studies have proposed a species B adenovirus receptor (sBAR) for species B1 and B2, and a specific receptor sB2AR exclusively for species

Table 19.2 Adenovirus proteins

Proteins from the capsid		
Name	Name	Known functions
II	Hexon monomer	Structural
III	Penton base	Penetration
IIIa	Associated to penton base	Penetration
IV	Fibre	Receptor attachment, Haemagglutination
V	Core: associated to DNA and penton base	Histone-like; Packaging?
VI	Hexon minor polypeptide	Stability/particle assembly?
VII	Core	Histone-like
VIII	Hexon minor polypeptide	Stability/particle assembly?
IX	Hexon minor polypeptide	Stability/particle assembly?
TP	Genome–Terminal Protein	Genomic replication

Adapted from T. Shenk and M.S. Horwitz.

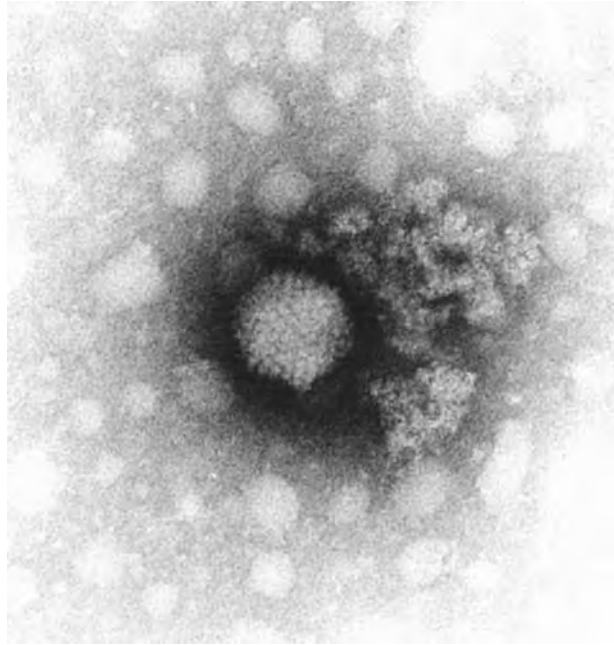


Figure 19.2 Electronic microscopy micrograph of negatively-stained adenovirus serotype 4. Magnification $\times 400\ 000$. Note the icosahedral shape of the virus. (Kindly provided by Dr Ludmila Asher.)

B2 (Segerman *et al.*, 2003). Other receptors include sialic acid saccharides, mostly used by adenovirus serotypes 8, 19 and 37 (species D) (Arnberg *et al.*, 2002).

After attachment, the virus migrates to clathrin-coated pits and is internalized in endosomes. Interaction between the penton base protein of the viral capsid and integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) is needed for efficient internalization. The interaction occurs through an arg-gly-asp (RGD) sequence present in the penton base. Since not all cell types produce α_v integrins, attachment can occur but no efficient replication is achieved.

Uncoating of the virus occurs and transport of the genome to the host nucleus is microtubule-mediated. The intracellular trafficking is different for species B and C. While adenovirus 5 (species C) rapidly translocates to the nucleus within one hour after infection, adenoviruses 7 (species B) is widely distributed in the cytoplasm. It has been suggested that the fibres do not only mediate the binding to the cell surface but also modulate intracellular trafficking (Miyazawa *et al.*, 1999).

Viral proteins are expressed, and viral DNA replication which occurs in the nucleus starts 12 hours post-infection in tissue culture. Structural proteins synthesized in the cytoplasm then move to the nucleus and form capsids, which encase the DNA and aggregate into crystalline arrays. After maturation, viruses remain localized in the nucleus (Figure 19.3). During a lytic

infection the cells die and the virus particles are liberated.

Virus DNA Replication

The replication cycle is divided into three phases: an immediate-early phase, a delayed-early phase, which precedes viral DNA replication, and a late phase, which follows DNA replication and is characterized by the expression of the structural proteins of the viral capsid. The five early transcription units are E1A, E1B, E2, E3 and E4. E1A and E1B stimulate the infected cell to transcribe and replicate adenovirus genes (Russell, 2000). E1A blocks interferon (IFN)-induced gene expression and the ability of the p53 and Rb growth-suppressor genes to suppress cell division. E1B proteins inhibit apoptosis in the stimulated cell. The E2 region codes for proteins necessary for viral DNA replication. Several E3 products prevent death due to receptor-mediated apoptosis. E3/14.7K seems to interfere with the cytolytic and pro-inflammatory activities of tumor necrosis factor (TNF), while E3/10.4K and 14.5K proteins remove Fas and TRAIL receptors from the cell surface by inducing their degradation in lysosomes. These and other functions that may affect granule-mediated cell death might drastically limit lysis by natural killer (NK) cells and cytotoxic T cells. In general, E3 gene products appear to facilitate immune evasion. E4 gene products are

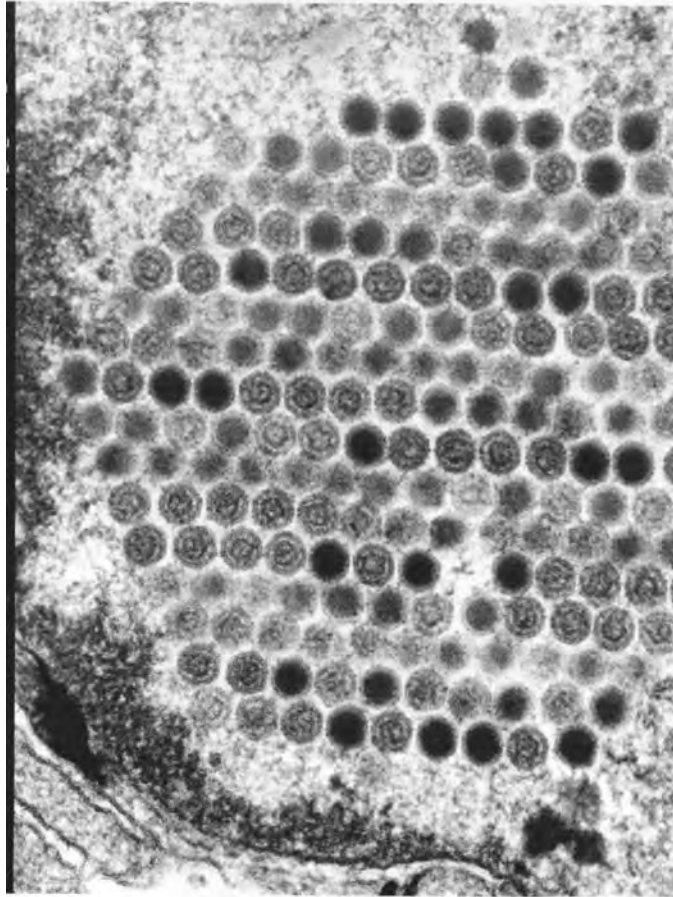


Figure 19.3 Electronic microscopy micrograph of adenovirus in a thin section of the infected cell (magnification $\times 108,000$) (Kindly provided by Dr Ludmila Asher.)

diverse and some are required for efficient tripartite splicing, used in the transcription of the late genes. The two delayed early units are IX and IVa2. One late unit is processed to generate five families of late mRNA (L1, L2, L3, L4 and L5). Not all the late genes are structural; there are also VA genes transcribed by the RNA polymerase III. The VA RNA is a short, highly-structured RNA that interferes with the cell's ability to produce IFN.

Almost all transcription units generate a complex set of alternately-spliced mRNAs that encode for multiple proteins. Interestingly, important molecular and cell biological processes such as RNA splicing and cell cycle regulation were discovered while studying adenovirus gene expression.

Assembly of the virion particles occurs in the nucleus, where they form crystalline aggregates like nuclear inclusion bodies (Figure 19.3). The replication cycle takes approximately 30 hours. From 10 000 to over 1 000 000

viral particles are produced per cell; only 1–5% are infectious.

Adenoviruses may cause at least three different types of infection. Productive lytic infection occurs mostly in epithelial cells. Latent or persistent infections occur primarily in the lymphoid mucosal tissue at sites such as the tonsils and adenoids, where they are maintained by unknown mechanisms and infrequently produce virus (Garnett *et al.*, 2002). Transformation occurs primarily in rodent cells infected with species A serotypes, which integrate into the host genome and induce cellular replication without producing infectious virus.

PATHOGENESIS

In cell culture, adenoviruses cause cell rounding, aggregation and accumulation of basophilic nuclear inclusions. Pentons have a toxin-like activity and isolated

penton bases produce the detachment of monolayers in cell culture after two hours of incubation. Adenoviruses have the capability to shut down host mRNA and synthesize adenovirus structural proteins, which accumulate in the nucleus, giving the characteristic histopathological intranuclear inclusions.

The reasons for the different organ tropisms and the production of such diverse diseases by adenoviruses serotypes have not been completely elucidated. Studies of the pathophysiology are limited by the lack of animal models that faithfully reproduce the diseases seen in humans. Studies on pathogenesis have been carried out in the cotton rat (*Sigmodon hispidus*) as it is susceptible to intranasal infection with adenovirus serotype 5 and develop pulmonary histopathology similar to that seen in humans, although the virus is not truly adapted to this animal.

In the cotton rat, the virus replicates in bronchiolar epithelial cells. *In situ* hybridization also shows early gene expression in macrophages/monocytes in alveoli and hilar lymph nodes (Ginsberg and Prince, 1994). The histopathological response consists of two phases. The early one is a mild-to-moderate damage of bronchiolar epithelial cells (including cytoplasmic vacuolation and loss of cilia) and diffuse infiltration of the peribronchiolar and alveolar regions with monocytes/macrophages, neutrophils and lymphocytes. The late phase consists of a peribronchiolar and perivascular infiltration composed almost entirely of lymphocytes. The predominant process is the response of the host to infection, rather than direct viral damage to cells.

Only early genes seem to be required to induce pathological changes in cotton rats. It seems that for complete viral replication it is essential only to infect a sufficient number of cells to yield an adequate expression of early gene functions. However, results from the cotton rat should be cautiously extrapolated to humans.

Whether or not adenoviruses causes a lytic infection still remains controversial. Earlier studies on the mechanism of adenovirus pathogenesis in cell cultures led to the hypothesis that the basis of cell killing is the intranuclear accumulation of viral proteins. Studies in mice and cotton rats showed that neither adenovirus 2- nor adenovirus 5-infected epithelial cells are lysed, although they are severely injured and probably die, because early adenovirus genes shut off DNA and protein synthesis in infected cells.

Studies on pathogenesis in children with fatal adenovirus infection have shown that TNF- α , interleukin (IL)-6 and IL-8 were detected in the serum, while these cytokines were not found in patients with moderate diseases (Mistchenko *et al.*, 1994).

Whether the cell-damaging effects of adenovirus infection or the host immune responses are responsible for the tissue pathology and clinical manifestations remains unclear.

Latency/Persistence

Adenoviruses devote a significant portion of their genome to gene products whose sole function seems to be the modulation of host immune responses. These mechanisms might play a role in maintaining the virus in a persistent state. Adenoviruses mount an apparently effective defense against the host immune response, as evidenced by the ability of the virus to persist at low levels in the host for long periods of time with periodic shedding of infectious virus in faeces and into respiratory secretions. Adenoviruses 1, 2, and 5 persist in tonsils for years through a low-grade replication.

Although the establishment of persistent infections is well documented in several epidemiological studies, the tissue site of replication during asymptomatic periods is enigmatic. In 1973 van der Veen *et al.* isolated adenovirus types 1, 2, 5 and 6 with a low frequency from tonsil- and adenoid-derived lymphocytes. One group reported the presence of adenovirus nucleic acid sequences in peripheral lymphocytes of healthy adults by *in situ* hybridization (Horvath *et al.*, 1986). However, amplification studies using PCR have demonstrated zero or low (1.7%) presence of adenovirus DNA in peripheral blood from healthy adult volunteers (Flomenberg *et al.*, 1997; Villamea *et al.*, 2002). Adenovirus species C persists in mucosal lymphoid tissues from tonsils and adenoids (Garnett *et al.*, 2002). Virus shedding in stool also supports a mucosal association of the virus. Thus, the virus may be strictly associated with the mucosal-lymphocyte compartment and rarely be found in circulation.

The gp 19kDa and protein 14.7kDa play a central role in the ability of subgroup C to produce persistent infections. Gp 19 prevents transport of the class I MHC to the surface of infected cells, reducing cytotoxic T-cell attack of the infected cells.

IMMUNE RESPONSE

The host has a range of efficient and well-orchestrated strategies to respond to adenovirus infections. Innate defenses, including recruitment of macrophages, activation of complement and NKs, the production of a range of pro-inflammatory cytokines and the orchestration of other signalling pathways, play a significant role in clearing adenovirus infection. The host also produces IFN, and the cell can redirect its metabolism to apoptotic circuits.

From the viral side, adenoviruses develop a number of mechanisms to evade the host immune response (see 'Immune Evasion').

In addition, cellular and humoral responses are critical in controlling adenovirus infections. T cells are effective defenses via both CD8 and CD4. CD4+ T-helper cells stimulate proliferation of B cells to synthesized immunoglobulins for the humoral response. It was suggested that CD4 T cells recognize conserved antigens among different serotypes and that the majority of individuals develop long-lived CD4+ T-cell responses to adenoviruses (Flomenberg *et al.*, 1995). This may play a role in modulating infections with a range of serotypes.

The humoral response is a major component in the control of adenovirus infections. After infection, most patients develop group- and type-specific antibodies to the infecting serotype. Group-specific antibodies do not neutralize viral infectivity but are useful for diagnosis. Type-specific antibodies are measured by neutralization or inhibition of haemagglutination. Adenovirus-neutralizing antibodies are directed against epitopes of the capsid, including the penton, the fibre and the hexon. These antibodies may protect against re-infection with the same serotype. This observation led to the development of effective vaccines for military recruits.

Immune Evasion

Adenoviruses have the capability to evade the immune system through several different mechanisms: (i) Inhibition of IFN functions by viral-associated (VA) RNA and E1A. IFNs are often the earliest host response to viral infection. dsRNA produced during viral infection induces antiviral cytokines. The dsRNA-dependent protein kinase (PKR) phosphorylates the translation factor eIF2 alpha. Adenoviruses encode VA RNAs that are transcribed by RNA polymerase III. Synthesis of VA RNA begins early in infection. This binds and inhibits PKR, preventing the induction of IFN type I. In addition, E1-A proteins also have the property to inhibit the function of IFN type I. (ii) Inhibition of TNF and Fas-mediated apoptosis. TNF is a pro-inflammatory cytokine secreted by activated macrophages and T cells. It can induce cell death. Adenoviruses encode four proteins (E1B19K, E314.7, E310.4K/14.5K) that protect adenovirus-infected cells from TNF and/or Fas-mediated apoptosis. (iii) Downregulation of surface class I MHC. Antiviral immune effector mechanisms rely on cytotoxic T lymphocytes (CTLs) to clear viral infected cells. CD8+CTL are triggered to lyse viral infected cells through recognition of viral peptides bound to class I MHC molecules on the cell surface. Adenoviruses encode proteins that inhibit class I presentation. E3 gp 19 and E1 A from adenovirus type 12 bind MHC class I and retain them in the endoplasmic reticulum, thus

reducing the transport of class I MHC to the cell surface (Mahr and Gooding, 1999).

Oncogenicity

Adenoviruses of species A are highly oncogenic, while those of species B are weakly oncogenic, in newborn hamsters. Despite extensive efforts, no evidence has been found concerning the aetiologic role of adenoviruses in human malignancies.

EPIDEMIOLOGY

Adenovirus infections are transmitted by direct contact, small-droplet aerosols, the faeco-oral route, water, ocular instruments and fomites. The mode of transmission in early life is thought to be primarily faeco-oral.

A child born in a family with relatives harbouring the virus in the intestine will eventually become an excretor. An infected child may excrete the virus initially from the respiratory tract and later from the gastrointestinal tract. Intermittent excretion in stools can be as prolonged as 906 days (Brandt *et al.*, 1969).

Adenovirus infections occur worldwide as epidemic, endemic and sporadic infections. The most common adenovirus serotypes causing respiratory infections are serotypes 1, 2 and 5 (species C) and serotypes 3 and 7 (species B). Adenovirus types 1, 2, 5 and 6 are mostly endemic, whereas 4, 7, 14 and 21 cause small epidemics. Respiratory outbreaks have been described in closed communities such as boarding schools, day-care centres and military training facilities. For respiratory epidemics (serotypes 4 and 7), spread by direct contact and aerosols are important.

The epidemiological pattern of adenovirus respiratory infection in civilian populations is different from the military. Recent publications described serotypes 4, 7 and 21 as the most prevalent types related to respiratory outbreaks among military recruits in the United States (Kolavic-Gray *et al.*, 2002).

Adenoviruses can cause ocular infections such as PCF and epidemics of keratoconjunctivitis (KC). PCF has been mostly associated with serotypes 1–7 and less frequently with serotypes 11–17, 19–21 and 29. Serotypes 3, 4 and 7 are commonly associated with swimming-pool outbreaks (Turner *et al.*, 1987). Adenovirus serotype 4 commonly causes ocular infections in Asia, but this is uncommon in Western countries.

Large epidemics of KC have been associated with serotypes 8, 9 and 37 (Keenlyside *et al.*, 1983). They spread by contact, through contaminated fingers or ophthalmologic instruments. These serotypes are often

endemic in poor hygienic conditions in developing countries, but in Western countries occur mostly in epidemics, which are sometimes nosocomial.

Infant gastroenteritis has mostly been associated with serotypes 40 and 41, which are therefore named 'enteric adenoviruses'. Adenovirus species A, B, C and D can also cause acute gastroenteritis. Serotypes 2 and 31 are more frequently followed by serotypes 1, 3, 5, 7, 12 and 18 (Filho *et al.*, 2007; Yolken *et al.*, 1982). Enteric types 40 and 41 spread via the faeco-oral route, and gastroenteritis occurs endemically throughout the world.

CLINICAL FEATURES

Adenovirus infections are widely distributed and common. Infections occur most frequently during childhood, where they tend to be self-limited and induce type-specific immunity following recovery. Adenovirus serotypes have different cell tropisms and cause distinct clinical manifestations (Table 19.3). In addition, some genotypes (e.g. 7h) appear to be more virulent, causing unusually severe respiratory manifestations and higher mortality rates (Kajon *et al.*, 1996).

Although adenovirus infections were traditionally associated only with respiratory, ocular and gastrointestinal diseases, many other clinical manifestations have now been associated with infection, especially in immunocompromized patients.

Clinical manifestations depend on the host and the serotype involved and include pharyngitis, PCF, conjunctivitis, pertussis-like syndrome, KC, bronchiolitis, pneumonia, acute HC and gastroenteritis (Ambinder *et al.*, 1986; Kajon *et al.*, 1996; Turner *et al.*, 1987; Yolken *et al.*, 1982). Less frequent clinical syndromes are hepatitis, meningitis, encephalitis, myocarditis, pericarditis, genital infections, pancreatitis and fatal disseminated disease (Davis *et al.*, 1988; Munoz *et al.*, 1998; Swenson *et al.*, 1995).

RESPIRATORY INFECTIONS

In Children

Most children become infected with one or more adenovirus serotype early in life. These infections are usually self-limited and mild. When symptomatic, usual signs and symptoms are fever, nasal congestion, coryza, pharyngitis and cough with or without otitis media. Adenoviruses may cause an exudative tonsillitis that is clinically indistinguishable from group A streptococcus. If conjunctivitis accompanies the syndrome, the disease is called PCF.

Upper-respiratory illnesses are mostly associated with species C (serotypes 1, 2, 5), while species B infections prevail in children with acute lower-respiratory disease from adenovirus (Kajon *et al.*, 1996). The frequency of adenoviruses in nasal-swab specimens from outpatient children with upper-respiratory infection showed 9% positivity using a generic PCR (Echavarría *et al.*, 2006). Interestingly, simultaneous infection with two different adenovirus species was observed in 19% of the cases.

After the onset of symptoms, adenovirus excretion in stools may be prolonged for months and this feature may account for the endemic presence of the virus in paediatric populations (Brandt *et al.*, 1969). On the other hand, adenovirus detection in nasal-swab samples by PCR showed a clearance of the virus within one to twelve weeks (Figure 19.4).

Lower-respiratory infections may be severe and require hospitalization. In children less than four years old, between 2 and 7% of acute respiratory infections and 10% of respiratory infections that require hospitalization are caused by adenoviruses. A higher prevalence has been documented in Argentina, which was dependent on the complexity of the hospital (Carballal *et al.*, 2001; Videla *et al.*, 1998). The syndromes include bronchitis, bronchiolitis, croup and pneumonia. Severe and even fatal pneumonia with a fatality rate around 16% or higher, which is primarily associated with adenoviruses types 3 and 7, can occur in infants and children (Murtagh *et al.*, 1993). In particular, genotype 7h, initially described in Argentina, was associated with a fatal outcome in 34% of cases during the acute stage of the disease (Murtagh *et al.*, 1993). Among survivors, residual lung damage secondary to bronchiolitis obliterans has been reported. Children less than two years old are more susceptible to severe primary infections, with occasionally fatal outcomes and long-term sequelae. In general, extra-pulmonary manifestations such as gastroenteritis, renal involvement, hepatosplenomegaly, meningoencephalitis and disseminated disease have been associated with a higher mortality rate. In bronchiolitis cases, intense air trapping with parahilar and peribronchiolar infiltrates with segmental or lobar atelectasis is observed. When multifocal pneumonia develops, fluffy or patchy and dense parenchymal infiltrates indicating alveolar consolidation scattered throughout both lungs with ill-defined margins are observed. With progression of the disease, hazy to totally opaque lungs can be observed. Air leaks can produce pneumothorax or pneumomediastinum (Murtagh *et al.*, 1993). Of those who recovered, most had residual chronic pulmonary sequelae. It was postulated that adenovirus genotype 7h may have increased virulence compared to other types (Kajon and Wadell, 1994). During 1984–1985, the predominant genotype in Argentina was 7c, but in 1986 it switched to 7h

Table 19.3 Adenovirus diseases, associated serotypes, hosts and clinical specimens for diagnosis

Diseases	Associated serotypes		Frequent hosts	Specimens
	Frequent	Infrequent		
Upper-respiratory illness	1-3, 5, 7	4,6,11,14,15, 18,21,29,31	Infants, children	NP ^a aspirate or swab
Lower-respiratory illness	3,4,7,21	1,2,5,7,8,11,35	Infants, children, IC ^b	NP aspirate or swab, BAL, lung tissue
Pertussis syndrome	5	1,2,3,12,19	Children	Throat swab
Acute respiratory disease	4,7	2,3,5,8,11,14,21,35	Military recruits	Throat swab, BAL, lung tissue, NP aspirate or swab
Acute conjunctivitis	1-4,7	6,9,10,11,15-17, 19,20,22,37	Children	Conjunctival swab or scraping
Acute haemorrhagic conjunctivitis	11	2-8,14,15,19,37	Children	Conjunctival swab or scraping
Pharyngoconjunctival fever	3,4,7	1,2,5,6,8,11-17, 19-21, 29,37	Children	NP aspirate or swab, throat swab, conjunctival swab or scraping
Epidemic keratoconjunctivitis	8,9,37	2,3,4,5,7,10,11, 13-17,19,21, 23,29	Any age	Conjunctival swab or scraping
Gastroenteritis	40,41	1,2,3,5,7,12-18, 21,25,26,29, 31, 52	Children	Stool
Haemorrhagic cystitis	11	7,21,34,35	Children, IC	Urine
Hepatitis	1-3, 5,7	4,31	Infants, children, IC	Liver tissue, blood
Myocarditis	7,21		Children	Heart tissue, blood
Meningoencephalitis	2,7	1,3, 5,6,11,12,32	Children, IC	Brain tissue, CSF
Veneral disease	2,37	1,5,7,11,18,19,31	Teens, adults	Lesion swab
Disseminated disease	1,2,5,11,34,35	3,6,7,14, 21,29-31, 37-39,43,45	IC, newborns	Blood, BAL, urine, involved tissue

^aNP, nasopharyngeal.^bIC, immunocompromised.

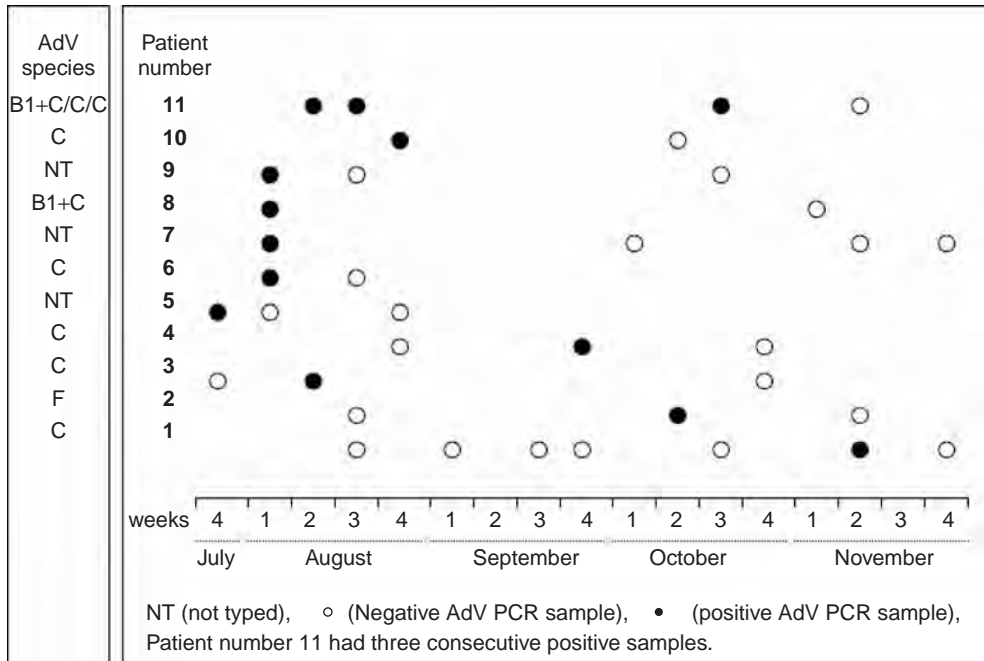


Figure 19.4 Longitudinal observation of adenovirus (AdV) detection by PCR in 11 patients with upper-respiratory infection and in several tested nasal-swab samples obtained during winter and spring.

(Carballal *et al.*, 2002). In Japan, the first report on 7h occurred in 1996 (Hashido *et al.*, 1999). In the United States, Australia and countries from Europe, the most prevalent genotype has been 7b, although the appearance of 7d2 and 7h in North America represents introduction of these viruses from previously geographically-restricted areas (Erdman *et al.*, 2002). A recent study evaluating the adenovirus prevalence in the United States from samples obtained in 2004–2006 using a molecular typing technique did not show a strong association between serotype 7 and higher morbidity compared to other serotypes. This could be due to the stabilization of circulating adenovirus serotype 7, with subsequent increases in herd immunity and reduction in these infections (Gray *et al.*, 2007).

Adenoviruses can be recovered throughout the year, although a higher frequency is observed in winter and spring.

Severe lung disease after measles infections has also been described. It is suggested that secondary infection with adenoviruses is responsible in some patients. Measles virus may render children more susceptible to serious complications from adenovirus infection. The many deaths from ‘measles pneumonia’ in developing countries and the occasional occurrence of post-measles bronchiec-

tasis in some countries may be due to secondary adenovirus infections (Warner and Marshall, 1976).

Pertussis-like Syndrome

Pertussis syndrome is usually caused by *Bordetella pertussis*. However, adenoviruses have frequently been isolated in patients with this syndrome. The rate of adenovirus infection was statistically significant in patients with pertussis syndrome (23%) versus control subjects (5%). The frequent association of adenoviruses with pertussis syndrome suggests that they may play a role in the pathogenesis of this disease. Adenovirus type 5 has been isolated from patients with severe whooping cough that ended fatally. However, it is not known whether adenoviruses co-infect with *B. pertussis* or whether that infection causes conditions favourable to reactivation of latent adenoviruses.

Acute Respiratory Disease in Military Recruits

Adenoviruses were first associated with respiratory outbreaks among military recruits undergoing basic training in 1953 (Hilleman and Werner, 1954). During the 1950s through the 1960s, adenovirus-associated ARD constituted one of the most important causes of medical morbidity among military recruits in the United States. The

serotypes most commonly associated with respiratory disease were serotypes 4 and 7, followed by 3, 14 and 21. Severe ARD required hospitalizations in 50% of cases at Fort Dix (Top, 1975) and fatal pneumonia with adenovirus type 7 was reported in three previously healthy military trainees (Dudding *et al.*, 1972).

Since the 1980s, the routine use of live oral vaccines to serotypes 4 and 7 among military recruits resulted in a significant decrease in ARD morbidity (Figure 19.3). However, in 1996, vaccine discontinuation resulted in a resurgence of adenovirus-associated respiratory-disease epidemics at military training centres (Kolavic-Gray *et al.*, 2002). Most recruits presented with fever, sore throat and persistent cough. Adenovirus 4 was the most prevalent type recovered, followed by types 3 and 21.

During the pre-vaccine era, recruits used to develop severe signs and symptoms of lower-respiratory-tract infection and pneumonia. During recent respiratory outbreaks (1997, 1998, 2000 and 2001), the hospitalization rates have ranged from 10 to 21%, but these have not been significantly severe (Kolavic-Gray *et al.*, 2002). The low level of pre-existing immunity for adenovirus 4 (12–26%) in young adults may reflect the lack of exposure to this serotype during childhood. Serotype 4 has been uncommonly associated with respiratory disease in adult civilians. The different epidemiology of adenoviruses among civilian and military groups is not completely understood. It may be due to conditions during military training, where individuals from different geographic areas and backgrounds are subjected to crowding and stress. In one study evaluating adenovirus carriage at military entry, no adenoviruses were recovered by culture in throat-swab samples and only one individual was weakly positive by PCR (Echavarría *et al.*, 2003).

Interestingly, adenovirus DNA type 4 was detected by PCR from air filters in sleeping barracks during respiratory outbreaks (Echavarría *et al.*, 2000). Positivity in air filters correlated with the number of hospitalizations due to adenovirus-ARD during the same period. Environmental control of airborne spread of adenoviruses may help

reduce the risk of adenovirus-associated outbreaks during periods of vaccine unavailability.

OCULAR INFECTIONS

Pharyngoconjunctival Fever

PCF is characterized by follicular conjunctivitis and lymphadenopathy, as well as fever, pharyngitis and malaise. Headache, diarrhoea and rash can also be present and 5–10% of patients may have involvement of the cornea. Either one or both eyes may be involved. Infected individuals can shed the virus for about 10 days. Complete recovery without sequelae usually occurs. PCF is seen in children and young adults in discrete outbreaks, but sporadic cases occur at all ages. Community outbreaks of PCF have been associated with inappropriate chlorination systems in swimming pools (Turner *et al.*, 1987). Illness was directly related to hours of exposure to the pool and swallowing pool water. Adenovirus serotypes 3 and 7 have been most associated with PCF. Adenoviruses types 1–7, 11–17, 19 and 21 have also been reported.

Epidemic Keratoconjunctivitis

In contrast to PCF, EKC is highly contagious and serious. EKC can start with conjunctivitis, which may be follicular, followed by oedema of the eyelids, pain, photophobia and lacrimation (Figure 19.5). Superficial erosions of the cornea may occur, followed by deeper sub-epithelial corneal infiltrates with a characteristic round shape. A mild respiratory infection may accompany the symptoms. This clinical entity was identified in 1941, before adenoviruses were initially isolated, in the marine shipyards at Pearl Harbour. ‘Shipyard eye’ was probably transmitted in the medical facilities caring for eye trauma. Adenovirus 8 was the cause of those infections.

The incubation time may be 4–10 days and conjunctivitis may resolve in 2 weeks, although reduced vision, photophobia and foreign-body sensation may persist for



Figure 19.5 Ocular characteristics from a patient in an outbreak of adenovirus keratoconjunctivitis.

months to years. Adenoviruses 8, 19 and 37 are the most prevalent serotypes associated with EKC. In Japan, adenovirus 4 has also been associated with this condition.

EKC outbreaks have been reported in many countries as an important cause of nosocomial morbidity in ophthalmology clinics. Risk factors for EKC were associated with exposure to contact tonometry (Keenlyside *et al.* 1983). Ophthalmic solutions, the hands of medical personnel and towels are also vehicles for the spread. Direct inoculation into the eye appears to be required to cause disease. EKC outbreaks have resulted from eye-fomite-eye inoculation, sharing towels or facecloths, direct contact between children and family members, and even among health-care workers.

GASTROINTESTINAL INFECTIONS

Adenoviruses are the second most common cause of viral gastroenteritis in children less than two years old. The incidence for this disease ranges from 4 to 12% of cases of diarrhoea and it occurs throughout the year. The route of transmission is faeco-oral. During the prodrome, 50% of patients have respiratory symptoms. Diarrhoea is usually watery, nonbloody and with no faecal leucocytes. The mean duration is 10 days. It may be associated with mild fever, vomiting and abdominal pain. Gastroenteritis may also be a sign of systemic infection related to serotypes 3 and 7 that causes respiratory symptoms. Subsequent to gastroenteritis due to adenoviruses, some patients become intolerant to lactose-containing products for up to five to seven months. Other gastrointestinal syndromes associated with adenoviruses have included intussusception, acute mesenteric lymphadenitis and appendicitis. Serotypes 40 and 41, the enteric adenoviruses, are the most prevalent types that cause acute gastroenteritis (van der Avoort *et al.*, 1989). In patients who develop adenovirus enteritis, the enteric strains seem to induce disease that persists longer than that cause by strains other than 40 and 41.

HAEMORRHAGIC CYSTITIS

Adenoviruses can cause acute HC in paediatric populations and in immunocompromised patients. A sudden onset of gross haematuria, dysuria and increased frequency of urination were described. Serotype 11 is the most prevalent type recovered in these patients (Ambinder *et al.*, 1986). Other serotypes, including 34 and 35, have also commonly been recovered from immunocompromised patients. Adenoviruses 11, 34 and 35 can cause persistent infection in the kidney and may be reactivated during

immunosuppression. HC has been highly associated with adenoviruses in bone marrow transplant (BMT) recipients and can represent the first clinical manifestation of a disseminated adenoviral disease (Echavarría *et al.*, 1999). HC has also been described in renal transplant recipients.

ADENOVIRUSES INFECTIONS IN IMMUNOCOMPROMIZED PATIENTS

Adenoviruses are increasingly recognized as viral pathogens that may cause severe disease, including fatal infections, in immunocompromised patients. In recent years, populations of immunocompromised patients have expanded due to advances in transplantation technology and treatment, cancer therapy and the AIDS epidemic. In immunocompromised patients, adenovirus infections tend to be more prolonged and more severe, and may result in fatal outcome. In these patients, infections may occur due to endogenous reactivation or primary infection. Clinical manifestations in immunocompromised patients depend on the underlying disease, affected organ system, patient age and virus serotype (Hierholzer, 1992). Clinical manifestations in these patients include pneumonia, hepatitis, HC, colitis, pancreatitis, meningoencephalitis and disseminated disease (Ambinder *et al.*, 1986; Blanke *et al.*, 1995; Davis *et al.*, 1988; Hierholzer, 1992; Ljungman *et al.*, 1989; Shields *et al.*, 1985). Adenovirus-associated case fatality rates in the immunocompromised have been reported to be as high as 60% for patients with pneumonia, and 50% for those with hepatitis, compared with fatality rates of 15% for pneumonia and 10% for hepatitis in immunocompetent patients (Hierholzer, 1992).

In BMT and haematopoietic stem cell transplantation (HSCT) recipients, adenovirus infections are especially frequent. The incidence of adenovirus infection in these patients has increased in recent years due to a variety of factors, including higher awareness of this pathogen, aggressiveness of conditioning regimens, greater sensitivity of diagnostic methods and systematic screening (Flomenberg *et al.*, 1994). The range for adenovirus infection varies from 5 to 47% depending on the age of patients, conditioning regimens, type of method for diagnosis and clinical sample analysed (Table 19.4). Some of the risk factors are: lower age (paediatric recipients are 2–3.5 times more likely than adults to become infected), allogeneic transplantation, T-cell depletion, unrelated or HLA-mismatched grafts and total body irradiation. The paediatric population may be more vulnerable because they are more likely to experience primary infections. The range for adenovirus disease varies from 10 to 89%, even among adult patients (Table 19.4). Some of the risk factors for adenovirus disease are: the number of sites from

Table 19.4 Prevalence of AdV infection and disease, and serotypes involved in bone marrow and haematopoietic stem cell transplant recipients

Publication (yr)	AdV infection (%)	AdV disease (%)	Species or serotypes
Shields <i>et al.</i> (1985)	5	20	1, 2, 5, 7, 11, 34, 35
Wasserman <i>et al.</i> (1988)	18	Nd	1, 2, 3, 4, 11, 12, 15
Ljungman <i>et al.</i> (1989)	6	Nd	34
Flomenberg <i>et al.</i> (1994)	Adult: 14 Children: 31	31	1, 2, 4, 5, 29, 35
Blanke <i>et al.</i> (1995)	14	10%	1, 11, 12
Hale <i>et al.</i> (1999)	6	46	5, 7, 11
Howard <i>et al.</i> (1999)	12	64	Nd
Venard <i>et al.</i> (2000)	20	61	1, 2, 3
Hoffman <i>et al.</i> (2001)	47	82%	Nd
Bordigoni <i>et al.</i> (2001)	12	60	1, 2, 3, 5, 8, 31
Echavarría <i>et al.</i> (2001)	12	45	1, 2, 5, 12/31
La Rosa <i>et al.</i> (2001)	3	89	Nd
Leruez-Ville <i>et al.</i> (2004)	18	Nd	2, 5, 6, 12
Kampmann <i>et al.</i> (2005)	41	Nd	A, B, C
Yusuf <i>et al.</i> (2006)	32	75	Nd

which adenoviruses can be detected, immunosuppressive therapy, lymphocytopenia, detection of adenovirus in blood and detection of rising viral load in blood. Severe lymphopenia (<300 cells/mm³) is a risk factor for progression to disseminated disease and is often fatal (Chakrabarti *et al.*, 2002).

Adenovirus infections can be asymptomatic with clinical manifestations. The disease can be localized to a single organ or disseminated. PCR in blood is usually a predictor of disseminated disease (Echavarría *et al.*, 2001a). Therefore, when adenovirus disease is considered, blood specimens should be tested by PCR. Surveillance in blood samples is currently a common practice among HSCT, especially in the child population. The virus can be detected in blood two to three weeks before clinical symptoms develop (Lion *et al.*, 2003). Follow-up and prognosis is better assessed with quantitative PCR methods. Increased viral-load measurements are associated with increased risk of death (Schilham *et al.*, 2002).

Adenovirus in HSCT patients is usually detected within 100 days post-transplant. Clinical manifestations include upper- and lower-respiratory disease such as interstitial pneumonitis, hepatitis, renal disease including HC or nephritis, gastrointestinal disease including haemorrhagic colitis, central nervous system (CNS) disease or disseminated disease (Ambinder *et al.*, 1986; Kampmann *et al.*, 2005; La Rosa *et al.*, 2001; Wasserman *et al.*, 1988; Yolken *et al.*, 1982). HC can be especially severe and may signal the start of dissemination (Echavarría *et al.*, 1999).

Adenovirus mortality varies from 6 to 70% in paediatric and adult transplant patients. A lower mortality rate (2%) was reported when pre-emptive therapy was

instituted (Yusuf *et al.*, 2006). Different adenovirus serotypes have been isolated from these patients, most commonly from species A, B and C (Table 19.4). More than a single serotype can be detected during the course of the infection. In fact, a recent study has shown that 36% of stem cell transplant recipients demonstrated two or more adenovirus strains after transplantation (Kroes *et al.*, 2007).

In the solid organ transplant recipient, the primary site of adenovirus disease is usually related to the transplanted organ. Some of the clinical manifestations described among lung, liver, renal and small bowel transplantations include pneumonia, hepatitis, nephritis, HC, enteritis and disseminated disease. Incidence data for adenovirus disease in solid organ transplant recipients are more limited than for stem cell transplant recipients. Most of the symptomatic and severe infections have been reported in paediatric transplant populations, in liver and in lung allograft recipients, in patients who receive anti-lymphocyte antibodies and in patients with donor-positive/recipient-negative adenovirus status.

In liver transplant recipients, the most frequent clinical manifestation is hepatitis, most commonly associated with adenovirus species C (serotypes 1, 2 and 5). The mortality rate can be as high as 53%.

In renal transplant recipients, the predominant symptom is HC, mostly related to adenovirus species B (serotypes 7, 11, 34 and 35) and a 17% fatality rate. In lung transplant recipients, adenoviruses can cause pneumonia and graft failure that can be fatal (Bridges *et al.*, 1998), and they are more frequent in children than in adults.

Adenovirus infections are frequent in HIV-infected patients and can range from 7 to 29%. The most frequent sites for adenovirus recovery in these patients are the gastrointestinal and urinary tracts. Clinical manifestations include pneumonia, hepatitis, meningoencephalitis, nephritis, gastrointestinal and disseminated disease, which may sometimes be fatal (Khoo *et al.*, 1995; Schnurr *et al.*, 1995). Prolonged adenovirus faecal excretion has been associated with low CD4 counts (Khoo *et al.*, 1995). Some of the fatal cases were caused by serotypes 1, 2 and 3. Co-infection with other micro-organisms including bacteria and fungus is common in these patients; therefore a direct association with adenovirus infection is sometimes difficult to establish. Most adenovirus serotypes infecting the gastrointestinal tract of HIV-infected patients belong to species D, including serotypes 9, 17, 20, 22, 23, 26, 27 and 42–51.

Adenovirus is infrequently found in the urine of immunocompetent patients. In contrast, the frequency of adenovirus detection in the urine of AIDS patients, especially in the pre-HAART (highly active anti-retroviral therapy) era, may achieve 20%, although usually without evidence of bladder inflammation or bleeding (de Jong *et al.*, 1983). This frequency is higher when molecular methods are applied (Echavarría *et al.*, 1998). Serotypes 11, 34, 35 and 42–47 were found in the urine of AIDS patients (Echavarría *et al.*, 2001). Persistence in urine can be as long as 12 months (Horwitz *et al.*, 1984). One study showed that patients with parenteral exposure to HIV were more likely to be adenovirus-positive in urine than those with sexual exposure. In addition, in this study the median time to death was shorter in adenovirus-positive patients than in adenovirus-negative patients (Echavarría *et al.*, 2001b).

Serotypes 43–51 were identified from HIV-infected individuals (de Jong *et al.*, 1999). It has been suggested that the long-term infection seen in AIDS patients and the potential co-infection with more than one serotype may provide the opportunity for mutations within a strain, or for recombination between co-infecting serotypes.

The introduction of HAART has dramatically reduced the incidence and mortality from opportunistic infections in general, including adenoviruses. However, the development of viral resistance and difficulties with compliance with HAART may lead to the re-emergence of adenovirus infections in this population.

OTHER CLINICAL MANIFESTATIONS

Adenovirus have been recovered from cerebrospinal fluid (CSF) and brain tissue from patients with meningoencephalitis. Adenoviruses 3 and 7 were the most prevalent serotypes associated with this syndrome. Severe

encephalitis has also been associated with adenovirus group B. A recent study has shown the presence of species C and F adenovirus in CSF from hospitalized children with CNS infection (Zimmermann *et al.*, 2007). Adenovirus should be included in the differential diagnosis of viral CNS infections.

Adenoviruses can also be the cause of myocarditis. Adenovirus genome has been detected by PCR in myocardial samples from patients with acute myocarditis.

Adenoviruses serotypes 2, 8 and 37 have been isolated in 0.3% of patients with genital lesions attending a sexually-transmitted-disease clinic (Swenson *et al.*, 1995). These findings have suggested that adenoviruses can be sexually transmissible.

Adenovirus 36 can cause obesity in animals. Studies done in rhesus and marmoset monkeys have demonstrated a significant longitudinal association of positive antibody status to adenovirus 36 with weight gain (Dhurandhar *et al.*, 2002). Further studies are needed to determine if such an association exists in humans.

DIAGNOSIS

The syndromes caused by adenoviruses are frequently not clinically distinguishable from other bacterial or viral causes; therefore the laboratory diagnosis of adenovirus infections becomes important. In addition, virus detection and characterization are essential for epidemiological studies and therapy interventions.

Adenoviruses have been isolated in tissue culture from stool, throat swabs, nasopharyngeal (NP) aspirates, conjunctival swabs, urine, cerebrospinal fluid, blood and a variety of biopsy specimens. Sample collection early in the illness is necessary for optimal recovery. Duration of excretion from the onset of symptoms is around one week in respiratory infections, two weeks from eye specimens in those with PCF or KC, and two weeks to twelve months or longer in urine and stool from immunocompromized patients. Adenoviruses are stable and can be transported at room temperature, although prompt shipping to the laboratory is always recommended. Swabs and biopsies should be transported in viral transport media containing serum, albumin or gelatin and antibiotics/antimycotics.

The choice of method for adenovirus diagnosis depends on the type of disease and sample obtained. Adenovirus diagnosis is mostly performed using direct methods. These include virus isolation in cell culture, antigen detection and genome detection with or without amplification, and to a lesser extent particle detection by EM. Indirect diagnosis using serology is limited due to the lack of sensitivity, heterotypic response or antibody production, especially in immunocompromized patients. Serology should therefore be reserved for epidemiologic investigations or

to confirm associations between virus detection and unusual clinical outcomes.

Conventional and molecular methods are used for direct detection of the virus. Some of the limitations of conventional methods are that culture may be prolonged and can be inhibited by neutralizing antibody or other interfering substances, while EM and antigen detection methods may be insensitive. In recent years, the development and application of molecular methods using DNA amplification by PCR has increased sensitivity and rapidity of diagnosis.

Direct Methods

Virus Isolation

Continuous human cell lines of epithelial origin such as A 549, Hep-2, HeLa, human embryonic kidney (HEK) and KB can be used for the recovery of adenoviruses from all clinical specimens (Huang and Turchek, 2000; Landry *et al.*, 1987). Blood rarely yields virus except by PCR, probably due to the presence of neutralizing antibody. All adenovirus serotypes, except the enteric 40 and 41, grow in human epithelial cell lines and produce a cytopathic effect (CPE) characterized by clumping and cell rounding with refractile intranuclear inclusion bodies. The rapidity of CPE depends upon the concentration of infectious particles in the sample, the sensitivity of the cell line and the adenovirus serotype involved. CPE may take from 2 to 28 days to become evident. CPE needs to be confirmed by indirect IF with monoclonal antibodies, by radioimmunoassay or by ELISA. Serotypes 40 and 41 are fastidious and replicate poorly in the previously-mentioned cell lines. They do replicate in Graham-293 adenovirus-5-transformed secondary HEK cell line and in Hep-2 under a special medium.

Cell culture remains the gold standard, although it can be insensitive with some clinical samples (blood), may have fungal or bacterial contamination, and may have inhibitors or be toxic to the host cells.

Histopathology

Histopathologic findings in lung are characterized by diffuse interstitial pneumonitis, necrosis of bronchial epithelial cells, bronchiolitis with mononuclear cell infiltrates and hyaline membrane formation. Infected cells have enlarged nuclei with basophilic inclusions, surrounded by a thin rim of cytoplasm called 'smudge' cells, which are typical of adenovirus infection.

Direct Antigen Detection

Immunofluorescence IF uses monoclonal antibodies, usually directed against the hexon protein. It is especially

useful for respiratory specimens, swabs or biopsies. The advantage of this method is rapidity, as a result can be obtained within two to four hours. However, it lacks sensitivity, depending on sample quality, timing and type of monoclonal antibody used. Sensitivities of 30–60% in comparison to cell culture have been described.

Enzyme Immunoassays EIAs are particularly useful for diagnosing enteric adenoviruses in faecal samples where the antigen is not intracellular. Genus-specific tests and type-specific assays for serotypes 40/41 are commercially available. Most EIAs utilize microtiter plates and adenovirus-specific MAbs as capture and detector reagents. Sensitivity of most enteric adenovirus EIAs compared to 293 cell culture or EM is >90%, but can be lower if variants are circulating. Specificity is usually >97%. Genus-specific assays are sensitive (>95%) when stool specimens are used. Sensitivity is lower (65–75%) with respiratory tract and eye specimens. Urine has the lowest detection rate (August and Warford, 1987; Gleaves *et al.*, 1993). Occasional false-positive reactions occur.

Other antigen-detection tests include immunochromatography (IC), where the specimen migrates laterally along a membrane strip, antigen binds to virus-specific antibodies and a coloured line develops within minutes. The sensitivity of a commercially-available adenovirus IC assay with NP specimens tested at the bedside was 84% compared to culture and PCR, and 95% when the same specimens were retested in the laboratory (Fujimoto *et al.*, 2004). Another antigen detection assay is latex agglutination (LA), which is used primarily for genus-specific detection of adenovirus in stools. LA has similar sensitivity to EIA, although up to 13% of specimens may yield uninterpretable results (Grandien *et al.*, 1987).

Immunohistochemistry This method is used on tissue sections using adenovirus monoclonal antibody directed against the hexon antigen. This method is specific but also lacks sensitivity.

Direct Particle Detection

Electron Microscopy The EM morphology of adenoviruses is unique, allowing rapid identification of the virus (Figure 19.2). EM is mainly used for diagnosis of adenovirus acute gastroenteritis, due to the high number of viral particles excreted (10^6 – 10^8 particles/ml). It requires an electron microscope and highly-trained personnel, and lacks sensitivity. Therefore it is not in current practice in most clinical virology laboratories.

Direct Genome Detection

DNA detection can be performed using molecular methods with or without amplification.

Hybridization Hybridization without amplification has proved to be an appropriate tool for diagnosis in tissue sections in particular, although its lack of sensitivity has been recognized. *In situ* hybridization has been proven to be useful not only for diagnosis but also for studies of pathogenesis.

Polymerase Chain Reaction (PCR) Amplification of the viral genome using methods such as PCR has increased sensitivity and rapidity compared to conventional classical methods. The usefulness and application of this method in the clinical setting has significantly increased in the last few years. It is especially useful when non-infectious virus is present, when viral load is too low to be detected by culture, when interference such as neutralizing antibody may inhibit growth or when results are needed quickly.

Until 1997, few PCR methods for stools and swabs were available for clinical diagnosis (Allard *et al.*, 1990; Morris *et al.*, 1996). Later, different generic and type-specific assays were developed and tested on a range of different clinical samples (Allard *et al.*, 2001; Echavarría *et al.*, 1998; Xu *et al.*, 2000). For clinical diagnosis, generic PCR should be used, and it should be capable of detecting all adenovirus serotypes. PCR primers from the hexon gene or fibre gene, or from the VARNA I and II regions, are usually chosen because they have areas well conserved among serotypes. The hexon gene is located between 0.51 and 0.6 of the genome and has three different segments: a central variable region (nucleotides 403–1356) and two highly-conserved regions flanking it. There are seven hypervariable regions inside the variable region, which differ in sequence and length. Hypervariable region 1 has the highest variability and is related to viral tropism (Crawford-Miksza and Schnurr, 1996).

PCR assays have proven to be highly specific in studying adenovirus in urine, respiratory samples and blood from healthy asymptomatic immunocompetent individuals (Echavarría *et al.*, 1998, 2003; Villamea *et al.*, 2002). Adenovirus DNA has been detected in virtually all clinical samples, so specimen selection depends largely on the associated disease (Table 19.3). In patients with disseminated disease, PCR detected virus in the bloodstream, while other methods could not (Echavarría *et al.*, 1999). The presence of adenoviral DNA in serum was found to be associated with severe or fatal adenovirus infection in BMT recipients (Echavarría *et al.*, 2001a). Since proper management of these patients is dependent on

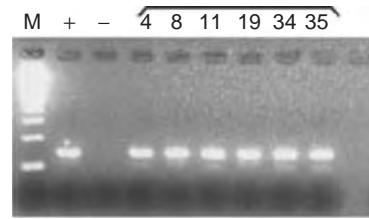


Figure 19.6 Home-brew PCR for adenovirus. Amplified products are detected in an agarose gel stained with ethidium bromide. M, marker; (+), positive control; (-), negative control. Samples 4, 8, 11, 19, 34 and 35 represent the corresponding adenovirus serotypes. A positive result corresponds to 139 bp product.

early diagnosis and differentiation from other conditions, PCR assays can be valuable as early markers for disease. Most conventional PCR tests are in-house tests where amplified products are detected in stained agarose gels (Figure 19.6) or further developed by southern blot. Some commercially-available PCR assays use ELISA detection (Vabret *et al.*, 2004).

Real-time PCR Real-time PCR assays are more rapid than conventional PCR because amplification and detection of amplified products occur simultaneously. Therefore, results can be obtained within hours, compared to one to two days for conventional methods. In addition, real-time PCR can be used for quantification of the virus. Since higher adenovirus levels in blood have been correlated with a fatal outcome, current studies are using ‘real-time’ amplification methods for the rapid determination and quantification of the virus in different clinical samples (Lankester *et al.*, 2002; Schilham *et al.*, 2002). Several in-house real-time adenovirus PCRs have been developed, initially for BMT or HSCT patients but recently for other patient populations as well (Echavarría *et al.*, 1998; Flomenberg *et al.*, 1997; Hierholzer, 1995; Nagafuji *et al.*, 2004; Sprangers *et al.*, 2003). A recent commercially-available real-time PCR assay performed satisfactorily in detecting all adenovirus serotypes (Figure 19.7). In general, more than one set of primers and several probes are necessary to detect all serotypes when using real-time PCR. All specimen types can be used to obtain a qualitative result by real-time PCR, but blood is frequently submitted to determine viral load in plasma or serum. Nevertheless, currently quantitative real-time adenovirus PCR has some limitations due to the lack of international standard calibrators. Inter-laboratory result variability is expected. Adenovirus DNA can be detected in peripheral cells, whole blood, plasma or serum, giving some different viral-load results. There is no clear cut-off value that predicts disease or outcome. It may

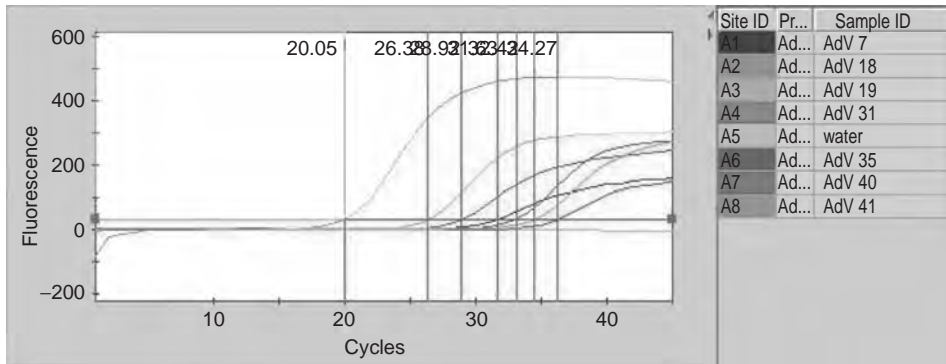


Figure 19.7 Primary curves from a real-time PCR assay with different adenovirus serotypes. The different curves represent the fluorescence level and the corresponding Ct for adenovirus serotypes 7, 18, 19, 31, 35, 40 and 41 with the commercial adenovirus r-gene mix (Argene, Inc). Water was used as a negative control.

therefore be preferable to analyse the viral kinetics for each individual case, considering the adenoviral viral load over time and establishing the relative change rather than the absolute value (Leruez-Ville *et al.*, 2004).

Typing Systems Adenovirus typing is used primarily for epidemiologic investigations, studies of pathogenesis or to reveal the cause of an unusual or especially severe infection. Serologic or molecular typing can be performed. Serologic typing requires an isolate and detects differences in epitopes on the fibre gene and hexon gene. Molecular typing can be performed on isolates and on the original specimen. The two systems differ fundamentally in target, so results do not always concur.

Serologic typing by HA provisionally determines the species of an isolate (Table 19.1); if the isolate is from stool its species is determined by a species F (serotype 40/41) EIA. This is followed by serotyping by haemagglutination inhibition (HAI) or serum neutralization (SN) using serotype-specific antisera. HAI is easier but SN is the definitive test for serotype. There are three major SN assays: a conventional seven-day test in human epithelial cells (or 293 cells for stool isolates); a three-day test in monkey kidney cells, which is the most simple and rapid assay; and a five-day micro-neutralization test using Vero cells (Hierholzer, 1995). Interpretation is not always straightforward, for example HAI and SN fail to agree with intermediate species D variants containing the fibre gene of one serotype and the hexon gene of another. Yet serotyping remains the gold standard against which newer tests are evaluated. Other serologic methods include an immunoperoxidase technique, immune-adherence hemagglutination (HA) and indirect IF with serotype-specific antisera.

Molecular typing is increasingly performed because it is rapid, utilizes molecular equipment and expertise

available now in many laboratories, and does not require expensive and difficult-to-obtain antisera. The restriction enzyme analysis was initially used. It can be applied to purified virus, virus-infected cells or stools from children with diarrhoea (Adrian *et al.*, 1986; Buitenwerf *et al.*, 1985). The starting material is first lysed and then digested with restriction endonucleases (e.g. Sma I), then serotype- and/or genotype-specific band patterns are visualized after agarose gel electrophoresis. Unfortunately, strains that defined the initial restriction enzyme analysis patterns are no longer circulating, so patterns of currently-obtained adenoviruses may be uninterpretable. Co-infections can also cause ambiguous results. restriction enzyme analysis (REA) is still useful for presumptive identification of new serotypes, identification of genotypes (e.g. 7h or 7d2) associated with severe disease, or to confirm results obtained by other means (de Jong *et al.*, 1999; Gray *et al.*, 2005). Other molecular-typing methods include single-stranded conformation polymorphism and heteroduplex mobility (Soares *et al.*, 2004).

Typing by PCR is now preferred by many laboratories. Extracted DNA from an isolate or purified virus is amplified using a generic or multiplex species-specific PCR and hexon-gene or fibre-gene primers (Lu and Erdman, 2006). Serotype is then indicated by measurement of product-length sizes or DNA sequence of fragments generated after cleavage with restriction enzymes, or by DNA sequence. Some schemes detect only limited numbers of serotypes, whereas others are more comprehensive and can demonstrate co-infections (Metzgar *et al.*, 2005; Sarantis *et al.*, 2004). Correlation between hexon-gene-sequence-typing methods and the classic serologic-typing method varies from 71 to 97% (Gray *et al.*, 2007; Kroes *et al.*, 2007). Genotypic analysis may provide an alternative for classical serotyping but some discordance occurs.

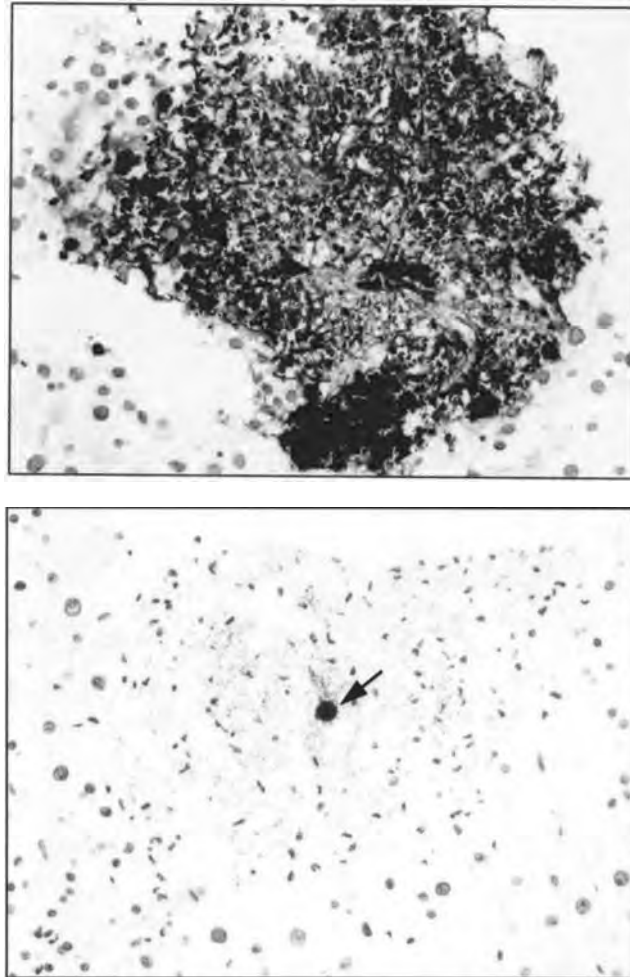


Figure 19.8 Clearance of adenoviral hepatitis in a paediatric liver transplant recipient. Histopathology and immunohistochemical stain for adenovirus in liver biopsies before and after ribavirin therapy. Top: before treatment: multiple foci of necrosis with acute inflammation and strong immunohistochemical staining for adenovirus. Bottom: after treatment: only one cell was positive for AdV by immunohistochemistry.

Indirect Methods

Serology

Adenovirus infections can be diagnosed by the presence of specific IgM or IgG seroconversion detected by EIA, IF, haemagglutination inhibition or neutralization. EIA and IF are the methods most frequently used in the diagnostic laboratory. SN assays or haemagglutination-inhibition assays are used for detecting specific antibodies against each adenovirus serotype.

Some of the limitations of serology are: (i) specific IgM is detected in only 20–50% of infections; (ii) false-negative results may occur due to a poor immune

response; (iii) false-positive results may occur due to heterotypic anamnestic responses; (iv) with seroconversion-based tests the diagnosis is retrospective. However, serology is useful and important for epidemiological studies.

TREATMENT

Although no antiviral drug has been formally approved for treatment of adenovirus infection, clinical observation and *in vitro* data have shown that some antivirals may be effective. The strongest antiviral activity was associated

with cidofovir, and a moderate effect was seen with ribavirin and ganciclovir (Arav-Boger *et al.*, 2000; Bordigoni *et al.*, 2001; Hoffman *et al.*, 2001). Clinical studies in immunocompromized patients have used cidofovir or ribavirin, but prospective randomized controlled trials have not been carried out. Ribavirin is a purine nucleoside analogue with *in vitro* activity against RNA and DNA viruses. Different mechanisms have been proposed, including the inhibition of RNA-capping activity, direct inhibition of viral polymerases and increased mutation in newly-synthesized DNA. But it has not been established which is the possible mechanism of action against adenoviruses. The most common adverse effect is reversible mild anaemia. Successful use was described in the treatment of adenovirus HC, pneumonia, enteritis after BMT and hepatitis in a liver transplant recipient (Arav-Boger *et al.*, 2000; Howard *et al.*, 1999) (Figure 19.8). Other authors, however, have described its therapeutic failure (Chakrabarti *et al.*, 1999; Hale *et al.*, 1999). Success seems to be related to early treatment and serotype since only strains from species C (serotypes 1, 2, 5 and 6) are sensitive to ribavirin (Morfin *et al.*, 2005). Therefore, early identification, for example by PCR, followed by typing may permit earlier antiviral treatment.

Cidofovir is an acyclic nucleoside phosphonate analogue used as a broad-spectrum antiviral agent. Incorporation of diphosphate cidofovir results in termination of DNA-chain elongation. All adenovirus serotypes are susceptible *in vitro*. Despite its significant side effects (nephrotoxicity, myelosuppression and uveitis), cidofovir is currently used among BMT recipients and solid organ recipients. Strict monitoring of renal function should be performed in treated patients. Treatment success, defined as disappearance of signs and symptoms and clearance of adenovirus infection, was noted in 20 of 29 (69%) patients in a multicentre study of patients with various clinical manifestations, including gastrointestinal disease, pneumonia, encephalitis and hepatitis (Ljungman *et al.*, 2003). Improvements were noted in 10 of 14 (71%) BMT patients with HC (Nagafuji *et al.*, 2004). Clinical improvement of diarrhoea, cystitis and fever and clearance of the virus was observed in 5 of 7 (71%) children who received cidofovir treatment, despite the persistence of their immunodeficiency measured by CD4 (Legrand *et al.*, 2001). An apparent increased effectiveness in 56 of 57 (98%) was observed when patients were aggressively screened and treated regardless of symptoms or viral load in blood (Yusuf *et al.*, 2006).

A drawback of most of these studies is the lack of data regarding immune recovery after transplantation and its relation to adenovirus infection. A strong association between immune reconstitution (measured by the absolute lymphocyte count and CD4) and development of

adenovirus disease was observed (Chakrabarti *et al.*, 2002).

Antiviral treatment should be considered in patients positive for adenovirus if immunosuppression can not be reduced or if the patient has severe lymphocytopenia (Chakrabarti *et al.*, 2004). Prospective studies on drug efficacy, including active surveillance and evaluation of the immune recovery of the patient, are urgently needed.

Pre-emptive therapy is based on the possibility that adenovirus reactivates initially without clinical symptoms. Early initiation of pre-emptive therapy may be useful in controlling viral replication before the inflammatory response and disease has been triggered. Symptomatic disseminated disease is preceded by a period of asymptomatic viremia. Some investigators have suggested that surveillance of high-risk HSCT recipients and early pre-emptive therapy with cidofovir would be more effective than treatment after symptoms develop. Therefore, the use of pre-emptive therapy with cidofovir is current policy in some hospitals.

Another therapeutic alternative is the use of adenovirus-specific adoptive immunotherapy. Donor lymphocyte infusion has been successfully applied to a few BMT patients with severe adenovirus infection (Chakrabarti *et al.*, 2000; Hromas *et al.*, 1994). *In vitro* production of specific CTL to control adenovirus infections constitutes an active area of research.

The use of intravenous immunoglobulin has been associated with successful recovery, but data is scarce and limited.

PREVENTION

Prevention of adenovirus infections is not yet possible in community or institutional settings, although feasible in hospitals and eye clinics. Adenovirus spread in hospitals can be reduced by isolating infected patients, using good hand-washing practices, cleaning and appropriately disinfecting instruments and equipment (Rutala *et al.*, 2006). Adequate chlorination of swimming pools has also prevented KC epidemics.

Vaccines

To prevent ARD among military recruits, live oral adenovirus type 4 and 7 vaccines were developed in the 1960s. The vaccine serotypes are packaged together in enteric capsules, which bypass the stomach and only replicate once the intestine is reached. These vaccines were licensed in 1980 to US recruits only, who were routinely immunized (Top, 1975). Until recently, these vaccines were highly successful in preventing adenovirus-associated ARD; however, with interruptions of the

vaccine supply due to the manufacturer's decision to terminate production, adenovirus-associated ARD has re-emerged at military basic training facilities (Kolavic-Gray *et al.*, 2002).

The use of adenovirus vaccines has not been extended to young children even though this age group is at particular risk. The lack of attenuation and concern about potential oncogenicity have prevented the use of routine vaccines in civilians.

Adenoviruses as Vectors for Vaccination and Gene Therapy

For gene therapy, several E1-, E3-deleted vectors containing foreign genes have been constructed. The advantages of using adenoviruses as vectors are that they can be produced in large amounts which transduce in both replicating and nonreplicating cells.

Adenovirus vectors for the treatment of genetic diseases carrying the cystic fibrosis transmembrane regulator (CFTR) gene and others with the dystrophin gene have been constructed. Human trials with the CFTR gene have not been particularly promising, due to inefficient gene delivery to target cells and short-lived expression. Since adenoviruses do not integrate into the host chromosome, the expression is usually transient (Russell, 2000).

Other studies for the treatment of cancer used the p53 inserted into adenovirus vectors to restore the anti-oncogenic effect. Although preliminary evidence of efficacy has been reported, definite results are not yet available (Russell, 2000).

A variety of recombinant adenoviruses expressing exogenous genes for vaccination use have been constructed for HIV and rabies. This is an active field of research and significant progress is expected in the future for both gene therapy and recombinant vaccines.

FUTURE PROSPECTS

Given the severity and prevalence of adenovirus diseases among immunocompromized patients, there is a need for effective and nontoxic anti-adenoviruses therapy. Large multicentre prospective controlled clinical trials in different patient populations will be useful to determine the benefits and adverse effects of different antiviral drugs and immunotherapy.

Adenovirus respiratory infections can also be severe and fatal, but still no specific treatment has been proposed for these patients. Some antiviral drugs with *in vitro* activity are used for disseminated disease in immunocompromized patients. Another severe condition occurs in patients who suffered from KC, who were left with sensation of 'foreign body' and vision loss for weeks or

months. The development of topic antiviral drugs for this infection is highly needed.

No adenovirus vaccines are available for civilian populations. The live oral vaccine approved only for US military recruits has been discontinued. Current studies to re-establish production and usage in the military population are under development. New studies are needed in order to develop new and safe vaccines, especially for young children and immunocompromized patients.

Adenovirus vectors show considerable promise for gene therapy and vaccination. Although no significant clinical success has yet been shown with earlier first- and second-generation vectors, third-generation ones are now being studied.

PCR is current practice in most centres for rapid diagnosis and intervention. The quantitation of adenovirus viral load in blood by real-time PCR for treatment and prognosis of disseminated disease is gaining wide acceptance. However, the lack of well-standardized FDA-cleared or -approved assays and the lack of international standards limits the widespread utilization of this test. Determination of the changes in the viral load over time by regular monitoring of the same specimen type in transplant patients may be more useful than determination of the absolute viral load in predicting adenovirus disease.

The presence of more than one adenovirus serotype concomitantly in some patients should be further evaluated as it may provide some insight about adenovirus pathogenesis and host response.

Knowledge of this virus is rapidly expanding. The greater awareness of the pathology, the significant development of diagnostic methods and typing, and the possibility of determining the viral load all help to achieve a better understanding of the pathogenic mechanisms.

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Rhinoviruses

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INTRODUCTION

Rhinoviruses (Figure 20.1) are the major cause of the common cold. Although the majority of infections produce only mild disease, their impact on overall morbidity and their economic cost worldwide is very considerable. More recently, their role in acute exacerbations of asthma and other airway disease has been demonstrated, revealing a more sinister nature than previously thought.

While the recorded history of the common cold is over 2000 years old, already known and commented on by Hippocrates around 400 bc, rhinoviruses were first propagated in 1953 in tissue cultures whose supernatants were able to produce common cold symptoms in volunteers. The first rhinovirus was initially isolated from patients with colds and named in 1957, while three years later cytopathogenic viruses were recovered using human embryo kidney cell cultures (Tyrrell and Parsons, 1960). Other susceptible cell lines were subsequently discovered and used to identify additional serotypes and to study rhinoviral biology and their role in disease. Recently, the genomes of several serotypes have been sequenced and three-dimensional structures have been revealed in atomic resolution. Molecular technologies are rapidly progressing in the development of novel diagnostic tools and, it is hoped, will aid the so far unsuccessful search for an effective treatment.

TAXONOMY

Rhinoviruses are small RNA viruses belonging to the picornavirus (pico = small + RNA) family. The *Picor-*

naviridae also include the enteroviruses, such as polioviruses, coxsackieviruses and echoviruses, the cardioviruses, such as the rodent encephalomyocarditis virus, and the aphthoviruses or foot-and-mouth disease viruses. Rhinoviruses are more closely related to enteroviruses than to the other genera. They are the most numerous of the picornaviruses, with over 100 serotypes identified and numbered by specific antisera in a collaborative programme supported by the World Health Organization (Hamparian *et al.*, 1987). New virus types are constantly emerging by a process of random mutation and immune selection (Lau *et al.*, 2007; Lee *et al.*, 2007b; McErlean *et al.*, 2007; Patterson and Hamparian, 1997; Savolainen *et al.*, 2002). Rhinoviruses are divided into major (90%) and minor (10%) groups, according to their cellular receptor usage (Uncapher *et al.*, 1991). Human rhinovirus 87 (HRV-87) is an exception and does not use either of the above receptors (Vlasak *et al.*, 2003). An alternative categorization, dividing the viruses into groups A and B, based on sensitivity to antiviral compounds and correlating with sequence similarities and pathogenicity has also been proposed (Andries *et al.*, 1990; Lau *et al.*, 2007). Several serotypes are now fully sequenced (Kistler *et al.*, 2007).

Structure

The Capsid

Rhinoviruses are among the simplest infectious agents. Their virion consists of a non-enveloped capsid surrounding a single-stranded positive-sense genomic RNA. In 1985, X-ray crystallography revealed the structure of rhinovirus 14 and poliovirus 1 at atomic resolution

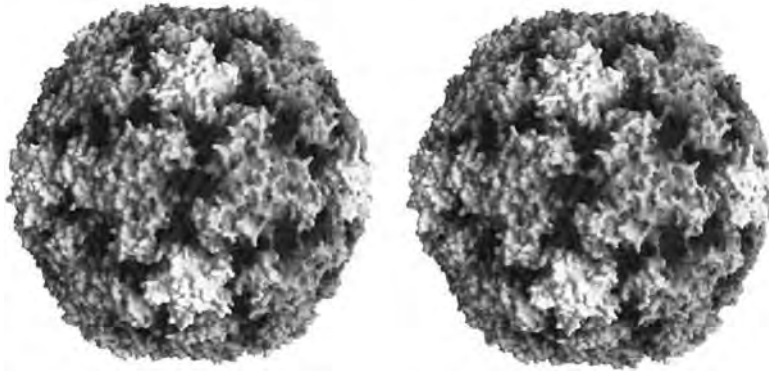


Figure 20.1 Three-dimensional computer-enhanced electron micrography of human rhinovirus 14. (Source: Courtesy of Dr J.Y. Sgro.)



Figure 20.2 Arrangement of the three main structural rhinovirus proteins. The fourth structural protein, VP4, is confined to the interior and hence is not shown.

(Rossmann *et al.*, 1985). These and subsequent studies also using cryoelectron microscopy and more recently hydrogen exchange mass spectrometry (Wang and Smith, 2005) showed considerable structural similarities amongst picornaviruses, such as prominent β -sheets forming a β -barrel, even though the sequence of each virus differs considerably from others in the group. The rhinovirus capsid is composed of 60 identical subunits arranged as 12 pentamers in an icosahedron. Each subunit consists of all four structural proteins of the virus named VP1–VP4, with molecular masses of 32, 29, 26 and 7 kDa respectively. VP1, 2 and 3 are surface proteins interacting with antibody and corresponding to the part of the genome with the highest variability. VP4 is confined to the interior of the capsid and is closely associated with the viral RNA. The arrangement of these structural proteins can be seen in Figure 20.2. Around the fivefold symmetry axis of the capsid there is a 2.5-nm-deep depression shaped by the five VP1 units, forming a ‘canyon’ (Figure 20.1). In most rhinoviruses, VP1 also contains a hydrophobic ‘pocket’

under the canyon, containing an incompletely characterized fatty acid ‘pocket factor’. This pocket is thought to be involved in virus uncoating and is the major target of antiviral compounds (Hadfield *et al.*, 1997). The structural organization of rhinoviruses is independent of serotype (Janner, 2006).

The conformation of the canyon, being physically unreachable by the immunoglobulin Fab, led to the hypothesis that this was the receptor-binding site. Evidence in favour of this hypothesis has been produced (Colonna *et al.*, 1988), demonstrating that the amino acid sequences at the base of the canyon are very well conserved, while the edges of the canyon and the most external portions of the virus capsid are hypervariable and correspond to neutralizing immunogenic sites (Rossmann and Palmenberg, 1988).

Antigenicity

Four such sites, localized in different areas of the capsid of HRV-14, along the edge of the canyon, were characterized by RNA sequencing of mutants that escaped neutralization by specific antisera (Sherry *et al.*, 1986). Nim-1a, Nim-1b on VP1 are positioned above the canyon, while Nim-2 on VP2 and Nim-3 on VP3, are positioned below. HRV-2 has a slightly different pattern, nevertheless, the antigenic sites are still located on the most protrusive regions of the virion. A peptide from VP2 (156–170) of HRV-2, structured like a random coil in solution, was shown to contain enough information to direct the production of neutralizing antibodies against the serotype (Molins *et al.*, 1998). High variability of these regions is implied by the large number of distinguishable serotypes and has been, to a certain extent, molecularly characterized (Horsnell *et al.*, 1995; Kistler *et al.*, 2007).

Expression of foreign peptides such as peptides from respiratory syncytial virus (RSV) or human immunodeficiency virus (HIV) in a rhinovirus vector, has been successful and resulted to an immune response against the specific peptide (Ding *et al.*, 2002; Zhang *et al.*, 1999). Such chimaeric viruses may become potent tools for mucosal immunization.

Receptors

An important function of the viral capsid is to deliver the genomic material intact into a cell following attachment to a cell surface receptor. Initially, it has been shown that almost 90% of rhinoviruses (major group) bind to one receptor on HeLa cells, while the remaining (HRV-1a, -1b, -2, -29, -30, -31, -44, -47, -49, -62) use another (Colonna *et al.*, 1986). The major group receptor was independently identified by three different groups in 1989 (Greve *et al.*, 1989; Staunton *et al.*, 1989; Tomassini *et al.*, 1989) as the intercellular adhesion molecule 1 (ICAM-1). ICAM-1 is a 95-kDa glycoprotein, a member of the immunoglobulin superfamily, physiologically acting as a receptor for the lymphocyte-function associated antigen 1 (LFA-1, CD11a/CD18) and Mac-1, found on leukocytes. It facilitates the interaction of lymphocytes with antigen-presenting cells as well as their migration to inflammatory sites. The binding sites of LFA-1 and major rhinoviruses on the ICAM molecule are distinct but significantly overlapping (Staunton *et al.*, 1990). Furthermore, cryoelectron microscopy proved that the rhinovirus-binding site is positioned in the canyon, verifying the 'canyon hypothesis' (Rossmann, 1994). This hypothesis suggests that one strategy for virus to escape the host immune's surveillance is to protect receptor attachment, as its site in the 'canyon' is largely inaccessible to antigens and resistant to accepting mutations that might inhibit receptor attachment. Recently, the three-dimensional atomic structure of the two N-terminal domains (D1 and D2) of ICAM-1 has been determined. Rhinovirus attachment is confined to the BC, CD, DE and FG loops of the N-terminal immunoglobulin-like domain (D1) at the end distal to the cellular membrane (Bella *et al.*, 1999). Binding of rhinoviruses to ICAM-1 blocks LFA-1/ICAM-1 interaction, possibly downregulating the local immune response and upregulating the viral receptor itself (Papi and Johnston, 1999), while there is evidence that at the same time rhinovirus-ICAM-1 binding induces conformational changes promoting viral uncoating and cell entry (Casasnovas and Springer, 1994). Rhinovirus appears to induce membrane-bound ICAM-1, while decreasing the soluble component, thereby facilitating and promoting cell infectivity (Whiteman *et al.*, 2003).

The minor group viruses bind to members of the low-density lipoprotein receptor (LDLR) family, including the very low-density lipoprotein receptor (VLDL-R) and α_2 -macroglobulin receptor/LDLR-related protein (α_2 MR/LRP) (Hofer *et al.*, 1994). The number of minor group human rhinoviruses has recently been extended to 12, since a recent study has demonstrated that HRV-23 and HRV-25, which were thought to bind to ICAM-1, are neutralized by MBP-V33333, bind LDLR and infect ICAM-1-deficient COS-7 cells (Vlasak *et al.*, 2005). The minor receptor binds to the small star-shaped dome on the icosahedral fivefold axis, in contrast to the major group where the receptor site for ICAM-1 is located at the base of a depression around each fivefold axis (Hewat *et al.*, 2000). Minor group viruses, exemplified by rhinovirus 2, transfer their genomic RNA to the cytoplasm through a pore in the endosomal membrane (Schober *et al.*, 1998). Interestingly, rhinovirus 89, a major group virus, was adapted in culture to replicate in ICAM-1-devoid cells, and LDLR-soluble fragments could not inhibit such replication, suggesting the possibility of additional receptors (Reischl *et al.*, 2001).

Replication

Rhinoviral RNA has a molecular weight of approximately 2 MDa, consisting of some 7200 nucleotides (Duechler *et al.*, 1987). A 5' non-coding region (NCR) of around 620 bases in length and a 3' NCR of 50 bases before a polyadenylated tail are characteristic of all currently sequenced serotypes (Figure 20.3). The long 5' untranslated region is highly conserved and contains several AUG codons upstream of that used to initiate translation. These sequences are within a complex structure, termed the internal ribosome entry site (IRES), which can bind ribosomal subunits directly, without the requirement for a free 5' end or a cap-binding protein (Stoneley *et al.*, 2000). The organization of the genome is shown in Figure 20.3. The structural proteins VP1-4 derive from the P1 region, while P2 and P3 code for the enzymes required for virus replication. The non-structural proteins, seven in total, include two proteases with specific viral cleavage sites (2A, 3C), an RNA-dependent RNA polymerase (3D) (Hung *et al.*, 2002) and a small protein Vpg (3B) which is covalently bound to the 5' end of the RNA and possibly acts as a primer for RNA synthesis (Rowlands, 1995). The remaining non-structural proteins (2B, 2C, 3A) are associated with RNA replication. Interestingly, an internal cis-acting replication element (cre) located within the genome segment encoding the capsid proteins, is required for RNA replication (McKnight, 2003).

The single-stranded, positive-sense RNA can act as messenger RNA and is infectious on its own. As in the other picornaviruses, the genome is translated into a

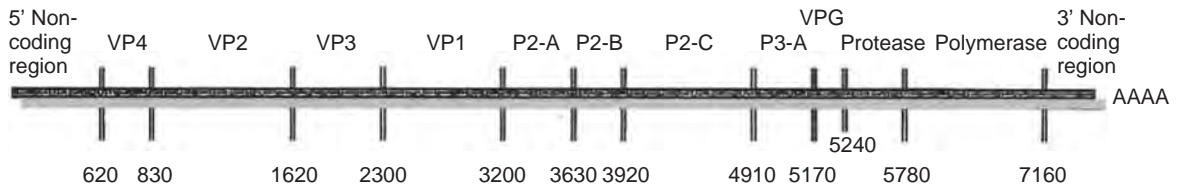


Figure 20.3 Diagrammatic representation of the rhinovirus genome.

polyprotein from a single, long open reading frame. This polyprotein is processed into the mature proteins by several cleavage steps performed by virus-specific proteases, 2A and 3C. The 2A protease makes the first cleavage between the structural and non-structural proteins, while 3C protease catalyses most of the remaining internal cleavages (Wang, 1999). In recent years, considerable efforts have been made in the development of antiviral compounds targeting 3C due its essential role in viral replication (Wanga and Chen, 2007). With the polymerase, a negative-sense copy of the genome is produced, from which further positive strands are made by viral RNA polymerase and these can act either as messenger RNA, which is subsequently translated, or can be incorporated as genomic RNA in the progeny virus.

Translation requires cellular trans-acting factors that are absent from, or limiting in rabbit reticulocytes, but are more abundant in HeLa cell extracts. At least two such factors have been identified: polypyrimidine tract-binding protein (PTB) and a complex of unr, an RNA-binding protein with five cold-shock domains, and a 38-kDa protein named unrip (unr-interacting protein) (Hunt *et al.*, 1999).

A recent study has reported that translation inhibition in infected cells can be mediated by the cleavage of host proteins, such as the eukaryotic initiation factor 4G (eIF4G) and poly(A)-binding protein (PABP), by viral proteases (Kerekatte *et al.*, 1999).

Rhinoviruses are capable of completing numerous replicative cycles within 6–8 hours with a yield of up to 100 000 viruses per cell (Belsham and Sonenberg, 1996).

PHYSICAL PROPERTIES

Rhinoviruses are small with a diameter of 28–34 nm. Their total molecular weight is approx. 8–8.5 MDa and their buoyant density in caesium chloride is 1.38–1.42 g/ml as fully infectious particles or 1.29 g/ml as empty capsids.

They are resistant to inactivation by organic solvents, including ether and chloroform, due to the absence of lipid envelope around the virion. They are also unaffected

by 70–95% ethanol, 5% phenol and trichlorofluorethane (Macnaughton, 1982). However, weak hypochlorite solution (common bleach) rapidly inactivates infectivity, provided that there is not a high concentration of organic matter present. Rhinoviruses are characteristically labile to extreme pH due to irreversible conformational changes, being inactivated at pH <5 as well as at pH 9–10. Acid sensitivity together with lipid solvent stability tests have been used for the identification of rhinoviruses. In addition, ultraviolet radiation is inactivating for rhinoviruses, while cationic substances such as MgCl₂, are favourable for the replication of most serotypes, appearing to stabilize the virions especially in high temperatures (Blough *et al.*, 1969).

Viability steadily decreases at room temperature, while it remains stable for days at 4 °C, months at –20 °C and indefinitely at –70 °C or lower. Rhinoviruses show considerable variation in their ability to resist heat inactivation at 50–56 °C, with some serotypes completely inactivated at lower temperatures and others quite resistant. Optimal culture temperature ranges from 33 to 35 °C (Killington, Stott and Lee, 1977), but most serotypes are able to productively infect and replicate in HeLa cells at 37 °C (Papadopoulos *et al.*, 1999).

INCUBATION AND TRANSMISSION

Infection with a rhinovirus can be initiated by small doses if the inoculum is delivered effectively to the nasopharynx. In experimental human transmission studies, the frequency of virus shedding after inoculation varied by inoculum size and the titre of the subject's serum-specific anti-serotype antibodies. Subjects with no neutralizing antibody could be infected with an inoculum of less than 10⁴ TCID₅₀ (tissue culture infectious dose₅₀) (Johnston and Tyrrell, 1995). However, susceptible patients, such as those with chronic obstructive pulmonary disease (COPD) may be successfully infected and a cold induced with doses as low as 10 TCID₅₀ (Mallia *et al.*, 2006). The mode of person-to-person transmission has been debated, but it is likely that both direct hand-to-surface-to-hand contact (Gwaltney and Hendley, 1978) and aerosol inhalation (Meschievitz *et al.*, 1984) are involved. A significant

positive relationship between lower levels of outdoor air supply in office buildings and increased frequency of virus detection in air filters has been reported (Myatt *et al.*, 2004). Rhinoviruses are capable of surviving on surfaces for several hours under ambient conditions (Sattar *et al.*, 1993) and transfer of rhinovirus through hand touch can occur in only a few seconds. This means that direct inoculation by rubbing the nares or eyes with infected hands can easily occur. The viral load in oral and pharyngeal secretions is considerably lower than that in nasal mucus. On the other hand there is good experimental evidence (Dick *et al.*, 1987) as well as epidemiologic evidence favouring the predominance of the inhaled route (Johnston *et al.*, 1996).

The incubation period for rhinovirus is one to four days, commonly two to three days. In general, viral shedding peaks two to four days after infection, and lasts for 7–10 days, however, it can persist for as long as three weeks. Viral clearance may be suboptimal in atopic subjects, with around half of them still infected two weeks after an experimental infection (Gern *et al.*, 2000), or more than six weeks after an acute asthma exacerbations (Kling *et al.*, 2005). The possibility of chronic infection has also been demonstrated in lung transplant recipients (Kaiser *et al.*, 2006). Natural transmission increases in relation to high virus titres in nasal secretions, increased symptoms, time spent in contact and social factors such as crowding and hygiene. In families, the interval between initial and secondary infections relates to the quantity and duration of shedding, ranging up to 10 days with an average of three days (Foy *et al.*, 1988). Transmission occurs most frequently from children to adults with a lag of 5–10 days (Contoli *et al.*, 2006).

HOST RANGE

In general, rhinoviruses are extremely host-specific. Human viruses have not been recovered from animals. Under experimental conditions, chimpanzees have been infected, developing a common cold-like disease, as well as gibbons which do not exhibit symptoms. Other primates are less susceptible. Mice develop an immune response to parenterally administered rhinoviruses (Hastings *et al.*, 1991), but intracellular blocks to rhinovirus replication have been described (Yin and Lomax, 1986). Major group rhinoviruses are reported not to bind mouse ICAM-1, thus 10% of the known rhinovirus serotypes use both the human and mouse LDLR to enter cells of either species. Recently, viral replication as well as inflammatory events similar to those seen in humans were observed in a transgenic murine model, in which mouse ICAM-1 extracellular domains 1 and 2 were substituted

with the human domains 1 and 2 (Bartlett *et al.*, 2008). In this model rhinovirus infection induced neutrophilic and lymphocytic airway inflammation and chemokine and pro-inflammatory cytokine production, as in humans. These models are therefore likely to provide a similar boost to research into diseases in which rhinovirus infection is implicated.

PATHOGENESIS

The nose is the main portal for rhinovirus entry, although the eye and oral inhalation may also serve as entry routes. Whether or not an infection can begin in the lower airways is not as yet clearly determined. The nasal epithelium is the primary site of infection. A series of upper airway biopsies suggested that rhinovirus infection may initiate in the nasopharynx, in the area of the adenoid (Winther *et al.*, 1986). A considerable proportion of surgically removed adenoids harbour rhinoviruses (Rihkanen *et al.*, 2004). Rhinovirus replication occurs primarily in the nose, as inferred from comparative titrations of nasal, pharyngeal and oral secretions as well as of droplets from coughs and sneezes. Nevertheless, the fact that rhinovirus replication occurs in the lower airways has been confirmed in several studies (Gern *et al.*, 1997b; Mosser *et al.*, 2002; Papadopoulos and Johnston, 2000) and evidence suggests that this is the norm rather than an exception following infection of the upper airways. Viraemia has not been detected in normal adults, but it may occur during rhinovirus respiratory infections in normal children and is common in the early course of acute asthma exacerbations (Xatzipsalti *et al.*, 2005).

Rhinovirus infection does not produce extensive cytopathology of the nasal mucous membrane; however, the extent correlates with the titre of recoverable virus. *In vitro*, exposure of human primary bronchial epithelial cells to rhinovirus may result in an extensive cytopathic effect (CPE), depending on serotype and culture conditions (Papadopoulos and Johnston, 2000; Schroth *et al.*, 1999). In an already compromised epithelium, such as in the case of asthma, rhinovirus may induce considerable epithelial cytotoxicity (Bossios *et al.*, 2005). Mucosal oedema with sparse infiltration of inflammatory cells, mainly neutrophils, are the predominant histologic changes. Nasal mucociliary clearance is reduced as a result of a reduced ciliary beat frequency and loss of ciliated epithelium (Wilson *et al.*, 1987).

Nasal secretions of infected individuals have increased plasma proteins, such as albumin and immunoglobulin G (IgG), as well as glandular proteins (lactoferrin, lysozyme and secretory IgA, 7F10 immunoreactive mucin) (Igarashi *et al.*, 1993a; Yuta *et al.*, 1998). These

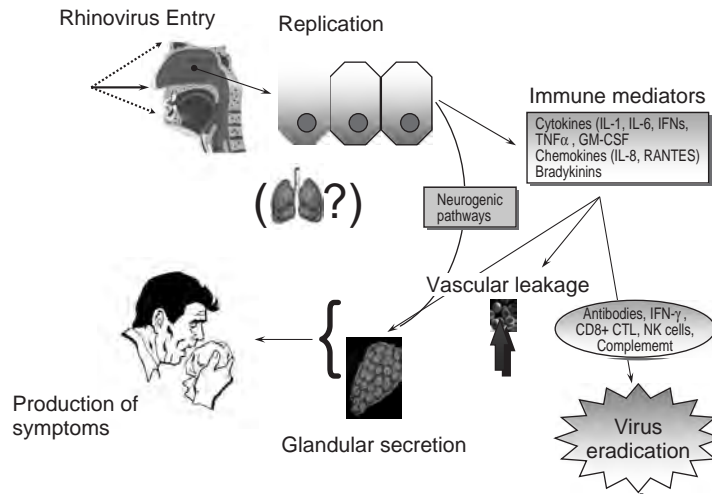


Figure 20.4 Schematic representation of pathogenetic mechanisms of rhinovirus infection. Rhinovirus entry and replication in the nasopharynx induces immune mediators and neurogenic pathways. Viruses are eradicated, but the concurrent vascular leakage and glandular secretion result in the well-known symptoms of the common cold.

findings reflect both increased vascular permeability, usually mediated by vasoactive amines, and glandular secretion commonly induced by cholinergic reflexes and neuropeptides. However, the detailed involvement of any such mediators remains to a great extent speculative (Figure 20.4). Kinins are generated in nasal secretions during natural and experimental colds, while their intranasal instillation causes a sore throat (Naclerio *et al.*, 1988). The list of both pro-inflammatory (interleukin (IL)-1 β , IL-2, IL-4, IL-6, IL-8, IL-11, IL-12, IL-13, IL-16, G-CSF, GM-CSF, TNF- α and interferon gamma (IFN- γ)) and anti-inflammatory (IL-1Ra, IL-10) cytokines induced either *in vitro* or *in vivo* by rhinovirus infections, is long and is growing (Einarsson *et al.*, 1996; Johnston *et al.*, 1998; Papadopoulos *et al.*, 2000; Teran *et al.*, 1997; Zhu *et al.*, 1996, 1999). Several chemokines able to attract neutrophils and eosinophils are also induced after rhinovirus infection of nasal and bronchial epithelium, including RANTES, Gro α , ENA-78, eotaxin and eotaxin-2 (Griego *et al.*, 2000; Papadopoulos *et al.*, 2001). Rhinoviruses are also able to directly activate airway smooth muscle cells *in vitro*, however, it is not clear whether the virus could reach these cells *in vivo* (Grunstein *et al.*, 2001). In a helper T cell type 2 (Th2)-dominant microenvironment, an increased production of eotaxin-1 after infection, by activating intracellular Toll-like receptor 3 (TLR-3) has been observed (Niimi *et al.*, 2007). Oxidative stress and the activation of inflammation-related transcription factors, such as nuclear factor kappa-B (NF- κ B), mediate the elaboration of several rhinovirus-induced cytokines and chemokines (Biagioli *et al.*, 1999; Papi *et al.*, 2002;

Zhu *et al.*, 1997). This is also the case for ICAM-1, which is also significantly upregulated by rhinovirus infection via an NF- κ B-mediated mechanism (Papi and Johnston, 1999). Possible pathways may also be through the stress-activated protein kinase p38 (Griego *et al.*, 2000), Syk (Wang *et al.*, 2006), phosphatidylinositol (PI) 3-kinase, interferon regulatory factor 3 (IRF-3) and activating transcription factor 2 (ATF-2).

The involvement of histamine is uncertain and conflicting results have been obtained from clinical trials of antihistamines. First-generation antihistamines could relieve sneezing and nasal discharge, but these compounds have H₁-blocking, anticholinergic and other central nervous system (CNS) activities, in contrast to second-generation agents, which are less effective in colds. Elevated histamine levels were detected after experimental and wild-type infection in allergic patients but not in normal subjects (Igarashi *et al.*, 1993a). Nevertheless, the second-generation antihistamines loratadine and desloratadine were able to inhibit rhinovirus-induced ICAM-1 upregulation in airway epithelial cells *in vitro* (Papi *et al.*, 2001). In mast cells and basophils, rhinovirus has been shown to increase histamine production *in vitro* (Hosoda *et al.*, 2002).

The arachidonic acid pathways are also involved, suggested by induction of 5-lipoxygenase- and cyclooxygenase-2-positive cells in bronchial biopsies of experimentally infected volunteers (Seymour *et al.*, 2002), as well as upregulation of leukotriene C₄ during experimental infection (Gentile *et al.*, 2003).

As far as the nervous system is concerned, parasympathetic blockade is able to reduce some symptoms of rhinoviral colds (Jacoby and Fryer, 1999). Double-stranded RNA may cause increased bronchoconstriction via increased release of acetylcholine from the vagus nerves because of loss of the muscarinic receptor 2 function on parasympathetic nerves in the lungs (Bowerfind *et al.*, 2002), although this has not been shown with rhinovirus. Furthermore, unilateral inoculation and infection results in bilateral symptomatology, favouring a neural pathway activation (Winther *et al.*, 1986).

Vascular leakage (Igarashi *et al.*, 1993b) and mucus secretion (He *et al.*, 2004; Inoue *et al.*, 2006) result in nasal blockage and stimulation of the sneeze and cough reflexes, resulting in the well-known common cold symptomatology.

Mucosal oedema resulting from vascular leakage, venous sinusoidal engorgement and mucosal inflammatory cell infiltration is a prominent feature, and may lead to complications such as otitis and sinusitis. Indeed, computed tomography (CT) scans obtained during and after acute rhinoviral infection have clearly demonstrated that paranasal sinus occlusion is a common event in rhinovirus colds (Gwaltney *et al.*, 1994).

The mechanisms of asthma exacerbations induced by viral infections in susceptible individuals are another currently investigated issue in rhinovirus pathophysiology. Rhinovirus infection seems to potentiate allergic responses (Calhoun *et al.*, 1994), however, the precise mechanisms have yet to be determined. Inflammatory mediators such as kinins, and pro-inflammatory cytokines induced by rhinovirus infection (see list above), altered cell-mediated immunity, virus-specific IgE and other mechanisms have been proposed, but the relative importance of each of these currently remains unclear (Papadopoulos and Johnston, 2001a). Fewer studies have examined the epidemiology of virus infection in COPD. Between 33 and 70% of exacerbations are associated with symptoms of the common cold. Persistent infection with viruses such as RSV, rhinovirus and adenovirus has been associated with the progression of COPD (Message and Johnston, 2004). Higher plasma fibrinogen, induced sputum IL-6 levels and increased airway eosinophilia have been found at exacerbation (Wedzicha, 2002).

Finally, it has been demonstrated recently that rhinovirus induces the production of angiogenic and pro-fibrotic mediators, which may contribute to airway remodelling. Infection of primary bronchial epithelial cells increases vascular endothelial growth factor (VEGF) (De Silva *et al.*, 2006; Psarras *et al.*, 2002), fibroblast growth factor 2 (FGF-2) (Volonaki *et al.*, 2006) and transforming growth factor beta (Dosanjh,

2006); production of such growth factors is amplified in an atopic environment (Psarras *et al.*, 2002; Xatzipsalti *et al.*, 2008).

IMMUNITY

Both cellular and humoral immunity are activated in response to rhinovirus infection. Virus-specific IgG and IgA serum antibodies remain low for the first week after inoculation and subsequently begin to increase, to reach their peak approximately a month later. IgG antibodies stay at high levels for at least a year, while IgA declines slowly but remains detectable during the same period. Nasal IgA is also produced, becoming detectable two weeks after inoculation, reaching its peak one week later and slowly declining to its original levels by one year (Barclay *et al.*, 1989). The late rise in antibody titres indicates that humoral immunity is not essential for recovery from viral illness. On the other hand, existing antibodies, especially in high titres, are associated with protection against reinfection with the same serotype and/or lessened signs and symptoms (Alper *et al.*, 1998). Possible mechanisms of antibody-mediated virus inactivation include virus aggregation, activation of the complement cascade, prevention of binding to receptor as well as inhibition of uncoating (Rowlands, 1995).

An important role in virus eradication has been attributed to cellular immunity, but the mechanisms involved are not yet understood in detail. In contrast to the high specificity of humoral immunity, rhinovirus-specific T cells can recognize both serotype-restricted and shared viral epitopes (Gern *et al.*, 1997a). Peripheral blood lymphocyte counts are decreased during infection, followed by recovery or even leukocytosis (Skoner *et al.*, 1993). Increased production of IL-2 and IFN- γ after mitogen stimulation as well as increased natural killer cytotoxicity has been found in peripheral blood mononuclear cells (PBMCs) after experimental rhinovirus infection (Hsia *et al.*, 1990). Lymphocytes can be activated both specifically and nonspecifically through a monocyte-dependent mechanism (Gern *et al.*, 1996b). Rhinoviruses infect human macrophages and can replicate efficiently in THP-1, a monocytic cell line which can be differentiated to a macrophage phenotype by treatment with phorbol myristate acetate (PMA) (Laza-Stanca *et al.*, 2006), indicating a potentially direct effect of these cells in antiviral immunity (Gern *et al.*, 1996a). Monocytes stimulated by rhinovirus were also shown to inhibit the allostimulatory capacity of myeloid dendritic cells (DCs), by producing large amounts of the immunosuppressive IL-10 (Stockl *et al.*, 1999). DCs infected by rhinovirus demonstrate a diminished T-cell stimulatory capacity, inducing a deep

anergic state through B7-H1 and sialoadhesin (Kirchberger *et al.*, 2005). The production of type 1 (IFN- γ , IL-12) as well as type 2 (IL-4 and IL-10) cytokines has been documented, and atopic asthmatic individuals appear to respond with relatively deficient type 1 cytokine production (Papadopoulos *et al.*, 2002a; Parry *et al.*, 2000). In a more complex model, PBMCs were exposed to rhinovirus and the resulting supernatants, containing various factors associated with the immune response to the virus, were used to treat epithelial cells during rhinovirus infection. When such conditioned media from normal or atopic asthmatic individuals were used, it was shown that in an 'atopic environment' epithelial inflammation was reduced, while virus replication and the resulting epithelial cytotoxicity were significantly increased (Xatzipsalti *et al.*, 2008). IFNs are critical regulators of a wide array of innate protective responses. Recently, it has been demonstrated that asthmatic bronchial epithelial cells show an impaired IFN- β and IFN- λ production and impaired virus-induced apoptosis, resulting in increased virus replication (Contoli *et al.*, 2006; Wark *et al.*, 2005). Strong lines of evidence support the role of TLRs in the induction of the IFN response. TLR-3 recognizes double-stranded RNA and has been found to be increased after rhinovirus infection in bronchial epithelial cells (Hewson *et al.*, 2005) and in cultured nasal cells (Lin *et al.*, 2007). Its role in human diseases and specifically in rhinovirus-induced respiratory pathology requires further characterization.

EPIDEMIOLOGY

The common cold is probably the most frequent illness afflicting humans and certainly the most common cause for primary care consultations and absenteeism from work or school. The average number of yearly infections in adults is estimated to be between two and five, while this number increases to around 12 in children. A simple estimation would reveal that a normal individual spends between one and two years of his or her life suffering from colds! At least a quarter of six-month-old infants have antibodies against rhinoviruses, while over 90% have such antibodies at the age of two years (Blomqvist *et al.*, 2002).

Rhinoviruses cause about 35–60% of common colds. Interestingly, in contrast to popular belief, early studies were unable to demonstrate any increase in susceptibility to rhinoviral infections after exposure to cold temperatures (Douglas *et al.*, 1967), although this possibility cannot be rejected (Eccles, 2002). In one report, acute chilling of the feet was suggested to induce onset of common cold symptoms in around 10% of subjects who were chilled

(Johnson and Eccles, 2005). Several other epidemiological factors are also involved. Age is certainly important. Infections increase significantly from the second year of life and throughout school age, decreasing subsequently, probably due to neutralizing antibodies induced by previous exposures (Monto, 1995). Increased morbidity and complications reappear in the elderly (Nicholson *et al.*, 1997). Apart from the age-related susceptibility to the virus, socio-economic factors such as nutrition and population density, but most importantly family structure strongly influence the incidence of rhinovirus infections. An infection is usually introduced by a child to other siblings and parents at home, increasing by this way the incidence of the disease in the members of the family (Oliver *et al.*, 2006). Mothers are more susceptible than fathers, possibly because of increased exposure. Transmission at school and daycare facilities is also very high, due to overcrowding, low immunity and children's unhygienic habits (Goldmann, 1992).

A seasonal pattern has been documented in temperate climates, with two peaks occurring: one in autumn, following the opening of schools, and another one in late spring. School attendance is the major factor in determining seasonal patterns; infections occur throughout the winter months, peaking in the first two to three weeks after children return to school (Johnston *et al.*, 1996). Depending on age, season and population, and on the sensitivity of the polymerase chain reaction (PCR) assay, between 3% and 30% of asymptomatic subjects may be found positive by PCR (Marin *et al.*, 2000; Nokso-Koivisto *et al.*, 2002).

All populations are affected. The prevalent serotypes vary from year to year. It is possible that a small number of serotypes may cause most of the illnesses (Monto *et al.*, 1987). Based on the sequence for the VP1 capsid protein of rhinovirus, 101 members of the rhinovirus genus can be subdivided into two distinct genetic species, rhinovirus-A, rhinovirus-B and a single serotype, HRV-87 (Ledford *et al.*, 2004). More recently, analysis of the 5' NCR sequences showed limited homologies between the different serotypes. Despite the development of new diagnostic assays, the variability and the genotypic diversity of rhinovirus clinical strains makes the specific identification of rhinoviruses that cause disease in humans very difficult (Deffernez *et al.*, 2004).

There is a well-documented epidemiological relationship between various aspects of psychological stress, emotional style (Cohen *et al.*, 2003) and the susceptibility to rhinovirus infection (Cohen *et al.*, 1997; Takkouche *et al.*, 2001), the mechanisms of which are still speculative.

The question of whether atopic individuals are more susceptible to colds, develop more severe colds or clear rhinoviruses less effectively than normal subjects is still

incompletely understood. There is evidence for incomplete virus clearance (Gern *et al.*, 2000; Papadopoulos *et al.*, 2002a) and augmented colds in atopic asthmatic subjects (Bardin *et al.*, 1994; Heymann *et al.*, 2005). A recent, well-designed longitudinal study could not find differences between atopic asthmatic and normal subjects with respect to frequency, duration, or severity of rhinovirus colds in terms of upper respiratory symptoms, though there was significantly increased severity and duration of lower respiratory symptoms and falls in lung function in the asthmatic subjects (Corne *et al.*, 2002). Epidemiological studies have confirmed a synergistic interaction between respiratory virus infections and allergen exposure for risk of asthma exacerbations in adults and children (Custovic *et al.*, 2005; Green *et al.*, 2002). Surprisingly, in one report, experimental allergen challenge was able to protect from subsequent rhinovirus infection, suggesting a more complicated interaction (Avila *et al.*, 2000). Hence, repeated low-dose allergen exposure and rhinovirus infection have been found to induce distinct inflammatory profiles within the airways in patients with asthma. No interaction between these two environmental triggers have been noticed, indicating that preceding allergen exposure is not a major determinant of the severity of rhinovirus-induced asthma exacerbations in patients with mild asthma (Johnston, 2003; de Kluijver *et al.*, 2003). The explanations for these apparently contradictory findings will require further study.

CLINICAL FEATURES

The symptoms of the common cold easiest to describe are the ones which everybody has experienced: rhinorrhoea, sneezing, nasal obstruction, sore throat and cough. General malaise and headache may occur, while fever is less common. Mood and mental functioning are also affected, with reduced alertness and slowed reaction times (Smith *et al.*, 1998). Sleep is also significantly impaired (Drake *et al.*, 2000). The course of the disease correlates with virus titres. Symptoms appear after an incubation period of 24–48 hours, reach their peak two to three days later and last for five to seven days in total, persisting occasionally for as long as two to four weeks. Symptom severity is highly variable; on many occasions the disease may be hardly noticed, while around 20% of non-influenza flu-like (fever $\geq 38^{\circ}\text{C}$ plus one respiratory and one constitutional symptom) illnesses can be attributed to rhinoviruses (Boivin *et al.*, 2002). In patients hospitalized with respiratory problems, rhinovirus-related clinical presentations include bronchiolitis and pneumonia at ages less than five years, asthma exacerbations

in older children and adults and pneumonia, COPD exacerbations and congestive heart failure in older adults (El-Sahly *et al.*, 2000). The presence of rhinovirus either alone or in combination with RSV may be related to a more severe clinical presentation of acute bronchiolitis in infants (Papadopoulos *et al.*, 2002b).

Rhinovirus infections may cause significant morbidity in certain patient groups. Infants with bronchopulmonary dysplasia may develop serious respiratory illness, necessitating intensive care unit admission and occasionally mechanical ventilation (Chidekel *et al.*, 1997). This is also the case for children with primary immunodeficiencies (Crooks *et al.*, 2000; Ison *et al.*, 2003). Myelosuppressed individuals may be associated with more severe pneumonia (Ghosh *et al.*, 1999). Pulmonary function abnormalities, disease progression and secondary bacterial infections can result in children with cystic fibrosis (Collinson *et al.*, 1996). Elderly people, especially residents of nursing homes, are also prone to severe disease that can prove fatal (Wald *et al.*, 1995).

Complications

Acute otitis media (AOM) is the most common complication of a viral upper respiratory infection in children. Negative middle ear pressure develops in the majority of common colds in healthy individuals (Winther *et al.*, 2002). Using PCR, rhinoviruses are detected in the middle ear fluid of 25–40% of episodes of AOM. Rhinoviruses were also present in 20% of middle ear fluids of patients with otitis media with effusion at the time of tympanostomy tube placement (Pitkaranta *et al.*, 1998). Rhinovirus is also the predominant virus recovered by reverse transcriptase polymerase chain reaction (RT-PCR) in the middle ear cavity of children with asymptomatic otitis medium with effusion (OME), especially those with a history of longstanding OME or repeated episodes (Chantzi *et al.*, 2006). As previously mentioned, sinus involvement is detected by CT scan in a high percentage of rhinovirus infections, while there is evidence suggesting a causal role of the virus in as many as 50% of episodes of community-acquired sinusitis (Pitkaranta *et al.*, 1997). Recently, the detection of rhinovirus in turbinate epithelial cells of chronic sinusitis patients has also been confirmed (Jang *et al.*, 2006). In all instances, the viruses may also facilitate or contribute to concurrent bacterial infections.

The role of rhinovirus infection in exacerbations of asthma has been well documented in many studies. Upper respiratory tract infections are associated with around 80% of asthma episodes in schoolchildren, with rhinovirus being the most commonly detected causative agent (Johnston *et al.*, 1995) (Figure 20.5). In adults, detection frequencies are lower, possibly due to diminished

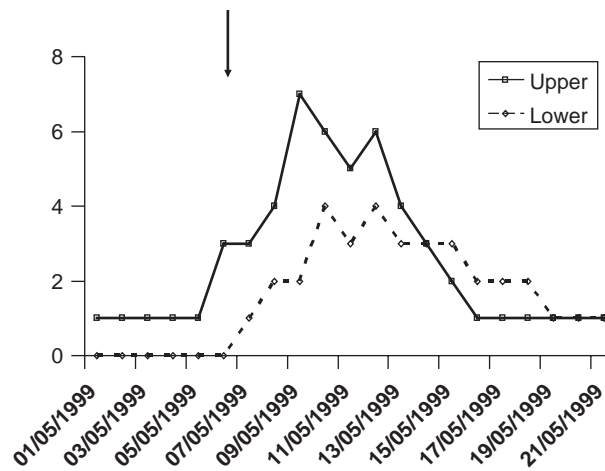


Figure 20.5 Upper and lower airway symptom score charts during rhinovirus infection. The arrow indicates the point at which a nasal wash was obtained and rhinovirus aetiology was confirmed with PCR.

viral shedding, although studies using sensitive molecular methodology indicate an involvement in at least half of asthma exacerbations (Nicholson *et al.*, 1993; Teichtahl *et al.*, 1997) and two recent studies using PCR to detect viruses in sputum samples have detected viruses in over three-quarters of asthma exacerbations in adults (Wark *et al.*, 2005). Time trend analysis confirms that viral infections are indeed important in exacerbations in adults as well as children (Johnston *et al.*, 1996). The mechanisms by which rhinoviruses exacerbate asthma include bronchial epithelium-mediated inflammation, induction of an abnormal immune response, neural mechanisms and more, reviewed elsewhere (Papadopoulos *et al.*, 2004).

Common colds are also associated with exacerbations of COPD. It has been suggested that the majority of exacerbations of COPD are associated with bacterial infections but many exacerbations occur after viral infections. The virus detection rates vary according to the methods of virus detection from 48.4% (Papi *et al.*, 2006) to 58.2% (Seemungal *et al.*, 2001). Respiratory viruses have been detected even when the patients are stable, and this suggests that chronic viral infection may occur as well.

Rhinoviruses are also implicated in acute and chronic bronchitis (Seemungal *et al.*, 2000), bronchiolitis and pneumonia (Papadopoulos and Johnston, 2001b; Papadopoulos *et al.*, 2002b). Clinical studies report that rhinovirus infection is the second most frequent agent associated with community-acquired pneumonia (Tsolia *et al.*, 2004) and bronchiolitis in infants and young children (Hayden, 2004; Papadopoulos *et al.*, 2002b).

DIAGNOSIS

Virus cultures, serological tests and more recently nucleic acid detection have been used in the detection of rhinoviruses for diagnostic and research purposes, although rhinovirus diagnostics are rare in clinical laboratories. Virus culture is cumbersome, time-consuming and insensitive, and its diagnostic value is very limited in a clinical setting. Serology is totally impractical as a result of the large number of serotypes. Nucleic acid techniques, mainly PCR, can potentially overcome these difficulties and become the methods of choice for viral detection; a number of protocols are currently under evaluation.

Virus Isolation

The optimal specimen for virus isolation is a nasal washing or a nasal aspirate. Throat or nasal swabs are used as an alternative in the field, or from children where nasal washings are difficult to obtain. Samples should be transported to the laboratory in virus transport medium as soon as possible and put immediately into culture or stored frozen at -70°C . Rhinoviruses replicate in several human embryonic and monkey tissues. However, they replicate best in human fetal lung fibroblasts (WI38, MRC5) and strains of HeLa cells. Samples are inoculated in cell cultures in tubes and are incubated in a roller drum at 33°C . They are then monitored under low-field magnification daily for the development of a CPE. Rhinoviral CPE is characterized by shrinkage and rounding of cells, cellular destruction and detachment of the cell layer (Figure 20.6).

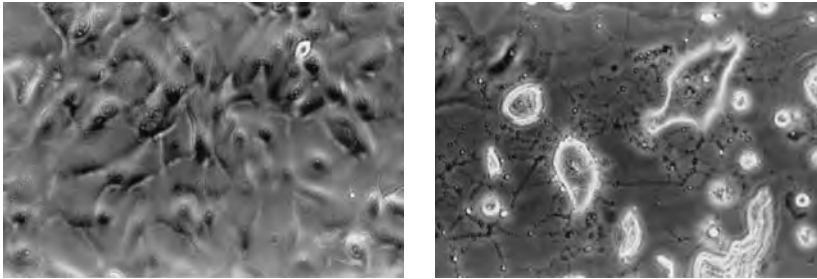


Figure 20.6 Rhinovirus-infected primary human bronchial epithelial cells developing a characteristic cytopathic effect (right), in comparison to control cells (left).

It can be noticed as early as 24 hours after inoculation, while the majority of positive samples develop CPE within eight days. A second passage after this time is usually performed to identify less potent strains (Johnston and Tyrrell, 1995). Confirmation of rhinoviral identity can be performed by acid stability testing and of individual serotypes by neutralization tests using serotype-specific antisera.

Serology

Serological methods can be used for the detection of antiviral antibodies. Neutralization tests can be done in combination with any culture system and have been the only standardized method for a number of years. They are used for antibody detection and quantification once the serotype is known, as in the case of experimental studies with volunteers inoculated with a known serotype, or with a virus isolated from the same patient. Pre- and post-infection serum titres are compared. Unfortunately, due to the large number of serotypes (>100), these tests are only of limited practical value and are not used in routine clinical diagnosis.

Haemagglutination inhibition tests are possible with a subset of rhinoviruses that agglutinate red blood cells. Their results are comparable to those of the neutralization assays, but they are not widely used as they require large amount of virus and can be applied only to a subset of serotypes.

Enzyme-linked immunosorbent assays (ELISAs) are faster and much easier to perform, offer better sensitivity and can be readily adapted to measure different antibody isotypes both in serum and nasal secretions (Barclay and Al-Nakib, 1987). However, as with the other serological assays ELISA is diagnostically useful only when the rhinovirus serotype is already known, or when ELISA is carried out using a virus isolated from the same patient.

Nucleic Acid Detection

Molecular methods for infectious agent identification are steadily becoming more widely applied in regard to a vast array of microorganisms, including rhinoviruses. Different approaches based on the PCR have been investigated in order to optimize the method for research as well as clinical purposes (Andeweg *et al.*, 1999; Blomqvist *et al.*, 1999; Steininger *et al.*, 2001). Most sets of primers derive from the 5' NCR, which is the most conserved between different serotypes. Sensitivity is markedly improved in comparison to virus culture (Hyypia *et al.*, 1998; Johnston *et al.*, 1993). A problem has arisen from the fact that in several instances these procedures could not distinguish rhinoviruses from the closely related enteroviruses. Several approaches, including nested designs, differential hybridization or restriction fragment length polymorphism (RFLP) analysis have addressed this problem successfully (Mori and Clewley, 1994) (Papadopoulos and Johnston, 1999) (Figure 20.7). Nucleic acid sequence-based amplification (NASBA) (Samuelson *et al.*, 1998) and real time PCR are also in development. Thin-film technology has been used to increase sensitivity and versatility of amplicon detection (Jenison *et al.*, 2001). Finally, multiplex PCR approaches for the simultaneous detection of different respiratory viral pathogens, including rhinoviruses, which would greatly facilitate epidemiological studies as well as clinical diagnosis, are also in development. New tests that can detect different respiratory virus types/subtypes (Mahony *et al.*, 2007) using sensitive microarray system (Quan *et al.*, 2007) or multiplex PCR chemistry coupled with high-throughput microsphere flow cytometry (Lee *et al.*, 2007a) have been developed. A database containing information on virus-specific oligonucleotides and primers is available in the internet (Onodera and Melcher, 2002). Multiplex kits for respiratory virus detection, including rhinoviruses, are also commercially available.

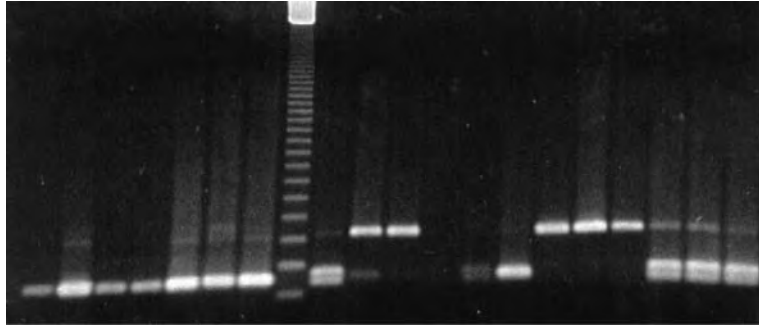


Figure 20.7 Agarose gel electrophoresis of PCR products reveal a band at 190 bp in the case of rhinovirus (lanes 1–7) and a 380 bp band or a double 180–200 bp band in the case of enteroviruses (lanes 9–20), after RFLP digestion of picornavirus PCR amplicons. Lane 8 is a ladder.

PREVENTION AND TREATMENT

Intensive efforts over the last 40–50 years on the development of treatment or prevention strategies against rhinovirus-induced common colds have been so far without encouraging results. Approaches that have been investigated include nonspecific and empirical therapies, antiviral and antimediator drug treatments and vaccines (Table 20.1). Frequently inappropriate remedies are applied, resulting in a very high cost burden (Bertino, 2002).

The major obstacle for vaccine development is the high number of serotypes that do not produce heterotypic humoral immunity. Initial attempts have achieved protection against a specific serotype in volunteers inoculated with inactivated viruses. A decavalent vaccine was also tested in humans, resulting in variable responses to the included serotypes and insufficient heterotypic immunity (Hamory *et al.*, 1975). The protective duration of any responses was also undefined. The design of peptide vaccines, especially against T-cell epitopes which seem to be more conserved, appeared promising but has not been progressed (Francis *et al.*, 1987).

Among the pharmacological antiviral agents, IFN- α was the earliest and most widely investigated. IFN is effective if given intranasally before or shortly after exposure to the virus, both in experimental infections in volunteers and in family outbreaks (Douglas and Betts, 1974). However, its high cost and local bleeding and discharge after long-term prophylactic usage have been the major drawbacks for its clinical use. Following the demonstration of an important role of IFNs β and λ in the epithelial response to rhinovirus, these agents are currently potential antiviral candidates.

A large number of compounds able to inhibit rhinoviral infection by preventing virus uncoating and/or cell entry have been investigated. Unfortunately, to date no effective

drug has emerged from this research due to problems in potency, delivery and toxicity of the compounds and the emergence of resistant viral strains (Arruda and Hayden, 1996).

Since much of the symptomatology of the common cold is produced through inflammatory mediators, drugs antagonizing these mediators have been tested. Little benefit was demonstrated by antihistamine treatment, a bradykinin antagonist showed negative results, while anticholinergic sprays are able to reduce rhinorrhoea and sneezing (Johnston, 1997). Corticosteroids were found to have a marginal effect on symptoms, but a rebound appeared when treatment was stopped; moreover they have been associated with increases in viral titres. Speculation on the potential use of macrolide antibiotics, due to their *in vitro* anti-inflammatory effects (Suzuki *et al.*, 2002), were not confirmed in a clinical trial (Abisheganaden *et al.*, 2000), although more recently telithromycin was shown to be effective against acute asthma exacerbations (Johnston *et al.*, 2006).

The inadequacies of specific therapies, in addition to the frequency of the problem, have supported the emergence of nonspecific treatments, including the use of ascorbic acid, zinc gluconate, echinacea, inhalation of humidified hot air or the traditional Japanese herbal medicine *hochu-ekki-to* (Yamaya *et al.*, 2007). For all these remedies the mechanisms are speculative and the evidence frequently contradictory. Despite several blinded studies and meta-analyses, the usefulness of zinc preparations is, at best, marginal (Turner and Cetnarowski, 2000). However, *in vitro* addition of zinc salts is able to potentiate the anti-rhinoviral activity of IFN- α by 10-fold (Berg *et al.*, 2001). Substances with antioxidant properties are also able to inhibit rhinovirus-induced ICAM-1 upregulation *in vitro* (Papi *et al.*, 2002), suggesting a possible mechanism of action for ascorbic acid. Ascorbic acid in

Table 20.1 Efforts towards an anti-rhinoviral therapy

General	
Ascorbic acid, zinc gluconate, inhalation of hot air, echinacea, non-steroidal anti-inflammatory drugs, <i>hochu-ekki-to</i>	Questionable benefit
Anti-inflammatory	
Corticosteroids (oral or inhaled)	Effective in reducing symptoms only in high doses, for a short period and with a rebound effect
Cromolyns	Reduce symptoms and duration of disease. Unknown mechanisms
Antihistamines	Little benefit. Some improvement in sneezing and rhinorrhoea
Anticholinergic nasal sprays	Reduce sneezing and rhinorrhoea
Macrolides	Reduces <i>in vitro</i> the RV-mediated production of pro-inflammatory mediators. Clinical efficacy questionable
Antiviral	
Interferon- α	Able to prevent symptoms, but expensive and with significant side effects
Combination therapies	Combination of interferon- α , ipratropium and naproxen was effective in one clinical trial
Experimental therapies	
Anti-rhinoviral compounds	Enviroxime, WIN compounds, chalcones, pyridazinamines and other. Prevent viral uncoating. Some are currently under clinical trials
Soluble ICAM-1	Inhibits viral attachment of major type viruses to their receptor. Promising in initial clinical trials
Antisense oligonucleotides, 3C anti-proteinases, DNA vaccines, interferons β and λ	Potential routes of future intervention

relatively high doses seems to have a modest benefit in reducing duration of cold symptoms, but potential prophylactic effects have not been confirmed (Hemilä *et al.*, 2007). Although promising results were obtained with the use of cromolyns, which were able to reduce cold symptoms and shorten the duration of the illness (Aberg *et al.*, 1996), these have not been reconfirmed or further exploited. A recent meta-analysis suggested echinacea extracts may reduce the frequency of common colds in experimental rhinovirus challenge studies (Schoop *et al.*, 2006).

A combination approach was also proposed (Gwaltney, 1992), involving the administration of antiviral (IFN- α), anticholinergic (ipratropium) and anti-inflammatory (naproxen) drugs. Although a significant therapeutic result was achieved, together with a decrease in viral shedding, this approach does not appear to have been followed up with further studies.

Advances in molecular biological techniques have renewed interest in novel therapeutic approaches. New antiviral compounds are synthesized by computer-aided design (Joseph-McCarthy *et al.*, 2001), such as drugs that

can fit in the hydrophobic pocket of VP1, stabilizing the capsid proteins and preventing uncoating, or rhinovirus protease inhibitors (Johnson *et al.*, 2002; Tsang *et al.*, 2001). Another possibility includes the use of antisense oligonucleotides which anneal *in vitro* to viral RNA which is subsequently degraded by RNases (Johnston, 1997). A considerable number of such compounds are currently evaluated *in vitro*, while some such as pleconaril and AG7088 (Witherell, 2000) are undergoing clinical trials. These compounds are reviewed elsewhere (McKinlay, 2001).

A soluble form of the major group rhinovirus receptor ICAM-1 can inhibit viral entry and inactivate the virus in most experimental settings. Although soluble ICAM-1 was able to prevent rhinovirus infection in chimpanzees (Huguenel *et al.*, 1997) and initial human trials gave promising results (Turner *et al.*, 1999), this approach appears to have been abandoned due to the expense and issues with frequency of dosing. Recombinant LDLR fragments are also able to inhibit minor group rhinovirus infection *in vitro* (Marlovits *et al.*, 1998), suggesting that

receptor blockage could be an important anti-rhinovirus strategy if such problems can be overcome.

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Coronaviruses and Toroviruses

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INTRODUCTION

The first coronavirus to be isolated was infectious bronchitis virus (IBV) from chickens (Beaudette and Hudson, 1937) and coronaviruses were first isolated in humans in the mid 1960s using organ cultures of human embryonic trachea or nasal epithelium and in primary human kidney cell cultures (Tyrrell and Bynoe, 1965). Human coronaviruses were usually thought of in the context of the common cold and indeed, the two human coronaviruses – 229E and OC43 – are responsible for approximately 25% of colds. The global outbreak of severe acute respiratory syndrome (SARS) caused by a novel coronavirus (SARS-CoV) led to a resurgence of interest in coronaviruses and to the discovery of number of other previously unrecognized human and animal coronaviruses. This has resulted in renewed interest in the virology and pathogenesis of these viruses and in efforts to develop vaccines and antivirals against them.

Toroviruses were first isolated from cases of diarrhoea in horses in 1972 (Berne virus, the type strain of *Equine torovirus* (EToV)) and neonatal calves 1982 (Breda virus, type strain of *Bovine torovirus* (BToV)) (Weiss and Horzinek, 1987). Subsequently particles resembling these two viruses were observed by electron microscopy in the stools of children and adults with diarrhoea (*Human torovirus* (HTV)) (Beards *et al.*, 1984). More recent studies have confirmed the presence of HTV, especially associated with gastroenteritis.

THE VIRUSES

Coronavirus and *Torovirus* are the two genera within the family *Coronaviridae*, order *Nidovirales*. Both genera have genomes that are nonsegmented, single-stranded RNA of approximately 30 000 nucleotides and positive polarity (Enjuanes *et al.*, 2000).

Coronaviruses are assigned to one of three groups (Table 21.1) on the basis of group-specific (usually non-structural) proteins, genome organization and RNA sequence identity, supported to some extent by antigenic analyses (Enjuanes *et al.*, 2000; Gonzalez *et al.*, 2003). However, with the discovery of SARS-CoV and a number of novel human and animal coronaviruses, an alternative phylogenetic classification system of coronaviruses perhaps needs to be considered (Figure 21.1). Currently, SARS-CoV is officially classified as a group 2b virus. The finding that many novel group 1 and 2 coronaviruses are endemic in bats has led to the suggestion that bats may be the reservoir from which many other animal group 1 and 2 coronaviruses have arisen (Figure 21.1) (Vijaykrishna *et al.*, 2007).

Electron Microscopic Appearance

Coronaviruses and toroviruses are pleomorphic, ether-labile, enveloped viruses with diameters of approximately 120 nm and a buoyant density in sucrose of 1.15–1.18 g ml⁻¹. While coronaviruses are round in shape, toroviruses have a doughnut shape sometimes seen within the virus particle which led to the use of the root 'toro',

Table 21.1 Representative group 1–3 coronaviruses.

Group 1	Human coronavirus 229E (HCoV-229E)
	Human coronavirus NL63 (HCoV-NL63)
	Canine enteric coronavirus (CCoV)
	Feline coronavirus (FCoV)
	Porcine transmissible gastroenteritis coronavirus (TGEV)
	Porcine epidemic diarrhoea coronavirus (PEDV)
	Bat coronaviruses (BtCoVs)
Group 2	Human coronavirus OC43 (HCoV-OC43)
	Human coronavirus HKU1 (HCoV-HKU1)
	Bovine coronavirus (BCoV)
	Canine respiratory coronavirus (CRCoV)
	Porcine hemagglutinating encephalomyelitis coronavirus (HEV)
	Murine hepatitis coronavirus (MHV)
	Feline infectious peritonitis virus (FIPV)
	SARS coronaviruses (SARS-CoVs)
Bat coronaviruses (BtCoVs)	
Group 3	Infectious bronchitis coronavirus (IBV)
	Turkey coronavirus (TCoV)
	Pheasant coronavirus (PhCoV)

from the Latin *torus*, the lowest convex moulding in the base of a column (Figures 21.2a–c). Particles of both genera have club-shaped surface projections or spikes (S) of up to 20 nm in length. The name coronavirus (Latin *corona*, crown) derives from the large spike-like surface projections found on the surface of these viruses which are reminiscent of a crown (Figures 21.2 and 21.3). Electron microscopy of BTV revealed few of the 20-nm spikes but rather an intact fringe of smaller spikes, some 7–9 nm in length (Cornelissen *et al.*, 1997). Toroviruses isolated from human faeces had an intact fringe of 10-nm spikes (Beards *et al.*, 1984). It is possible that the 10-nm spikes are the haemagglutinin esterase (HE) protein. BTV has an HE protein which forms a fringe of spikes approximately 6 nm in length (Cornelissen *et al.*, 1997).

Coronavirus particles within infected cells have a diameter of approximately 85 nm in thin sections of infected cells and are seen within the Golgi or endoplasmic reticulum. There is usually no budding from the plasma membrane and the virus particles typically found attached to the surface of the cell membrane are probably those disgorged from cytoplasmic vesicles (Figure 21.2d).

Composition of Virions

Coronaviruses contain an envelope spike (S) glycoprotein, a smaller integral membrane glycoprotein (M) largely embedded in the virus envelope, and small amounts of a third membrane-associated non-glycosylated protein (E, envelope), and a nucleocapsid protein (N) that surrounds

the genome (Figure 21.3). Some group 2 coronaviruses have an HE protein (see above). By contrast, toroviruses have three structural proteins – S, M, N (Figure 21.2) – and lack the E protein (Figure 21.4).

The S protein of coronavirus is a type I glycoprotein which forms mono- to trimeric complexes for receptor binding and membrane fusion. The membrane-spanning region is near the C-terminus, the actual C-terminus being within the lumen of the virion. The S protein is highly glycosylated (N-linked glycans) and might be proteolytically processed into S1 (N-terminal) and S2 subunits. The S proteins of group 2a and group 3 coronaviruses, for example, IBV, contain a multiple basic motif and are efficiently cleaved into two subunits, S1 and S2 (S2 being a little longer than S1). Most of the S protein of HCoV-OC43 grown in a human rectal tumour cell line is not cleaved but addition of trypsin cleaves S into S1 and S2. This indicates that the extent to which the S protein of HCoV-OC43 is cleaved in various cell types depends in part on the presence of the relevant protease within cells which cleave adjacent to a basic S1–S2 connecting peptide, a situation which also applies to murine hepatitis coronavirus (MHV). Although the S protein of group 1 and 2b coronaviruses lacks a highly basic sequence such as occurs in IBV at the S1–S2 cleavage site, some of the viruses (e.g. SARS-CoV) in these groups could be cleaved by cellular proteases. Protease cleavage is not absolutely necessary for the replication of all coronavirus.

The M protein, a type III glycoprotein, has a short (20 amino acids or so) glycosylated N-terminus at the

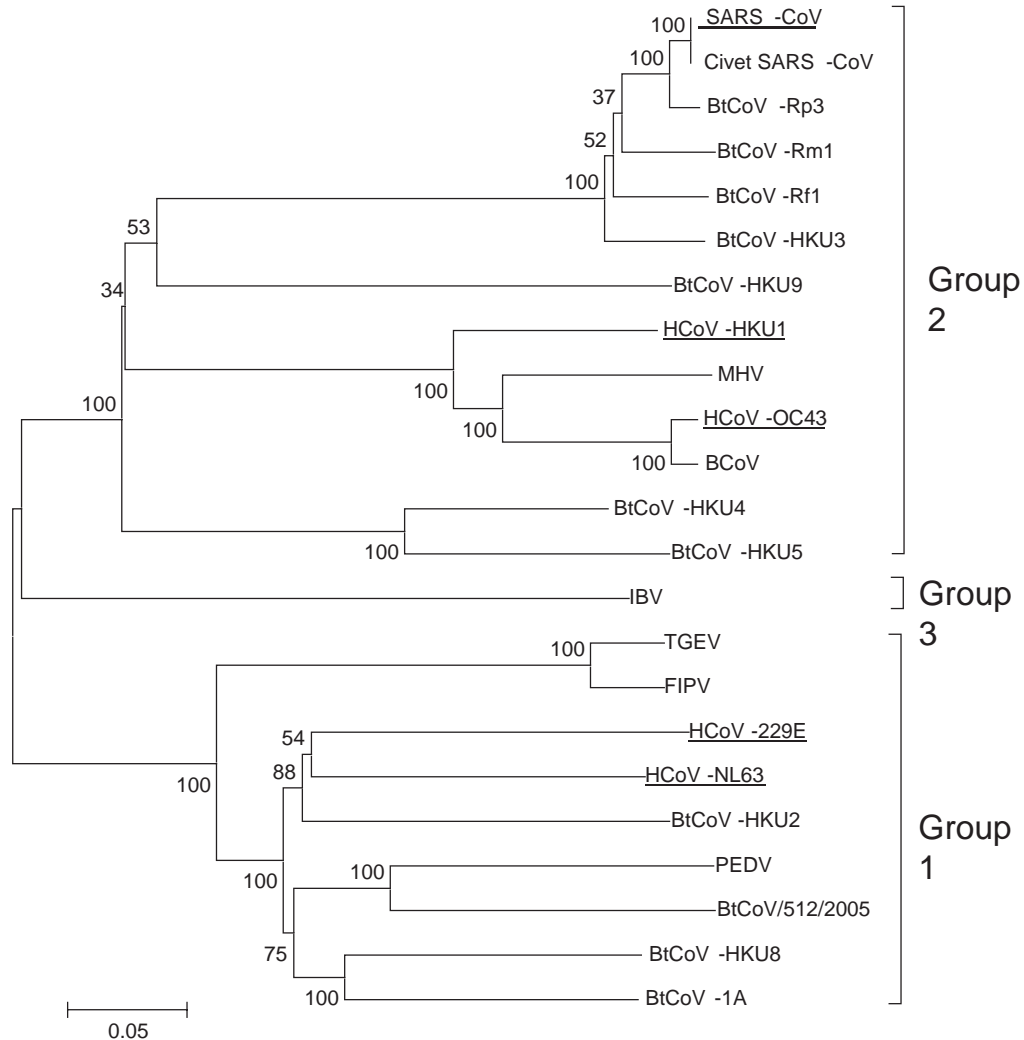


Figure 21.1 Phylogenetic tree of RNA-dependent RNA polymerase encoding nucleotide sequences from representative coronaviruses. The phylogenetic tree was built using the neighbour joining method using Poisson correction. Human coronaviruses are underlined. Refer to Table 21.1 for the abbreviations used. Additionally, abbreviations for bat coronaviruses closely related to SARS-CoV include BtCoV-Rp3: *Rhinolophus pearsoni*, BtCoV-Rm1: *Rhinolophus macrotis* and BtCoV-Rf1: *Rhinolophus ferrumequinum*. Civet SARS-CoV represents virus detected in civet cats (*Paguma larvata*).

surface of the virion, followed by three hydrophobic membrane-spanning regions, an amphipathic region and finally a C-terminal tail in the lumen of the virion. The amphipathic and tail regions may be referred to as the endodomain. Cryoelectron microscope visualization of unstained transmissible gastroenteritis coronavirus (TGEV) has revealed the presence of a core structure. It has been proposed that the TGEV virion comprises (i) a core, made of the nucleocapsid (N protein plus genome) and the

endodomain of M and (ii) the envelope, containing S, E and M.

The E protein is present in only trace amounts in virions. The E is known to be critical for the virion formation of coronaviruses. In addition, the E of SARS-CoV and MHV can induce apoptosis. Recently, the E proteins from some of the coronaviruses have been shown to have ion channel activities.

The N protein is a multipurpose protein that plays a vital role during virion assembly. It binds the viral

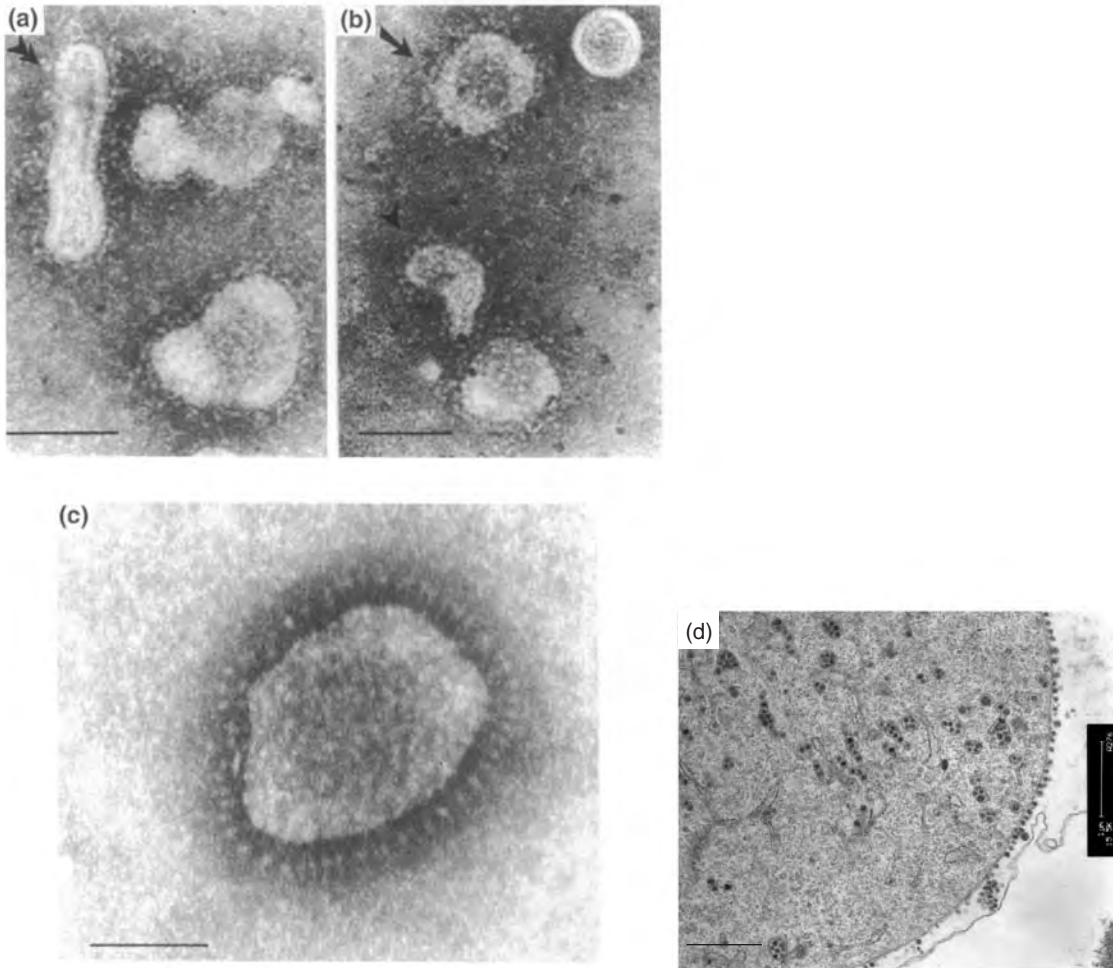


Figure 21.2 Negatively stained electron micrographs of (a, b) human enteric torovirus showing torus (arrow), crescent (arrowhead) and rod (double arrowhead), (c) a human enteric coronavirus with spikes which are more prominent and readily discernible than those of the torovirus. The shorter (10 nm) projections on the torovirus particles may be the haemagglutinin esterase protein, Bar = 100 nm, and (d) transmission electron micrograph of FRhK-4 cells infected with SARS-CoV, Bar = 1 μ m.

genomic RNA to form a complex of ribonucleoprotein, which is then packaged into progeny viral particles.

Many group 2a coronaviruses including HCoV-OC43 (but not SARS-CoV which is a group 2b coronavirus) contain an additional HE glycoprotein, which forms a 5-nm layer of surface projections. BoTV has an HE protein which is absent from EToV as this virus has an incomplete HE gene. The BoTV HE protein has approximately 30% amino acid sequence identity with that of coronaviruses and influenza C virus. The HE protein is not essential for virus replication *in vitro*.

Comprehensive reviews of these structural and non-structural viral proteins have been extensively summarized by others (Gorbalenya *et al.*, 2006; Lai and Cavanagh, 1997; Masters, 2006).

Viral Transcription and Replication

Both homologous (with viruses of the same species and other coronaviruses) and less commonly heterologous (with viruses of other genera) genetic recombination is a feature of coronaviruses and toroviruses (Lai and Cavanagh, 1997).

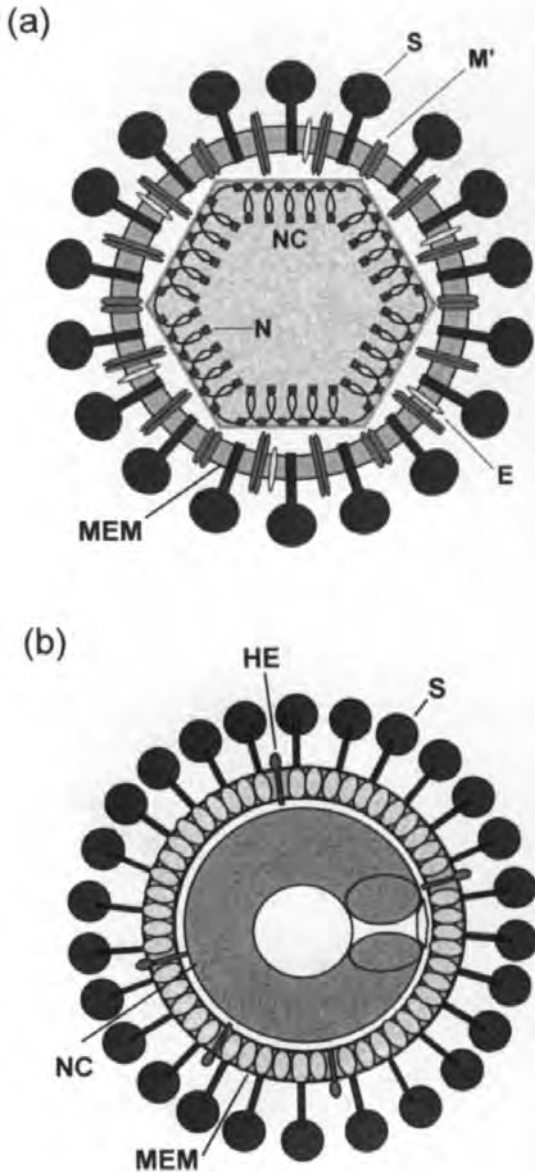


Figure 21.3 Diagrammatic representation of the virions of (a) a coronavirus and (b) a torovirus. Both types of virus have a lipid membrane (MEM), prominent spike proteins (S) and an integral membrane protein (M). HCoV-OC43, but not HCoV-229E, and some toroviruses have a haemagglutinin esterase protein. Coronaviruses, but not toroviruses, contain a small number of molecules of an E protein. (a) Cryoelectron microscopy has indicated the presence of a core structure in coronaviruses, comprising a nucleocapsid (NC: N protein plus genomic RNA) and the endodomain of the M protein. (b) The nucleocapsid of toroviruses has the appearance of a torus.

The transcription and replication of viral RNA is believed to occur on virus-induced double-membrane vesicles in the cytosol of infected cells. The genome organization of EToV and human coronaviruses are shown in Figure 21.4a. They all differ with respect to the number and location of genes that encode proteins believed to be nonstructural that is, not present in virus particles. In HCoV-OC43 all the genes are monocistronic whilst HCoV-229E and SARS-CoV have one and four dicistronic genes respectively.

Members of each coronavirus group tend to have similar nonstructural protein genes located in the same positions of the genome and hence they are referred to as group-specific genes. Their roles are not known. Deletion of one or more of these non-structural genes of group 2 coronaviruses had little effect on virus replication *in vitro*. Consequently they have also been referred to as accessory genes. It is believed, however, that they play a role *in vivo*; deletion of these genes from MHV attenuated the pathogenicity of the virus. Rearrangement of the gene order had little effect on infectivity of MHV (de Haan *et al.*, 2002). Toroviruses do not have any nonstructural protein genes, other than gene 1 (Figure 21.4).

Gene 1 of the coronaviruses (~20 000 nucleotides) and toroviruses encodes proteins required for RNA replication and transcription. In both genera, it comprises two overlapping open reading frames (ORFs), the second one, 1b, being translated by a frameshifting mechanism. These, and the subsequent processing of the gene polyproteins, have been reviewed (Gorbalenya *et al.*, 2006; Masters, 2006).

Transcription in both genera involves the production of a 3' co-terminal nested set of mRNAs (Figure 21.4b). It is generally believed that the transcription involves a template switch (also known as discontinuous RNA synthesis) during the negative-stranded RNA synthesis. The negative copy of the subgenomic (sg) mRNA is then used as the template for sg mRNA production. At the 5' end of each coronavirus gene is a transcription regulatory sequence (TRS) (known as body TRS) while at the 5' terminus of the genome is a leader sequence which also contains a TRS. The sg mRNAs have a copy of the leader sequence fused at the TRS region. The sequence of the TRS varies among the coronaviruses (Thiel *et al.*, 2003). During negative-stranded sg mRNA synthesis, the RNA polymerase is thought to pause after encountering a body TRS. This polymerase may subsequently 'jump' to the leader TRS of the same template to complete the nascent negative-stranded sg mRNA transcription. Thus, this discontinuous RNA transcription would add a common leader sequence to each sg mRNA. The template switching process is determined by the strength of interaction between the nascent negative-stranded

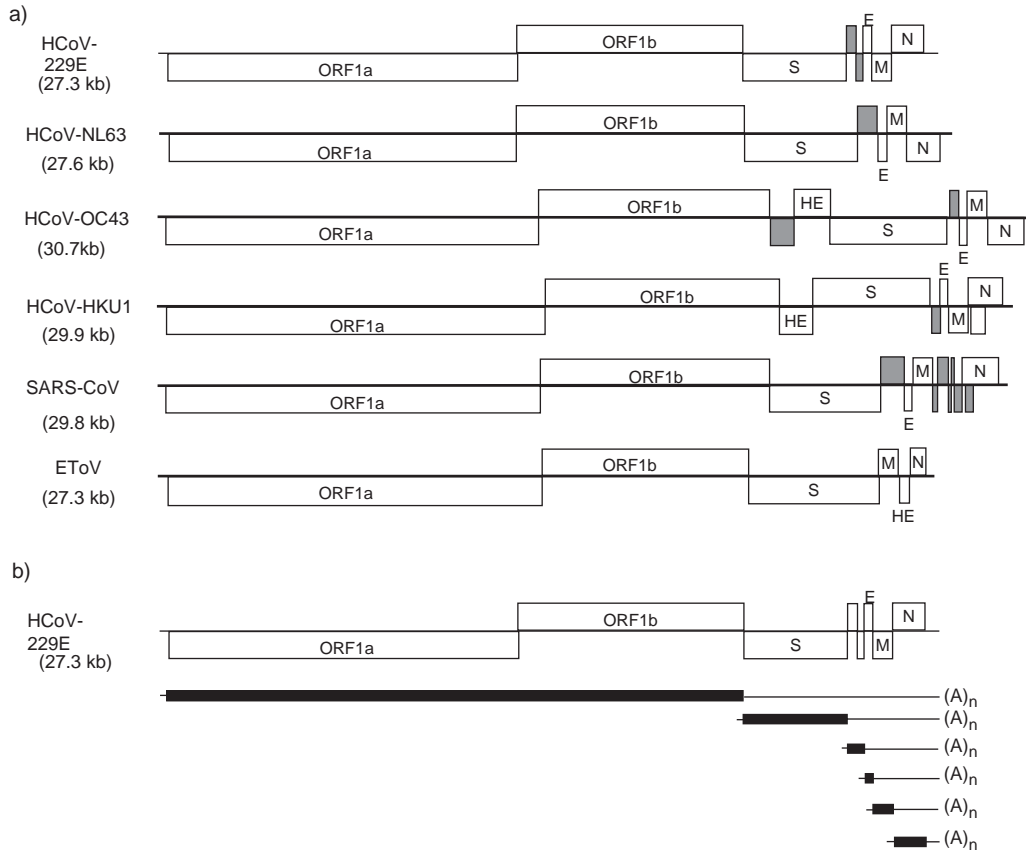


Figure 21.4 (a) Genome organization of the HCoV-229E (group 1), HCoV-NL63 (group 1), HCoV-OC43 (group 2), HCoV-HKU1 (group 2), SARS-CoV (group 2) and equine torovirus. The replicase/polymerase-encoding gene 1, structural protein genes and nonstructural protein genes are shown. Genes for accessory proteins are indicated by grey rectangles. (b) The 3' co-terminal nested set arrangement of coronavirus mRNAs is illustrated for HCoV-229E. The thick black lines indicate the part of each mRNA that is translated.

sg mRNA transcript and the leader TRS (Sola *et al.*, 2005).

Toroviruses have TRS-like sequences at the 5' end of the M, HE and N genes but, unlike coronaviruses, the mRNAs corresponding to these genes do not have a leader sequence fused at their 5' end. However, the S gene, which does not have an obvious TRS sequence, gives an mRNA with a leader sequence, albeit a short one (18 nucleotides) from the 5' end of the genome (van Vliet *et al.*, 2002).

Assembly of Virions

The coronavirus glycoproteins are synthesized at the rough endoplasmic reticulum and are then translocated to the endoplasmic reticulum–Golgi intermediate com-

partment. This is the budding compartment where immature virus particles containing the viral ribonucleoprotein (genome plus nucleocapsid protein) form (Salanueva *et al.*, 1999). The particles progress to the *cis*, medial, *trans*-Golgi compartments and then to the *trans*-Golgi network. Secretory vesicles then transport the virus particles to the cell surface and release them presumably by fusing with the plasma membrane. The coronavirus M protein is not translocated beyond the Golgi compartments, hence this is where virus particles form, not at the plasma membrane. The M protein of toroviruses is also thought to determine the site of virus particle formation. In the case of the coronavirus, E protein is also essential for the formation of virus particles (Raamsman *et al.*, 2000).

Antigenic Structure

Virus-neutralizing and haemagglutination-inhibiting antibodies are induced by both the S and HE proteins of coronaviruses and toroviruses and some antibodies against the coronavirus M protein neutralize virus in the presence of complement.

Epitope mapping of coronaviruses has been done mostly for S protein and has shown that virus-neutralizing epitopes are formed largely by the N-terminal S1 half of the protein, which has much greater amino acid sequence variation (Siddell, 1995). Removal of glycans from the S protein of IBV greatly diminished the binding of virus-neutralizing monoclonal antibodies.

The S protein has been shown to be a major inducer of protective immunity (Buchholz *et al.*, 2004) although the other structural proteins, particularly the nucleocapsid protein, contribute to cell-mediated protection. In the case of feline infectious peritonitis virus (FIPV), the immune response to S protein is believed to exacerbate the disease. FIPV attached to anti-S antibody remains infectious and is more readily taken up by macrophages (antibody-dependent enhancement), within which FIPV replicates (de Groot and Horzinek, 1995). Similar antibody-dependent enhancement has not been reported for human coronaviruses, although there is a report that SARS-CoV spike-bearing viral pseudotypes can enter human B cell lines that lack the virus receptor angiotensin-converting enzyme 2 (ACE2) via FcγRII in the presence of antibody (Kam *et al.*, 2007) and one needs to be alert to this possibility.

Immune electron microscopy has revealed relationships between the toroviruses of humans, equines and bovines. Much remains to be done to establish the extent of variation among human toroviruses.

INITIATION OF INFECTION AND PATHOGENESIS

Cell Attachment Proteins

The S protein serves to attach the virus to cells. The extent to which the HE protein, when present, is also responsible for cell attachment is not clear. The HE proteins, in contrast to the S proteins, of both coronaviruses and toroviruses are not required for replication *in vitro* (Popova and Zhang, 2002). Amino acids 417–547 of the HCoV-229E S protein were required for binding to its cell receptor and the receptor-binding motif of SARS-CoV's S protein has been mapped to the amino acid residues 424–494 (Li *et al.*, 2005b; Wentworth and Holmes, 2007). The role of S protein as a determinant of host cell range has been demonstrated using recombinant coronaviruses

expressing heterologous S proteins. For example, MHV expressing the feline coronavirus (FCoV) S protein was able to successfully infect and replicate in feline cells previously refractory to MHV infection. It is the S1 part of S protein that determines receptor specificity. Experiments with recombinant MHVs showed that the extent of replication of the virus, and the degree of hepatitis in the liver, was determined largely by the S protein. Gallagher and Buchmeier (2001) have reviewed virus entry and pathogenesis for MHV.

Cell Receptors for Coronaviruses

The specificity of virus attachment to host cells can be a major factor in determining host range, tissue tropism and hence pathogenesis. Three cellular proteins – aminopeptidase N (APN), carcinoembryonic antigen cell adhesion molecule 1 (CEACAM-1) and ACE2 – have been identified as the principal receptors for binding group 1 and 2 coronaviruses. The transfer of coronavirus receptors from susceptible cells to other cells can render the latter susceptible to infection (Wentworth and Holmes, 2007).

Human APN has been identified as the receptor for HCoV-229E. This protein is a metalloprotease located on the surface of epithelial cells, including those of the intestine, lung and kidney. Human cells that were not susceptible to canine coronavirus (CCoV) or FCoV became susceptible when transfected with a human/canine chimera of APN. The critical, C-terminal domain was formed by amino acids 643–841 of the canine APN. When amino acids 255–348 of porcine APN were replaced by amino acids 260–353 of human APN, the resulting chimeric protein was able to function as a receptor for HCoV-229E. Thus different parts of APN molecules may function as receptors for most group 1 coronaviruses. Expression of feline APN in rodent cells rendered the cells susceptible not only to FCoV but also to HCoV-229E, CCoV and TGEV. Previous work had shown, however, that human APN would bind HCoV-229E but not TGEV, while porcine APN would bind TGEV but not human coronavirus (HCoV). Surprisingly, HCoV-NL63, also a human group 1 coronavirus, uses ACE2 for receptor binding (Hofmann *et al.*, 2005).

Much less work has been done on receptors for group 2a human coronaviruses. The cellular receptor for MHV (a group 2a coronavirus, like HCoV-OC43), is a member of the CEA family of glycoproteins in the immunoglobulin superfamily (Lai and Cavanagh, 1997). CEACAM-1, is a 424-amino-acid glycoprotein with four immunoglobulin-like domains. A soluble form of this protein has been crystallized and its atomic structure has been deduced (Tan *et al.*, 2002).

The immunoglobulin-loop of human CEACAM-1 confers virus-binding specificity. Different isoforms of

murine CEACAM-1 protein exist. These have extensive differences in the N-terminal immunoglobulin-like domain to which MHV binds and so bind MHV to different extents. Amino acids 38–43 were key elements for binding MHV and for virus-induced membrane fusion (Rao *et al.*, 1997). When a persistent infection of MHV was established in murine 17 Cl 1 cells, which express very low levels of the CEACAM-1 receptor, there was selection of mutant MHVs that were better able to use other molecules as receptors (Schickli *et al.*, 1997).

Human ACE2, an 805-amino-acid glycoprotein, is the primary receptor for SARS-CoV and HCoV-NL63. It is a carboxypeptidase which cleaves angiotensin II to angiotensin-(1-7). In addition to playing a role in regulating the blood pressure and cardiovascular and renal function, the renin-angiotensin system is believed to modulate acute lung injury. The crystal structure of the protein complex comprising the human ACE2 and SARS-CoV receptor-binding domain has been elucidated. The adaptation of the SARS-CoV-like civet virus to humans was associated with key changes in the spike protein which enhance binding to the ACE2 receptor. In comparison to the human SARS-CoV, the civet SARS-CoV-like virus spike has two amino acid substitutions in the spike (N479K; T487S) that adversely affect its interaction with human ACE2 (Li *et al.*, 2005b). Similarly, the civet-like virus that re-emerged to infect humans in late 2003 also had the T487S amino acid substitution and an additional change at L472P compared with human SARS-CoV and this probably contributed to its poor pathogenicity and transmissibility in humans. Thus a small number of spike mutations may be critical for facilitating efficient interspecies transmission. The spike of the SARS-like coronavirus found in bats is markedly different in this region and will not bind human ACE2.

Apart from ACE2, other cellular proteins such as DC-SIGN, L-SIGN and L-SECtin are also able to facilitate virus binding of some coronaviruses (Wentworth and Holmes, 2007). The expression of L-SIGN in cells lacking ACE2 expression allows SARS-CoV binding but not virus replication. It is thus a binding receptor rather than a functional receptor for SARS-CoV.

Involvement of Glycans on Receptors

The S protein of some coronaviruses and the HE protein of group 2a coronaviruses bind to sialoglycoconjugates on cell surfaces and these interactions may also be relevant for virus entry and tissue tropism. Attachment of coronaviruses might be a two-step process. Primary attachment might be mediated by a first receptor, for example, *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac₂) for some coronaviruses, a second receptor for example, APN or CEACAM-1 proteins, bringing the virus

and cell membranes closer together for subsequent membrane fusion. Some receptors might fulfil both functions for some coronaviruses.

Both the S and HE proteins of bovine coronavirus (BCoV) bind to cell surface components, an important one being the glycan component Neu5,9Ac₂. This residue acts as a receptor not only on erythrocytes but also on susceptible cell cultures (Schultze and Herrler, 1992). The S protein binds more efficiently than HE to Neu5,9Ac₂ and it has been proposed that the BCoV S protein is responsible for the primary attachment to cells.

HE proteins from different group 2a coronaviruses have different specificities to bind or cleave sialoglycoconjugates. For instance, the HE protein of HCoV-OC43 prefers to bind to Neu5,9Ac₂ and it is also a sialate-4-*O*-acetylsterase. Thus by contributing to both binding and removal of the virus from the cell surface, the HE protein may help virus release as well as virus entry.

Host Range of Coronaviruses

Interspecies transmission of coronaviruses is well recognized in animals. The closely related group 1 coronaviruses, porcine transmissible gastroenteritis virus, canine and FCoV can infect pigs with some disease expression. BCoV can naturally infect dogs and humans and even cause disease in avian hosts. The HCoV-OC43 has >99% amino acid identity in its S and HE proteins with the corresponding proteins of BCoV. A recently discovered respiratory CCoV is a group 2 coronavirus, the S protein of which had 96 and 95% amino acid identity with that of BCoV and HCoV-OC43, respectively. Cross-infection by these viruses has not been studied but a broad host range for them is a possibility (Saif, 2006).

Coronaviruses can undergo dramatic changes in virulence and tissue tropism. Porcine enteric transmissible gastroenteritis virus caused a highly virulent disease in pigs. A spontaneously occurring spike gene deletion mutation led to the emergence of a virus (porcine respiratory coronavirus) with a tropism for the respiratory tract and reduced virulence in pigs. These changes in the spike were responsible for the altered tissue tropism (Ballesteros *et al.*, 1997). Interestingly these deletion mutants arose independently in Europe and North America as these viruses have differences in the size of the spike deletion.

Thus it is no surprise that a civet SARS-like virus was able to establish itself and to transmit and cause disease in humans. In addition to the changes in the civet virus spike that led to a dramatic change, the binding affinity for the human ACE2 receptor also altered. Another striking difference observed in human SARS-CoV is a 29-nucleotide deletion which interrupts the ORF8ab of the civet virus

(Guan *et al.*, 2003). The biological significance of this deletion is still unclear.

Fusion Processes

The S protein of coronavirus is a class 1 fusion protein. The fusion processes for coronavirus viral entry is not completely understood. During the fusion, the S protein undergoes a series of conformational changes. The binding of S to the receptor could induce the separation of S1 from S2, and alter the conformation of S1, generating alternative disulfide linkages within S1. The fusion peptide in the S2 region is then exposed to host cell membrane. This is followed by a series of conformational changes of the S2 protein and the hydrophobic heptad repeat regions (i.e. HR1 and HR2) in the S2 domains of the trimeric complex to form a six-helical bundle at the final stages of fusion. It is believed that a small region rich in tyrosine and tryptophan residues before the transmembrane domain of S2 may help to destabilize lipid bilayers, thereby leading to pore formation and membrane fusion (Wentworth and Holmes, 2007).

EPIDEMIOLOGY

Seroepidemiology indicates that antibodies to 229E and OC43 appear early in life and are ubiquitous by adulthood (McIntosh *et al.*, 1970). Limited data based on enzyme-linked immunosorbent assay (ELISA)-based assays using portions of the recombinant nucleocapsid and pseudotyped virus expressing the NL63 spike protein suggest that the same is true of NL63 (Hofmann *et al.*, 2005; Shao *et al.*, 2007). There is limited seroepidemiology data on HKU1 as this virus still cannot be cultivated efficiently. Preliminary ELISA assays using recombinant HKU1 nucleocapsid protein showed evidence of seroconversion in patients with HKU1 disease but a low (2%) seroprevalence in the community (Woo *et al.*, 2005a).

Community studies have shown that the incidence of, for example, coronavirus 229E infections may vary from year to year from as low as 1% to as high as 34% (Hamre and Beem, 1972). Coronaviruses appear to have a winter–spring seasonality in temperate regions. In subtropical Hong Kong, the seasonality appears to be similar except that NL63 may be found in early summer and autumn (Chiu *et al.*, 2005; Lau *et al.*, 2006). Unlike many other respiratory viral infections, all age groups appear to be uniformly infected with HCoV. Reinfection of individuals with the same HCoV serotype often occurs within months of the first infection, suggesting that even homologous protection is short-lived. Available data suggest that antibodies to one HCoV group may not be protective against infection with viruses from the other HCoV group.

Severe Acute Respiratory Syndrome

SARS emerged as a pneumonic respiratory disease in Guangdong Province, China in November 2002 and within weeks had spread to affect about 8000 patients in 29 countries across five continents, associated with an overall fatality rate of 9.6% (Peiris *et al.*, 2007). A novel coronavirus, SARS-CoV was identified as the aetiological agent (Drosten *et al.*, 2003; Ksiazek *et al.*, 2003; Peiris *et al.*, 2003b). The lack of antibody to SARS-CoV in asymptomatic healthy people in the community suggested that it was a disease that emerged from an animal reservoir (Ksiazek *et al.*, 2003; Peiris *et al.*, 2003b). The earliest human cases of SARS in late 2002 had epidemiological links with live animal markets (Xu *et al.*, 2004). A virus closely related (99.8% nucleotide identity) to SARS-CoV was detected in palm civets (*Paguma larvata*) and other small mammal markets in southern China where live game animals are sold as exotic foods for human consumption (Guan *et al.*, 2003). Humans working in these markets had high prevalence of antibody to SARS-CoV although they had no history of a SARS-like disease, suggesting that they had been infected with the less pathogenic precursor SARS-CoV-like virus. Furthermore, when SARS re-emerged to infect humans in Guangdong in late 2003 and early 2004, the virus was more closely related to the civet-like virus rather than to the human adapted virus that spread worldwide in mid 2003. It was of low pathogenicity and failed to transmit from human to human (Liang *et al.*, 2004), probably as a consequence of the poor affinity of the virus–receptor interaction (see above). Thus it is likely that these animal markets were the interface which allowed the precursor virus to adapt and amplify in animals such as civets and infect humans. However, civets were not the natural reservoir. A SARS-like coronavirus has been found in wild caught bats (*Rhinolophus* spp.) and these appear to be the natural reservoir from which SARS-CoV emerged (Lau *et al.*, 2005; Li *et al.*, 2005a). The nucleotide identity between the bat precursor virus and human SARS-CoV is only around 90% and this precursor virus had to undergo significant adaptation before becoming efficiently transmissible in humans. As bats, civets and a range of other animal species are sold for human consumption within the markets of Guangdong, this provided the ideal milieu for such adaptation to occur (Peiris *et al.*, 2007).

While many SARS patients did not transmit infection at all, a few patients accounted for a disproportionately large number of secondary cases, the so-called ‘super-spreading incidents’. Although host factors may have played a role in these super-spreading events, in many instances it was a combination of host factors and environmental circumstances that facilitated transmission. Overall, the basic reproduction number of SARS was estimated to

be approximately 2–4 (Lipsitch *et al.*, 2003; Riley *et al.*, 2003).

Respiratory droplets were believed to be the major vehicle of transmission and the use of effective droplet precautions did protect healthcare workers (Seto *et al.*, 2003). However, true air-borne transmission probably did occur in some instances through the use of aerosol-generating procedures such as intubation, suction, high-flow oxygen therapy and the use of nebulizers. Infectious virus was also present in faeces (Peiris *et al.*, 2003b) and the largest super-spreading incident at Amoy Gardens in Hong Kong which led to over 300 cases is believed to have occurred through aerosolization of infected faeces in the sewage system in a high-rise apartment (Yu *et al.*, 2004). SARS-CoV retains viability for much longer periods than 229E, suggesting that fomites may also be an important means of virus transmission (Rabenau *et al.*, 2005).

Unlike many other respiratory viral infections, relatively little SARS transmission occurred early (first five days) in the illness (Lipsitch *et al.*, 2003). This was probably related to the low viral load in the upper respiratory tract (and faeces) early in the illness with a peak of viral load at around day 10 of disease (Peiris *et al.*, 2003a). These disease characteristics probably contributed to the dramatic spread in the hospital setting (by which time patients were highly contagious) and the relatively lower transmission within the family setting. Healthcare workers comprised 21% of all cases (Peiris *et al.*, 2007).

Another unusual feature of SARS was that asymptomatic infections was uncommon (Leung *et al.*, 2004b). The absence of large number of asymptomatic transmitters of the disease and the limited transmission in the first five days of illness were fortuitous features of this disease that allowed its successful containment through the application of public health measures such as case detection and isolation.

It is relevant to consider whether SARS may return. Since the interruption of the major global outbreak in July 2003, there is no evidence of continued circulation of the human-adapted SARS-CoV in humans or animals. However, this virus exists in laboratories and there were three laboratory infections in 2003 and 2004, in one instance, leading to transmission in the community. Fortunately, these outbreaks were successfully contained. The SARS-CoV-like civet virus led to four new cases in December 2003 and January 2004, many of whom had a history of contact with the animal market trade, but these civet SARS-CoV were not well adapted to efficient human transmission (see above). The sale of civets in big animal markets in southern China was subsequently banned. While it is possible that an illegal trade continues, this

will probably not provide the same opportunities for interspecies transmission and sustained circulation of virus as may happen in the large organized animal market setting. The original precursor virus still exists in bats and it is conceivable that it may re-emerge, again re-adapt to human transmission and lead to human outbreaks (Peiris *et al.*, 2007).

CLINICAL FEATURES

Association of human disease with coronavirus 229E and OC43 has been conclusively established by infecting human volunteers with cultured virus and documenting ‘common cold’-like symptoms in association with virus replication in the upper respiratory tract (Bradburne *et al.*, 1967; Hamre and Procknow, 1966) thereby fulfilling Koch’s postulates. These studies also established that the incubation period was around two days with peak symptoms occurring at three to four days. Virus was detectable in the respiratory tract around the time of symptom onset and continued to be detectable for around five days. Coronaviruses (229E and OC43) account for approximately 25% of common colds and are second only to rhinoviruses as the causative agent (Makela *et al.*, 1998). The symptoms are those of a nasal discharge, mild sore throat, sneezing, general malaise, perhaps with headache and lasts for an average of six to seven days. Fever and coughing may be exhibited in 10 and 20% of cases, respectively. No difference is observed between 229E and OC43 strains in the pathology of infection. Generally symptoms are indistinguishable from those of colds caused by rhinoviruses. Subclinical or very mild infections are common. HCoV were found in 10% of children with otitis media with effusion, respiratory syncytial virus being associated with approximately 30% of cases (Pitkaranta *et al.*, 1998).

Ethical considerations have precluded human volunteer studies being done with the more recently discovered coronaviruses NL63 and HKU1 or for SARS-CoV. Thus disease association has been indirect, by detecting the virus in the respiratory tract in association with disease. The aetiological link has been conclusive with SARS where the virus and seroconversion to it was almost exclusively associated with clinical disease and there was minimal asymptomatic infection (see above). Furthermore, the virus was demonstrated at the site of pathology, viz. lung and gastrointestinal tracts (Nicholls *et al.*, 2006; Peiris *et al.*, 2003b). Experimental transmission of the virus to cynomolgous macaques led to SARS-like lung pathology (Fouchier *et al.*, 2003). Taken together, these findings satisfy Koch’s postulates for an association of a pathogen and disease. However, in the case of NL63

and HKU1, the association with human disease depends on the virus being more frequently found in the respiratory tract of patients with disease than in matched controls. While detection of these viruses in association with respiratory disease has been observed in many studies worldwide, fewer studies have compared these data with concurrent age-matched controls. These viruses (and also HCoV-229E or HCoV-OC43) can occasionally be found in 'healthy' controls and furthermore, finding multiple respiratory viruses in patients with disease makes it difficult to establish the contribution of each one to the illness (van der Hoek, 2007). Unlike SARS-CoV, neither of these viruses has relevant animal models that can be used to fulfil Koch's postulates. In studies that have used a control group for comparison, 229E and OC43 were associated with 11% of children with acute respiratory tract disease compared with 0.37% of controls ($P < 0.01$), confirming the data from volunteer studies (van Elden *et al.*, 2004). NL63 and HKU1 were not sought in this study. In patients aged 60 years or older, 229E and OC43 were found in 17% of 107 elderly subjects with acute respiratory disease and only 2% of controls. Rhinovirus was more commonly detected (32% of patients and 2% of controls) while influenza was less common (7% of cases and 0% controls) (Graat *et al.*, 2003). In a study of children and young adults, 229E and OC43 were detected in both patients (3.6% of flu-like illness; 7.7% of other respiratory illness) and in controls (3.9%) and there was no statistically significant association with disease (van Gageldonk-Lafeber *et al.*, 2005).

Coronaviruses have also been associated with exacerbations of wheezing in asthmatic children (McIntosh *et al.*, 1973). Coronavirus was detected in approximately 5% of children (Freymuth *et al.*, 1999) and 22% of adults hospitalized because of asthma (Atmar *et al.*, 1998). Allergic patients with a common cold, associated with a number of viruses, including HCoVs (25%), had prolonged nasal eosinophil influx (van Bentem *et al.*, 2001). Whether that would increase the risk of subsequent allergen-induced hypersensitivity reactions is not known.

An experimental model has been established for viral wheeze, involving volunteers (some atopic, others not) with and without a history of viral wheeze (McKean *et al.*, 2001). Over half developed colds after inoculation with HCoV-229E. The viral wheezers reported more upper respiratory tract symptoms than controls. Over half of the wheezers, but none of the controls, reported lower respiratory tract symptoms (wheeze, chest tightness and shortness of breath).

NL63 has been found significantly more often in patients with croup than in controls (17% vs. 4%) and appeared to be an even more important cause of this disease than parainfluenza virus (van der Hoek *et al.*, 2005).

However, studies of cases and controls for association of NL63 with other features of acute respiratory disease have been inconclusive (Boivin *et al.*, 2005; Dare *et al.*, 2007). An alternative, but less definitive approach of establishing disease associations is to identify patients with the virus of interest without any other relevant respiratory pathogen. NL63 was detected as the sole pathogen in 0.4–9.3% of patients with respiratory infection and HKU1 in between 0.3 and 4.4% of patients (van der Hoek, 2007). The symptoms found in patients with NL63 alone included fever, cough, rhinorrhoea, pharyngitis, croup, bronchiolitis, pneumonia and febrile seizures. Some patients had underlying disease. A association of NL63 with Kawasaki disease has not been confirmed. HKU1 was associated with a similar spectrum of disease as seen with NL63 although it was not associated with croup. Febrile seizures were more common in HKU1 infected patients than those with OC43 (Woo *et al.*, 2005b). Between 50 and 80% of patients had underlying diseases (van der Hoek, 2007).

In hospitalized patients, 229E, OC43, NL63 and HKU1 have all been found in upper as well as lower respiratory tract disease, especially in children (Arden *et al.*, 2005; Boivin *et al.*, 2005; Chiu *et al.*, 2005; Sloots *et al.*, 2006; Woo *et al.*, 2005b). These coronaviruses have also been detected in bronchoalveolar lavages of immunocompromised and noncompromised adults, providing additional evidence of their pathogenic potential in the lower respiratory tract (Garbino *et al.*, 2006). Follow-up bronchoalveolar lavage specimens were available from some patients and these were often negative, indicating that the virus was temporally associated with the clinical symptoms.

HKU1 has also been detected in respiratory specimens from patients with diarrhoeal symptoms and in stool specimens in association with fever, otitis and convulsions (Vabret *et al.*, 2006). Thus, it is possible that, as with SARS, HKU1 may lead to disseminated disease.

Coronaviruses have been associated with nosocomial outbreaks of disease in children in neonatal units and in premature babies (Esper *et al.*, 2005; Sizun *et al.*, 1995). Neonates with coronavirus infections had bradycardia, apnoea, hypoxaemia, fever or abdominal distention. Chest X-ray revealed diffuse infiltrates.

As with influenza and respiratory syncytial virus, coronaviruses are also associated with respiratory illness in the elderly (Falsey *et al.*, 2002; Walsh *et al.*, 1999).

Severe Acute Respiratory Syndrome

The incubation period of SARS is estimated to be 2–14 days. SARS typically presents as fever, myalgia, malaise, chills or rigour and fever of acute onset leading in some patients to a rapidly progressing viral pneumonia. A dry cough is common but rhinorrhoea and sore throat are

less prominent. Watery diarrhoea was reported in a proportion of patients. Radiological abnormalities were seen in >60% of cases at presentation. Typically the chest radiograph showed ground glass opacities, and focal consolidation over the periphery and subpleural regions of the lower zones of the lung. One or both lungs may be involved and the radiological changes may shift location. High-resolution computed tomography (CT) scanning allowed detection of abnormalities in a proportion of patients with initially unremarkable chest radiographs. While approximately one-third of patients improved, with defervescence and resolution of the radiographic changes, others progressed to develop increasing shortness of breath, tachypnoea and oxygen desaturation. Extrapulmonary manifestations included hepatic dysfunction, a marked lymphopenia involving both B and T cells (CD4 and CD8 subsets) and NK cells and less commonly, central nervous system (CNS) manifestations (Cheng *et al.*, 2007; Peiris *et al.*, 2003c, 2007).

About 20–30% of patients required management in intensive care and many of them needed mechanical ventilation. The terminal events were severe respiratory failure associated with acute respiratory distress syndrome and multiple organ failure. The overall case fatality rate was 9.6%.

The clinical features of SARS were not pathognomonic and a contact history and virological evidence of infection were important in establishing the diagnosis. The occurrence of clusters of cases of a rapidly progressing pneumonia in a family or hospital setting was suggestive of the disease.

The severity of illness and increasing case fatality was associated with increasing age and presence of other co-morbidities. A higher viral load in nasopharynx and serum early in the disease was an independent risk factor for mortality (Chu *et al.*, 2004b). Children had a milder disease course (Leung *et al.*, 2004a). The elderly may present atypically without fever or respiratory symptoms and failure to recognize such atypical presentations led to catastrophic nosocomial outbreaks (Chow *et al.*, 2004).

Residual impairment of lung function persists into late convalescence including restrictive lung function abnormalities due to lung fibrosis, impairment in diffusion capacity, muscle weakness, and reduced exercise capacity. Psychological sequelae including depression and post-traumatic stress have been reported in survivors (Cheng *et al.*, 2007).

Coronaviruses and Toroviruses Associated with the Human Enteric Tract

Coronavirus-like (non-SARS) viruses have been isolated from faecal specimens from humans (Duckmanton *et al.*, 1997). Some of these viruses were isolated from infants

with necrotizing enterocolitis, patients with nonbacterial gastroenteritis and from homosexual men with diarrhoea who were symptomatic and seropositive for human immunodeficiency virus. Some isolates were shown to be serologically related to OC43. The discovery that a protein found in enterocytes functions as a receptor for HCoV-229E strengthens the likelihood that coronaviruses might replicate in the human alimentary tract.

Evidence has increased that toroviruses are associated with gastroenteritis in humans. In a case–control study of children, an antigen-capture ELISA revealed torovirus in stools from 27% (9/33) of children with acute diarrhoea, 27% (11/41) with persistent diarrhoea and none in controls (Koopmans *et al.*, 1997). Enteroggregative *Escherichia coli* was commonly found in association with the torovirus. In another childhood study electron microscopy revealed a torovirus incidence of 35% (72/206) and 15% in gastroenteritis cases and controls, respectively (Jamieson *et al.*, 1998). Those infected with torovirus were more frequently immunocompromised (43 vs. 16%) and nosocomially infected (58 vs. 31%); experienced less vomiting (47 vs. 68%); had more bloody diarrhoea (11 vs. 2%).

Coronaviruses in the Central Nervous System

Multiple sclerosis (MS) is a chronic disease of the CNS involving multifocal regions of inflammation and myelin destruction. Coronaviruses are one of the many infectious agents that have been proposed as causes for triggering MS and associated with demyelination in humans and rodents (Stohlman and Hinton, 2001). Coronavirus RNA and evidence of virus replication have been detected in the brains of humans and many human neural cell lines have been shown to support the replication of both OC43 and 229E types of HCoV. Murine hepatitis virus infection in mice and rats has long been a model for coronavirus-induced demyelination, although the exact mechanism(s) by which MHV induces demyelination and the role of the immune system in the pathology is not known. Inflammatory mediators, for example, interleukin 1 β , tumour necrosis factor, IL-6 and an MHV-induced immune response that cross-reacts with myelin proteins, have been proposed as possible mechanisms. Peripheral cross-reactive T-cell clones recognizing both HCoV and a myelin antigen have been detected in MS patients. It has also been hypothesized that HCoV RNA might sometimes lead to a low level of viral protein synthesis that could be involved in the stimulation of immune responses within the CNS, exacerbating the effect of coronaviral infection in MS patients (Arbour *et al.*, 2000). Persistent OPC43 and 229E infections have been established in some human neural cell lines.

Pathology and Pathogenesis

There are limited pathology data from patients infected with the non-SARS human coronaviruses because they have been generally mild and those rare instances of fatal disease have not been investigated at autopsy. There is a report of electron microscopic changes in the nasal mucosa of a child with coronavirus infection. There was minimal cytopathology but evidence of active virus replication with virus particles seen within cytoplasmic vesicles and the Golgi apparatus (Afzelius, 1994).

Severe Acute Respiratory Syndrome

Although the major adverse pathology was on the respiratory tract, SARS was a disseminated infection with virus being isolated from the upper respiratory tract, the lungs, faeces and urine (Chan *et al.*, 2004) and with reverse transcription polymerase chain reaction (RT-PCR) evidence of viral RNA in the plasma and serum (Ng *et al.*, 2003). Viral RNA has also been detected in the lymph nodes, spleen, liver, heart, kidney and skeletal muscle (Farcas *et al.*, 2005). Autopsy findings in the patients who died in the first 10 days of illness revealed diffuse alveolar damage, air-space oedema, desquamation of pneumocytes, inflammatory infiltrate and hyaline membrane formation (Franks *et al.*, 2003; Nicholls *et al.*, 2003). Virus antigen was detectable by immunohistochemistry in alveolar epithelial cells and also in macrophages and bronchial epithelium within the first 10 days of illness but rarely after that (Nicholls *et al.*, 2006). This correlates with *in vitro* studies showing that SARS-CoV replicates in differentiated type 2 alveolar epithelium and in ciliated cells in primary human airway epithelium but not in undifferentiated alveolar epithelial cell lines such as A549 (Mossel *et al.*, 2007; Sims *et al.*, 2005). Intestinal epithelial cells had viral particles demonstrable by electron microscopy with no obvious cytopathology and this is consistent with the watery diarrhoea observed clinically (Leung *et al.*, 2003).

Patients with SARS had high serum levels of a range of pro-inflammatory chemokines (IL-8, CCL2, CXCL10) and cytokines (IL-1, IL-6) (Wong *et al.*, 2004) and these same immune mediators were elicited from virus-infected macrophages *in vitro* (Cheung *et al.*, 2005). It is not clear whether these mediators are a reflection of the severe tissue damage or are contributing to the exacerbation of pathology induced by the virus.

Genetic polymorphisms associated with susceptibility to SARS have been identified. Two independent studies have reported an increased susceptibility to SARS associated with low serum levels of mannose-binding lectin (MBL) and that MBL neutralizes the infectivity

of SARS-CoV *in vitro* (Ip *et al.*, 2005; Zhang *et al.*, 2005). Although other genetic polymorphisms have also been reported to be associated with SARS (HLA-B*4601; HLA-B*0707, ICAM-3, CLEC4M), they have not been independently confirmed.

DIAGNOSIS

Diagnosis of HCoV infections was not routinely done in most diagnostic virus laboratories in the past because these viruses are not readily culturable *in vitro* and well-standardized reagents for immunological detection were not available. Antigen-capture ELISAs have been used to detect HCoV antigens in nasal and throat swabs, and nasopharyngeal aspirates, and to detect HTVs in stools (Koopmans *et al.*, 1997). Furthermore, the human coronaviruses 229E and OC43 were considered nothing more than the cause of the 'common cold'. The discovery of SARS and other novel human coronaviruses, the realization that human coronaviruses can cause clinically significant disease and the more widespread use of PCR or molecular diagnostics in clinical virology laboratories is leading to a reassessment.

The specimens for diagnosis of coronavirus infections are typically respiratory specimens (nasopharyngeal aspirates, nasopharyngeal swabs, throat and nose swabs and, when available, bronchoalveolar lavages) and paired sera for seroconversion when appropriate. In the case of SARS, viral RNA was also detectable in faecal specimens and in serum. HKU1 has also been detected by RT-PCR in faeces.

It is notable that the discovery of a novel coronavirus as the cause of SARS relied heavily on the classical virological techniques of virus isolation and electron microscopy, subsequently followed by molecular diagnostics and genome sequencing (Drosten *et al.*, 2003; Ksiazek *et al.*, 2003; Peiris *et al.*, 2003b). The use of virus detection microarrays also independently identified the cell culture isolate as a coronavirus (Wang *et al.*, 2003). Initial partial sequence information was obtained using random primer-based RT-PCR (Drosten *et al.*, 2003; Peiris *et al.*, 2003b) and by consensus primer RT-PCR methods (Ksiazek *et al.*, 2003). The discovery of the new human coronavirus NL63 was achieved by virus isolation followed by a novel approach VIDISCA (virus discovery based on cDNA-AFLP) to identify the virus isolate by deriving fragments of genetic sequence (van der Hoek *et al.*, 2004). HKU1 and novel bat coronaviruses were discovered using consensus primer RT-PCR methods (Poon *et al.*, 2005a; Woo *et al.*, 2005a). Virus detection microarrays are likely to be a strategy of the future (Kistler *et al.*, 2007).

Virus Isolation

HCoV-OC43 and related strains were considerably more difficult to propagate in cell culture than HCoV-229E-related strains and required the use of organ cultures, hence the letters 'OC'. Now, however, cell lines are available for the propagation of both laboratory-adapted HCoV types, although primary isolation remains difficult. 229E and related strains can be isolated in roller culture monolayers of human embryonic lung fibroblasts such as W138 and MRC5 cells and the human embryonic intestinal fibroblast cell MA177. In virus-positive cultures, a gradual cytopathic effect consisting of small, granular, round cells appears throughout the monolayers, especially around the periphery of the monolayers. However, cell sheets are rarely destroyed completely on initial isolation (Myint, 1995).

Vero E6 cells and fetal rhesus kidney cells have been used for the isolation of SARS-CoV (Drosten *et al.*, 2003; Ksiazek *et al.*, 2003; Peiris *et al.*, 2003b). Passage of SARS-CoV in cells leads to the emergence of amino acid substitutions in the spike and M genes related to adaptation to the host cells (Poon *et al.*, 2005b).

HCoV-NL63 can be cultured in tertiary monkey kidney cells and in LLC-MK2 cells. HCoV-HKU1 is difficult to grow but has been isolated in HuH7 cells (Vabret *et al.*, 2006). Human torovirus has not been grown *in vitro*.

Electron Microscopy

Electron microscopy is not routinely used for diagnosis of HCoV causing respiratory disease although it has been sometimes used to detect HCoVs in epithelial cells shed from the nasopharynx of patients after attempted isolation in cell culture (229E strains, SARS-CoV) and organ culture (OC43 type). Immune electron microscopy has been used with convalescent sera from patients.

Enteric HCoVs and HTVs have also been sought in faeces using electron and immune electron microscopy (Duckmanton *et al.*, 1997). The two types of virus are of similar size. HTVs are pleomorphic, sometimes exhibiting a torus (doughnut) appearance and an irregular rod shape. The spikes of coronaviruses, and possibly of toroviruses, are not always clearly revealed by negative staining. Thus the two viruses can be confused if electron microscopy alone is used; corroborative evidence is required (Cornelissen *et al.*, 1998).

Serology

Complement fixation, enzyme immunoassay, immunofluorescence or neutralization tests have been used for serodiagnosis of coronaviruses (Myint, 1995). Immunofluorescence tests on virus-infected cells fixed

in ice cold acetone or virus neutralization tests were used for detecting seroconversion to SARS-CoV and for seroepidemiological studies. Neutralizing antibodies against SARS-CoV peak at around four months after disease onset and decline gradually thereafter. Around 16% of survivors had undetectable levels of neutralizing antibody three years later (Cao *et al.*, 2007). There may be cross-reactions in other serological tests. For instance, immunofluorescent antibody responses to SARS-CoV appear to be associated with anamnestic responses to other human coronaviruses (OC43, 229E) although the converse was not true, possibly because there was no prior immunological memory to SARS-CoV to give rise to the anamnestic responses (Chan *et al.*, 2005). ELISA assays using recombinant nucleocapsid antigens have been described, but positive results must be confirmed by immunofluorescence or microneutralization tests (Woo *et al.*, 2004).

HTV purified from the stool of a patient was used in a haemagglutination-inhibition assay to detect antibodies to HTV in nosocomial cases of gastroenteritis; acute and convalescent sera were examined, revealing rises in antibody titres (Jamieson *et al.*, 1998). Bovine antisera to BTV cross-reacted with the human toroviruses, showing that BTV antisera could be used for human diagnostic purposes. Human convalescent HTV serum reacted with the HE protein of BTV in western blots.

RT-PCR

RT-PCR assays are increasingly used for the diagnosis of respiratory viruses, including coronaviruses (Vabret *et al.*, 2001). Given the limited sequence data available on 229E and OC43, as well as prototype viruses isolated many years ago, it is possible that the incidence of these viruses is underestimated through primers not detecting variant strains. This situation will improve with the availability of more virus sequence data. The design of consensus primers that potentially detect viruses within the coronavirus genus has been useful in our hands. Modifications based on the primers reported by Ksiazek (Ksiazek *et al.*, 2003; Stephensen *et al.*, 1999) targeted at ORF1b appear to perform well, although they are not as sensitive as virus-specific primers for directly detecting HCoV in clinical specimens (Chiu *et al.*, 2005; Poon *et al.*, 2005a).

The application of PCR technology for the detection of HTVs awaits gene sequence data.

Microarray Analysis

Viral microarrays that detect a wide range of known and unknown viral pathogens are being used to identify

pathogens in clinical specimens. These are providing a better insight into the global viral flora present in health and disease and also leading to the detection of novel pathogens (Kistler *et al.*, 2007; Quan *et al.*, 2007; Wang *et al.*, 2002). However, as with PCR-based methods, it is important to have relevant age-matched controls in these studies to be able to interpret the clinical significance of such findings.

PROPHYLAXIS: ACTIVE AND PASSIVE IMMUNIZATION

Active Immunization

There are no vaccines for the group 1 and 2 HCoV, but following the SARS outbreak in 2003 there has been interest in developing a vaccine for SARS. There have been coronavirus vaccines for veterinary use, but they have had limited success and it is perhaps relevant to learn from the successes and limitation of these veterinary vaccines. They have been developed against three group 1 coronaviruses – TGEV, FCoV and CCoV – and the group 3 coronavirus IBV. With the possible exception of IBV vaccines, the coronavirus vaccines have been only marginally effective in the field (Saif, 2005). Live vaccines have been more effective than killed vaccines for TGEV in pigs and IBV in chickens. Serum neutralizing antibodies generally fail to correlate with protection but immunoglobulin A (IgA) antibody in the colostrum is a better correlate of protection against TGEV. For TGEV and IBV, priming with a live attenuated vaccine and boosting with a killed vaccine led to increased mucosal immunity. Vaccinating cats with FCoV vaccines actually led to enhanced disease with the antibody to the spike leading to immunopathology (Olsen, 1993). Vaccines for IBV in chicken are widely used but antigenic variability of the virus poses a challenge because cross-immunity is generally poor (Cavanagh and Naqi, 2003).

Work towards a vaccine for SARS has been reviewed (Cheng *et al.*, 2007; Gillim-Ross and Subbarao, 2006). Experimental challenge studies in mice immunized with DNA vaccines and the use of adoptive transfer and T-cell deletion established that antibodies rather than T cells are the key effectors of vaccine-mediated immune protection (Subbarao *et al.*, 2004). A range of vaccine strategies including the use of inactivated whole virus vaccines, subunit vaccines (including baculovirus-expressed S1 subdomain, trimeric spike protein expressed in mammalian cells), DNA vaccines expressing the spike, the N, M or E proteins, vectored vaccines based on modified vaccinia Ankara (MVA) virus, vesicular stomatitis virus, parainfluenza type 3 or adenovirus vectors, have been

evaluated. An attenuated parainfluenza virus 3 vector individually expressing SARS-CoV spike, E, M and N proteins showed that only the recombinants expressing spike protein were effective in inducing neutralizing antibodies and protecting hamsters from experimental challenge (Buchholz *et al.*, 2004).

Induction of neutralizing antibody responses and, where relevant, T cell responses, were measured. Some of these experimental vaccines have been evaluated in experimental animal models with challenge using infectious SARS-CoV. Experimental challenge of vaccinated animals did not convincingly reveal evidence of immune-mediated disease enhancement, as has been seen with FCoV. As SARS is no longer circulating in the human population, there has been little incentive to take these experimental vaccines to human clinical trials.

These vaccines have all been prepared using the human SARS-CoV. However, it is likely that a re-emergent SARS will arise from re-adaptation of a precursor virus from the animal reservoir. As none of these bat or civet SARS-like CoVs can be cultured *in vitro*, cross-protection of these vaccines against the wild-type animal precursor viruses has not been tested. However, reverse genetics has allowed the reconstruction of animal (civet-like) SARS-CoV and these viruses are less efficiently neutralized by human convalescent antisera to SARS-CoV (Rockx *et al.*, 2007). This possibly reflects that fact that, while the receptor-binding domain is the major SARS-CoV neutralization determinant (Yi *et al.*, 2005), the SARS-CoV from civets does not efficiently bind the human ACE2 receptor. This raises questions whether a vaccine developed against the human SARS-CoV will be effective in protection against a future re-emergent animal SARS-like CoV. More recently, human monoclonals that neutralize both SARS-CoV as well as GDO3 and civet viruses SZ3 and SZ16 have been reported (Zhu *et al.*, 2007). While this provides evidence of the existence of antibody paratopes that will cross-neutralize both human and closely related animal precursors, this does not imply that many of the vaccine strategies used hitherto will predominantly induce antibody to such conserved protective epitopes.

Passive Immunization

Passive immunization with human monoclonal antibodies to the SARS-CoV spike protein have been successful in protecting mice and ferrets from challenge with SARS-CoV (ter Meulen *et al.*, 2004). While some of these monoclonal antibodies failed to cross-neutralize closely related animal viruses, antibodies that cross-neutralize SARS-CoV and strains of civet origin are now available and protect mice from challenge with reconstructed viruses with spike protein that represents both human and

civet viruses (Rockx *et al.*, 2007, 2008). Pools of such antibodies have promise for passive immunization and also perhaps for immunotherapy of patients with SARS-like disease.

THERAPY

Notwithstanding research in this area, antiviral drugs are not currently in use for control of the 'common cold' or other respiratory manifestations caused by HCoV. SARS was a severe pneumonia caused by what was initially an unknown pathogen and the global spread of the disease was contained in a relatively short period of time. As such, there are no controlled clinical trial data on which to base treatment recommendations. Clinical management of SARS is primarily supportive care. Since SARS has no pathognomonic features and presents as a community-acquired pneumonia, appropriate antimicrobial coverage should be given until the diagnosis of SARS is virologically confirmed, at which point antimicrobials could be stopped unless there is reason to suspect secondary bacterial complications.

Initially, given the lack of knowledge of the causative virus or its antiviral susceptibility, ribavirin was used as a broad-spectrum antiviral therapeutic option for SARS. Subsequently there were contradictory results on the *in vitro* antiviral susceptibility of SARS-CoV to ribavirin (Morgenstern *et al.*, 2005; Tan *et al.*, 2004). The discrepant findings may be related to the cell lines used (Vero, Caco-2, pig kidney cells). Interferon (IFN)- α 1, IFN- α 3, leukocytic iIFN and IFN- β have evidence of antiviral effect *in vitro*. The HIV protease inhibitor nelfinavir and a number of other compounds, including glycyrrhizin, baicalin and chloroquine, ACE2 analogues, antiviral peptides targeting the heptad repeat region 2 of the S2 subunit of the spike protein and small interfering RNA (siRNA) have been shown to have antiviral effects *in vitro* (reviewed in (Cheng *et al.*, 2007; Haagmans and Osterhaus, 2006). Some of these compounds have been tested in experimentally infected animals. Pegylated IFN- α significantly reduced lung virus titres in cynomolgus macaques (Haagmans *et al.*, 2004). Interferon- α BD at higher doses and the IFN inducer Ampligen (poly(I):poly(C)) inhibited virus replication in BALB/c mice but nelfinavir, chloroquine and ribavirin did not (Barnard *et al.*, 2006a, 2006b).

Clinical data have suggested that the use of the recombinant consensus IFN- α preparation alfacon-1 together with corticosteroids (Loutfy *et al.*, 2003) and that the use of the protease inhibitor nelfinavir/lopinavir together with ribavirin (Chu *et al.*, 2004a) appears to improve clinical outcome compared to historical controls. Passive

immunotherapy with convalescent plasma containing high neutralizing antibody titres to SARS-CoV was used in some patients with no adverse effects and perhaps some clinical benefit in comparison to historical controls (Cheng *et al.*, 2005). However, these were not controlled clinical trials and the use of historical controls may be misleading.

Immunomodulators including corticosteroid therapy were used in patients with SARS in view of their previous use in acute respiratory distress syndrome. There is, however, no documented evidence of clinical benefit and they may increase viral load in the plasma (Lee *et al.*, 2004) and increase the risk of adverse effects, including avascular necrosis of bone.

In summary, the IFNs appear to be the only class of antiviral agents that has evidence of *in vitro* activity and efficacy in experimental animal models.

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Measles Virus

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INTRODUCTION

Acute measles is normally a mild disease contracted by children and young adults as a result of infection by the highly contagious measles virus (MV) (ter Meulen and Billeter, 1995). MV is an efficient pathogen, persisting in nature in populations large enough to support it, even though it causes an acute infection in any individual only once in a lifetime. Despite this, the virus is distributed worldwide and is antigenically stable. With the advent of molecular epidemiology, however, the existence of MV genotypes has been confirmed. MV has no animal reservoir, and although monkeys are susceptible to infection, transmission from animals is not an important means of introducing the disease into a human community. MV may persist for years in a single individual, yet these infections are rare and not associated with periodic shedding of infectious virus as seen with herpesviruses. A single attack of measles is sufficient to confer lifelong immunity to clinical disease upon reinfection, even in the absence of re-exposure to the virus. Consequently, in order to remain endemic in a given community, the virus must rely on the infection of the young who are still susceptible. So efficient is the process that the first known report of measles (in Egyptian hieroglyphics) failed to recognize the infectious nature of the illness, and described it as a normal part of child growth and development.

In the pre-vaccine era in developed countries the maximum incidence of measles was seen in children aged five to nine years. Infections and epidemics centred around elementary schools, and younger children acquired measles as secondary cases from their school-age siblings. By the age of 20, approximately 99% of subjects tested had been exposed to MV. With the introduction of the measles vaccine the age incidence and percentage of cases in

different age groups has changed markedly. In countries with an optimal vaccine utilization, measles infection has shifted to the teenage group, whereas in areas with ineffective vaccine programmes children up to four years of age reveal a high primary measles attack rate (Centers for Disease Control and Prevention, 1991, 1996). In contrast, in developing countries measles has its greatest incidence in children under two years of age. Here, case fatality rates range from 1 to 5% and can reach 30% in refugee camps and comparable settings, with malnourishment, crowding and intensity of exposure being major determinants of severity and mortality (Moss, 2007). Although a safe and efficient vaccine has been available for over four decades, measles still is a leading cause of death for young children. The Measles Initiative, a partnership between the World Health Organization (WHO), the United Nations, the US Centers for Disease Control and Prevention (CDC), the American Red Cross and other organizations, was launched in 2001 and has since been developing and implementing comprehensive strategies for sustainable measles mortality reduction. As a result, measles deaths worldwide fell from an estimated 873 000 (1999) to 345 000 in 2005 (Wolfson *et al.*, 2007; www.measlesinitiative.org/index3.asp).

THE VIRUS

Although measles has been known for centuries, it was only with the isolation of the virus by Enders and Peebles in 1954 that experimentation became possible. The development of tissue culture systems, the availability of monoclonal antibodies and molecular biological approaches then permitted insight into viral structure and replication. Functional studies on MV control regions and

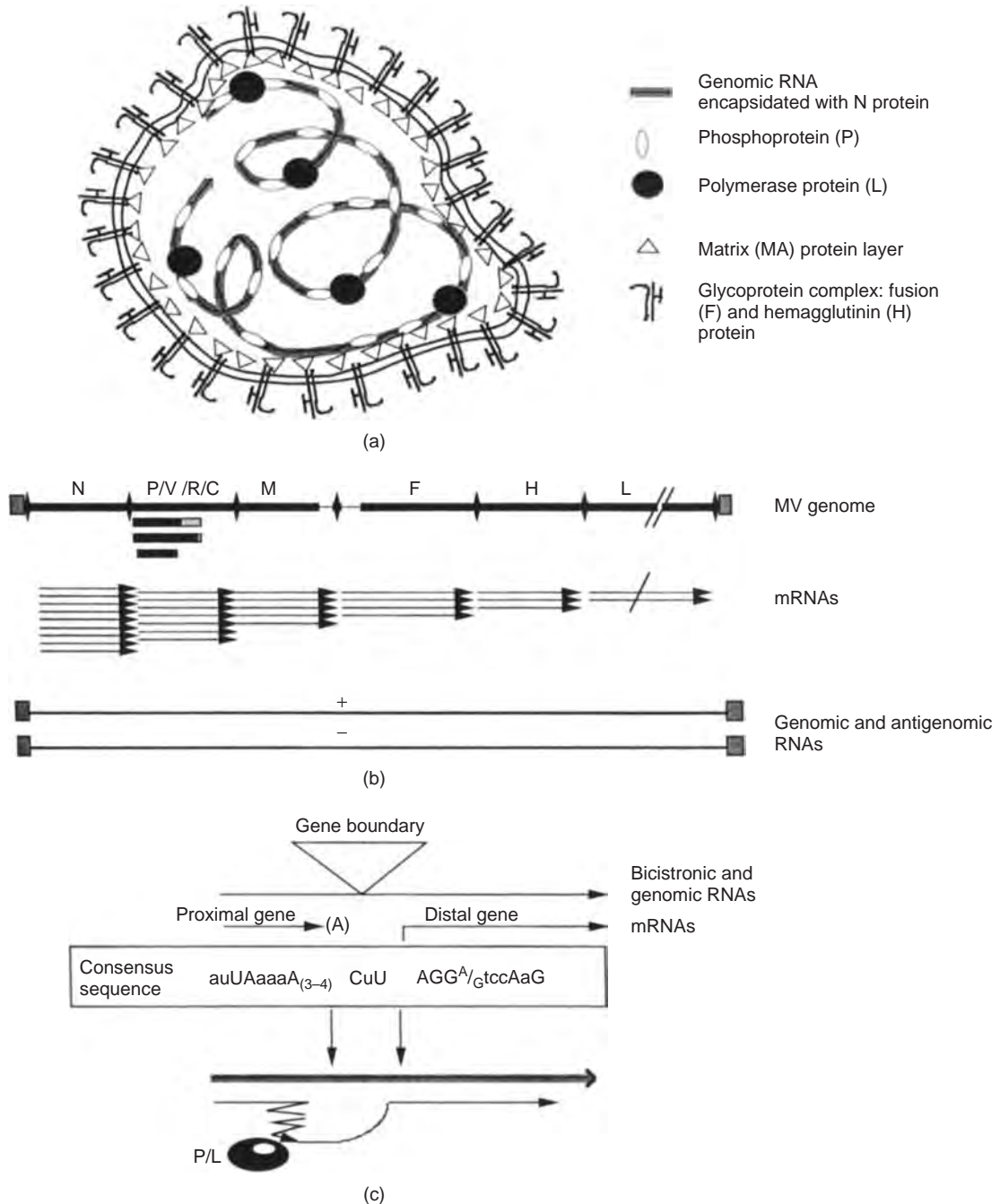


Figure 22.1 (a) Diagrammatic representation of the measles virus particle. (b) MV mRNAs are sequentially transcribed from the genome with decreasing efficiency and encode the structural proteins. In addition, the second gene (the P gene) encodes three nonstructural viral proteins C, R and V. (c) At the gene boundaries, MV genes are separated by conserved intergenic regions where the polymerase complex stutters to polyadenylate the proximal mRNA to subsequently reinitiate transcription of the downstream gene. Alternatively, these signals are neglected by the polymerase when bi- or polycistronic mRNAs are transcribed as well as during genome replication.

proteins extensively benefited from the establishment of a plasmid-driven reverse genetic system which allowed construction and rescue of infectious MVs in tissue culture that carry stable alterations in their genomes (Radecke *et al.*, 1995). Thus, within limitations imposed by the fitness of the mutated recombinant virus, the contribution of single viral gene products within the viral life cycle in tissue culture, but also in experimental infection in animals can be addressed. Meanwhile, a plethora of recombinant MVs have been generated, expressing extra reading frames such as fluorescent or enzymatically active marker genes, which allow tracing of viral replication after experimental infection, and cytokine genes. Moreover, targeted interaction of recombinant MV with its natural or other, desired host cell receptors has been widely studied to gain insight into basic determinants of viral tropism, but also for potential application of systemic, targeted delivery in cytoreductive regimens for malignancies (Iankov *et al.*, 2007). The use of recombinant MV carrying heterologous viral genes (e.g. simian immunodeficiency virus (SIV) or human immunodeficiency virus (HIV) genes) as multivalent vectors for immunization has been explored, although this has been limited to animal studies so far (Tangy and Naim, 2005; Zuniga *et al.*, 2007).

MV is a member of the *Mononegavirales* which comprises the *Rhabdoviridae*, *Filoviridae*, *Henipaviridae*, *Bornaviridae* and *Paramyxoviridae*. As a paramyxovirus, MV has structural and biochemical features associated with this group, but because it lacks a virion-associated neuraminidase activity it is grouped into a separate genus, *Morbillivirus*, of which *Measles virus* is the type species. Other members include: *Peste-des-petits-ruminants virus* (PPRV), which infects sheep and goats, *Rinderpest virus* (RPV), which infects cattle, *Canine distemper virus* (CDV), which infects dogs, *Phocine distemper virus* (PDV), which infects seals and sea-lions, *Dolphin morbillivirus* (DMV) and *Porpoise morbillivirus* (PMV). All these viruses exhibit antigenic similarities, and all produce similar diseases in their host species. Both MV and CDV can persist in the central nervous system (CNS) in their natural hosts and produce chronic neurological diseases.

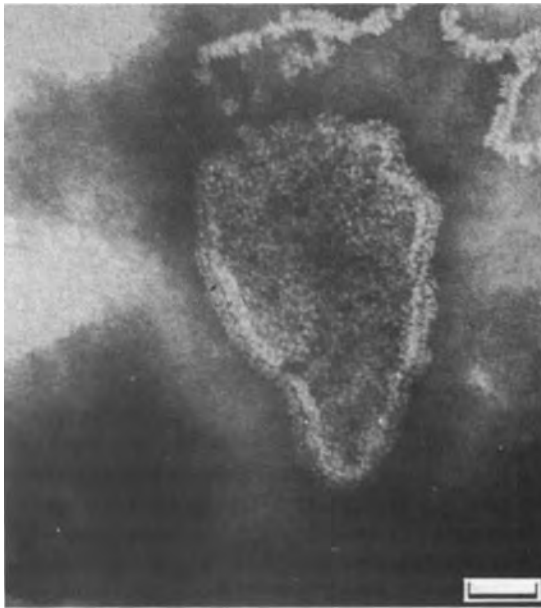
VIRUS MORPHOLOGY

Measles virus particles consist of a lipid envelope surrounding the viral ribonucleoprotein (RNP) complex, which is composed of genomic RNA associated with proteins (Figure 22.1a). The MV transmembrane fusion (F) and haemagglutinin (H) proteins project from the envelope surface of the particle, extend through the lipid bilayer into the cytosol. It is the N-terminus of the H

protein that protrudes through the cytoplasmic and viral membranes (type II glycoprotein), while the F protein is anchored near the C-terminus (type I glycoprotein). One or both of the cytoplasmic domains are believed to interact physically and functionally with the matrix (M) protein (Tahara *et al.*, 2007), which, in turn, links the envelope to the RNP core structure. The viral genomic RNA is fully condensed with N (nucleocapsid) protein to form the RNase-resistant RNP core structure. *In vitro* experimentation suggests that the virion is able to package more than one genome as long as the 'rule of six' (see below) is maintained (Rager *et al.*, 2002). As the viral genome cannot serve as mRNA, the viral polymerase complex consisting of the P (phospho-) and L (large) proteins is part of the RNP core. Their location within this complex is as yet unknown, as is that of cellular actin, known to be also packaged into the virion structure. The virions are highly pleomorphic, with an average size of 120–250 nm. In an electron micrograph the virion is bounded by a lipid envelope which bears a fringe of spike-like projections (peplomers) 5–8 nm long (Figure 22.2a). The membrane below the spikes is 10–20 nm thick and encloses the helical viral RNP core which has a diameter of 17 nm and a regular pitch of 5 nm. Immediately below the membrane M proteins appear as a shell of electron-dense material.

GENOME STRUCTURE

The viral genome is a nonsegmented RNA molecule of negative polarity that is about 16 kb in length. The genome encodes six structural genes for which the reading frames are arranged linearly and without overlap in the following order: 3' nucleoprotein (N, 60 kDa), phosphoprotein (P, 70 kDa), matrix protein (M, 37 kDa), fusion protein (F, disulfide-linked 41 kDa F₁ and 22 kDa F₂ proteins, cleavage products of a 60 kDa precursor F₀ protein), haemagglutinin protein (H, 80 kDa, existing as disulfide-linked homodimer) and the large protein (L, 220 kDa) encoded on its 5' end (Figure 22.1b) (Rima *et al.*, 1986). The genome is flanked by noncoding 3' leader and 5' trailer sequences that contain specific encapsidation signals and the viral promoters used for viral transcription and/or replication (Parks *et al.*, 2001). The genomic RNA molecule is entirely complexed with N protein with one N molecule covering six nucleotides. This is thought to be the reason why only viral genomes (also including recombinant MVs carrying extra transcription units) whose number of nucleotides is a multimer of six are efficiently replicated by the viral polymerase. Transcription of the viral monocistronic mRNAs is initiated within the 3' leader sequence. The coding regions of the viral genome are separated by



(a)



(b)

Figure 22.2 (a) Electron micrograph of the measles virion (bar = 50 nm). (b) Electron micrograph of purified MV nucleocapsids (bar = 50 nm); both are negatively stained with phosphotungstic acid. (Source: Reproduced with permission from Meadeley, C.R. (1973) *Virus Morphology*, Churchill Livingstone, Edinburgh.)

intergenic regions which consist of a polyadenylation signal at the 3' of each gene, a trinucleotide (CUU, except for CGU at the H/L gene boundary) and a reinitiation signal for the distal gene (Figure 22.1c) (Parks *et al.*, 2001). From the P gene, three nonstructural proteins, C (20 kDa), V (46 kDa) and R (46 kDa) are expressed. Whereas the C protein is encoded within a separate reading frame, V protein is translated from edited P mRNAs where a non-encoded G residue is co-transcriptionally inserted at a particular site by an intrinsic activity of the MV polymerase. It thus shares the N-terminal domain with P protein, yet has a unique cysteine-rich C-terminal domain. About 50% (to yield V protein) of the P mRNAs are edited. At the M-F gene boundary a GC-rich region of about 1 kb in length spans the 3' end of the M gene and the 5' end of the F gene. Several open reading frames have been predicted for this region which could only be accessed by translation from a bicistronic transcript by ribosomal reinitiation. None of the putatively encoded proteins has yet been detected in infected cells.

MV PROTEIN FUNCTIONS

The Viral RNP and Nonstructural Proteins

The fully encapsidated MV genomic RNA serves as a unique target for the viral polymerase to initiate transcription and replication. The N protein, which is phosphorylated at serine and threonine residues, is the most abundant of the MV proteins and acts to condense the viral genomic RNAs into a smaller, more stable and more readily packaged form. This gives the nucleocapsid its helical form and 'herringbone' appearance in the electron micrograph (Figure 22.2b). When expressed in the absence of viral RNA, N proteins self-aggregate into nuclear and cytoplasmic nucleocapsid-like structures. The N-terminal 398 amino acids are important for self-aggregation and RNA interaction, while the C-terminal 125 amino acids protrude from the nucleocapsid. This particular domain reveals an intrinsically disordered structure (Longhi *et al.*, 2003) and interacts with cellular proteins such as Hsp72 and IRF-3. It is by formation of high-affinity protein complexes with the phosphorylated P protein, that self-aggregation and nuclear localization of N proteins are prevented during replication of the viral genome. Similar, interaction with the P protein is essential for folding of the C-terminal domain of the N protein (Johansson *et al.*, 2003). Both the C-terminal and N-terminal domains of P protein are important for N/P complex formation, while another C-terminal motif is essential for oligomerization of the P protein. Phosphorylation on serine residues is crucial for its polymerase cofactor activity in transcription and replication. Stable complexes between P and L are equally

important for these processes. The L protein is a multi-functional RNP-specific RNA polymerase producing mRNAs, replicative intermediates and progeny viral genomic RNAs. Capping, methylation, editing and polyadenylation are thought to be mediated by the polymerase protein in addition to initiating, elongating and terminating ribonucleotide polymerization. Active sites within the protein have not yet been determined, but conserved motifs were identified which suggest a linear arrangement of the functional domains.

Although largely considered as typical cytosolic and nonstructural (such as V protein), C protein was found to shuttle between nucleus and cytoplasm and also to associate with viral particles (Devaux and Cattaneo, 2004; Nishie *et al.*, 2007). Recombinant MVs defective in either V or C protein functions apparently replicated well in tissue culture cells (Radecke and Billeter, 1996; Schneider *et al.*, 1997), yet there is evidence that these proteins regulate the efficiency of MV replication in primary cells or modulate both viral gene expression and induction of or sensitivity to the cellular interferon (IFN) response (Cruz *et al.*, 2006; Escoffier *et al.*, 1999; Nakatsu *et al.*, 2006; Palosaari *et al.*, 2003; Takeuchi *et al.*, 2003). More recently, P protein was found to inhibit IFN signalling as well (Devaux *et al.*, 2007). Not surprisingly, absence of V and C proteins affects MV virulence in animal models (Tober *et al.*, 1998; Valsamakis *et al.*, 1998).

The Envelope Proteins

The RNP core structure is enclosed by a lipid envelope which contains two glycosylated proteins on its external surface, the H and the F protein, which are organized as functional complexes. The H protein mediates binding to cellular receptors, the F protein causes fusion of viral and cell membrane at neutral pH. Both H and F protein are protease-sensitive and can be isolated by gentle detergent lysis of virions, although F protein tends to remain strongly associated with cellular actin. The tendency of spikes to aggregate most likely resides in the hydrophobic tail of each molecule, which normally serves as anchor in the lipid bilayer.

The H protein can be isolated as a tetrameric complex from the cell membrane and efficiently agglutinates red blood cells from sheep and monkeys, but not humans. Glycosylation occurs within a region of 70 amino acids, and is essential for haemadsorption, probably by stabilizing the highly complex tertiary structure of the protein. Analogous to that of its Newcastle disease virus (NDV) orthologue, the ectodomain of the MV H protein is thought to be organized into a membrane proximal stalk and a membrane distal globular head region composed of a six-wing propeller (Crennell *et al.*, 2000). Seven residues located within the globular head domain are

essential for oligomerization and folding of the H protein, and a cysteine residue in the stalk regions for formation of disulfide-linked dimers. In addition, amino acids involved in binding of the H protein to the MV receptors were identified. These, located in the ectodomain, include amongst others, residues 451, 481, 546 and 473–477 (Vongpunsawad *et al.*, 2004).

As revealed by transfection experiments, the H protein also exerts a helper function in F-mediated membrane fusion, probably by directing the fusion domain into the optimal distance to the target cell membrane and stabilizing the interaction. Synthesized as a precursor protein (F₀), F protein is cleaved in the Golgi compartment by subtilisin-like proteases to yield two disulfide-linked subunits, F₁ and F₂. Glycosylation of the F₀ precursor is an essential prerequisite for cleavage, and all the potential N-glycosylation sites reside within the F₂ subunit. Mutations of any of these sites affect cell surface transport, proteolytic cleavage, stability and fusogenic activity of the F protein. Indeed, the contribution of the F₂ subunit to MV fusion has been directly documented (Plempner and Compans, 2003). The F₁ subunit reveals an N-terminal stretch of hydrophobic residues (the fusion domain) and two amphipathic α -helical domains, one of which is adjacent to the fusion domain (HRA) and the other (containing a leucine-zipper motif) is next to the N-terminal of the transmembrane region (HRB). The fusogenic domain within the F₁ subunit is masked during intracellular transport by intramolecular folding, and this most likely prevents fusion of internal membranes (Doyle *et al.*, 2006). A central region mediates interaction with the H protein. Homo- and hetero-oligomerization of the glycoproteins occurs in the endoplasmic reticulum and both interaction strength and fusogenicity of the complex are influenced by their cytoplasmic tails, both independent and dependent of their interaction with the M protein (Plempner *et al.*, 2002). The fully processed F_{1/2} protein is incorporated into the cell membrane as an oligomer. In the intact virion the active site of each protein is presumably carried at the tip of the spike and orientated outwards, away from the hydrophobic tail and towards any possible target cell.

The M protein interacts with the viral RNP and with the plasma membrane. When expressed in the absence of other MV proteins, the protein promotes formation of virus-like particles confirming that, in analogy to its functional orthologues, it essentially triggers late steps in the viral life cycle such as assembly site and budding (Pohl *et al.*, 2007). Consequently, recombinant MVs carrying deletions within the M gene bud highly inefficiently. Physical interaction between M and other viral structural proteins has been difficult to demonstrate, however, M protein modulates the fusogenic activity of the F/H complex and interactions with the glycoprotein cytoplasmic

tails allow the M protein to co-segregate to the apical surface in polarized cells. A recombinant MV defective for the expression of the MV glycoproteins (expressing the glycoprotein of vesicular stomatitis virus instead) revealed the requirement of the MV glycoproteins for packaging the M protein into mature budding virions.

THE REPLICATION CYCLE

Measles Virus Receptor Usage and Tropism

One of the most important parameters determining viral tropism is the availability of specific surface receptors on susceptible cells. MV naturally only replicates in primate hosts. *In vivo* it reveals a pronounced tropism for cells of the haematopoietic lineage, but may, however, at later stages, also replicate in a variety of cell types as it does in tissue culture. Thus, the receptor would be expected to be expressed by most human cells both *in vivo* and *in vitro*. This is true for the first MV receptor identified, CD46 (Griffin and Bellini, 1996), which reveals a wide tissue distribution *in vivo*, and, notably, is expressed on monkey but not on human erythrocytes. Several isoforms of CD46 (due to alternative splicing of a precursor mRNA)

are expressed in a tissue-specific manner and all of them support MV uptake. CD46 contains four repetitive conserved subunits within its ectodomain, with the two most membrane distal being essential for binding of the MV H protein. The molecule's physiological ligand(s), complement components C3b/C4b, bind to membrane-proximal domains (Figure 22.3). As a member of the regulators of complement (RCA) gene family, CD46 is involved in protecting uninfected cells from complement-mediated lysis by recruiting the C3b/C4b components, thereby rendering them accessible to degradation by serum proteases. It is considered of pathogenic importance that CD46 is downregulated from the surface of infected cells or following interaction with MV H protein, as these cells are significantly less protected against complement-mediated lysis *in vitro* (Schneider-Schaulies *et al.*, 1995). The inability of certain MV strains, particularly those isolated and passaged exclusively on lymphocytes, to use CD46 as receptor soon indicated the existence of additional MV receptor(s), and this led to the identification of CD150 (also referred to as signalling lymphocyte activation molecule, SLAM), a CD2-like molecule of the Ig superfamily, as MV receptor (Tatsuo and Yanagi, 2002). CD150 is expressed by activated and memory T and B cells,

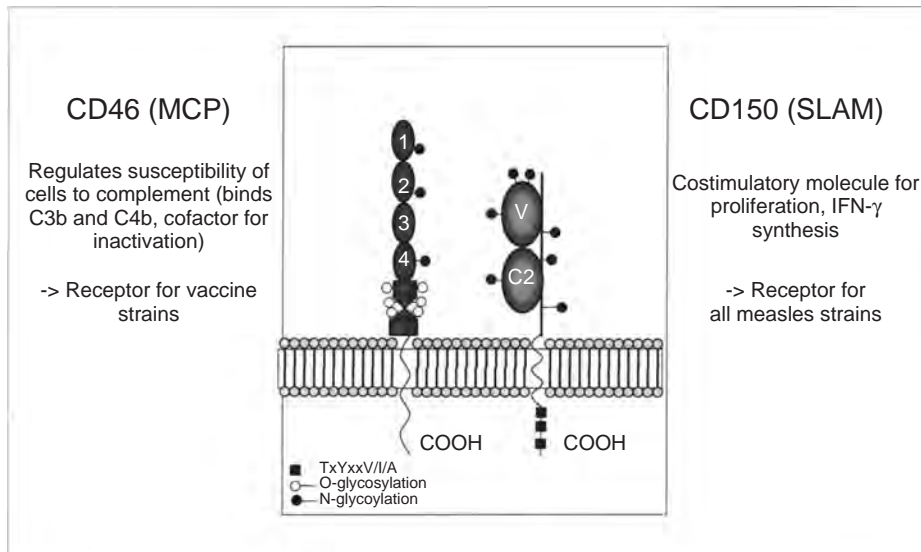


Figure 22.3 Schematic representation of CD46 (MCP, membrane cofactor protein) (left), the major protein receptor for attenuated MV strains. MV-binding sites are located within the short consensus repeat (SCR) domains 1 and 2, whereas complement components C3b/C4b bind to SCR 3 and 4, respectively. Proximal to the transmembrane domain, oligosaccharide-rich serine/threonine/proline (STP) domains are located. CD150, a member of the Ig superfamily, (right) is the receptor of all MV strains tested as yet. MV binding occurs at the membrane distal domain (the V domain). Glycosylation sites in the extracellular domains are indicated as are residues in the cytoplasmic domain identified as important for signalling.

and immature thymocytes, but not by freshly isolated monocytes and immature dendritic cells, where expression of this molecule is inducible. For monocytes, this can occur by interaction of the H protein of wild-type MV strains with Toll-like receptor 2 (TLR2), which itself does not serve as an entry receptor (Bieback *et al.*, 2002). As for CD46, the most membrane-distal portion of CD150, the V domain, is important for MV binding, and CD150 is also downregulated in response to infection or H protein interaction. CD150 supports entry of all MV strains known, yet its expression is confined to haematopoietic cells, and thus, alternative MV entry receptors should exist, promoting uptake of wild-type MV into cell types such as endothelial, epithelial (during acute measles) and brain cells (as prerequisite for CNS persistence) (Yanagi *et al.*, 2006).

The contribution of the MV receptors to MV tissue tropism and pathogenicity is not yet understood. Rodents genetically modified to express CD46 or CD150 are not susceptible to MV infection unless the virus is intrathecally applied. Most likely, as yet unknown intracellular factors efficiently restrict MV replication in rodent cells. Infection of brain cells *in vivo*, on the other hand, can occur independently of CD46 expression as documented in mice or rats after intracerebral infection with an attenuated, rodent brain adapted MV strain. In cotton rats which are susceptible to intranasal infection with both attenuated and wild-type MV strains (as revealed by virus isolation from peripheral blood mononuclear cells (PBMCs), development of interstitial pneumonia and immunosuppression) tissue distribution of potential CD150 and CD46 ortho-

logues has not been evaluated due to the lack of appropriate reagents. It has been shown, however, in these animals that infection with the wild-type MV strain or recombinant viruses expressing the wild-type MV H glycoprotein leads to preferential infection of secondary lymphoid tissues and pronounced immunosuppression. These findings lend strong support to the essential role of the interaction of the MV H protein with its receptors in MV pathogenesis *in vivo*.

Intracellular Replication

The time taken for MV replication in a host cell is variable and becomes shorter as the virus adapts to growth *in vitro*. For instance, the Edmonston strain replicates well in Vero cells, an African green monkey kidney cell line, where growth is complete within 6–8 hours and accompanied by effective inhibition of host cell macromolecular synthesis. Other strains, particularly freshly derived isolates, grow more slowly and replication times of 7–15 days are not uncommon. Such viruses often have very little inhibitory effect on the biosynthesis of the host cell. The origin and activation stage of the host cells also influence the efficiency of MV replication as does their ability to produce type I IFN or, in turn, the ability of the virus strain to prevent IFN production and/or signalling.

Following delivery of the viral RNP complex into the cytoplasm, viral transcription is initiated after specific attachment of the polymerase complex to the promoter located within the 3' end of the genome and progresses to the 5' end by transcribing mono- and bicistronic mRNAs (Figure 22.4). At each gene boundary, the polymerase complex resumes transcription of the distal

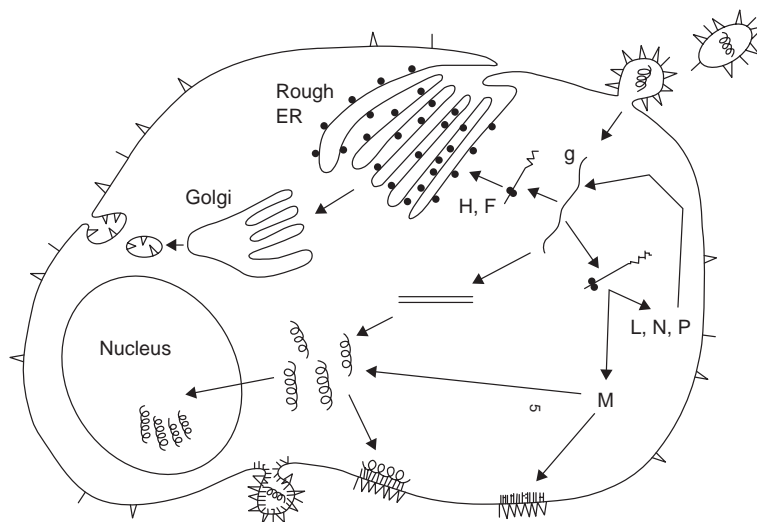


Figure 22.4 Schematic representation of the events occurring in measles virus replication.

gene or leaves the template. As a consequence, a polar gradient is established for the frequency of viral mRNAs with the N-specific mRNA being the most abundant and the L-specific mRNA the least represented (Figure 22.1b). At the 3' end of each gene, poly(A) tracts are added to the mRNA transcripts, most probably by a polymerase stuttering mechanism at the termination signals (Figure 22.1c). Bi- and polycistronic polyadenylated transcripts spanning two or more adjacent genes are also produced. In the replication mode, the polymerase complex reads through the intergenic boundaries to yield a positive-sense replicative intermediate which is about 100-fold less abundant than that of negative polarity. Transcripts of positive polarity containing the encapsidation signal at their 5' end joined to the N gene sequence are indicative of antigenome replication. Replication of, but not primary transcription from viral genomic RNA is dependent on protein synthesis, and the switch to the replication mode is possibly determined by the accumulation levels of N protein that has to encapsidate the nascent genome and may act as an anti-terminating protein. Not surprisingly, expression and/or function of the L protein are currently targeted by RNA interference approaches or non-nucleoside inhibitors *in vitro* in order to exploit inhibition of the polymerase for therapy (White *et al.*, 2007).

The viral mRNAs direct the synthesis of viral proteins which are translocated and, in case of the glycoproteins, modified through the Golgi apparatus (acquisition of N-linked glycosylation and proteolytic cleavage of the F₀ protein) and finally inserted into the plasma membrane. The F protein associates preferentially with membrane microdomains (also referred to as lipid rafts) and most likely drags the H protein into these structures from where the virus subsequently buds. Nascent RNA genomes condense with N protein to form the nucleocapsid, and P and L proteins bind to these structures in the perinuclear area. Late in infection, nucleocapsids may also enter the cellular nucleus. M protein combines with both cytoplasmic nucleocapsids and plasma membrane resident virus glycoproteins, and possibly interacts with cytoskeletal components during intracellular transport of viral RNPs. Progeny nucleocapsid structures line up beneath these modified areas of the membrane and are pinched off in the budding process. The ability of M protein to aggregate in a crystalline array most likely enables distortion of the membrane into an outward-facing bulge, and ultimately budding of the nucleocapsid inside a small vesicular structure—the new virion (Figure 22.4). During the replication process the large amount of glycoprotein inserted into the cell membrane causes it to develop the capacity to attach to adjacent cells, while the F protein promotes fusion with adjacent cells. Multinucleate

giant cells are thus formed which are pathognomonic for measles infection. Host cells are rapidly killed by fusion, and if this is prevented both survival and virus production are increased.

BIOLOGICAL PROPERTIES OF THE MEASLES VIRUS

Stability

The structure of the virion explains much of the early data concerning the stability and infectivity of the virus, which depends on the integrity of the envelope. Hence the virus is sensitive to any procedure which disrupts this structure, such as detergents or other lipid solvents, including acetone or ether. Particles are acid labile and inactivated below pH 4.5, although they remain infective in the range pH 5–9. The virus is also thermolabile. It may remain infective for two weeks at 4 °C, but it is completely inactivated after 30 min at 56 °C. At 37 °C it has a half-life of 2 hours. Thermolability is probably due to an effect on the internal structure of the particle since haemagglutinin is relatively temperature-resistant. Virus can be stored for prolonged periods at –70 °C and also freeze-dries well. These properties have important consequences for the transport and storage of vaccine.

Haemagglutinin

MV displays haemagglutination activity for erythrocytes from rhesus, patas and African green monkeys and baboons, but not from humans. The H protein mediates attachment to susceptible cells, and consequently, the ability to cross-link erythrocytes which do not support virus replication represents an unnatural process in virus multiplication. Thus the inability to agglutinate erythrocytes from the primary host, which is based on the lack of the major MV receptor component CD46 on these cells, is not surprising. Morbilliviruses have no neuraminidase activity and do not attach to receptors containing sialic acid. Consequently, once attached to a red blood cell, MV does not re-elute rapidly.

H protein inserted into any membranous structure is active in the haemagglutination test. Virus particles separated by isopycnic centrifugation have a buoyant density of 1.23 g cm⁻³ and haemagglutination activity is detected in this area of the gradient. A large amount of haemagglutinating material is also found in the upper regions of the gradient (termed light haemagglutinin) which probably represents H protein inserted into empty membranous fragments of infected cells, or defective virus particles. Haemagglutinating activity in this fraction can exceed

that associated with the intact particles. Notably, MV wild-type strains isolated on B lymphoid cells and not adapted to grow on Vero cells have very low haemagglutination activity. This may be caused by the presence of an additional *N*-linked glycosylation site within their H proteins, but is best explained by their inability to interact with CD46 on monkey erythrocytes. H protein is the major immunogen of the virus, and antibodies directed against this protein have both haemagglutination inhibition (HAI) and virus-neutralizing activities (neutralizing test, NT). However, these antibodies cannot prevent the progressive viral cell-to-cell spread mediated by the F protein. The function of H protein as the major viral attachment protein and downregulation of host cell receptor(s) has already been outlined above. Moreover, receptor ligation by H protein can trigger signalling pathways in host cells, and this may relate to certain aspects of MV-induced immunosuppression (see below).

Haemolysis

The ability to lyse red blood cells once the virus has bound is mediated by the viral F protein. This ability is artificial in the same way that haemagglutination is artificial, since the F protein is not normally called upon to lyse a target cell before productive infection is accomplished. Nevertheless, haemolysis (HL) provides a convenient measure of F protein activity which is more sensitive to pH and temperature than haemagglutination. The optimum temperature for HL is 37°C and optimum pH is 7.4. The ability of paramyxoviruses to fuse at neutral pH accounts for their characteristic cytopathic effect (CPE), the formation of giant cells. Proteolytic activation of the F protein is vital for its activity; although uncleaved molecules can be inserted into mature virus particles, these do not fuse with target cells and are therefore not infectious. Insertion of the N-terminal hydrophobic fusion domain of the F₁ subunit is thought to have a destabilizing effect on the local structure of the target cell and synthetic peptides with similarity to this region but also those corresponding to the heptad repeat regions (which inhibit back-folding of the F₁ subunit into its fusion active conformation after MV receptor interaction) efficiently impair cell fusion. Antibodies directed against the F protein are required for effective containment of virus infection, which can be maintained locally by cell-to-cell fusion. Given the importance of this protein for cell entry, it is not surprising that structurally conserved microdomains within the F protein are exploited as potential target sites for rationally designed antiviral compounds (Plempner *et al.*, 2004, 2005; Sun *et al.*, 2006).

EPIDEMIOLOGY AND RELATEDNESS OF DIFFERENT VIRUS ISOLATES

The efficient spread of the virus is mediated by aerosol droplets and respiratory secretions, which can remain infectious for several hours. The disease incidence in the northern hemisphere tends to rise in winter and spring when lowered relative humidity would favour this form of transmission. In equatorial regions epidemics of measles are less marked but can occur in the hot dry season. Acquisition of the infection is via the upper respiratory tract, the nose and, possibly, the conjunctivae. Virus is also shed in the urine but this is unlikely to be an important means of transmission.

The spread of measles is a convenient example to illustrate the principles of epidemiology; based on calculations, any community of less than 500 000 is unlikely to have a high enough birth rate to supply the number of susceptible children required for the continuous maintenance of the virus in a population. In fact, complete elimination of measles from isolated groups has been documented; these remain free of the disease until MV is reintroduced from outside, and susceptible individuals are once more at risk. Measles often leads to a more serious disease in such communities experiencing the illness for the first time, because all age groups are susceptible to the infection. In general, measles mortality is highest in children under two years of age and in adults. Death from uncomplicated measles is rare in the developed world, yet introduction of the virus to the Fiji Islands in 1875 resulted in an epidemic with a fatality rate of 20–25% and into Greenland in 1951 produced an epidemic which infected 100% of susceptibles and resulted in a death rate of 18 per 1000.

MV isolates have been obtained from many different locations and from patients with different clinical conditions. In serological terms, the virus is monotypic and thus infection by any MV confers immunity to them all. With monoclonal antibodies, antigenic differences between vaccine and wild-type viruses can be observed, though these could not be linked to pathogenicity, replication or transmission. The monotypic nature of MV has masked the existence of a set of genotypes which accumulate mutations continuously. Extensive studies on the molecular epidemiology of MV failed to reveal the existence of MV strains with different pathogenic potential (lymphotropism and neurovirulence, the latter more likely to cause subacute sclerosing panencephalitis (SSPE)) or potential antigenic drift in wild-type MV strains that may impair the protective effect of the current vaccine. They have been highly instrumental in populations where mass vaccination campaigns have been undertaken, because they allow us to define whether any single measles case is due to an imported virus or represents a still inadequate

vaccine coverage or vaccine failure. Thus, in 1995, 60% of the 309 cases of measles reported in the United States were either directly imported or were found to be directly linked to an imported case by routine investigation or molecular epidemiology methods (Rota *et al.*, 1992).

Sequence analysis of vaccine and wild-type MV strains as well as SSPE isolates has allowed classification of these various MVs into lineage groups, referred to as 'clades' (numbered A–H), and within those, different genotypes can be distinguished. Genotyping of a given measles strain is based on the C-terminal 151-amino-acid sequence of the N protein, where up to 10.6% divergence in the amino acid sequence between unrelated strains can occur. For most of the recent isolates, the sequence of the H gene is also available. While some MV clades are more or less extinct (i.e. have not been isolated for at least 15 years), others are still co-circulating (see www.cdc.gov/ncidod/dvrd/revb/measles/index.htm). The activity within a clade is directly mirrored by the heterogeneity of genetically related recent isolates which are drifting on a genetic level. It is indicative of MV re-importation into regions where transmission of indigenous MV is interrupted that MVs of different, genetically unrelated genotypes are isolated during an outbreak. Genetic characterization of MV is a powerful adjunct to the standard epidemiological techniques applied to study measles transmission as it helps to confirm the sources of virus or suggest a source for unknown source cases, or to establish links, or lack thereof, between cases and outbreaks. Molecular surveillance is most beneficial in tracing local genotypic changes over time, and this, analysed in conjunction with standard epidemiological data, has helped to document the interruption of transmission of endemic measles and to measure the effectiveness of measles control programmes. MV vaccine strains (which all fall within clade A) differ widely from wild-type isolates, and SSPE-derived sequences were closely related to those of wild-type viruses. It was even possible to identify wild-type MVs that had circulated in a given population as likely infectious agents found later in SSPE brain material. In the late 1980s and early 1990s, re-importation of wild-type MV of a known genotype into the United States caused about 50 000 cases. In a recent study, seven cases of SSPE were noted which could clearly be assigned to the wild-type MV having caused the acute infection 10 years ago (Bellini *et al.*, 2005). These findings resulted in three important conclusions: first, SSPE develops more frequently than thought (Bellini *et al.*, 2005; Takasu *et al.*, 2003), second, SSPE develops after infection with a wild-type MV and not following vaccination, and third, circulating wild-type viruses and not particular neurotropic strains access the CNS. Thus, the development of complications is not determined by

the virus, but rather by the susceptibility, age and immune status of the host at the time of infection. The biological importance of the fact that all vaccine strains have a genotype substantially different from the currently co-circulating wild-type viruses is unclear. There is, however, no evidence to suggest that the currently used vaccines are not able to control MV infection with viruses of differing genotypes.

CLINICAL MANIFESTATIONS

Acute Measles

Measles was an inevitable disease of childhood prior to the vaccine era, and thus clinical features are well documented. The course of acute measles is illustrated diagrammatically in Figure 22.5. The virus first gains entry into the body through the upper respiratory tract or conjunctiva. Replication is assumed to occur at the site of entry. It is unknown as yet whether epithelial cells in the respiratory epithelium support MV replication, or if the first target cells are professional antigen-presenting cells (APCs) (such as monocytes or tissue-resident macrophages or dendritic cells) which acquire virus by CD150-dependent infection. Whether the interaction with DC-SIGN, a lectin receptor, shown *in vitro* supports entry of MV into dendritic cells (DCs) or internalization *in vivo* is unknown. APCs most likely mediate transport of MV to secondary lymphatics, where MV replicates and causes tissue destruction. The virus then spreads to the rest of the reticuloendothelial system and respiratory tract through the blood (primary viraemia). Giant cells containing inclusion bodies (Warthin–Finkeldy cells) are formed in lymphoid tissues and also on the epithelial surfaces of the trachea and bronchi.

About five days after the initial infection the virus overflows from the compartments in which it has previously been replicating, to infect the skin and viscera, kidney and bladder (secondary viraemia). Giant cells are formed in infected tissues, which are also characterized by lymphoid hyperplasia and inflammatory mononuclear cell infiltrates.

After 10–11 days incubation the patient enters the prodromal phase, which lasts from two to four days. The initial symptoms consist of fever, malaise, sneezing, rhinitis, congestion, conjunctivitis and cough, which increase over the next days and are quite troublesome. A transitory rash of urticarial or macular appearance can sometimes develop within the prodromal phase, but this disappears prior to the onset of the typical exanthem. At this time giant cells are present in the sputum, nasopharyngeal secretions and urinary sediment cells. Virus is present in blood and secretions, and the patient is highly infectious. During this period Koplik's spots, the

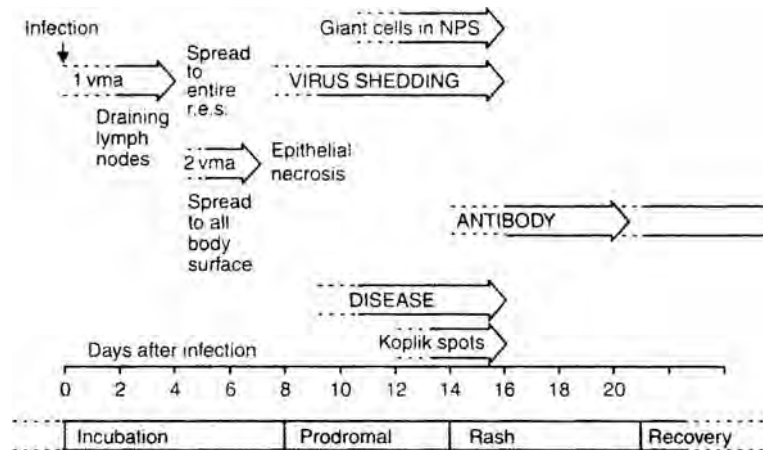


Figure 22.5 The course of clinical measles. The events occurring in the spread of the virus within the body are shown in the lower case lettering. As the virus spreads by primary and secondary viraemia (vma) from the lymph node to the entire reticuloendothelial system (r.e.s.) and finally to all body surfaces, epithelial cell necrosis occurs and disease. The characteristics of the disease are given in upper case lettering.

pathognomonic enanthem of measles, appear on the buccal and lower labial mucosa opposite the lower molars. These raised spots with white centres are characteristic of measles and begin to fade some two to four days after the onset of the prodromal phase as the rash develops. The distinctive rash appears about 14 days after exposure and starts behind the ears and on the forehead. From there the exanthem spreads within three days and involves the face, neck, trunk and upper and lower extremities. Once the entire body is covered, the rash fades on the third or fourth day and a brownish discoloration occurs, sometimes accompanied by a fine desquamation. Histologically, the rash is characterized by vascular congestion, oedema, epithelial necrosis and round cell infiltrates. Once the exanthem has reached its height, fever, conjunctivitis and respiratory symptoms subside. Antibody titres rise and virus shedding decreases from this point, and the patient shows a rapid improvement.

Continuation of clinical symptoms of the respiratory tract or fever suggests complications. In partially immunized children, such as infants with residual maternal antibodies, individuals who have received immune serum globulin or, occasionally, in the course of live vaccine failure, the disease follows a modified course. Then, the illness is generally mild and follows the regular sequence of events seen in acute measles yet with a very reduced symptomatology.

Atypical Measles

This condition is established after incomplete measles vaccination prior to exposure to wild-type MV. The

majority of reported cases received several doses of inactivated vaccine or a combination of inactivated vaccine followed by attenuated live vaccine. Because development of atypical measles was clearly linked to application of the formalin-inactivated vaccine, the latter was abandoned in the late 1960s. Since then, cases of atypical measles have not been reported and thus the disease has disappeared.

Atypical measles presented with sudden high fever, headache, abdominal pain and myalgia, often accompanied by a dry cough and a pleuritic chest. The rash spread, atypically, from the distal, often oedematous extremities, later became purpuric, vesicular or urticarial and involved the palms and soles with a prominent appearance on the wrists and ankles. The majority of cases developed pneumonia with a lobular or segmental appearance accompanied by pleural effusion resulting in respiratory distress with dyspnoea. Recovery from the pulmonary symptomatology was slow, and pulmonary lesions could sometimes be seen by X-ray months after onset of the disease. Marked hepatosplenomegaly, hyperaesthesia, numbness or paraesthesia were occasionally found. The pathogenesis of this disease is still not fully understood. Initially, production of inefficient F protein-specific antibodies unable to block viral spread by cell to cell fusion, possibly because the inactivation procedure changed the immunogenicity of the protein, was considered to be important. This hypothesis was largely dismissed based on findings in monkeys which, upon challenge with wild-type MV, produced F-specific antibodies with all biological properties even though they had been vaccinated with the killed vaccine. These animals developed a strong anamnestic humoral antibody response, although the antibodies were

preferentially of low avidity, complement-fixing and non-protective. Deposition of immune complexes in the lung and a pronounced eosinophilia was also noted (Polack *et al.*, 2003).

Complications of Measles Infection

Complications of acute measles are relatively rare, and result mainly from opportunistic secondary infection of necrotic surfaces such as those in the respiratory tract. Bacteria and other viruses can invade to cause pneumonia or other complications such as otitis media and bronchitis. The most severe complications caused directly by MV are giant cell pneumonia and measles inclusion body encephalitis (MIBE), which occur in the immunocompromised patient, and acute measles encephalitis (AMPE) and SSPE with no precipitating factors known. Other unusual manifestations that may complicate acute measles are myocarditis, pericarditis, hepatitis, appendicitis, mesenteric lymphadenitis and ileocolitis. If measles infection occurs during pregnancy spontaneous abortions, congenital malformations, stillbirth and low birth weight may occur.

Complications in the Immunocompromised

In immunocompromised patients, MV itself may lead directly to a life-threatening pneumonia (giant cell (Hecht) pneumonia) characterized by the formation of giant cells, squamous metaplasia of the bronchiolar epithelia and alveolar lung cell proliferation. Measles infection in such patients is thus a serious threat and is usually severe with a protracted course and frequently fatal. The same applies to MIBE which is, for instance, seen in children with leukaemia undergoing axial radiation therapy. The incubation period ranges from a few weeks to six months, and the patients often present without rash since generation of the latter requires an antiviral immune response. Clinically, the condition resembles and is therefore frequently confused with SSPE and commences with convulsions, mainly myoclonic jerks which are often focal and localized to one site. Other findings include hemiplegia, coma or stupor depending on the localization of the infectious disease process within the CNS. The disease progresses much faster than SSPE and proceeds to death within weeks or a few months. Furthermore, no or only low titres of measles antibodies are detectable in the cerebrospinal fluid (CSF). This kind of infection is probably best regarded as an opportunistic MV infection.

Complications in the Immunocompetent: Acute Measles Post-infectious Encephalitis

Acute encephalitis during measles is a severe complication that occurs at a frequency of about 1 per 1000 of

5000 cases. Although the figures vary among the available reports, fatality can be as high as 15%, and 20–40% of those who recover are left with lasting neurological sequelae. Encephalitis usually develops when exanthem is still present, but it can occasionally also occur during the prodromal stage. The encephalitis is characterized by resurgence of fever, headache, seizures, cerebellar ataxia and coma. In common with post-infectious encephalitis induced by other viruses, demyelination, perivascular cuffing, gliosis and the appearance of fat-laden macrophages near the blood vessel walls can be seen on histology. Petechial haemorrhages may be present and in some cases inclusion bodies have been observed in brain cells. CSF findings in measles encephalitis consist usually of mild pleocytosis and absence of measles antibodies. Long-term sequelae include selective brain damage with retardation, recurrent convulsive seizures, hemi- and paraplegia.

Subacute Sclerosing Panencephalitis

Subacute sclerosing panencephalitis (SSPE) is a rare, fatal, slowly progressing degenerative disease of the brain. It is generally seen in children and young adults and follows measles after an interval of six to eight years, although SSPE cases have also occurred up to 20–30 years after primary infection. Boys are more likely to develop SSPE than girls, and, according to recent reports, the overall incidence is 1 case per 10⁴ cases of acute measles (Bellini *et al.*, 2005; Takasu *et al.*, 2003). No unusual features of the acute disease have ever been demonstrated and other factors are presumed important. Predisposing factors remain unknown, yet immaturity of the immune system upon primary infection may contribute since half of SSPE patients have contracted measles before the age of two years, which is a remarkably high figure considering the proportion of actual measles cases in these young children. Genetic factors may also play a role as indicated by high incidence of SSPE in Papua New Guinea (Takasu *et al.*, 2003) and recent reports on the occurrence of the disease in siblings (Tuxhorn, 2004; Vieker *et al.*, 2003).

The course of SSPE varies considerably, but usually starts with a generalized intellectual deterioration or psychological disturbance which may not be recognized as illness until more definite signs appear. These are neurological or motor dysfunctions and may take the form of dyspraxia, generalized convulsions, aphasia, visual disturbances or mild, repetitive simultaneous myoclonic jerks. In 75% of cases the invasion of the retina by the virus leads to a chorioretinitis, often affecting the macular area followed by blindness. Finally the disease proceeds to progressive cerebral degeneration leading to coma and death. The progression of the disease is variable, remissions are

common and some stages may overlap. The illness inevitably leads to death within one to three years, though this can also occur in a matter of months.

Neuropathological investigations reveal a diffuse encephalitis affecting both the white and grey matter, characterized by perivascular cuffing and diffuse lymphocytic infiltration. Glial cells may proliferate, and fibrous astrocytes, neurons and oligodendroglial cells contain intranuclear inclusion bodies which may occupy the entire nucleus. These, also referred to as Cowdry bodies, contain MV nucleocapsid structures. Giant cell formation or membrane changes consistent with virus maturation have not been observed. The neuropathological lesions lead to characteristic EEG changes consisting of periodic high-amplitude slow wave complexes which are synchronous with myoclonic jerks recurring at 3.5- to 20-second intervals. These periodic complexes (Radermecker) are remarkably stereotyped in that their form remains identical in any given lead. They are bilateral, usually synchronous and symmetrical. Moreover, they usually consist of two or more delta waves and are biphasic or polyphasic in appearance. The pathophysiology of this abnormal EEG pattern is as yet poorly understood, but most investigators regard these complexes in SSPE as characteristic and even pathognomic. It is noteworthy that this EEG pattern is variable within the course of the disease and from one patient to another. Moreover, these typical complexes may disappear as the disease progresses.

Another important and pathognomic finding is the state of hyperimmunity against MV as well as the prominent gammaglobulin increase in CSF. High measles antibody titres except against the M protein are present both in serum and CSF. The isotypes of MV-specific antibodies found in CSF include IgG, IgA and IgD, and in this compartment the immune response is of restricted heterogeneity. Isoelectric focusing experiments have indicated that it is oligoclonally restricted. This is thought to be due to a restricted number of antibody-secreting plasma cells that have migrated into the CNS and synthesize their antibodies there.

Osteitis Deformans and Otosclerosis

Viral-like nuclear and cytoplasmic inclusions that react with antibodies against paramyxoviruses, including MV, have been detected in multinucleated osteoclasts, in osteoblasts, osteocytes, fibroblasts and lymphomonocytes of patients with Paget's disease (Basle *et al.*, 1986). The mechanisms responsible for viral persistence are still unclear, particularly as the disease persists for many years and remains highly localized with new lesions rarely, if ever, developing in previously unaffected bones. One report describes successful amplification of MV N-specific transcripts from mononuclear cells and osteoclast-like

multinucleated cells formed in long-term bone marrow cultures, osteoclast precursors and PBMCs of patients but not of controls (Reddy *et al.*, 1996). Ultrastructural and immunohistochemical studies suggested the presence of MV-like structures and MV antigens and, by reverse transcriptase polymerase chain reaction (RT-PCR), MV N-specific transcripts in bone material from patients with otosclerosis, a disease that shares clinical and histopathological findings with Paget's disease (Arnold *et al.*, 1996). The aetiology of both Paget's disease and otosclerosis is largely unknown, and it is unclear whether detection of MV in bone tissue is of pathogenic significance or just an epiphenomenon.

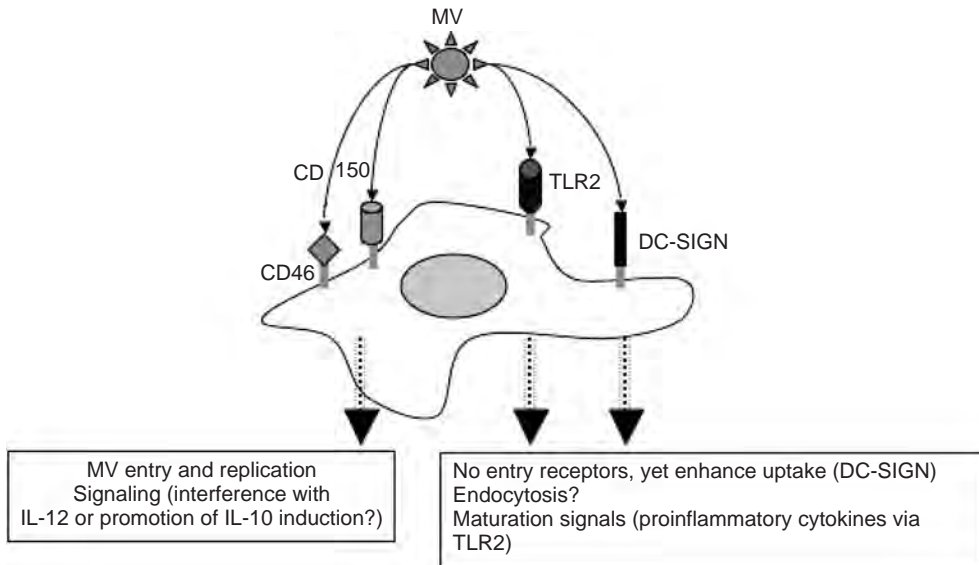
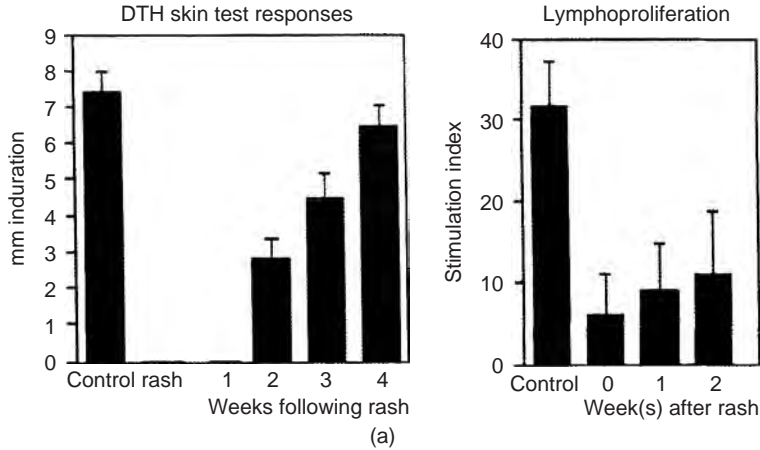
THE PATHOGENESIS OF MEASLES AND ITS COMPLICATIONS

MV is a lymphotropic virus, and its interaction with the immune system is itself responsible for some of the key features of the disease. A delayed hypersensitivity reaction is implicated in the production of the rash, and could also be involved in the tissue damage observed in AMPE. Furthermore, its interaction with the immune system may be responsible for the modification of the disease process observed in SSPE.

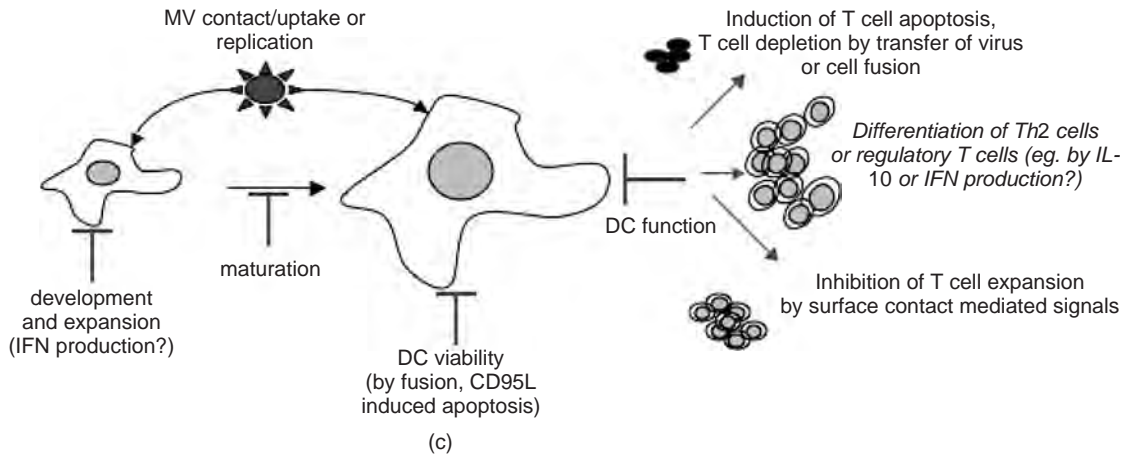
MV pathogenesis is not easily assessed in animal models. As yet, only primates have been found permissive for MV following intranasal infection and develop clinical measles, whereas attempts to induce measles-like disease processes in small animals by this route have largely failed. Cotton rats reveal a certain permissivity to infection by the intranasal route. In addition, CNS infections can be experimentally induced by intracerebral MV infection in mice and rats and these have led to a better understanding of both virological and immunological parameters of MV-induced CNS diseases.

Acute Measles

Characteristic features are a pronounced lymphopenia and a defect in cell-mediated immunity (CMI), as demonstrated in tuberculin-positive individuals who become tuberculin negative. These effects result from virus interactions with cells of the lymphoid system. Following uptake, MV exhibits a pronounced lymphotropism, and replication is detected in the draining lymph nodes rather than at the site of entry. *In vitro*, replication of MV requires mitogen activation of lymphocytes, whereas the ability of monocytes to support productive replication is limited. *In vivo*, the virus remains mainly cell associated and can be isolated from lymphocytes in the early stages of infection, and this is greatly assisted if the cells are



(b)



mitogenically stimulated. Only a minor proportion of the patient's PBMCs are infected, and this includes B and T cells and monocytes. Particularly in late stages of the infection, MV is almost exclusively found in monocytes.

Following extensive replication in the lymphoid tissue, virus is spread through a secondary viraemia, and replication continues in the epithelia of the lung and buccal cavity. The epithelia of the respiratory tract and conjunctiva are relatively thin, with about one or two cell layers. These soon begin to break down, and inflammatory reactions lead to the symptoms which mark the onset of the prodromal phase –runny nose, conjunctivitis, malaise and fever. The thicker mucosal surfaces of the buccal cavity are then affected and Koplik's spots appear about 11 days after infection. The appearance of these spots marks the commencement of a delayed-type hypersensitivity reaction (DTH) similar to that which gives rise to the rash. The spots fade some three days after their appearance as the rash itself develops.

Unlike other sites of replication, virus antigen is absent from skin lesions themselves, but rather concentrated near blood vessels and in the endothelial cells of the dermal capillaries. The rash is characterized by vascular congestion, oedema, epithelial necrosis and round cell infiltration, but giant cells are absent. Virus replication does not break through the skin and virus is not shed from this surface. The containment of infection in the skin is thought to be due to the development of cytotoxic T cells which destroy infected tissue and to IFN production which acts to promote cellular resistance to infection. The rash itself results from accumulated damage to the vascular walls caused by this DTH, and is thus mostly not observed in the immunosuppressed. As a typical self-limiting infection, acute measles is controlled and lastly resolved by an efficient cellular immune response. The latter is highlighted by an early activation of a Th1-response which later shifts into a Th2-predominated profile. In addition, the importance of CD8⁺ T cells in controlling the acute infection has been directly demonstrated in experimentally infected rhesus monkeys (Permar *et al.*, 2003a). In support of the importance of T-cell responses in controlling

acute measles, patients with underlying T-cell deficiencies do not develop rash and develop severe complications, while those with agammaglobulinaemia handle measles normally and recover. Antibody titres are usually rising at this stage of the illness, yet are not thought to be the major factor in promoting recovery. MV-infected cells are lysed inefficiently by the classic pathway of the complement activation, although more so by the alternative pathway.

MV-induced Immunosuppression

At the same time as it triggers an efficient virus-specific immune response in the immunocompetent host leading to viral clearance and establishment of a lifelong immunity, measles infection causes a general suppression of responsiveness to other pathogens. This was recognized long before the virus was isolated and almost exclusively accounts for the high morbidity and mortality rates still associated with acute measles. Typically, the patients reveal a marked lymphopenia affecting both B and T cells and are highly susceptible to opportunistic infections. Mechanisms underlying lymphopenia are not entirely clear. Experimental studies suggest that MV may target CD34⁺ stem cells and/or induce thymocyte apoptosis (Manchester *et al.*, 2002), yet when directly analysed in patients, thymic output of lymphocytes was unaffected (Permar *et al.*, 2003b). Possibly, lymphocytes are lost due to viral infection which may initially proceed quite extensively. In addition, it has been proposed that activated T cells expressing high levels of lymphocyte function-associated antigen 1 (LFA-1) are preferentially lost from the peripheral blood due to aberrant homing to tissues. Immunosuppression, however, is still observed up to weeks after the onset of the rash when the lymphocyte counts have returned to normal and MV-infected cells are present with only low frequency or are no longer detectable.

Key features of MV-induced immunosuppression are inhibition of delayed-type hypersensitivity responses and a restricted ability of lymphocytes to proliferate in response to recall antigens, or allogenic and mitogenic stimulation (Figure 22.6a). As only a few infected cells are

Figure 22.6 (a) In the course of and following measles, both delayed-type hypersensitivity reactions (DTH) as measured by tuberculin test and *in vitro* proliferative responses of lymphocytes to mitogen stimulation are suppressed. (Source: Data from Tamashiro *et al.*, 1987 and Hirsch *et al.*, 1984.) (b) Schematic representation of surface receptors on dendritic cells (DCs) interacting with MV. CD46 and CD150 support uptake with subsequent replication of predominantly attenuated (CD46) or all (CD150) MV strains. Uptake can be enhanced by DC-SIGN, a pattern recognition receptor usually endocytosing its ligands. In addition, the ligation of CD46, CD150 and TLR2 can induce or modulate signalling pathways involved in cytokine production. (c) A schematic representation of current models to explain MV-induced immunosuppression at the level of interference of DCs. Inhibitory mechanisms supported by experimentation (such as block of development, maturation and viability as well as functional impairments leading to T-cell loss by apoptosis and/or infection and inhibitory signals provided by surface contact) are shown in standard, those suggested, but not yet proven, in italic letters.

usually detected, several hypotheses have been put forward to explain this finding, and these involve interference of MV with differentiation, function and viability of professional APCs, particularly DCs, which then promote depletion, aberrant differentiation or anergy of contacting T cells (Abt *et al.*, 2006; Schneider-Schaulies and Dittmer, 2006). Though infection of these cells *in vivo* has not yet been verified in humans, MV can interact with DCs *in vitro* independently (via pattern recognition receptors (PRRs) such as TLRs or DC-SIGN) or dependent of infection (because these cells express CD150 and CD46 *in vitro*) (Figure 22.6b). Interaction with PRRs expressed on the surface or internal membranes or in the cytoplasm modulates both the maturation of APCs and their cytokine profile and this is linked to upregulation of major histocompatibility complex (MHC) and costimulatory molecules (Bieback *et al.*, 2002; De Witte *et al.*, 2006), but also promote a release of cytokines which favour differentiation of Th2 cells (Atabani *et al.*, 2001; Fugier-Vivier *et al.*, 1997; Karp *et al.*, 1996). It is currently unclear as to whether interaction with DC-SIGN does affect DC maturation directly or promotes internalization of MV by an endocytic route, however, this molecule clearly enhances MV uptake and thereby subsequent viral replication in these cells (De Witte *et al.*, 2006). *In vitro* infection of DCs has been linked to apoptosis of these and/or depletion of contacting T cells by viral transmission or apoptotic signalling (Servet-Delprat *et al.*, 2003). Independent of T-cell depletion, MV-infected DCs were found to inhibit rather than to promote expansion of co-cultures allogenic T cells, and this has been related to the accumulation of the viral glycoprotein complex on the DC surface membrane (Dubois *et al.*, 2001; Klagge *et al.*, 2000; Schnorr *et al.*, 1997) (Figure 22.6c). *In vitro* and *in vivo* experimentation revealed that signalling by the MV glycoprotein complex interferes with the activation of intracellular signalling cascades in uninfected T cells in a contact-dependent manner (Niewiesk *et al.*, 1997; Schlender *et al.*, 1996; Schneider-Schaulies *et al.*, 2003). As a consequence, these cells arrest in the late G₁ phase of the cell cycle. Thus, MV-infected DCs most likely confer a negative rather than a positive signal to lymphocytes in the T-cell areas of the lymph node and this could play a central role in the induction of a widespread immune suppression. It appears particularly important in this setting that wild-type MV strains and recombinant MVs expressing the wild-type MV H protein reveal a pronounced tropism for secondary lymphatic tissues and a higher immunosuppressive activity (Ohgimoto *et al.*, 2001).

The role of the MV receptors in immunosuppression is not yet understood. The ability of certain MV strains

(either by surface contact of infection) to downregulate CD46 from the cell surface enhances susceptibility to complement-mediated lysis of the affected cells, and this might limit viral spread by cellular depletion. As this property is mainly confined to laboratory-adapted strains, this may represent an attenuation marker rather than contributing to immunosuppression. Consequences of MV-mediated downregulation of CD150 have not been established as yet. This molecule is predominantly expressed on activated lymphocytes, suggesting that these might be efficiently eliminated by MV infection. Both CD46 and CD150 have also signalling properties and thus MV interaction with either of them could probably alter cellular signalling independent of infection. In T cells, however, antibody ligation of either of these molecules was found to provide costimulatory rather than inhibitory signals. It is, however, essentially clear that induction of T-cell unresponsiveness by surface interaction with the MV glycoprotein complex does not require CD46 or CD150.

Acute Measles Post-infectious Encephalitis

It is likely that CNS involvement, even in uncomplicated measles, is common. Transient abnormality of the EEG is detected in about 50% of patients, headache is common and CSF pleocytosis is also observed. The question of whether and how MV targets the CNS in the course of the acute infection is still unknown. It is only exceptionally that MV can be isolated from the brain tissue of AMPE patients, while in the majority of cases MV antigen or RNA are not detectable in the CNS. Therefore, virus-induced autoimmune reaction leading for myelin breakdown is considered as most likely in the pathogenesis of AMPE, and in support of this, myelin basic protein (MBP) as well as T cells expanding in response to this protein *ex vivo* have been found in the CSF of patients. Such MBP-specific lymphoproliferative responses have also been seen for post-infectious encephalomyelitis caused by other viruses such as rubella, varicella or after rabies immunization (Johnson and Griffin, 1986). The latter disorder is probably the human equivalent of experimental allergic encephalitis (EAE) since such patients received rabies vaccine prepared in brain tissue. Since AMPE is characterized by demyelinating lesions in association with blood vessels as in EAE it is not surprising that an MBP-specific lymphoproliferative response is considered to be of pathogenetic importance. How MV leads to a T-cell-mediated autoimmune response is unknown, however, molecular mimicry or a deregulation of autoreactive cells occurring secondary to viral infections may be involved.

Measles Inclusion Body Encephalitis

This condition arises in patients with underlying immunodeficiencies, and is thus very rare and usually not accompanied by intrathecal antibody synthesis. The unprotected cells develop massive unclear and cytoplasmic inclusion bodies consisting of virus nucleocapsids. Infectious virus has not been isolated by conventional methods from brain tissue, suggesting defects in replication. This assumption has been supported by immunohistological and molecular biological studies on brain tissue of a case of MIBE. All viral mRNA species were detectable in brain-derived total RNA samples although the mRNAs for the envelope proteins were highly underrepresented. Both *in vivo* and *in vitro*, viral mRNAs directed synthesis of detectable amounts of N and P protein, yet not of the envelope proteins, indicating these are specifically restricted in MIBE (Baczko *et al.*, 1988). Sequences analyses revealed a high rate of mutations distributed over the entire MV genome, and within M gene, affected the initiation codon which explains why this protein is not expressed in infected MIBE brain tissue (Cattaneo *et al.*, 1987). Defects in MV mRNA transcription and envelope protein synthesis apparently do not largely affect the activity of the RNP complex, which spreads to different areas of the patient's brain. Because production of infectious virus and giant cell formation may never occur, due to the restriction of the envelope proteins, RNP spread is thought to occur by microfusion events.

Subacute Sclerosing Panencephalitis

MV was first implicated in the aetiology of SSPE by immunofluorescence in 1967 and this has since been confirmed by electron microscopy, immunoelectron microscopy (IEM), and finally upon rescue of virus by co-cultivation. Despite this, the manner in which the persistent infection is established in the brain and exactly how this leads to the production of disease are still ill-defined. The virus may enter the CNS during viraemia in acute measles, but once there, replication proceeds slowly and a widespread encephalitis is not established. It is also not known to what extent virus replication *per se* is responsible for the development of lesions, nor what part is played by the immune system.

Virological Aspects

MV replication is usually associated with giant cell formation and release of infectious progeny. In SSPE, free infectious virus has never been isolated either from the brain or from CSF, and histopathological examinations have consistently failed to reveal the morphological changes associated with virus maturation (Schneider-Schaulies and ter

Meulen, 1992). As with MIBE, giant cells and thickening of the plasma membrane indicative of budding sites are absent, indicating aberrant trafficking or absence of viral envelope proteins. Viral nucleocapsids present in the cytoplasm are randomly scattered and show no sign of regular alignment beneath the plasma membrane. Thus, the infection may spread slowly strictly in a cell-associated manner and not involve particle formation. As opposed to the polyclonal response in serum, MV-specific antibodies in the CSF are oligoclonal, suggesting their local production by plasma cells which have invaded this compartment in response to antigens (Dörries *et al.*, 1988). It is only MV-specific antibody titres that are tremendously elevated, while titres against other viruses remain normal. Antibodies specific to all MV proteins are present in serum and CSF, except for M protein which is barely (by serum antibodies) or not recognized (by CSF antibodies).

As pointed out for MIBE, only N and P proteins are consistently detectable in SSPE brain sections by immunohistochemistry, and extensive transcriptional and translational alterations affecting mainly envelope protein-specific genes are observed (ter Meulen and Billeter, 1995; Schneider-Schaulies *et al.*, 2005) which highly impair the synthesis of the corresponding gene products *in vitro* (Figure 22.7). A high rate of mutations all over the MV genome is found, although different genes are affected at different levels. The highest number of alterations accumulate in the M gene, followed by F, H, P and N genes, which are mutated to about the same extent, whereas the L gene is most conserved. Mutations introduced are either point mutations most probably accumulating due to the infidelity of the viral polymerase, or appear as clustered transitions which are thought to result from the activity of a cellular enzyme complex, now referred to as ADAR (adenosine deaminase, RNA-specific). As a result, translation from viral mRNAs is abolished or leads to the synthesis of truncated or unstable MV proteins. These molecular biological data explain the absence of infectious MV particles and the lack of budding and cell fusion processes in SSPE brain tissue. Yet virus has occasionally been rescued from brain tissue obtained post mortem by co-cultivation (Wechsler and Cody Meissner, 1982), and these so-called SSPE isolates can remain cell-associated, spreading through the culture with a gradually enlarging area of CPE or promote particle formation. Sequence analyses revealed that some SSPE isolates are likely contaminations and thereby rather ordinary laboratory MV strains. With the others, it is still unclear whether they represent a small subpopulation of replication/maturation competent viruses or revertants that were selected by isolation and may thus not be representative of the dominant virus population in the infected brain.

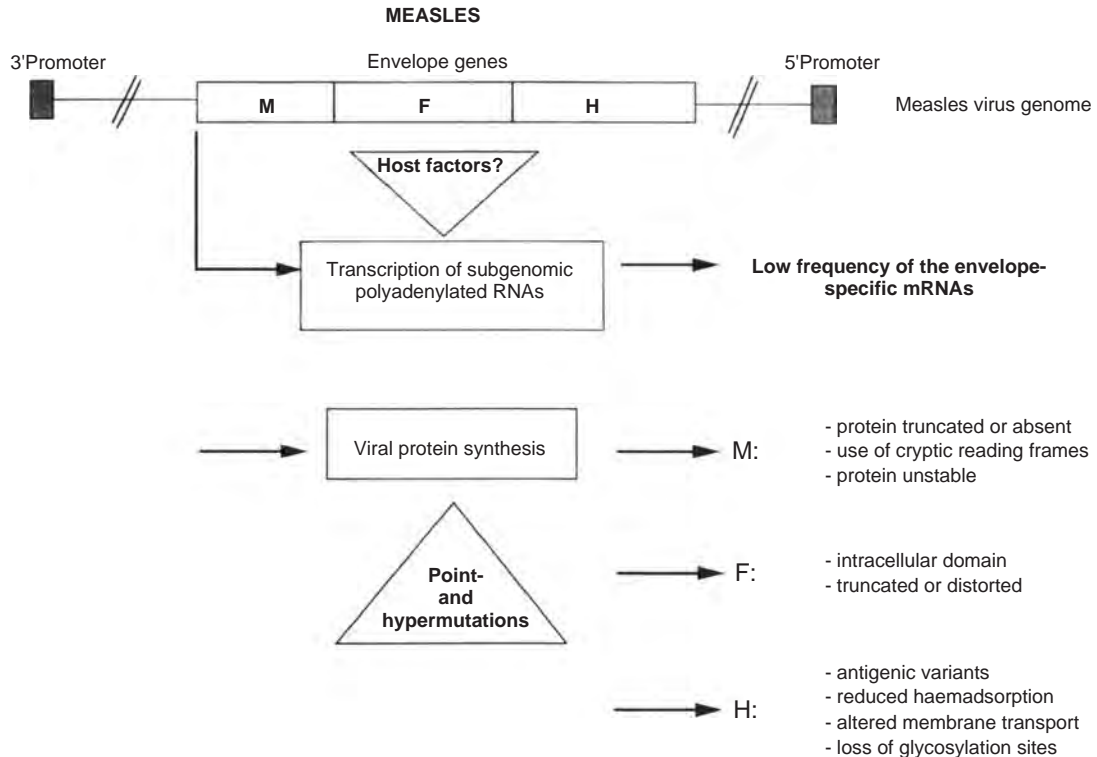


Figure 22.7 Schematic representation of restrictions of MV gene expression found in SSPE brain tissue. MV mRNAs encoding the viral envelope proteins are expressed at low frequency (most probably due to host cell control mechanisms) and, in addition, harbour mutations that prevent the synthesis of functional translation products.

Factors involved in the establishment of persistence by a non-defective MV in brain tissue are ill-defined. In neural tissue culture cells or brain material of experimentally infected animals, intracellular factors can intimately control the efficiency of MV replication and this may be more pronounced with differentiation of the host cells and age of the animals (Schneider-Schaulies *et al.*, 1990). This particularly applied to attenuation of viral transcription and translational control exerted predominantly on MV-specific rather than host cell mRNAs. In addition, ADAR activity actively modifying the primary sequence of viral RNAs has been demonstrated in these cells. Thus, intracellular factors present in brain cells possibly slow down viral replication after primary infection, thereby preventing rapid host cell destruction. Whether these mechanisms efficiently control the maintenance of the persistent infection or other factors, such as mutations within the viral genome, are required has not been resolved.

Immunological Aspects

As for virological factors, immunological features associated with the establishment of SSPE are at best vaguely

understood. Transient immunosuppression and immaturity of the immune system on primary exposure are thought to contribute, yet this has not yet been attributed to specific lymphocyte compartments (Oldstone *et al.*, 2005). In experimentally infected mice, primary MV CNS infection is controlled by CD4, CD8 and B cells in conjunction, rather than by any single subpopulation (Tishon *et al.*, 2006), yet it remains unclear whether defective responses of either of those would favour persistence, as it is unknown if, when and to what extent MV enters the brain during or after acute measles. Nevertheless, given the importance of CMI in the control of MV infection, considerable interest has focused on this response in SSPE patients, which, however, generally revealed a normal immune competence. T cells are present in normal amounts, lymphoproliferation and interleukin synthesis in response to antigenic stimulation are normal and skin grafts are efficiently rejected. The response to MV antigens can be impaired since anamnestic skin tests for these are often negative. Depending on the test system and on the potency and purity of the virus antigens used, a minor inhibition or normal reaction of CMI in comparison to controls can be observed

in SSPE patients. Once the disease becomes clinically visible, the humoral hyperimmune response to MV antigens in serum and CSF is the hallmark of and diagnostic for SSPE. Though this also involves neutralizing antibodies, these are unable to control the infection, and hence, have been proposed to support rather than eliminate persistence. MV-specific antibodies act to cross-link viral proteins on the cell surface which subsequently aggregate to form 'cap structures', also containing M protein, which are internalized from the cell surface. Since the concentration of complement components in the brain is low, complement-mediated lysis there is of low efficiency, and thus, the major effect of antibodies would be to promote clearance of antigen from brain cell surfaces. There is, however, also evidence that the antibody-induced capping process directly regulates viral protein synthesis. Thus, influenza haemagglutinin-specific antibodies can exert an inhibitory effect on the viral polymerase activity. Similar observations were made in cell lines persistently infected with MV or in MV-infected rats where passive transfer of neutralizing H protein-specific monoclonal antibodies prevented acute and promoted the development of a subacute MV encephalitis (Liebert *et al.*, 1990). Thus, the host immune response possibly supports establishment and/or maintenance of SSPE.

DIAGNOSIS

The symptoms of acute measles are so distinctive that a virus laboratory has been rarely called upon for diagnosis. As the vaccination programme takes effect, physicians in areas of high vaccine coverage may, however, become less familiar with the disease. Furthermore, as more patients are placed on immunosuppressive regimens, the need for diagnosis of measles may well increase.

Microscopy

Production of multinucleate giant cells with inclusion bodies is pathognomonic for measles during the prodromal phase. Such cells are detectable in the nasopharyngeal secretions. Clear identification of giant cells and detection of Cowdry inclusion bodies is facilitated if the smear is formalin-fixed prior to haematoxylin and eosin staining. Direct and indirect immunofluorescence have been widely used to stain cells shed in nasal secretions, although it may be necessary to remove antibodies which already coat virus antigens with a low pH buffer. Stained cells include macrophages and ciliated cells as well as giant cells. Urinary sediment cells can be successfully used as well, as immunofluorescence-positive cells are shed in the urine from two days before to up to five days after the appearance of the rash, which makes this specimen superior

to nasopharyngeal secretion in the later stages. Such cells may also be present in the urine 4–16 days after vaccination with the live vaccine. Immunofluorescence is useful for the diagnosis of measles in the pre-eruptive phase or in children vaccinated with killed vaccine, where rash development is atypical. Immunoperoxidase histochemical stains are also used, and the use of monoclonal antibodies has improved sensitivity and reliability of virus detection.

Serological Methods

In common with other infections, diagnosis of measles is made if antibody titres rise by more than four-fold between the acute and the convalescent phases or if measles-specific IgM is found. Traditional tests such as HAI, plaque reduction neutralization (PRN), haemolysin inhibition (HLI), complement fixation (CF) and enzyme-linked immunoassay (EIA) have been extensively used for serological diagnosis of measles. Because sensitive and specific commercial kits are available, EIA has become the most widely used test format. These tests also have the ability to measure measles-specific IgM as well as IgG responses and therefore have particular importance in diagnosis as well as measles control programmes. The recommended laboratory method for the confirmation of clinically diagnosed measles is a serum-based IgM EIA, and several commercially available serum-based capture and indirect IgM EIAs are used worldwide. In the case of SSPE, it is important that CSF is also tested.

Virus Isolation and Detection of Viral RNA

Virus isolation and RT-PCR-based detection of viral RNA are usually limited to specific instances such as suspected infection of the immunosuppressed in the absence of rash, developing pneumonia or unexplained encephalitis. Finally, these techniques can be attempted as a means of retrospective diagnosis.

The lack of sensitive cell lines for efficient growth of MV made cell culture isolation difficult, even when optimal specimens for virus isolation were available. Previous tissue culture cell lines, such as primary monkey kidney cells and Vero cells, were considered as permissive for measles infection. It is now recognized that these cells do not express the appropriate receptor(s) for most wild-type viruses, which explains the low frequency of isolation with these cell types. An Epstein–Barr virus-transformed marmoset B lymphocyte cell line (B95a), a human B lymphoblastoid (BJAB) and, more recently, an umbilical cord cell line proved to be up to 1000 times more sensitive than Vero cells and are currently used for MV isolation (Kobune *et al.*, 1990, 2007). Best success is attained when specimens (usually washing, swabs, Ficoll

gradient purified blood cells stimulated with mitogens or urinary sediments) are collected within five days of rash onset. Samples are mixed with buffered salt solution (pH 7.2) containing antibiotics before application onto tissue culture cells. CPEs develop usually between 48 hours and 15 days, and consist either of a broad syncytium or of a stellate form, both of which can reveal nuclear and cytoplasmic inclusion bodies. If CPE is slow or not clear, MV antigens or RNA can be detected by appropriate methods. RT-PCR analyses using MV N- and/or F-gene specific primers are the most commonly employed in a wide variety of specimens from which RNA is extracted and used as template, including serum, nasopharyngeal aspirates and urine sediment cells. Tissue samples including lung, kidney, skin and brain are also amenable for testing by RT-PCR following appropriate chaotropic salt treatment and nucleic acid extraction.

Diagnosis of SSPE

Formerly, brain biopsy was performed routinely for diagnosis of SSPE and tissue thus obtained was examined for inclusion bodies and virus antigen by immunofluorescence. After recognition of the characteristic intrathecal antibody synthesis, determination of measles antibody titres in the CSF may be sufficient with, if necessary, demonstration of MV-specific heterogeneity by isoelectric focusing in combination with an immunoblot technique (Dörries and ter Meulen, 1984). Virus isolation from SSPE brain tissue is complicated and often fails. As MV replication only occurs within brain cells and viral particles are not released, MV-specific sequences can generally not be amplified by RT-PCR in CSF samples.

MANAGEMENT

As an acute self-limiting disease, measles will run its course in the absence of complications without the need for specific intervention. Treatment is supportive and there are no proven effective antiviral compounds available. Infection of the undernourished, the immunocompromised or children suffering from chronic debilitating diseases is more serious. Such patients, children under one year of age and pregnant women after household contacts can be protected by the administration of human anti-measles gammaglobulin (0.25–0.5 ml kg⁻¹). If given within the first three days after exposure, it is usually effective, yet the effectiveness is partially or completely lost if the globulin is given four to six days, or later, respectively, after exposure.

Ribavirin has been used to treat pneumonitis and SSPE. Respiratory tract infections cause considerable damage

to the ciliated epithelium and this may lead to superimposed bacterial infections. For pneumonia, distinction between primary viral infections and the superimposed bacterial infection can sometimes be difficult, and thus, treatment with antibiotics is required. Treatment of acute post-infectious measles encephalitis (APME) is symptomatic and supportive, and does not differ from that for any other post-infectious encephalitis. Careful attention to fluid and electrolyte balance is essential. Seizure control requires anticonvulsive drugs. Application of steroids did not prove to be beneficial. No specific therapy is known for MIBE. A variety of approaches have been attempted for treatment of SSPE, but as yet there has been no convincing success and almost all cases have proved fatal. The effectiveness of treatment is extremely difficult to evaluate because the course of SSPE is highly variable and spontaneous remissions are common. In addition, being a rare disease, clinical trials are inevitably based on a very small number of patients. Isoprinosine (inosiplex) and IFN (also applied in combination) have been widely used. In the case of isoprinosine, it seems likely that treatment may lengthen survival time if given early in the disease. This effect becomes less pronounced if the drug is administered later.

As mentioned above, RNA interference-based approaches to downregulate synthesis of MV proteins essential for replication (such as the L protein) or development of peptide inhibitors targeting viral entry or, again, polymerase functions, are being exploited *in vitro*, and certainly will be used in suitable animal models once administration and targeting strategies have been established. Whether either of these approaches will make it to clinical application cannot be predicted at present, however, it is likely that application of those will probably be confined to situations which require specific intervention.

PREVENTION

The measles virus has no animal reservoir and is thus an obvious target for a controlled campaign aimed at eradication (Mitchell and Balfour, 1985). In the United States and Canada, where vaccination of all children is required at or before commencing school, startling results have been achieved. Case reports have fallen by over 99% but eradication has not yet been achieved. In other industrialized countries, where vaccination is not mandatory, Germany, Italy and the United Kingdom, distrust of vaccination has led to a lower acceptance rate and consequently, local epidemics continue to occur (e.g. 1000 cases in the Düsseldorf area in Germany in 2006). In developing countries, where the consequences of measles infection are most severe, considerable progress has been

made in controlling acute measles and reducing related mortality by the implementation of mass immunization campaigns.

Any vaccine used must be safe, as measles is not normally a severe illness, and cheap enough for mass administration. Its effectiveness should also be long lasting. Natural immunity is known to last for at least 65 years in the absence of re-exposure to the virus. In 1781 measles disappeared from the Faroe Islands following an epidemic, and was not reintroduced until 1846. Individuals old enough to have experienced the disease 65 years earlier were still protected. The unusual persistence of immunity even in the absence of boosting has suggested that MV may normally persist inside the body, and thus re-stimulates immunity from within.

Inactivated Vaccine

Inactivated vaccine was intended for use in young children less than one year of age, who are most prone to serious complications, to avoid the use of a live vaccine. With the formalin-killed virus preparation, at least three vaccinations were required to elicit a suitable antibody response, which, however, was comparatively low and soon waned. This left vaccinees open to virus attack, and their partial immunity led to serious hypersensitivity reactions to infection and a modified disease course (see section on Atypical measles). Therefore, this vaccine, administered in 1963–1967, was withdrawn from the market soon after notification of the first cases of atypical measles (Clements and Cutts, 1995).

Live Vaccine

The first attenuated vaccine strain was the Edmonston B strain produced by serial passage in human kidney cells, human amnion cells, chick chorioallantoic membrane and, finally, duck embryo cells. When administered intramuscularly or subcutaneously 12–18 months after the disappearance of maternal antibodies, this vaccine achieved seroconversion in 95% of recipients, but side effects of mild measles were common (5–10%). To oppose this, gammaglobulin was administered at doses appropriate to prevent side effects but not to promote vaccine failure. In 1966 a Medical Research Council (MRC) trial conducted in the United Kingdom followed 36 000 children. Measles incidence fell 84% in the first nine months, and decreased by 14% in the next two years. A follow-up study showed that the vaccine remained effective for at least 12 years.

The Edmonston B vaccine was further attenuated and thus rendered less reactogenic by passage in chick embryos at lowered temperature to yield the Schwartz and Moraten (Enders) strains. These indeed lowered the

incidence of post-vaccination febrile convulsions from 7.7 to 1.9 per 1000 recipients, and produced a 95% seroconversion rate, although antibody titres induced by the Schwartz strain declined more rapidly yet also remained at protective levels. Following passage in human diploid cells, a further attenuated strain, the Edmonston Zagreb strain, was obtained which produced higher seroconversion rates than the Schwartz vaccine. Similar observations were made with the AIK-C vaccine, produced in Japan. Titres of protective antibodies were seen to decline in isolated communities but were still detectable 14 years later. Children living in an open environment showed serological evidence of subclinical re-exposure which acted to boost their immunity. The measles vaccine is administered subcutaneously usually between the ages of 9 and 20 months for primary vaccination, either alone or in combination as measles, mumps and rubella (MMR) vaccine. According to recommendation by the American Academy of Pediatrics (AAPs) and the Immunization Practices Advisory Committee (ACIP) of the United States, in industrialized countries the first dose of measles vaccination should be administered at 15 months of age, as delayed primary measles vaccination (at 15 months of age or later) significantly reduces measles risk at later ages. Initial vaccination at 12 months of age is recommended for children living in high-risk areas (areas with a large inner-city urban population where more than five cases among preschool-aged children occurred during each of the last five years or with recent outbreaks among unvaccinated preschool-aged children). Of course, initial vaccination of infants 12–14 months of age is also recommended before travelling to areas in which measles is endemic or epidemic. A second dose is recommended with an interval of at least two months after primary immunization up to four to six years of age by ACIP that is expected to provide protection to most persons who do not respond to their initial vaccination.

Humoral immune responses, as defined by HAI, NT and ELISA, are good and protective as long as the vaccine is given after waning of the maternal antibodies. As with acute measles, the major isotype of MV-specific antibodies is IgG1. Levels of antibodies induced are generally lower than after measles and may decay more rapidly, but are still measurable in most individuals 15 years after immunization in the absence of boosting infections. Activation of CMI is generally thought to be similar to that of acute measles in that both MV-specific CD4 and CD8 T cells are stimulated, although cytotoxic T lymphocyte (CTL) responses after re-stimulation *in vitro* are considerably lower than after natural infection. As in acute measles, administration of live measles vaccine is associated with transient lymphopenia, loss of DTH skin test

responses to recall antigen and decreased *in vitro* proliferative responses to mitogens. Both mild leukopenia and atypical lymphocytosis have also been found after re-vaccination. In general, however, the immunosuppression observed after vaccination is marginal, and is usually not associated with complications. Reports that both measles (contracted early in childhood) and measles or MMR vaccine predispose towards the later development of intestinal bowel diseases –such as Crohn’s disease or ulcerative colitis –or autism have not been substantiated (Afzal *et al.*, 2006; Feeney *et al.*, 1997; Madsen *et al.*, 2002).

In spite of the effectiveness of the vaccine currently in use, which is at least equally effective when administered by aerosol rather than the subcutaneous route (De Swart *et al.*, 2006; Dilray *et al.*, 2007), new candidate measles vaccines are being explored in terms of efficacy (particularly in the presence of maternal antibodies) and other aspects, such as thermostability, standardization, production costs and application mode in animal models. Candidates being evaluated include expression of MV antigens in the context of live viral (like pox and adenovirus) or bacterial (like *Shigella flexneri* and *Salmonella typhimurium*) vectors, subunit or synthetic vaccines (like iscoms, biodegradable particles, lipopeptides) and nucleic acid vaccines (plasmid DNA). In addition, recombinant MV’s various heterologous viral antigens are being tested as vectors for multi-vaccine strategies, yet again so far only in animal studies.

Side Effects of Live Vaccination, Adverse Reactions, Precautions and Contraindications

The measles vaccine has an excellent safety record (National Vaccine Advisory Committee, 1991). While the vast majority of recipients remains asymptomatic, in rare instances a transient rash or low-grade fever, sometimes associated with moderate febrile seizures, is observed after 5–12 days in some vaccinees. Although children with a history of seizures are generally at increased risk for developing idiopathic epilepsy, febrile seizures following vaccinations do not increase the probability of subsequent epilepsy or neurological disorders. Most convulsions following measles vaccination are simple febrile seizures, and they affect children without known risk factors. Nevertheless, parents of children who have a personal or family history of seizures should be advised of the small increased risk of seizures following measles vaccination. CNS conditions such as encephalitis and encephalopathy have been reported with a frequency of less than 1 per million doses administered, an incidence which is lower than that of encephalitis of unknown aetiology. This finding suggests that the reported severe neurologic disorders temporally associated with measles vaccination were not caused by the vaccine.

Live measles vaccine should not be administered to women who are or are considering becoming pregnant within the next three months because of the theoretical risk of fetal infection. The decision to administer or delay vaccination because of a current febrile illness depends on the cause of the illness and the severity of symptoms. Hypersensitivity reactions following administration of live measles vaccine are rare and usually occur at the injection site. People with a history of anaphylactic reactions following egg ingestion should, however, be vaccinated with extreme caution, and individuals who have experienced anaphylactic reactions to neomycin should not be given the vaccine. Unlike with natural measles, exacerbation of tuberculosis has not been observed after measles vaccination.

Vaccination of Immunocompromised and HIV-infected Individuals

Replication of vaccine viruses can be enhanced in immunocompromised recipients, for example with leukaemia, lymphoma, generalized malignancy or therapy with alkylating agents, antimetabolites, radiation or large doses of corticosteroids. Thus, patients with such conditions or therapies (except for HIV infection) should not be given live measles vaccine. Short-term corticosteroid therapy does not contraindicate live measles vaccination. The large number of infants and preschoolers with HIV infection in certain countries has directed special attention to the appropriate immunization of these children. Asymptomatic children in need of measles live vaccination should receive it without prior evaluation of HIV infection state. Moreover, vaccination should be considered for all symptomatic HIV-infected children, including those with acquired immune deficiency syndrome (AIDS), since measles in these children can be severe. Limited data on measles vaccination among both asymptomatic and symptomatic HIV-infected children indicate that the vaccine has not been associated with severe or unusual adverse events (Moss *et al.*, 2002). Exposed symptomatic HIV-infected (as well as other immunocompromised) individuals should receive high doses of measles Ig regardless of their previous vaccination status.

Effectiveness of Vaccination in the Control of Measles

In industrialized countries that have achieved high levels of vaccine coverage, the interval between measles outbreaks increases and sufficiently high levels of vaccination can interrupt endemic transmission. With vaccination, the age distribution of cases is determined by which groups are likely to lack vaccine or measles-induced immunity.

In the pre-vaccine era, an estimated 4–5 million cases occurred annually in the United States and by the age of 15 years 95% of the population had seroconverted. Following the rigorous implementation of the MV vaccination programme, the case reports have fallen dramatically from 500 000 annually to 26 000 in 1978 and 1500 in 1983. From 1984 to 1988 only 3700 cases were registered. As a result of measles vaccination, the mortality and APME have declined and the available experience indicates that this also applies to SSPE. The United States experienced a dramatic increase in acute measles in 1989 and 1990 with 18 193 and 27 786 notified cases, respectively (Atkinson and Orenstein, 1992). The major causes for this resurgence of measles were an unappreciated low vaccine coverage among preschool-aged children, particularly in urban settings, and vaccine failure (waning immunity) in a low percentage of school-aged children who received only a single dose of vaccine. As a result of this measles epidemic and to address the issue of possible waning immunity, the AAP and the ACIP of the United States recommended a change from a one-dose to a two-dose schedule for measles vaccination.

The two-dose schedule is only one strategy used to ensure high vaccine coverage in the quest for measles elimination, particularly in developing countries where malnutrition, crowding and intensity of exposure aggravate measles infection and secondary infections are common and take severe courses in young infants (Aaby, 2007). Therefore, it is especially important to develop and implement vaccine programmes that are effective at conferring protection of the very young and at interrupting transmission to minimize the risk of exposure of the unprotected. As a result of the activity of the Measles Initiative launched in 2001, the incidence of measles-associated fatalities has been reduced by 60% worldwide between 1999 and 2005, and this has even surpassed the targets set by the UN in 2002 to reduce deaths due to measles by half within this period. This has been achieved by implementation of a four-part key strategy involving (i) provision of one dose of measles vaccine to all infants by 12 months of age via routine health services, (ii) a second opportunity for immunization for all children from 9 months to 15 years, generally through mass vaccination campaigns, to cope with primary and secondary vaccine failures, (iii) establishment of effective surveillance and (iv) improvement of clinical management of complicated cases, including vitamin A supplementation, which can effectively reduce measles morbidity. These measures increased vaccine coverage with the first routine dose from 71 to 77% globally between 1999 and 2005, and over 360 million children aged below 15 years received immunization through mass vaccination campaigns. Within this period, reduction of the estimated measles mortality

was most effective in the Western Pacific region (81%), followed by Africa (75%) and the Eastern Mediterranean Region (62%). Overall, more than 7.5 million deaths from measles were efficiently prevented.

Based on these encouraging figures, the Measles Initiative has set new targets to further reduce measles deaths primarily by ensuring that children receive a second dose of the vaccine shortly after the first, which will hopefully effectively meet the goal – elimination from the then 52 member states of the European Region by 2010 – set in 2005 by the WHO Regional Office for the European Region. In other regions, particularly in the 47 ‘priority countries’ that account for approximately 98% of global measles deaths, the four-part strategy will continue to be implemented and there, reduction of measles death by 90% by 2010 at a global level, and of the under-five mortality by two thirds by 2015 is envisaged. It is particularly in the 47 priority countries that national and district immunization plans are lacking, and poor management has led to high dropout rates. In addition, the highly infectious nature of the virus demands that governments build a long-term vision and plan since measles has repeatedly demonstrated its ability to quickly infect areas with low vaccination coverage.

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Rubella

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HISTORICAL INTRODUCTION

Rubella was known initially as 'German measles' because it was first described by two German physicians in the mid eighteenth century. It is generally a mild disease and therefore received comparatively little attention until 1941, when its association with congenital defects was recognized by N. McAlister Gregg, an Australian ophthalmologist (Gregg, 1941). Other studies in Australia and elsewhere confirmed these findings and suggested that a very high proportion of mothers who had had rubella during pregnancy delivered infants with congenital malformations (reviewed by Hanshaw *et al.*, 1985). Gregg, in a more extensive survey, reported that 122 of 128 (95%) children whose mothers had had rubella before the 16th week of gestation had congenital defects. However, these early retrospective studies, in which the starting point for investigations was an infant with congenital malformations should be interpreted with caution. Calculation of the true risk of rubella in pregnancy required that rubella in pregnant women should be confirmed serologically; more recent studies involving pregnant women with serologically proven rubella are discussed on pages 566–567.

Early work on the characteristics and transmission of rubella was carried out in human volunteers. Experimental studies using human volunteers showed that the incubation period of rubella ranged from 13 to 20 days and that subclinical infection occurred.

Rubella virus (RV) was not isolated in cell cultures until 1962, when two groups simultaneously published different methods. Parkman *et al.* (1962) inoculated primary

vervet monkey kidney (VMK) cultures with throat washings obtained when rash was present. Although no cytopathic effect (CPE) was observed, when cultures were challenged 7–14 days after inoculation with echovirus 11, the distinct CPE produced by this virus was not observed, suggesting the presence of an interfering agent. The interfering agent was neutralized by rabbit antiserum raised against one of the isolates. This interference assay was used extensively for diagnostic purposes and serological surveys. Weller and Neva (1962) used primary human amnion cultures to isolate the virus from blood and urine taken from typical cases of rubella. After specimens were passed once or twice in these cell cultures, a characteristic CPE with cytoplasmic inclusions was observed. These effects were neutralized by serum obtained from patients convalescent from rubella. The agents isolated in the different cell cultures by these two groups of workers were exchanged and found to be identical.

Studies conducted in different countries showed that the acquisition of rubella antibodies was related to age, social class and geographical location, and that approximately 80% of women of child-bearing age living in urban areas in western countries were immune. A haemagglutination inhibition (HAI) test was not developed until 1967, when it was shown that serum lipoproteins were inhibitors of HAI and must be removed from culture medium and test sera (Stewart *et al.*, 1967).

Clinical and virological investigations carried out during the extensive rubella epidemic in the United States during 1963–1964 led to a greater understanding of the pathogenesis of congenital rubella syndrome

Table 23.1 Key historical events—discovery of rubella and development of rubella control programmes

1881	Rubella accepted as a distinct disease by International Congress of Medicine
1941	Teratogenic effects of rubella first recognized by Gregg in Australia
1962	Rubella virus isolated in cell cultures. Neutralization test developed
1965–1967	Development of attenuated vaccine strains and first vaccine trials
1967	Rubella virus first visualized by electron microscopy
1969	HPV77.DE5 and Cendehill vaccine strains licensed in United States. Vaccination offered to all preschool children
1970	Cendehill vaccine strain licensed in Europe. Vaccination offered to 11- to 14-yr-old schoolgirls
1971–1972	RA27/3 vaccine strain licensed in Europe
1979	RA27/3 replaced other vaccine strains in United States
1986	Rubella genome sequenced
2000	WHO recommends immunization policies for elimination of CRS
2006	123 of 212 (58%) of countries/territories include rubella vaccination in national immunization programmes

(CRS), as well as a further appreciation of its clinical features and sequelae. During this epidemic about 30 000 rubella-damaged babies were delivered (Cooper, 1975). Studies at the time revealed that multisystem involvement was common and the range of abnormalities much wider than previously reported. When maternal rubella occurred in early pregnancy, a generalized infection developed in the fetus, which persisted during the remainder of gestation and into infancy, despite the presence of rubella antibodies. Many infected infants excreted virus and transmitted virus to susceptible contacts. The main historical events associated with rubella are summarized in Table 23.1 and reviewed by Best *et al.* (2005) and Banatvala and Peckham (2007).

THE VIRUS

Classification

Rubella virus is classified as a togavirus and is the only member of the genus *Rubivirus*. The genome structure and replication strategy of RV is similar to viruses of the *Alphavirus* genus of the *Togaviridae*, such as Semliki Forest and Sindbis (Frey, 1994). No antigenic relationship has been shown between RV and any alphavirus or flavivirus.

Structure of the Virus

Electron micrographs show that the RV virion is pleomorphic. The virus particle is about 70 nm in diameter, with a nucleocapsid containing the genomic RNA and a lipid envelope. The virion has two membrane-bound envelope glycoproteins and a nonglycosylated nucleocapsid protein: E1 (58 kDa), E2 (42–47 kDa) and C (32 kDa), respectively. The structure of the RV particle has not been determined, but the structures of alphaviruses, whose

envelope and nucleocapsid proteins are probably similarly organized in the virus particle, have been determined (Mukhopadhyay *et al.*, 2006). The E1 and E2 proteins of these viruses form heterodimers which arrange themselves into trimeric spikes (i.e. three copies each of E1 and E2 in each spike) in the membrane, above the icosahedral nucleocapsid. The main difference between the alphavirus and RV virion components is that the C proteins of RV retain the putative signal peptide of the E2 protein, and are thus bound to the virion membrane by their C-termini.

Genome Structure and Function

The RV genome comprises a positive-sense single-stranded RNA, which is capped at the 5' end and polyadenylated at the 3' end (Frey, 1994). The cap serves as a ribosome recognition site and is required for efficient translation. The RNA is infectious when extracted under appropriate conditions. The base composition of the genome is G 30.8, C 38.7, A 14.9 and U 15.4%. The G+C content of the RNA is very high, which makes some work difficult (e.g. reverse transcription polymerase chain reaction (RT-PCR)). There are some small variations between genomes of various RVs, but most are 9762 nucleotides (nt) in length, with a 40-nt 5' untranslated region, a 5' proximal 6351-nt open reading frame (ORF) coding for the nonstructural proteins (NSPs), including an RNA-dependent RNA polymerase, a 120-nt junction region, a 3' proximal 3192-nt ORF coding for the structural proteins and a 59-nt 3' untranslated region (Zhou *et al.*, 2007). The gene order is 5'-p150-p90-C-E2-E1-3' (Figure 23.1).

Genetic Variation

The level of diversity among RVs is low when compared to some other RNA viruses, such as human immunodeficiency virus (HIV) and poliovirus, and there is no

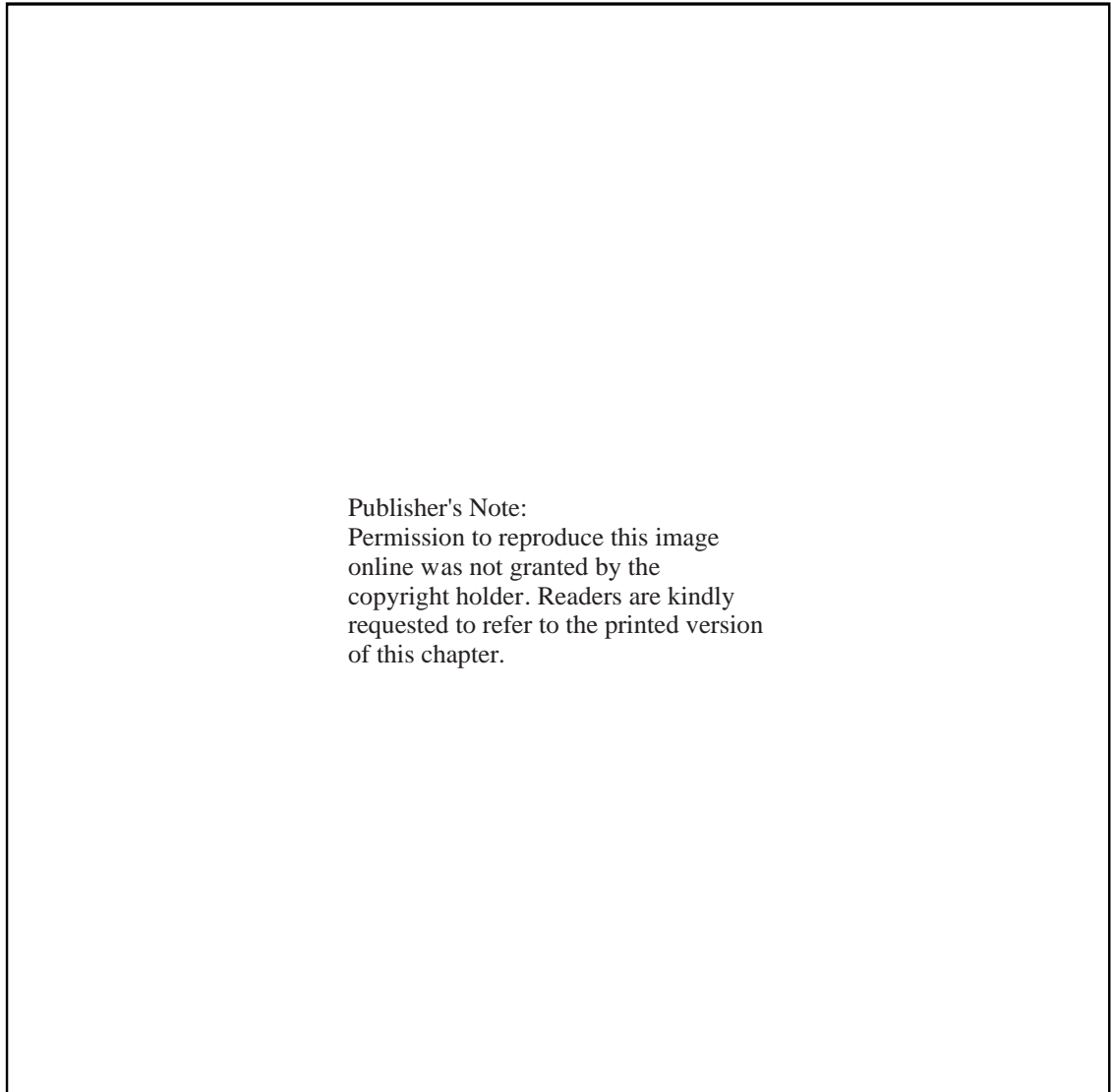


Figure 23.1 Schematic representation of the transcription and translation of rubella virus structural and non-structural proteins. (Source: Reproduced from Best, Cooray & Banatvala (2005) by permission of Edward Arnold (Publishers) Ltd.) The RV genome consists of two long non-overlapping open reading frames (ORFs), the 5' ORF encodes the nonstructural proteins and the 3' ORF encodes the structural proteins. A p200 polyprotein precursor is translated from the 5' ORF of the genomic RNA. This is subsequently cleaved by a virus-encoded protease to produce the nonstructural proteins p150 and p90. The p200 protein is the principal replication protein for negative-strand RNA synthesis. The negative strand acts as a template for the transcription of the positive-strand 40S genomic and 24S subgenomic RNAs. Synthesis of positive-strand RNAs is dependent on cleavage of p200. The 24S subgenomic RNA is translated into a p110 polyprotein precursor which is proteolytically cleaved by a host-encoded protease and post-translationally modified to produce the structural proteins C, E2 and E1. Within the 5' nonstructural ORF are a number of amino acid sequence motifs of functional significance: methyltransferase (M), papain-like cysteine protease (P) and the macro, Appr-1''-pase-like family (which is conserved among alphaviruses) (X) domains within p150; and helicase (H) and replicase (R) domains within p90.

evidence of more than one serotype even in highly vaccinated populations (Frey *et al.*, 1998). Nevertheless, there is sufficient variability in currently circulating RVs to allow them to be divided into two clades and 13 genotypes (World Health Organization, 2006, 2007) (Figure 23.2). Genetic analysis of RVs has been used to support rubella control and elimination activities (Icenogle *et al.*, 2006). The most commonly used vaccine strain of RV, RA27/3, differs from the most closely related RVs by coding and noncoding nucleotide changes. Thus, the RA27/3 virus can be identified in clinical specimens (Pugachev *et al.*, 1997). There is no evidence that reinfection is due to an antigenic variant, but two viruses isolated from joints exhibit changes in antigenic epitopes in E1 (Bosma *et al.*, 1996; Frey *et al.*, 1998). A comparison of NSP-coding sequences shows that the 1970–2360 nt region of p150 is highly variable.

Replication

Rubella virus probably enters the cell by receptor-mediated endocytosis, but the cellular receptor for the virus has not been identified. The reproductive cycle takes place in the cytoplasm and probably resembles that of the alphaviruses. In alphaviruses, the virion is transported to the endosomal compartment, where the low pH brings about the uncoating of the viral genome and fusion of the E1 and E2 glycoproteins with the endosomal membranes, allowing the viral genome to be released into the cytoplasm. However, the disassembly process may well be different for RV, since the capsid protein is membrane associated. In vertebrate cell cultures the replication of RV is slow and less efficient than that of the alphaviruses. In Vero cells virus

production reaches a peak at 48 hours after infection at high multiplicities of infection (Hemphill *et al.*, 1988). No effect on total cell RNA or protein synthesis has been noted within 72 hours after infection.

The single-stranded genomic RNA and the subgenomic RNA, which codes for the structural proteins, are found in RV-infected cells. The 40S genomic RNA is translated to produce the 2116-amino-acid polyprotein (p200) encoded by the 5' proximal ORF. This polyprotein is cleaved by a viral protease to give two products of 150 and 90 kDa. The p150 contains the proposed methyltransferase and protease motifs, while the p90 contains amino acid motifs indicative of RNA-dependent RNA polymerase and helicase activity (Figure 23.1). An X motif of unknown function is also present, which is a short region of homology between RV and the alphavirus NSP3. p200 is required for synthesis of the negative-sense genome. This is used as a template for the production of both full-length 40S progeny genomes and 24S subgenomic RNA, for which cleavage of p200 is required. The 24S subgenomic RNA, which is capped, methylated and polyadenylated, is translated to produce a 110-kDa polyprotein, which is cleaved by a host cell signal peptidase to produce the three structural proteins C, E2 and E1 (Figure 23.1). All three structural proteins are transported to the Golgi complex. E1 and E2 form heterodimeric complexes, while the C protein forms dimers, which are stabilized by disulfide bond formation shortly before virus release. Glycosylation, transport and assembly of RV proteins all occur in the Golgi and the C protein is known to influence RNA replication. Thus, it is difficult to sort out the precise steps in RV RNA replication and assembly of virions (Chen and Icenogle, 2007). In cell cultures RV is released by budding from intracellular membranes (e.g. Golgi, endoplasmic

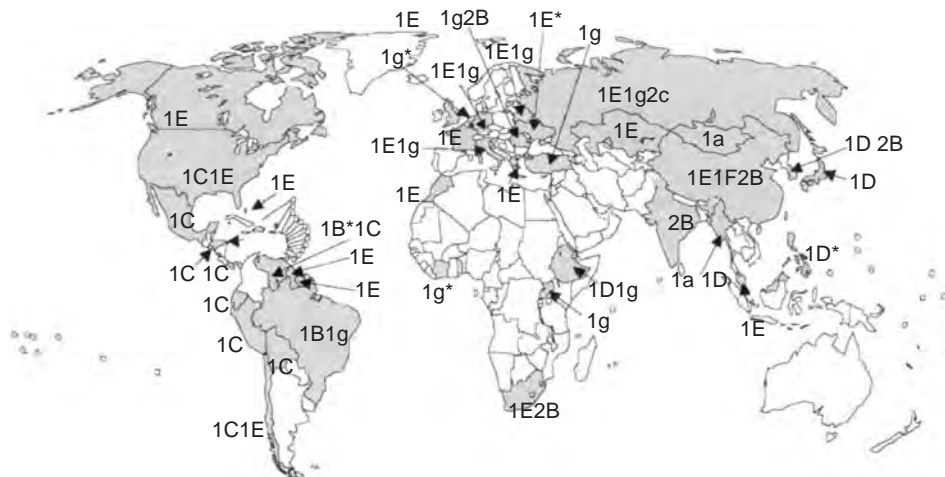


Figure 23.2 Global distribution of indigenous rubella viruses, 1995–2006. (World Health Organization, 2006).

reticulum) and from the plasma membrane. The ability to bud from intracellular vacuoles may enable RV to establish persistent infections (Chantler *et al.*, 2001).

The structure and replication of RV have been reviewed in detail by Chantler *et al.* (2001) and Chen and Icenogle (2007).

Physical and Chemical Properties of the Virus

Physical properties of RV have been reviewed in detail by Horzinek (1981) and Best *et al.* (2005). The virus is stable at 4 °C for over seven days, but is inactivated at 0.1–0.4 log₁₀ TCID₅₀/0.1 ml h⁻¹ at 37 °C and at 1.5–3.5 log₁₀ TCID₅₀/0.1 ml h⁻¹ at 56 °C. It is stable at –70 °C and when stored freeze dried at 4 °C. Stability is enhanced by the addition of proteins to the suspending medium. RV is inactivated by detergents and organic solvents, since the viral envelope contains lipid. The effects of these and other chemicals have been extensively reviewed elsewhere (Frey, 1994; Herrmann, 1979).

Antigenic Characteristics

Only one serotype of RV has been identified, although an antigenic site in the E2 protein recognized by a monoclonal antibody is not conserved among some circulating RVs (Zheng *et al.*, 2003). Vaccination with the RA27/3 vaccine affords protection against all circulating RVs. Although the E1 and E2 proteins are glycosylated, no evidence that glycosylation plays a role in the antigenicity of the virion has been reported.

Humoral and cell-mediated responses are produced against all three structural proteins, although E1 appears to immunodominant (Chaye *et al.*, 1992; Cusi *et al.*, 1989). Information on immunoreactive regions within the structural proteins has been obtained using monoclonal antibodies to map epitopes and by measuring antibody reactivity and T-cell proliferative responses to synthetic peptides and recombinant proteins. E1 has been shown to have at least six independent linear epitopes including two or three neutralizing epitopes within the amino acid region 214–285. The E2 glycoprotein in the mature virion appears to be relatively inaccessible to the immune response, but a number of B-cell epitopes have been identified (Mitchell *et al.*, 1993; Wolinsky *et al.*, 1991). Human sera react with synthetic peptides comprising residues 214–285 of the E1 protein and a recombinant protein containing these epitopes was recognized by most rubella antibody positive sera (Cordoba *et al.*, 2000; Starkey *et al.*, 1995). At least 17 T-cell epitopes have been described on the structural proteins. Conformation-dependant epitopes, which are likely play an important role in the induction of immune responses, have not been identified. Antigenic characteristics of RV have been reviewed in more detail by Chantler *et al.* (2001).

Growth in Cell Cultures

Although humans are the only natural host, RV grows in a wide range of primary and continuous cell cultures (Herrmann, 1979). Wild-type RVs often produce CPE in such continuous cell cultures as RK13 (rabbit kidney) and SIRC (rabbit cornea), but do not produce a reproducible CPE in Vero cells when first isolated. Although a characteristic CPE is usually produced in RK13 cells, the virus may be identified more reliably in both these and Vero cell cultures by immunofluorescence (IF), immunocolorimetric assays or polymerase chain reaction (PCR) (Chen *et al.*, 2007; Zhu *et al.*, 2007). The CPE has been shown to be due to rubella-induced caspase-dependent apoptosis in a number of continuous cell lines (Duncan *et al.*, 2000; Hofmann *et al.*, 1999). The exact mechanism of apoptosis has not been elucidated, but it is dependent on virus replication and is initiated within 12 hours of infection (Cooray *et al.*, 2003). Domegan and Atkins (2002) suggest that the p53 pathway is not involved. Gene expression using gene chip analysis has been compared in a primary cell line of fetal fibroblasts and a cell line of adult lung fibroblasts following infection with RV (Adamo *et al.*, 2008). Gene regulation favoured apoptosis in adult fibroblasts, but not in fetal fibroblasts, in which it appeared that gene expression was antagonistic to apoptosis.

Pathogenicity for Animals

RV infects rhesus (*Macaca mulatta*), vervet (*Cercopithecus aethiops*) and *Erythrocebus patas* monkeys, marmosets (*Sanguinus* species), chimpanzees, baboons, suckling mice, hamsters, ferrets and rabbits (reviewed by Banatvala and Best, 1990; Herrmann, 1979). Monkeys usually develop a subclinical infection with viraemia, virus excretion and an immune response, similar to that in humans. A viraemia and humoral and cell-mediated immune responses have been detected in six to eight week old BALB/c mice, which remain asymptomatic (Chantler *et al.*, 2001). Persistent infection has been established in suckling mice, adult hamsters, ferrets and rabbits.

Attempts to reproduce the teratogenic effects of RV in an animal model have produced inconsistent results. In such studies insufficient attention was given to the use of adequate controls and such factors as virus passage history, species adaptation, route of inoculation, nutrition and the effects of increased handling of the animals.

POSTNATALLY ACQUIRED INFECTION

Epidemiology in the Pre-vaccine Era

Humans are the only known hosts for RV, which had a worldwide distribution before the introduction of rubella

vaccination programmes. In temperate climates outbreaks usually occurred in the spring and early summer. Infection was uncommon in preschool children, but outbreaks involving school children and young adults living in institutional population groups were common. Women of child-bearing age were often infected as a result of exposure to children within their household or as a result of occupational exposure. Occasionally, extensive worldwide pandemics occurred, for example, in the early 1940s and again between 1963 and 1965, when a high incidence was reported in the United States, the United Kingdom and Australia. More commonly, rubella exhibited an increased incidence every three to four years, although within a particular country outbreaks might be localized.

Seroepidemiological studies have provided assessments of the incidence of rubella in different age groups and different geographical areas. Surveys have produced remarkably consistent results. In general, about 50% of 9- to 11-year-old children and about 80–85% of women of child-bearing age had rubella antibodies. Estimates of the basic reproductive number based on this information are $R_0 = 6.1$ (CR 4.3–9.2) (Kanaan and Farrington, 2005).

Rubella in Developing Countries

In an early World Health Organization study more than 80% of women of child-bearing age in mainland areas had HAI antibodies, although antibody prevalence was much lower in some rural areas and island populations (Dowdle *et al.*, 1970). In a more recent review of studies from 45 countries, Cutts *et al.* (1997) reported that less than 10% of women were susceptible to rubella in 13 countries, 10–26% in 20 countries and >25% in 12 countries, including island populations in the Caribbean, which have since adopted rubella vaccination programmes. There may be considerable variation in susceptibility in different parts of each country. The average age of infection varied from two to eight years of age.

Although CRS is now a rare disease in those developed countries that have adopted comprehensive rubella vaccination programmes, the burden induced by CRS imposes a considerable strain on scarce health and educational resources in many developing countries, which was insufficiently appreciated until recently. Although outbreaks of rubella may not always be recognized in developing countries, or rubella-induced rashes misdiagnosed, the incidence of CRS has recently been reported to have been considerably higher (range 0.6–2.2/1000 live births) than during the early years of the rubella vaccination programme in Britain (0.14/1000 during epidemics; at other times, 0.08/1000). Mathematical modelling estimated that there were approximately 110 000 (range 14 000–308 000) cases of CRS in developing countries during 1996 (Cutts

and Vynnycky, 1999); following epidemics this may show a tenfold increase.

Clinical and Virological Features of Primary Infection

Pathogenesis

Rubella virus is spread mostly by droplet from the upper respiratory tract. High concentrations of virus ($>10^5$ TCID₅₀/0.1 ml) may be excreted, but close and prolonged contact is usually required for virus to be transmitted to susceptible individuals. The epithelium of the buccal mucosa and the lymphoid tissue of the nasopharynx and upper respiratory tract probably represent the site of initial virus replication, following which RV spreads to the lymphatic system and establishes a systemic infection. It is likely that mononuclear cells are involved in dissemination of virus to different parts of the body although extracellular virus may be detected in serum.

Clinical Features

The rubella rash appears after an average incubation period of 14 days with a range of 12–21 days. Lymphadenopathy may appear up to a week before the rash and persist for 10–14 days after the rash has disappeared. Lymphadenopathy may be generalized, but the suboccipital, post-auricular and cervical lymph nodes are affected most frequently. Among children, onset is usually abrupt with the appearance of rash and constitutional symptoms usually mild or absent. The rash is at first discrete, and is in the form of pinpoint macular lesions. It appears first on the face and spreads rapidly to the trunk and then to the limbs. Lesions may coalesce, but the rash seldom lasts for more than three days; in many cases it is fleeting. Adults may experience a prodromal phase with such constitutional features as malaise, low-grade fever, headache, coryza, cough, sore throat, anorexia and mild conjunctivitis lasting for one to five days before rash develops. At this time an erythematous pinpoint enanthem may be visible on the soft palate. Rubella may occasionally present with a more severe fever and constitutional symptoms similar to measles.

Complications

Joint involvement is the most common complication of naturally acquired rubella and rubella vaccination, and usually develops as the rash subsides. Although relatively uncommon among pre-pubertal females and males, it may occur in up to 70% of post-pubertal females. Symptoms may vary in severity from a transient stiffness of the joints to an arthritis with pain, swelling and limitation of movement. This usually persists for three to four days, but occasionally for up to a month. The finger joints,

wrists, knees and ankles are most frequently affected. It has been suggested that rubella or rubella vaccination might be a cause of chronic joint disease, as RV or its antigens have been detected in the synovial fluid or synovium of patients with rheumatoid arthritis and seronegative arthritis (rheumatoid factor negative), but more recent studies have failed to confirm these findings (Bosma *et al.*, 1998; Geier and Geier, 2002). However, RV may persist in the synovium as virus has very occasionally been detected in both the synovial fluid and cells of patients with chronic joint disease (Bosma *et al.*, 1998).

Very rarely rubella is associated with other complications. A post-infectious encephalitis may develop in about 1 in 6000 cases within a week of onset of rash; it may also occur in cases without rash. In contrast to measles, the prognosis is usually good with recovery within 7–30 days and death is rare (Chantler *et al.*, 2001). The cerebrospinal fluid (CSF) usually contains cells, mostly lymphocytes, but CSF protein levels are normal. The encephalitis may be immune mediated, since infectious virus or its nucleic acid has been detected only rarely (Frey, 1997), and is not

associated with demyelination or inflammatory damage which is present in other post-infectious encephalitides.

Thrombocytopenia occurs in about 1 in 3000 rubella cases; a purpuric rash, epistaxis, haematuria, subconjunctival haemorrhage and gastrointestinal bleeding have been reported in such cases. Gullain–Barré syndrome, bone marrow aplasia, haemophagocytic syndrome, autoimmune haemolytic anaemia, optic neuritis, Fuchs heterochromic uveitis and relapsing encephalitis are other rare complications of rubella.

Differential Diagnosis

In 20–50% of cases, rubella infection is subclinical. Conversely, typical rubelliform rashes may result from infection by other viruses (e.g. enteroviruses, measles, human herpes viruses 6 and 7). Infections by such viruses as human parvovirus B19 and some arboviruses (e.g. Dengue, Chikungunya and Ross River viruses) may cause both rubelliform rashes and arthralgia (Table 23.2). Because clinical diagnosis is unreliable, it is essential that laboratory tests are conducted on all women who have been

Table 23.2 Differential diagnosis of postnatal rubella in different geographical regions

Virus infection	Geographical distribution								Key features
	Africa	Asia	Australia	Europe	North America	Central America	South America	Pacific	
Rubella	+	+	+	+	+	+	+	+	Erythema infectiosum
Parvovirus B19	+	+	+	+	+	+	+	+	
Human herpes viruses 6 and 7	+	+	+	+	+	+	+	+	Exanthem subitum. Predominantly <2 yr
Measles	+	+	+	+	+	+	+	+	Prodrome with cough, conjunctivitis, coryza
Enteroviruses	+	+	+	+	+	+	+	+	Echovirus 9, Coxsackie A9 most frequent
Dengue	+	+	+	–	–	+	+	+	Joint and back pain, haemorrhagic complications in children
West Nile fever	+	+	–	+	+	–	–	–	Joint pains
Chikungunya	+	+	–	–	–	–	–	–	
Ross River	–	–	+	–	–	–	–	–	
Sindbis	+	+	+	+	–	–	–	–	

Source: Reprinted from Banatvala, J.E. and Brown, D.W.G. Rubella. *The Lancet*, 363, 1127–37. Copyright (2004), with permission from Elsevier (Banatvala and Brown, 2004).

exposed to, or develop rubella-like illnesses in pregnancy; a past history of rubella without laboratory confirmation of the diagnosis must never be accepted as indicative of previous infection and consequent immunity.

Virological and Immunological Features

The relationship between the clinical and virological features of infection is shown in Figure 23.3. Patients are potentially infectious over a prolonged period; pharyngeal excretion may be present for up to a week before the onset of rash, and for seven to ten days thereafter. Although virus may be recovered from the stools and urine, excretion from these sites is more transient. Such specimens are therefore less suitable for virus isolation and do not play an important role in the transmission of virus. Viraemia is present for about a week before the onset of

rash, but, as rubella antibodies develop, viraemia ceases. IgG antibodies detected by enzyme immunoassay (EIA), and radial haemolysis (RH) usually develop six to seven days after onset of rash, while antibodies detected by HAI and neutralization appear a few days earlier. IgM antibodies detected by EIA develop over the first five days after rash (Helfand *et al.*, 2007). The predominant IgG response is IgG1; IgG3 is also detected, but IgG4 is rare (Thomas and Morgan-Capner, 1988).

Rubella induces a mild, transient immunosuppression, as demonstrated by a fall in total leukocytes, T cells and neutrophils and a depression in lymphocyte responsiveness to mitogens and antigens (e.g. PHA and PPD) and suppression of BCG-induced hypersensitivity. Lymphoproliferative responses develop a few days after onset of rash; a mixed Th1/Th2 response is seen (reviewed by Best and Reef, 2009).

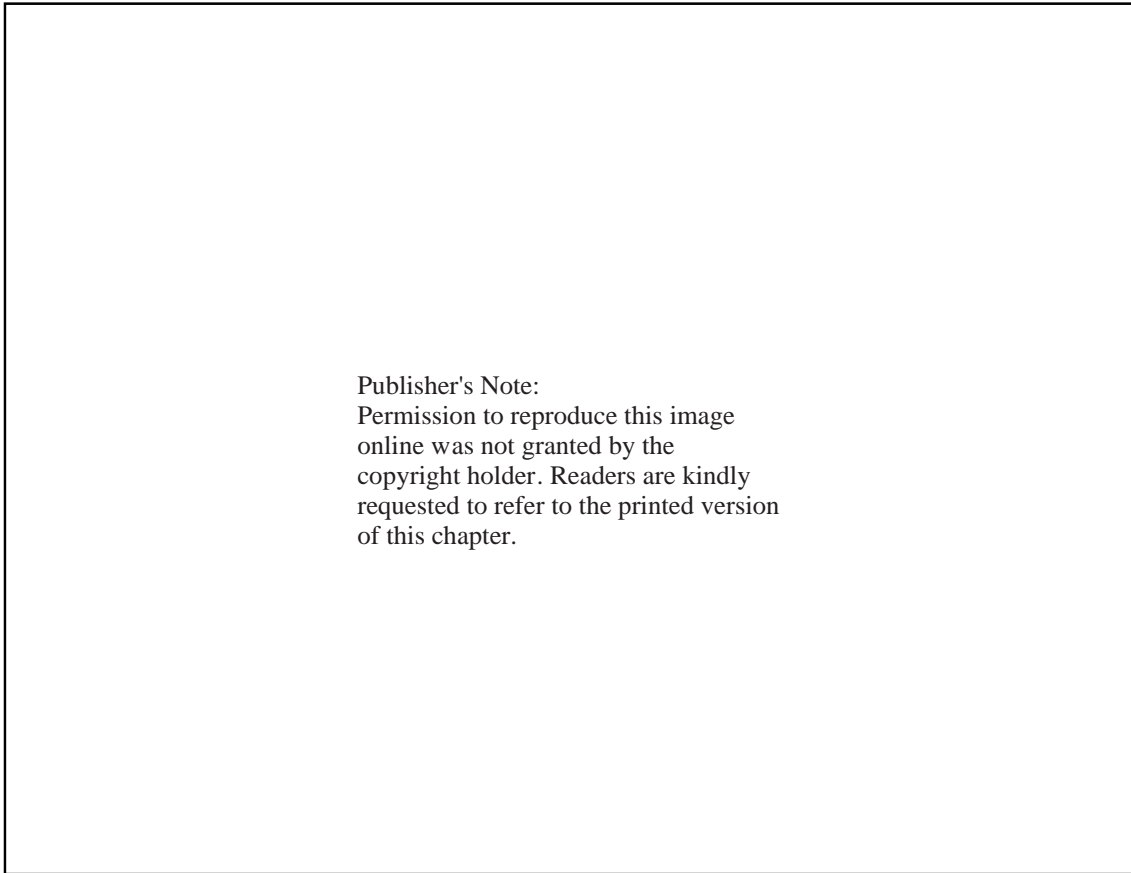


Figure 23.3 Relationship between clinical and virological features of postnatally acquired rubella. (*Source:* Reproduced from Best, Cooray and Banatvala (2005), by permission of Edward Arnold (Publishers) Ltd.) HAI, haemagglutination inhibition; Neut., neutralizing; SRH, single radial haemolysis; EIA, enzyme immunoassay.

Reinfection

Natural infection is usually followed by protection from reinfection. When reinfection does occur, it is usually subclinical and is defined by a significant rise in rubella IgG concentration, and/or detection of specific IgM in a patient with pre-existing antibodies. It is more likely to occur in those with vaccine-induced immunity than in those who have been naturally infected. It may be difficult to distinguish between primary infection and reinfection, particularly if blood was not obtained shortly after contact or if sera taken prior to contact (e.g. for screening purposes) are not available.

Reinfection would provide a hazard to the fetus if associated with viraemia. A few well-documented cases of rubella reinfection have been reported where RV was isolated from the products of conception or from children born with congenital rubella (Best *et al.*, 1989; Bullens *et al.*, 2000; Fogel *et al.*, 1996). Morgan-Capner *et al.* (1991) have calculated that the risk of fetal infection is approximately 8% following asymptomatic reinfection in the first 16 weeks of pregnancy, but fetal malformations are rare. The risk of symptomatic reinfection is probably greater. It is not clear why some individuals are susceptible to reinfection as some cases have occurred in women with antibody concentrations >15 IU ml⁻¹. However, it may be due to a failure to produce epitope-specific antibodies. There is no evidence that reinfection is caused by genotypic variants (Bosma *et al.*, 1996; Frey *et al.*, 1998).

CONGENITALLY ACQUIRED INFECTION

Pathogenesis

If acquired at an early gestational age, rubella is likely to induce a generalized and persistent fetal infection, resulting in multisystem disease. This reflects the inefficiency of the placenta to act as a barrier to infection and the fact that the fetus is unable to mount an immune response to eliminate virus early in gestation. Töndury and Smith (1966) demonstrated that the earliest lesions are found in the placenta, which is almost certainly infected during the maternal viraemic phase. However, since RV is also excreted via the cervix for at least six days after the onset of rash, and since it is possible that virus may multiply elsewhere in the genital tract, placental infection by direct contact from an ascending genital infection cannot be excluded. They suggested that RV enters the fetus via the chorion, since necrotic changes to the epithelial cells as well as in the endothelial lining of the blood vessels were present as early as the tenth day after maternal rash. Damaged endothelial cells may then be desquamated into the lumen

of the vessel and transported into the fetal circulation in the form of virus-infected 'emboli', to infect the various fetal organs. Damage to fetal endothelial cells may be extensive, and is the result of viral replication rather than any immunopathological mechanism, due to the lack of immune response in early gestational age before fetal immune responses have developed. Following rubella in the early gestational period the marked absence of any inflammatory cellular response was characteristic. Anomalies were present in 68% of 57 fetuses when maternal rubella was contracted during the first trimester. Eighty per cent were abnormal when rubella was contracted during the first month of pregnancy, with sporadic foci of cellular damage in the heart, lens, inner ear, teeth and skeletal muscle. Additional damage to such malformed organs as the liver, myocardium and organ of Corti resulted from the damage caused to endothelial cells, which may result in haemorrhages in small blood vessels causing tissue necrosis over a prolonged time.

In addition to virus-induced tissue necrosis, RV induces a retardation in cell division. It has been suggested that this is due to a rubella-specific protein, which reduces the rate of mitosis in infected cells. If this occurs during the critical phase of organogenesis, the organs will contain fewer cells than those of uninfected infants and multiple developmental defects are likely to occur. The exact mechanism of teratogenesis has not been determined. However, the lack of RV-induced apoptosis in fetal cells, and persistent interferon and chemokine responses may enable RV persistence (Adamo *et al.*, 2008). Teratogenesis may be caused by retardation of cell division and the disruption of cell survival and proliferation pathways by the noncytotoxic, persistent RV infection.

Virus Persistence

Following intrauterine infection in the first trimester, RV persists throughout gestation and can be isolated from most organs obtained at autopsy from infants who die in early infancy with severe and generalized infections (Bosma *et al.*, 1995a; Cradock-Watson *et al.*, 1989). In infancy RV may also be recovered from nasopharyngeal secretions (NPSs), urine, stools, CSF and tears. RV can be isolated from NPS of most neonates with severe congenitally acquired disease, but by the age of three months the proportion excreting RV declines to 50–60% and by 9–12 months to about 10% (Best and Enders, 2007; Cooper and Krugman, 1967). During the first few weeks after birth, those with severe disease may excrete high concentrations of virus and readily transmit infection to rubella-susceptible contacts. RV excretion may sometimes continue for 12 months or more and RV may

persist in infants with congenitally acquired disease in some sites for even longer. Thus, RV has been recovered from a cataract removed from a three-year-old child and detected by RT-PCR in lens aspirates from children with CRS up to the age of 12 months (Bosma *et al.*, 1995a). RV has also been detected in the CSF of children with CNS involvement up to the age of 18 months. Rubella antigen was detected by IF in the thyroid from a five-year-old child with Hashimoto's disease, and by co-cultivation techniques RV was recovered from the brain of a child who developed rubella panencephalitis at the age of 12 years. Experimental studies have shown that within the CNS the astrocyte is the main cell type in which virus replicates, high levels of virus being expressed. Intrauterine infection involving these cells may perhaps induce focal areas of necrosis resulting in the pattern of neurological deficit observed in CRS (Chantler *et al.*, 1995).

RV is isolated infrequently from neonates whose mothers acquired rubella after the first trimester, probably because fetal immune mechanisms can then effectively terminate infection. Evidence for fetal infection following post-first trimester rubella, comes from serological evidence of fetal infection obtained in 25–33% of infants whose mothers acquired rubella between weeks 16 and 28 of gestation (Cradock-Watson *et al.*, 1980; Miller *et al.*, 1982).

Cell-mediated Immunity

Postnatally acquired rubella causes a transient reduction in lymphocyte responses to phytohaemagglutinin as well as a decrease in the numbers of T cells. CRS might be expected to cause an even greater reduction in responsiveness. Indeed, significantly diminished lymphoproliferative responses to phytohaemagglutinin and rubella antigen, as well as diminished interferon synthesis have been demonstrated (Buimovici-Klein *et al.*, 1979). Impairment of cell-mediated immunity (CMI) responses was related to the gestational age at which maternal infection occurred, and was greatest in infants whose mothers acquired rubella in the first eight weeks of pregnancy. Hosking *et al.* (1983) suggested that children with nerve deafness due to CRS could be distinguished from those with immunity due to postnatally acquired rubella by their failure to produce lymphoproliferative responses to rubella antigen. O'Shea *et al.* (1992) reported that 10 of 13 (80%) children with CRS under the age of three years failed to mount a lymphoproliferative response. Congenitally infected infants have also been shown to have impaired natural killer cell activity and persistent T-cell abnormalities. It is of interest that the defective CMI responses may persist into the second decade of life.

Risks to the Fetus

Maternal Rubella in the First Trimester

Maternal rubella may result in spontaneous abortion, the delivery of a severely malformed infant, an infant with minimal damage or a healthy infant and occasionally in fetal death. The incidence of defects following maternal infection in the first 10 weeks of gestation is 90%, but the risk declines significantly after 12 weeks gestation (see below). If maternal rubella is acquired during the first eight weeks of pregnancy, spontaneous abortion may occur in up to 20% of cases. The rubella-induced congenital anomalies detected are related to the gestational age at which infection occurred (Figure 23.4). Cardiac and eye defects are likely to result when maternal infection is acquired during the critical phase of organogenesis, in the first eight weeks of pregnancy, whereas retinopathy and hearing defects are more evenly distributed throughout the first 16 weeks of gestation.

Maternal Rubella after the First Trimester

Serological studies confirm that a high proportion of infants are infected following maternal post-first trimester rubella, rubella-specific IgM being detected in 25–33% of infants whose mothers had rubella between 16 and 20 weeks of pregnancy (Cradock-Watson *et al.*, 1980; Miller *et al.*, 1982). However, because organogenesis is complete by 12 weeks and in more mature fetuses immune responses may limit or terminate infection, such infants rarely have severe or multiple anomalies. The results of four studies conducted in different countries are shown in Table 23.3. Most surveys have shown that deafness and retinopathy, which *per se* does not affect vision, are likely to be the only anomalies commonly associated with post-first trimester rubella (reviewed by Best *et al.* 2005). Table 23.3 shows that deafness is usually the sole clinical manifestation of fetal infection occurring between 13 and 16 weeks, and is relatively common, but deafness or any other defect is only rarely encountered after this time. These findings emphasize the importance of conducting careful follow-up studies on infants with serological evidence of intrauterine infection as a result of maternal infection occurring between 13 and 18 weeks, particular importance being directed towards the recognition of hearing defects.

Most studies on the risk of rubella in pregnancy have included women who have had symptomatic rubella. Although it has been suggested that the risk of subclinical rubella is less, this has not been confirmed.

Pre-conceptual Rubella

The definitive study conducted in Germany and Britain showed that pre-conceptual rubella did not result in

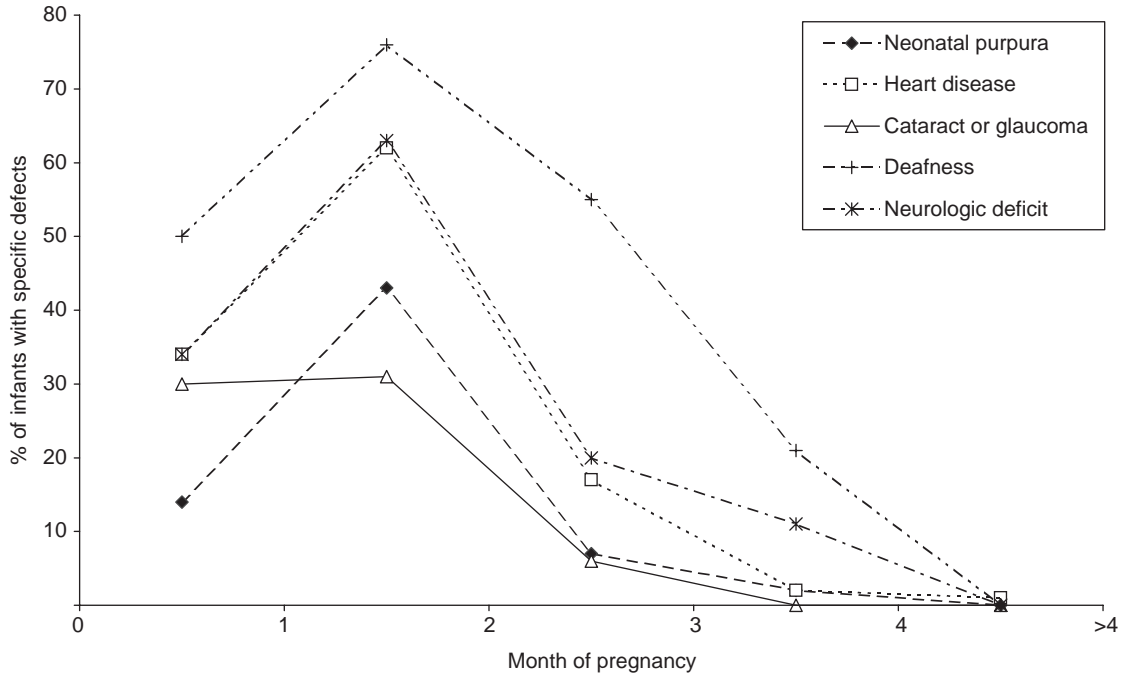


Figure 23.4 Relationship between the gestational age at time of maternal infection and clinical manifestations of congenital rubella. (Source: Extrapolated from Cooper *et al.*, 1969.)

Table 23.3 Incidence of rubella-induced defects in infants infected *in utero* after the first trimester

	Gestational age (wk)		
	13–16	17–20	>20
Peckham (1972) ^a	7/73 (10%) (3.9–18.8) ^b	4/40 (10%) (2.8–23.7)	1/11 (9.1%) (0.2–41.2)
Vejtorp and Mansa (1980) ^c	0/4 (0%) (0–60.2)	1/14 (7%) (0.2–33.9)	1/16 (6.2%) (0.2–30.25)
Miller, Cradock-Watson and Pollock (1982)	9/26 (35%) (17.2–55.7)	0/10 (0%) (0–30.8%)	0/53 (0%) (0–6.7)
Grillner <i>et al.</i> (1983)	4/17 (23%) (6.8–49.9)	2 ^d /54 (3.7%) (0.5–12.7)	0/35 (0%; 0–10.0)
Total	20/120 (16.7%) (10.5–24.6)	7/118 (5.9%) (2.4–11.8)	2/115 (1.7%) (0.2–6.1)

^aThese children were normal at birth.

^b95% confidence interval.

^cChildren followed up for 6 mo only.

^dOne additional case of deafness reported since 1983.

transmission of RV to the fetus (Enders *et al.*, 1988). Thus, there was no serological or clinical evidence of intrauterine infection in 38 infants whose mothers' rash appeared before or within 11 days after their last menstrual period (LMP). However, fetal infection occurred when there was an interval of 12 days between LMP and rash and all 10 mothers who developed rash three to six weeks after their LMP transmitted infection to their fetuses.

Clinical Features

The frequency and importance of congenital defects involving the heart, eyes and ears were emphasized in the early retrospective as well as in most prospective studies. However, following the extensive 1963–1964 epidemic in the United States, as well as in subsequent epidemics in other parts of the world, a much broader range of rubella-induced congenital anomalies were observed. This

phenomenon is more likely to be due to more careful and prolonged observation than to any change in the biological behaviour or teratogenic potential of the virus. Thus, careful examination of the case notes of infants with congenitally acquired disease who were born before this outbreak showed that such anomalies as thrombocytopenic purpura and osteitis occurred fairly frequently, even though not recorded in the literature. In addition, careful and prolonged follow-up studies showed that CRS was not a static disease, since some features of intrauterine infection might not be apparent for months or years (e.g. perceptive deafness or insulin-dependent diabetes mellitus (IDDM)).

Clinical features of the CRS have been categorized into those that are transient or permanent, some of which may be delayed to appear months or even years later (Table 23.4). The frequency of defects has been reviewed (Reef *et al.*, 2000). The World Health Organization

classifies suspected cases of congenital rubella according to the criteria shown in Table 23.5. Criteria used in the United States were updated in 2001 (Centers for Disease Control and Prevention, 2001a).

Transient Anomalies

Transient anomalies usually present during the first few weeks of life, do not recur and are not associated with permanent sequelae. Their pathogenesis has not been established, but such features as intrauterine growth retardation (small-for-dates babies), cloudy cornea, thrombocytopenic purpura (Figure 23.5), hepatosplenomegaly and haemolytic anaemia are common. About 60% of infected infants fall below the 10th, and 90% below the 50th growth percentile. The above features, which often result from infection acquired at an early gestational age,

Table 23.4 Clinical features of congenital rubella syndrome

	Early transient features	Permanent features some recognized late	Use in surveillance ^a
<i>Ocular defects</i>			
Cataracts (uni-/bilateral)		+	A
Glaucoma		+	A
Pigmentary retinopathy		+	A
Microphthalmia		+	
Iris hypoplasia		+	
Cloudy cornea	+		
<i>Auditory defects</i>			
Sensorineural deafness (uni-/bilateral)		+	A
<i>Cardiovascular defects</i>			
Persistent ductus arteriosus		+	A
Pulmonary artery stenosis		+	A
Ventricular septal defect		+	A
Myocarditis	+		
<i>Central nervous system</i>			
Microcephaly		+	B
Psychomotor retardation		+	
Meningoencephalitis	+		B
Behavioural disorders			
Speech disorders			
<i>Other</i>			
Intrauterine growth retardation	+		
Thrombocytopenia, with purpura	+		B
Hepatitis/hepatosplenomegaly	+		B
Bone 'lesions'	+		B
Pneumonitis	+		
Lymphadenopathy	+		
Diabetes mellitus		+	
Thyroid disorders		+	
Progressive rubella panencephalitis		+	

^aFor surveillance, a clinically confirmed case is defined as one in which two complications from group A or group B or one from group A or one from group B are present (World Health Organization, 1999).

Source: Adapted from Banatvala, J.E. and Brown, D.W.G. Rubella. *The Lancet*, **363**, 1127–37. Copyright (2004), with permission from Elsevier (Banatvala and Brown, 2004).

Table 23.5 WHO provisional case definition for congenital rubella syndrome

Suspected CRS case: Any infant less than 1 yr of age in whom a health worker suspects CRS. A health worker should suspect CRS when an infant aged 0–11 mo presents with heart disease and/or suspicion of deafness and/or one or more of the following eye signs: white pupil (cataract), diminished vision, pendular movement of the eyes (nystagmus), squint, smaller eye ball (microphthalmus) or larger eye ball (congenital glaucoma). A health worker should also suspect CRS when an infant's mother has a history of suspected or confirmed rubella during pregnancy, even when the infant shows no signs of CRS

Clinically confirmed CRS case: An infant in whom a qualified physician detects at least two of the complications listed in (a) below or one in (a) and one in (b):

- (a) Cataract(s), congenital glaucoma, congenital heart disease, loss of hearing, pigmentary retinopathy
- (b) Purpura, splenomegaly, microcephaly, mental retardation, meningocephalitis, radiolucent bone disease, jaundice that begins within 24 hours after birth

Laboratory confirmed CRS case: An infant with clinically confirmed CRS who has a positive blood test for rubella-specific IgM (100% of such infants are positive at the age of 0–5 mo; 60% are positive at 6–11 mo). Where special laboratory resources are available the detection of rubella virus in specimens from the pharynx or urine of an infant with suspected CRS provides laboratory confirmation of CRS (60% of such infants shed rubella virus at the age of 1–4 mo; 30% at 5–8 mo; 10% at 9–11 mo)

Congenital rubella infection (CRI): If a mother has suspected or confirmed rubella in pregnancy her infant should have a rubella-specific IgM blood test. An infant who does not have clinical signs of CRS but who has a positive rubella-specific IgM test is classified as having CRI

Source: Reproduced with permission from World Health Organization (2003c).

seldom occur without such other manifestations of congenital disease such as heart and eye defects, and reflect extensive infection which may result in high perinatal mortality rates. Infants with thrombocytopenic purpura have platelet counts ranging from 3 to $100 \times 10^9/l$ (normal = $310 \pm 68 \times 10^9/l$). This is associated with a decrease in the number of megakaryocytes in the bone marrow, although they are morphologically normal. If severely affected infants survive, their platelet count rises spontaneously during the first few weeks of life; rarely, infants may die from such complications of thrombocytopenia as intracranial haemorrhage.

Bony lesions may be present in about 20% of congenitally infected infants. The metaphyseal portion of the long bones are usually involved and radiologically appear as areas of translucency. These lesions, which result from a disturbance in bone growth rather than an inflammatory response, usually resolve without residual sequelae within the first one to two months of age.

About 25% of infants who present at birth with clinical manifestations of CRS also have CNS involvement, usually meningoencephalitis. At birth, these infants may be either irritable or lethargic with a full fontanelle and CSF changes consistent with a meningoencephalitis. Although about 25% of infants presenting at birth with a severe neonatal encephalitis may, by the age of 18 months, be severely retarded and have communication problems, ataxia or spastic diplegia, some infants progress well neurologically despite poor development during their first few months of life.

'Late Onset Disease' Between the ages of about 3 and 12 months, some congenitally infected infants may develop a chronic rubella-like rash, persistent diarrhoea and pneumonitis, which is referred to as 'late onset disease'. Mortality is high, but some infants improve dramatically if treated with corticosteroids. Circulating immune complexes are probably responsible for inducing this syndrome (reviewed by Hanshaw *et al.*, 1985).

Permanent Defects

The major permanent defects which may be present at birth are cardiac and ocular defects and damage to the organ of Corti. These and other defects are listed in Table 23.4. Cardiovascular and ocular anomalies provide the greatest problems.

Cardiac Defects Cardiac defects are responsible for much of the high perinatal mortality associated with CRS, the commonest lesions being the persistence of patent ductus arteriosus, proximal (valvular) or peripheral pulmonary artery stenosis or a ventricular septal defect. In association with such anomalies, a neonatal myocarditis may occasionally occur. RV may also cause proliferation and damage to the intimal lining of the arteries and this may cause obstructive lesions of the pulmonary and renal arteries.

Ocular Defects Most of the classical ocular defects seen in CRS were described by Gregg (1941), who



Figure 23.5 Purpuric rash in newborn infant with congenitally acquired rubella, who was subsequently found to have congenital heart disease and cataract. (Source: Reproduced from Best, Cooray and Banatvala (2005), by permission of Edward Arnold (Publishers) Ltd.)

drew particular attention to the pigmented retinopathy and cataract. Although usually present at birth, cataracts may not be visible until several weeks later. They are unilateral in about 50% of patients, and may be subtotal, consisting of a dense pearly white central opacity (Figure 23.6), or total, with a more uniform density throughout the lens. Microphthalmia may be present in eyes with cataract. Glaucoma occurs much less frequently than cataract, but glaucoma and cataract do not seem to be present in the same eye. Retinopathy is present in about 50% of congenitally infected infants and its presence may provide a useful clinical diagnostic marker. Retinopathy is not the result of an inflammatory response but is due to a defect in pigmentation and usually involves the macular areas. Hyperpigmented and hypopigmented areas give the retina a 'salt and pepper' appearance. Chronic uveitis, choroidal neovascularization, corneal hydrops and keratoconus have also been described. The mechanisms involved may include damage induced by viral persistence causing reduced cell growth rate and life span, virally induced vascular damage or autoimmune phenomena (reviewed by

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Figure 23.6 Congenital rubella cataract in a nine-month-old infant. Cataract was also present in the left eye but was removed surgically. (Source: Reproduced from Best, Cooray and Banatvala (2005), by permission of Edward Arnold (Publishers) Ltd.)

Arnold *et al.*, 1994). A recent case series from India has suggested that cataracts are the only clinical eye finding that is useful for screening (Vijayalakshmi *et al.*, 2007).

Hearing Defects Rubella-induced deafness may be unilateral with no characteristic audiometric pattern. Prior to the introduction of vaccination programmes CRS probably represented the commonest cause of congenital deafness in most developed countries, its impact being insufficiently appreciated since there may have been no history of maternal rubella. Deafness may be the only anomaly present, particularly if maternal infection occurred after the first trimester. Thus, in a study on a large number of children aged six months to four years who attended the Nuffield Hearing and Speech Centre in London, it was estimated that about 15% of all cases of sensorineural deafness were the result of CRS.

Delayed Manifestations

Congenital rubella is a progressive disease due to persistent virus infection. Some defects may take months or years before becoming apparent, but persist indefinitely (Cooper and Alford, 2006). Failure to recognize them may not merely be the result of difficulty in their detection, for there is evidence to show that such defects as perceptive deafness, some CNS anomalies and some ocular defects may actually develop later or become progressively more severe.

Hearing Defects On audiological examination, some infants, although they have apparently normal hearing at 9–12 months of age, are found to have severe sensorineural deafness months or even years later. Improvements in technology, such as otoacoustic emissions and auditory

brainstem responses, may be of value in assessing hearing defects in early infancy (van Straaten *et al.*, 1996).

Ocular Defects Glaucoma may develop between 3 and 22 years of age. Other late-onset eye defects are keratic precipitates, keratocornus, corneal hydrops and spontaneous lens absorption. Visual difficulties may develop due to subretinal neovascularization.

Insulin-Dependent Diabetes Mellitus IDDM (juvenile-onset; type 1) is the most frequent endocrine disorder among patients with congenital rubella. It was thought originally to be a rare complication of congenital rubella, but it is now recognized that clinical manifestations may be delayed until adolescence or adult life. A follow-up study of patients in New South Wales who were born with CRS in the 1940s showed that 20% eventually developed IDDM. Follow-up studies in New York on children, most of whom were infected *in utero* during the 1963–1964 epidemic, have shown that 30 of 242 (12.4%) had developed IDDM, although a higher proportion (20%) had pancreatic islet cell antibodies, suggesting that more of these patients were likely to develop IDDM (reviewed in Burke *et al.*, 1985). The mean age of children developing IDDM in this US study was nine years, while all of the Australian patients were in their twenties. Lymphocytic infiltration of the pancreas of an infant with CRS but without IDDM may suggest that RV can initiate a train of events that subsequently results in IDDM in later life. RV has been shown to induce a depression of immunoreactive secreted insulin in human fetal islet cells without being cytolytic (Numazaki *et al.*, 1990). Several experimental studies support the role for autoimmune mechanisms. Thus, Karounos *et al.* (1993) showed that immunoreactive epitopes in the RV capsid shared antigenicity (molecular mimicry) with islet β -cell protein. Ou *et al.* (1999), using clones of T cells from patients with CRS, have described human leukocyte antigen (HLA)-restricted cytotoxic responses to a β -cell autoantigen, GAD65. The HLA types in patients with IDDM are typical of those with autoimmune disease, there being a significant increase in prevalence of HLA-DR3 (reviewed in Burke *et al.*, 1985). Islet cell antibodies have been detected in 20% of these patients; these antibodies have a cytotoxic effect on cultured islet cells and predict the diabetic state. The mechanism by which RV may trigger autoimmune responses remains to be established, since studies on pathogenesis are limited by the lack of a suitable animal model.

Other Endocrine Disorders Thyroid autoantibodies have been detected in 20–40% cases of CRS, about 5% of cases have thyroid dysfunction, including hypothyroidism, hyperthyroidism and thyroiditis. Growth hormone deficiency has also been reported in CRS, suggesting pituitary

dysfunction. Poor growth and early cessation of growth have been reported.

Central Nervous System Disorders Mental retardation, autism and behavioural problems may develop and be progressive. Occasionally children with previously clinically stable congenitally acquired disease may develop a widespread subacute panencephalitis, which is invariably fatal (Chantler *et al.*, 2001; Frey, 1997). About 50 cases have been described in association with CRS and very rare cases following postnatally acquired rubella. Clinical and laboratory features are analogous to measles-induced subacute sclerosing panencephalitis, since, in addition to intellectual deterioration, spasticity and ataxia occur. Histological studies revealed a panencephalitis with a perivascular inflammatory response as well as a vasculitis. RV has been isolated from the brains of such patients, and has also been recovered from their lymphocytes. Rubella antigens have not been detected by IF in sections of brain. High levels of rubella-specific IgG and, occasionally, IgM may be present in the serum, while in the CSF there are elevated levels of protein and immunoglobulin, and oligoclonal bands are found. There is also a high CSF:serum rubella antibody titre ratio. It has been postulated that post-rubella panencephalitis may be a disease mediated by immune complexes or by RV-mediated autoreactivity to brain antigens.

Outlook for Children with Congenital Rubella

As a result of the extent and severity of rubella-induced congenital malformations, any surviving children require continuous and specialized medical care, rehabilitation and education. However, assessment of 25-year-old patients whose mothers acquired rubella during the extensive epidemic in Australia in the early 1940s showed that many had developed far better than had been anticipated in early childhood, despite the presence of hearing and eye defects. Most were of average intelligence, employed, and some had married and had normal children (Menser *et al.*, 1967). In contrast, studies on children with CRS resulting from the 1963–1964 US epidemic showed that they had fared less favourably (Cooper, 1975). This difference is probably due to the survival of children with some of the more severe manifestations of congenital infection, which reflects more modern, vigorous and sophisticated methods of treatment that were not available previously. More recent studies have reported a prevalence of diabetes, thyroid disorders, early menopause and osteoporosis, which is higher than the general population (Forrest *et al.*, 2002; Munroe, 1999). Precocious puberty has also been observed.

Management of Infants and Children with CRS

Clinical Management These children require care by a multidisciplinary team, as many lesions are not apparent at birth and long-term follow-up is required. There are few reports of attempted treatment of CRS; available chemotherapeutic agents have not affected the clinical course of disease (reviewed by Cooper and Alford, 2006).

Isolation Precautions These should be initiated as soon as a congenital infection is suspected. Infants with CRS excrete large amounts of RV initially and may remain infectious for 12 months or more. RV may be readily transmitted to rubella-susceptible individuals. Women of child-bearing age, some of whom may be in early stages of pregnancy, should be dissuaded from visiting such infants unless serological tests confirm that they are immune. It is important that midwives and nursing staff who may have to care for such infants are also shown to be immune to rubella; especially those midwives and nurses who originate from countries without rubella vaccination programmes.

LABORATORY TECHNIQUES AND DIAGNOSIS

Serological Techniques Used for Rubella Antibody Screening

Specific antibodies are associated with protection from rubella (Best and Reef, 2009). A minimum immune titre of 15 IU ml⁻¹ was adopted in the United Kingdom in 1978 in order to avoid the false-positive results obtained in some HAI tests (PHLS Working Party on the Laboratory Diagnosis of Rubella, 1988). More recently, this has been reduced to 10 IU ml⁻¹ for EIA and latex agglutination (LA) assays (Department of Health, 2003; Skendzel, 1996).

Extensive rubella antibody screening of adult women is carried out in order to identify susceptibles who require vaccination. In some countries all antenatal women are tested and those shown to be susceptible are offered a rubella-containing vaccine post partum or post pregnancy. It is particularly important that immigrant women and women planning *in vitro* fertilization are tested. Blood should be obtained from pregnant women for rubella antibody screening when they first book at antenatal clinics. It is advisable to store these specimens for at least a year, so that this pre-exposure specimen can be tested in parallel with later serum samples should a patient subsequently give a history of exposure to a rubella-like illness. In addition, should an infant be delivered with clinical features compatible with an intrauterine infection,

serum obtained in early pregnancy can be tested in parallel with that obtained after delivery and the infant's cord or neonatal blood (Department of Health, 2003). Tests may be carried out for evidence of congenital infection not only by rubella but also by such organisms as cytomegalovirus, parvovirus B19 and *Toxoplasma gondii*.

EIA is used most frequently for screening purposes, while RH and LA may be used for confirmatory purposes. HAI is not recommended since it is time-consuming, labour-intensive and false-positive results may occur due to failure to remove all serum lipoprotein inhibitors from test sera (PHLS Working Party on the Laboratory Diagnosis of Rubella, 1988). EIAs are widely used because they are sensitive, and can be used in automated antenatal screening. LA has the advantage of providing a result within a few minutes. Negative results should be confirmed using a different assay in order to identify sampling errors and monitor the first assay (Department of Health, 2003). Some women fail to produce antibody levels >10 IU ml⁻¹ even after several vaccinations. These women may be considered to be immune if they have a well-documented history of more than one vaccination.

Serological Techniques Used for Diagnosis

Detection of Rubella-specific IgM

Serological methods are used for the diagnosis of rubella. A diagnosis is usually made by detection of rubella-specific IgM using a commercial EIA, but in the case of a pregnant woman it is necessary to confirm that diagnosis by demonstrating a rise in specific IgG concentration, by detecting low-avidity IgG or specific IgM in a second serum. Care should be taken to ensure that the rubella IgM test employed has a high level of sensitivity and specificity (Tipples *et al.*, 2004). The performance of commercial assays may differ, but it is possible usually to detect specific IgM antibodies within four days of onset of rash and for 4–12 weeks thereafter.

Detection of a Significant Rise in Antibody Concentrations

A significant rise in antibody titre can be detected by a quantitative EIA, HAI or LA titration. Seroconversion can be detected by RH. Although HAI antibodies may develop one to two days after onset of symptoms, IgG antibodies detected by EIA, LA or RH may be delayed until seven to eight days (Figure 23.3).

Measurement of Rubella IgG Avidity

The avidity, or strength of binding of rubella IgG to antigen, increases with time after primary infection (Thomas

and Morgan-Capner, 1990). Several EIA methods have been developed to measure rubella IgG avidity. The diethylamine (DEA) wash method (Böttiger and Jensen, 1997) has the advantage that it detects low-avidity antibody for longer after primary infection than the urea wash method, and it is easier to perform than the DEA shift value method (reviewed by Best and Enders, 2007). Commercially available tests have been evaluated by Mubareka *et al.* (2007).

Tests for rubella IgG avidity are useful to distinguish primary rubella from reinfection, and to determine whether rubella IgM detected in the absence of rash or rubella contact, is a consequence of primary rubella, non-specific IgM or persistence of IgM antibodies.

Use of Oral Fluid and Dried Blood Spots for Surveillance

Rubella-specific IgG and IgM antibodies may be detected in oral fluid using antibody capture immunoassays and results correlate well with serum antibodies (Ramsay *et al.*, 1998). The optimum time for detecting specific IgM is one to five weeks after onset of illness. Using oral fluid, it has been possible to demonstrate that rubella-like illnesses in children under one year of age are due to other viruses, such as parvovirus B19 (Ramsay *et al.*, 2002). Optimum methods for collection, extraction and preservation of samples have been established (Mortimer and Parry, 1991). A commercially available assay suitable for detecting rubella IgM in serum and oral fluid is now available (Vijaylakshmi *et al.*, 2006).

Oral fluid can also be used for reverse transcription nested PCR (RT-nPCR). RV RNA can be reliably detected in samples collected within seven days of onset of rash (Jin *et al.*, 2002). Such samples may provide RV RNA for molecular epidemiological studies.

Dried blood spots and oral fluid have been used successfully for seroepidemiological studies (Hardelid *et al.*, 2008; Perry *et al.*, 1993), but these samples have not been evaluated for screening or management of individual patients. Dried blood spots are particularly useful if the cold chain is unreliable (Helfand *et al.*, 2007).

Detection of Rubella Virus

Rubella virus may be detected in clinical samples by isolation in cell culture, or by RT-nPCR, but these techniques are only available in specialized laboratories. RT-nPCRs are of value for postnatal and prenatal diagnosis of congenital rubella (see below) and have been described in detail (Bosma *et al.*, 1995a, 1995b; Jin and Thomas, 2007; Revello *et al.*, 1997; Zhu *et al.*, 2007). Real-time PCR has also been developed (Rajasundari *et al.*, 2008).

RV can be identified by CPE in RK13 or certain sublines of Vero cells (reviewed by Best and Enders, 2007; Best and O'Shea, 1995). Since the CPE may be difficult to detect, RT-PCR, RT-nPCR or indirect IF are often used to identify RV after two passages in Vero or RK13 cells.

Serological Assessment of Women Developing or Exposed to Rubella-like Illnesses in Pregnancy

Development of a Rubella-like Illness

Guidelines on the management of women with rash illness in pregnancy have been published in the United Kingdom (Morgan-Capner and Crowcroft, 2002). Access to laboratory tests to confirm or refute a diagnosis of rubella is critical, since a clinical diagnosis is unreliable. Blood collected from pregnant women with features of a rubella-like illness as soon as possible after onset of symptoms should be tested for rubella IgM and IgG. Rubella IgM can usually be detected by three to six days after onset of rash, while IgG antibodies detectable by EIA may not be present until six to seven days after onset of rash (Figure 23.3). A second serum should be taken 5–10 days later to confirm the IgM result and to demonstrate seroconversion or a significant rise in specific IgG concentration in positive cases. A significant rise in IgG antibody titre can often be detected within seven days, although occasionally the response may be delayed, in which case it may be necessary to test additional blood samples. Detection of rubella IgM in a single serum is not sufficient to confirm a diagnosis of rubella in the first 20 weeks of pregnancy. Results should be interpreted with caution, taking into account accurate information on the likelihood, date and duration of exposure, date of onset of illness, including presence and distribution of rash, lymphadenopathy and arthralgia and history of rubella vaccination and results of previous rubella antibody screening tests. It is more difficult to make a diagnosis when the pregnant woman presents more than four weeks after onset of symptoms, unless a earlier serum is available. Tests for rubella IgG avidity and immunoblotting would be helpful (Best and Enders, 2007). In such cases all relevant information is required to interpret results.

Although the presence of rubella-specific IgM used to be considered indicative of a recent primary infection, it is now recognized that if sensitive techniques are employed, low and transient concentrations of rubella-specific IgM may persist for several years and may also be detected in reinfection (Best and Enders, 2007). Such findings, and the occurrence of occasional false-positive rubella IgM results, emphasize the importance of assessing clinical and serological data carefully before making a diagnosis of

primary rubella infection, which may result in termination of a precious pregnancy (Best *et al.*, 2002).

Contact with a Rubella-like Illness

Women who give a history of contact with a rubella-like illness should be investigated unless they have rubella antibodies detected in two previous sera, two documented doses of a rubella-containing vaccine or one dose of vaccine followed by detection of rubella antibodies (Morgan-Capner and Crowcroft, 2002). They are much more likely to acquire infection if exposure is close and prolonged, for example, within their own household. It is important that blood is obtained as soon as possible after contact. If rubella IgG antibodies are detected in serum obtained within the incubation period (i.e. 12–14 days of last exposure) it can be assumed that the antibodies result from infection in the past. Patients who present more than 14 days after exposure should be tested for rubella IgM and IgG. Women who are seronegative should be followed up for one month after date of last contact to ensure that seroconversion has not occurred. Seronegative women may experience considerable anxiety during this time, which may be allayed by testing the index case for rubella IgM in serum or oral fluid to determine whether the infection was rubella. Women who remain seronegative should be offered measles, mumps and rubella (MMR) vaccine in the post-partum period.

Diagnosis of Reinfection

Rubella reinfection may be diagnosed by a significant rise in rubella IgG concentrations, sometimes to very high levels, and/or detection of specific IgM in a patient with pre-existing antibodies. If serum samples obtained before reinfection are not available for re-testing, evidence of pre-existing antibody may be accepted if there are at least two laboratory reports of antibodies $>10 \text{ IU ml}^{-1}$ obtained by a reliable technique (not HAI). A documented history of rubella vaccination followed by at least one test for rubella antibodies $>10 \text{ IU ml}^{-1}$ is also acceptable (Best *et al.*, 1989).

It is often possible to distinguish reinfection from primary infection by examining the avidity of rubella IgG antibodies. Sera taken from cases of recent primary rubella reinfection have low IgG avidity, while sera taken from people with distant infection, including cases of rubella reinfection, have higher avidity (Thomas and Morgan-Capner, 1990). Low-avidity IgG can also be detected in oral fluid. Immunoblotting may also be useful as antibodies to E2 do not appear until about three months after primary infection (Meitsch *et al.*, 1997). Diagnosis of reinfection has been discussed in detail by Best and Enders (2007).

Management of Rubella in Pregnancy

Close collaboration between the laboratory and those caring for pregnant women is essential in order that investigations are carried out as quickly as possible. In all cases involving potential rubella in a pregnant woman, the risk of exposure to wild-type RV infection must be considered (e.g. was there travel to a rubella endemic area?). Laboratory test results must be evaluated considering the predictive values of the tests performed. Once a diagnosis of rubella has been established (see above), it is necessary to discuss the risks of congenital infection with both parents. Information on the gestational age at infection is required in order to assess the risk to the fetus. The pregnant woman may wish to consider a termination of pregnancy if infection has occurred in the first 12 weeks of pregnancy when the risk of defects is very high, but this decision may be different after 12 weeks gestation. Prenatal diagnosis may be of value when maternal infection has occurred at 12–18 weeks gestation, when reinfection has occurred in the first 12 weeks of gestation and when equivocal serological results are obtained in the first trimester. Invasive prenatal diagnosis is not indicated when primary rubella is confirmed before the 12th week of gestation, when long-persisting IgM antibodies are detected, as they are not associated with congenital infection (Best and Enders, 2007), and when a seronegative woman has inadvertently received a rubella-containing vaccine. There are no official guidelines for the use of invasive prenatal diagnosis for the diagnosis of congenital rubella infection.

The use of human immunoglobulin may be considered if a susceptible woman has close contact with confirmed rubella infection and a termination is not acceptable. However, the concentration of rubella antibodies in such preparations may be too low to prevent viraemia and its use may mask clinical symptoms, giving a false sense of security (Cooper and Alford, 2006). Serological follow-up is essential to determine whether infection has occurred (Salisbury *et al.*, 2006).

Virological Diagnosis of Congenitally Acquired Infection

Postnatal Diagnosis

Congenital rubella has become a rare disease in countries with effective vaccination programmes, but it should not be forgotten as immigrants and travellers may still be at risk of acquiring rubella in pregnancy. Neonates born to women who have had rubella at any stage of pregnancy and neonates born with clinical features suggestive of congenital rubella should be tested for evidence of congenital rubella infection. In addition, results of laboratory tests are required for case classification

of congenital rubella (Table 23.5). The most common methods for diagnosing congenital rubella are described below.

Detection of Rubella-specific IgM in Cord Blood or Serum or Oral Fluid Taken in Infancy

Rubella-specific IgM antibodies synthesized by the fetus *in utero* are present at birth. The detection of rubella-specific IgM in cord, neonatal or infant sera by an IgM-capture assay is the method of choice for the diagnosis of CRS. Rubella IgM can also be detected in oral fluid (Eckstein *et al.*, 1996; Rajasundari *et al.*, 2008). Specific IgM can be detected in most cases of CRS during the first three months of life, when sensitive methods are used. In the few studies carried out by reference laboratories, specific IgM has been detected in about 85% of infants tested at three to six months of age, in 30–71% at 6–12 months, 27–75% at 12–18 months and 6–67% at 18–24 months. However, it should be noted that the numbers of older children were small and a very sensitive MACRIA was used in some of these studies (Best and Enders, 2007; Chantler *et al.*, 1982; Thomas *et al.*, 1993). The sensitivity of commercially available EIAs have not usually been established for the diagnosis of congenital rubella. However, the absence of specific IgM by IgM-capture assays in the neonatal period virtually excludes symptomatic congenital rubella. If a low or equivocal result is obtained by any assay, a further specimen of serum should be examined and other techniques employed.

Specific IgM is usually detected in infants with asymptomatic congenital rubella infection up to three months of age, but rarely after that time (G. Enders, personal communication, 2006).

Detection of a Persistent Rubella IgG Response in the Infant

The detection of specific IgG may be of value when tests for specific IgM have not been conducted in early infancy. Maternally derived rubella-specific IgG antibodies as well as the infant's specific IgG will be present at birth. The presence of rubella antibody at a time beyond which maternal antibody would normally have disappeared (approximately six months of age) is suggestive of congenital infection. Since rubella is uncommon under the age of one year, specific IgG detected between 7 and 11 months of age may be suggestive of congenital rubella. However, each case must be assessed individually, taking into account such factors as age, maternal vaccination history, presence of clinical findings suggestive of or compatible with congenital rubella, and rubelliform illnesses since birth.

Detection of Rubella Virus in Samples from Infected Infants

Rubella virus may be detected by isolation in cell culture, or more rapidly by RT-PCR-based methods. NPS, urine, oral fluid, EDTA-blood, CSF, lens aspirates and post-mortem tissues may be tested for RV. Specimens can be sent on dry ice or in formal saline for RT-nPCR in a distant laboratory. This has been used in our laboratory to test lens aspirates and to confirm the diagnosis of congenital rubella in children in India (Bosma *et al.*, 1995a, 1995b). FTA filter paper (Whatman) may also be used to collect fluids for RT-PCR (Chibo *et al.*, 2005).

Cooper and Krugman (1967) isolated RV from NPS from most neonates with severe CRS, but by the age of about three months the proportion excreting virus had declined to 50–60%; and to 3% at 13–20 months of age. More recent results demonstrate that RT-nPCR is more sensitive than virus isolation for this purpose. Under three months of age RV RNA was detected in 16/19 (84%) NPS by RT-nPCR and 7/19 (37%) by virus isolation and in 19/25 (76%) urine samples by RT-nPCR and 9/25 (36%) by virus isolation (Best and Enders, 2007). Virus excretion is infrequent in children under one year of age, but has occasionally been detected up to the age of 27 months (Best and Enders, 2007; L. Grangeot-Keros, personal communication, 2007). Rajasundari *et al.* (2008) showed a good correlation between RT-nPCR and detection of rubella IgM and detected a high copy number of RV in lens samples by real-time PCR. Some countries require the documentation of the absence of infectious virus in a CRS infant to lift isolation requirements. In asymptomatic congenital rubella infection RV RNA was detected less frequently and only up to three months of age (G. Enders, personal communication, 2006).

Other Tests

Low-avidity rubella IgG1, lack of antibodies to the SP15 E1 peptide and the E2 glycoprotein, and poor cell-mediated immune responses have been reported in children with CRS under the age of three years (reviewed by Best and Enders, 2007). Rajasundari *et al.* (2008) reported that 18 of 27 (66.6%) infants aged 0–11 months had low-avidity IgG, but further evaluation of the other techniques is required.

Loss of Rubella Antibodies in Congenital Rubella

Although rubella-specific IgG detected by RH and EIA may persist indefinitely, studies on 223 children with congenital rubella following the 1963–1964 US epidemic showed that the HAI antibodies declined more rapidly among congenitally infected children than among their

mothers; by the age of five, 20% of infants with congenital rubella no longer had HAI antibodies (Cooper *et al.*, 1971). Nevertheless, seronegative children failed to develop an HAI response or excrete virus when challenged with rubella vaccine. More recent studies suggest a decreased production of antibodies to the epitopes on the E1 glycoprotein which induce HAI antibodies in some children with congenital rubella, and Forrest *et al.* (2002) reported that at 60 years of age 41% patients with congenital rubella had no detectable rubella antibodies when tested by EIA. Thus, in order to determine the immune status of such children in later life it may be necessary to test sera by EIA, LA or immunoblotting (Meitsch *et al.*, 1997).

Prenatal Diagnosis

A prenatal diagnosis may be made by detecting rubella RNA by RT-nPCR in amniotic fluid or fetal blood obtained by cordocentesis (Best and Enders, 2007; Macé *et al.*, 2004; Revello *et al.*, 1997). When RT-nPCR is used a result can be available within 48 hours. Rubella-specific IgM may also be detected in fetal blood. It is advisable to use all available assays to exclude a diagnosis of CRS (Best and Enders, 2007). There are few reports on the use of ultrasound to detect congenital rubella abnormalities, but Bailão *et al.*, (2005) have recently shown that key ultrasound findings are micrognathia, microcephaly, dystrophic calcification, cataracts, microphthalmos, hepatosplenomegaly and intrauterine growth restriction.

Amniotic fluid is the preferred sample as it is easier to collect than fetal blood. The detection of rubella RNA in amniotic fluid by RT-nPCR has a sensitivity of 86% and a specificity of 91% (Best and Enders, 2007). Ideally, amniotic fluid should be taken seven to eight weeks after onset of maternal rubella infection and not before 21 weeks of gestation. Samples should be tested in triplicate as levels of RV may be low. Chorionic villus biopsies have been used less frequently and therefore detection of virus in these biopsies should be interpreted with caution, as the detection of RV in the placenta may not always reflect fetal infection (Bosma *et al.*, 1995a).

Fetal blood collected in EDTA (not heparin) may be used for detection of RV by RT-nPCR and detection of specific IgM. It should be collected seven to eight weeks after maternal infection and not before 21 weeks gestation. It is advised to test fetal blood for rubella-specific IgM by more than one assay, as levels of IgM may be low.

It should be remembered that there are few published studies on the use of prenatal diagnosis which have included the outcome of pregnancy. False-positive and false-negative results have occurred in all studies and therefore a negative result on a single sample should be interpreted with caution. Repeat testing and collection of

further samples is often necessary (Tang *et al.*, 2003). As fetal infection is not always associated with congenital defects, it is important that the limitation of prenatal diagnosis and the risks of the sampling techniques are explained to the parents. The technical risk of fetal loss associated with amniocentesis is 0.2–1% and with the collection of fetal blood 0.5–2%. Collection of fetal blood is technically demanding and is only carried out in expert fetal medicine units. Prenatal diagnosis has been discussed in detail by Best and Enders (2007).

PREVENTION—RUBELLA VACCINATION

Development and Use of Attenuated Vaccines

The RA27/3 vaccine strain was originally isolated from the fetal kidney of a rubella-infected conceptus and is now the most widely used vaccine worldwide. In Japan and China locally developed attenuated strains are used. The development and use of rubella vaccines has been reviewed (Best and Reef, 2009). Rubella vaccine is usually administered with measles (MR), measles and mumps, (MMR) or measles, mumps and varicella (MMRV) vaccines. Two doses are required to give satisfactory protection against all diseases.

Immune Responses

About 95% of vaccinees develop an immune response some 20–28 days post vaccination although occasionally it may be delayed for up to two months. Rubella-specific IgG and IgA responses can be detected, while virus-specific IgM is detected in about 70% of vaccinees between three and eight weeks after immunization and may occasionally persist for six months and rarely for up to four years (O'Shea *et al.*, 1985). Rubella-specific 7S IgA responses persist for up to 10–12 years but the oligomeric 10S response is transient. The RA27/3 vaccine induces a secretory IgA response which can be detected in NPS for up to five years post vaccination. Antibodies against E1 and C recombinant proteins can readily be detected after RA27/3 immunization (Nedeljkovic *et al.*, 1999), but antibodies to recombinant E2 are detected in <60% of vaccinees (Best and Enders, 2007).

Serum antibodies are long lasting and it is expected that they will provide life-long protection in most vaccinees. Thus, antibodies detected by HAI and RH have been shown to persist for at least 21 years, although in about 10% of vaccinees they decline to low (<15 mIU ml⁻¹) or even undetectable levels within five years. Up to 23% of young women in the United States and Nova Scotia, where there has been little circulation of rubella, have been shown to lack antibodies to rubella. Nevertheless,

when 19 volunteers with low or undetectable antibody levels were challenged intranasally with high-titre RA27/3, only one was viraemic, this being transient and at a low level (O'Shea *et al.*, 1983). Immune responses including cellular responses after rubella vaccination have been reviewed in more detail by Best and Reef (2009).

Protective Efficacy and Reinfection

During outbreaks, the clinical protection afforded by vaccination has been shown to be 90–100% (reviewed by Best and Reef, 2009). Subclinical reinfection may occur in about 7–10% of vaccinees who have received the RA27/3 rubella vaccine. Although specific antibodies correlate with immunity, to date it has not been possible to identify any type or level of antibody which is invariably associated with protection. An antibody concentration of ≥ 10 IU ml⁻¹ is considered to provide protection to the majority of individuals. People with antibodies < 10 IU ml⁻¹ may respond to infection or vaccination with a secondary immune response due to immunological memory.

Evidence of reinfection is usually obtained serologically by demonstrating a significant rise in antibody titre. Experimental studies suggest that reinfection is more likely to occur in those whose immunity is vaccine-induced rather than naturally acquired (reviewed in Best and Enders, 2007; Best and Reef, 2009). A transient rubella-specific IgM response may be detected if serum is tested by a sensitive technique within four to six weeks of exposure. Viraemia has very occasionally been detected in vaccinees who have been reinfected naturally or experimentally. The risk of such reinfection resulting in fetal damage is small.

Virus Excretion

Provided sensitive assays are used, RV may be detected in the NPS of virtually all vaccinees 6–29 days post vaccination. Vaccine strains may also be detected in the breast milk of lactating women. However, vaccine strains are not easily transmitted. This may reflect the low concentrations of virus excreted, or attenuation may result in alteration of the biological properties of the virus to make it less transmissible.

Vaccine Reactions

Rubella vaccines are generally well-tolerated. Lymphadenopathy, rash and joint symptoms may occur some 10–30 days post vaccination although they are usually much less severe than following naturally acquired infection. Lymphadenopathy is often not noticed by vaccinees and, should rash be present, it is usually faint, macular and evanescent. Joint symptoms are rare in

children of both sexes, but up to 40% of post-pubertal females may develop an arthralgia. The small joints of the hands are most commonly affected but such other joints as the wrists, knees and ankles may also be involved. Some vaccinees may experience a vaccine-induced arthritis with swelling and limitation of joint movement. Symptoms rarely persist for longer than about a week and, although recurrences may occur, this is a rare event.

An association between MMR vaccine and chronic inflammatory bowel disease and autism has been suggested by a group at the Royal Free Hospital. This generated considerable public concern about the safety of MMR and led to a decrease in vaccine uptake. However, it should be emphasized that a range of studies have failed to support the association and a number of expert groups who have examined the evidence did not support it (Institute of Medicine, 2001; Salisbury *et al.*, 2006; World Health Organization, 2003a). There is no justification for using single vaccines rather than MMR.

Vaccine Failures

Rubella is a labile virus and therefore inactivated by exposure to heat and light. Manufacturers recommend that vaccine be stored at 2–8 °C and that after reconstitution it should be kept at that temperature, protected from light and used within one hour. Failure to adhere to these instructions is the most frequent reason for vaccinees failing to seroconvert. Approximately 5% of vaccinees fail to respond for unexplained reasons, but usually respond satisfactorily if revaccinated. A few may fail to do so, or respond poorly, because they have a pre-existing low level of antibody, which is undetectable by some techniques. Seroconversion after vaccination may be assessed at about eight weeks after vaccination if required. Passively acquired antibody, whether from blood transfusion, immunoglobulin or maternally acquired, may interfere with vaccine uptake. Vaccination should be delayed for three months following blood transfusion or administration of immunoglobulin (but see below).

Contraindications

As with other live vaccines, patients who are severely immunocompromised as a result of disease or its treatment (including cytotoxic drugs, corticosteroids or radiotherapy) should not be vaccinated. Contraindications should also be extended to those with thrombocytopenia. Many patients with such minor immunodeficiencies as Di-George syndrome and children with juvenile idiopathic arthritis using methotrexate may receive MMR. Children who have had chemotherapy for cancer may be vaccinated with one vaccine dose six months after cessation of chemotherapy (Patel *et al.*, 2007). HIV-positive patients

may be vaccinated unless they are severely immunocompromised (Centers for Disease Control and Prevention, 2006; Salisbury *et al.*, 2006).

If another live vaccine is to be administered at the same time, both should be given simultaneously but at different sites (except in the case of MMR). Alternatively, both vaccinations should be separated by an interval of at least three weeks. A three-week interval should also be allowed between the administration of rubella and BCG. If the patient is suffering from a febrile illness, it is better to delay rubella vaccination. The manufacturer's leaflet should be studied carefully when patients with known hypersensitivity are to be vaccinated, since rubella vaccines contain traces of antibiotics (neomycin and/or kanamycin or polymyxin). There is increasing evidence that MMR vaccine can be given safely to children with an allergy to egg. Pregnancy should be avoided for one month after administration of rubella-containing vaccines (see below). Although passively acquired antibodies may interfere with antibody responses following rubella vaccination, if anti-D is required, it may be given at the same time, but at different sites and from different syringes. Anti-D does not interfere with vaccine-induced antibody responses (Salisbury *et al.*, 2006).

Vaccination During Pregnancy

If a pregnant woman is inadvertently vaccinated there is no indication for therapeutic abortion or prenatal diagnosis (Centers for Disease Control and Prevention, 2001b;

Salisbury *et al.*, 2006). Examination of the products of conception of rubella-susceptible women inadvertently vaccinated during pregnancy has shown that RV may be recovered from the placenta, kidney and bone marrow for up to 94 days after vaccination, indicating that vaccine virus can cross the placenta and establish persistent fetal infection. Follow-up studies on women who elected to go to term following inadvertent vaccination during pregnancy, or within three months before conception, have shown that none of 833 infants had an abnormality compatible with CRS (Table 23.6). However, a specific-IgM response provided virological evidence of congenital infection in 26 of the 708 (3.7%) babies tested. In the US study 212 of the 324 babies were born to women who had received the RA27/3 vaccine. It should be noted, however, that only 293 of 833 (35.2%) women in the combined series were vaccinated in the high-risk period between two weeks before and six weeks after conception (Table 23.6). If rubella vaccines were to induce congenital defects, it may be necessary for infection to occur during a much shorter period than following naturally acquired infection. Many of the cases included in these follow-up studies were vaccinated within the three months before conception, which is probably of minimal risk. These findings have been confirmed by a more recent prospective controlled study in Canada, where 94 vaccinated women were followed up (Bar-Oz *et al.*, 2004). In Iran, 35 infants were born to susceptible women who were vaccinated in pregnancy, two were rubella IgM-positive at birth, but none

Table 23.6 Risk of rubella vaccination in pregnancy: combined data for risk of CRS in infants born to rubella-susceptible women who received rubella vaccination during pregnancy.

Country	Live births to women receiving rubella immunization			
	Within 3 mo of conception or during pregnancy	In the high-risk ^a period	Evidence of infection ^b	Abnormalities compatible with CRS ^b
United States	324	113	6/222 (2.7%)(1–5.8)	0/324 (0%) (0–1.1)
Germany	280 ^c	155	6/280 (2.1%)(0.8–4.6)	0/280 (0%) (0–1.3)
Sweden	5	NK	0/5 (0%) (0–52.2)	0/5 (0%) (0–52.2)
United Kingdom	75 ^d	25	4 ^e /52 (7.7%) (2.1–18.5)	1 ^f /75 (0%) (0–7.2)
Brazil	149	NK	10/149 (6.7%) (3.3–12)	0/149 (0%) (0–2.4)
Total	833	293 (35.2%)	26/708 (3.7%) (2.4–5.3)	1 ^f /833 (0%) (0–0.67)

NK = not known.

^aUnited States used between one week before and four weeks after conception, United Kingdom used one to six weeks after last menstrual period. In Germany 69 cases were between one week before and four weeks after conception and 86 cases were between two weeks before to six weeks after conception.

^bNumber with evidence of infection/number tested (percentage and 95% confidence interval).

^cGisela Enders, personal communication, 2001.

^dPat Tookey, personal communication, 2000. Includes one set of twins.

^eThree of the rubella IgM-positive infants were born to mothers who were inadvertently immunized within the six weeks after last menstrual period.

^fOne rubella IgM-positive infant had a heart murmur which had resolved by 2 mo of age.

had CRS abnormalities (Hamkar *et al.*, 2006). Further evidence comes from Costa Rica (Badiilla *et al.*, 2007) and Brazil (Minussi *et al.*, 2008) where 170 and >1000 susceptible pregnant women were vaccinated with MR during mass vaccination campaigns and no congenital abnormalities were detected. The theoretical maximum risk of rubella-induced major malformations among infants delivered of susceptible mothers who were vaccinated in the high-risk period has been calculated to be 1.3% (Centers for Disease Control and Prevention, 2001b). This is less than the risk of major malformation occurring in 'normal' pregnancies (approximately 3%). The lack of teratogenicity may well be due to a lower viral load rather than a difference in teratogenicity of the attenuated viruses. One case of vertical transmission with prolonged virus shedding from the infant has been described, but the infant had no signs of CRS.

Vaccination Programmes

Rubella vaccination programmes have had a major impact in preventing CRS in countries that have implemented them.

Vaccination in the United States

Initially, the United States adopted universal childhood immunization. This programme resulted in more than 99% decrease in the number of cases of rubella reported between 1969 and 1988. However, as a number of rubella outbreaks occurred among adolescents and young adults in the late 1970s, further emphasis was placed on vaccinating susceptibles in these older age groups, which resulted in a further decline in rubella notifications. Since 1978 the decline in the incidence of CRS in the United States paralleled the decline in postnatally acquired rubella. Although there was substantial underreporting of CRS, it was anticipated that CRS was on the verge of elimination in the United States in the 1980s. However, in 1989–1990 there was a resurgence of CRS in southern California and among the Amish population in Pennsylvania. This was the result of missed opportunities for rubella screening or vaccination; many of the cases of maternal rubella could have been prevented if post-partum immunization had been carried out following previous pregnancies. Rubella was declared to have been eliminated in the United States in 2004, although occasional imported cases continue to occur (Centers for Disease Control and Prevention, 2005). The US policy results in a financial saving: cost–benefit analysis shows that the cost of rubella in an unvaccinated population is approximately 11 times more than the cost of the vaccination policy.

Vaccination in the United Kingdom

The United Kingdom and other European countries initially adopted a selective vaccination programme, in order to protect women during their child-bearing years. In the United Kingdom from 1970 to 1988, vaccination was directed towards pre-pubertal schoolgirls, and rubella-susceptible women of child-bearing age. The augmentation of the rubella vaccination programme in 1988, in which MMR vaccine was offered to preschool children of both sexes, resulted in a marked reduction in the incidence of rubella with the lowest ever number of cases reported in 1992 (Figure 23.7) (Miller *et al.*, 1993). A rubella outbreak in 1996 was largely confined to 17- to 24-year-old males who had never been offered rubella vaccine. Fortunately, transmission of rubella to pregnant women was limited since 98–99% were immune, either as a result of naturally acquired infection or vaccination as schoolgirls or post partum (Miller *et al.*, 1997). The incidence of rubella has remained low in the United Kingdom since 1996. Vaccination of susceptible women continues, but vaccination of pre-pubertal schoolgirls has been discontinued.

In order to eliminate rubella, it is necessary to monitor the efficacy of rubella vaccination programmes (Vyse *et al.*, 2002). In the United Kingdom this is done by seroprevalence studies in different age groups, including pregnant women, reporting of congenital rubella cases and terminations due to rubella in pregnancy, and monitoring uptake of MMR in children. In the United Kingdom in 1994–1995 only 2% of nulliparous and 1.2% of parous women were rubella susceptible and this, together with the decline in circulation of rubella among young children has resulted in only occasional terminations due to maternal rubella and cases of CRS (Figure 23.8) (British Paediatric Surveillance Unit, 2007; Miller *et al.*, 1997; Tookey and Peckham 1999). However, women who come to the United Kingdom from countries that do not have effective rubella vaccination programmes are more likely to be susceptible. In North London between 1996 and 1999, it was shown that susceptibility was about 5% in women born outside the United Kingdom, with the highest rates of susceptibility among women from Asia and Africa; 23.3% of Sri Lankan women in their first pregnancy were susceptible to rubella (Tookey *et al.*, 2002). Many of the recent reported cases have acquired rubella abroad. Between 1997 and 2006, 14 congenital rubella births were reported in the United Kingdom and Ireland. Nine of the mothers acquired rubella abroad and most of the cases were not diagnosed during pregnancy (British Paediatric Surveillance Unit, 2007). This emphasizes the importance of questioning women who come from developing countries when pregnant, about any illness experienced in early pregnancy, so that appropriate tests can be carried out (Department of Health, 2003).

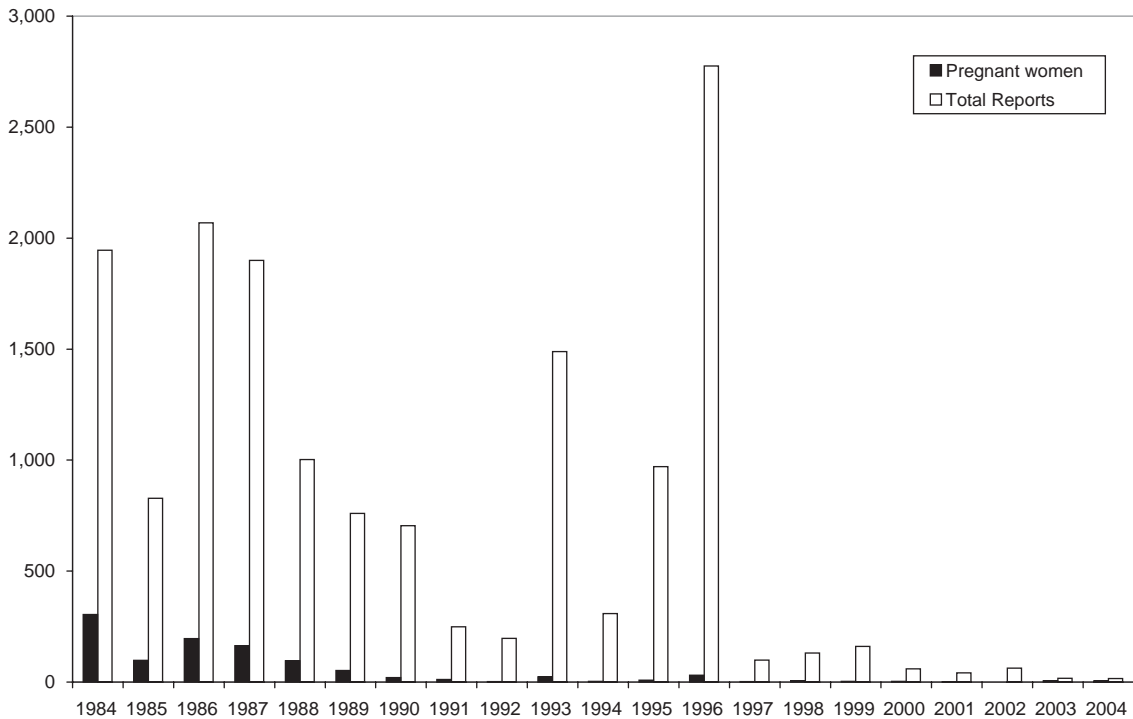


Figure 23.7 Laboratory reports of rubella in England and Wales, 1984–2004. (Source: Data kindly provided by Dr E. Miller, Health Protection Agency, UK.)

Although the uptake of MMR among children was 92% in 1995, it dropped to 84% in England and Wales in 2002, due to unnecessary public concern about the safety of MMR. This has resulted in measles outbreaks in 2002–2008 and may put unvaccinated girls at risk of rubella in the future.

Current United Kingdom recommendations can be found in ‘Immunization against infectious disease’ (Salisbury *et al.*, 2006). It is recommended that the first dose of MMR be given at 13 months of age and the second dose at any time from three months after the first dose, but is normally given before school entry. All children should have received two doses of MMR before they leave school. MMR vaccine can be given at any age. It should be offered to rubella-seronegative women of child-bearing age, and on entry into college, university, military service or prison if there is no history of MMR vaccination. It is particularly important that healthcare workers are vaccinated. MMR can be given to those with a history of infection with MMR or if they have previously received single vaccines. If there are concerns about vaccination, specialist advice should be obtained rather than leave the child or woman unvaccinated.

Other European Countries

The WHO European region consists of 52 member states, which are culturally and economically diverse. This region aims to eliminate rubella and to reduce the incidence of CRS to $<1 : 100\,000$ live births by 2010. Initially selective vaccination programmes were adopted in countries in Western Europe. Subsequently MMR has been introduced into childhood vaccination programmes and most countries now offer a second dose. In 2004, 47 (90%) countries used a rubella-containing vaccine. Sweden and Finland have already eliminated rubella by use of two dose MMR vaccination programmes. Countries such as Italy have strengthened their vaccination programmes and surveillance of rubella and CRS. Countries in Eastern Europe have undergone significant economic change, which has had an adverse effect on vaccination programmes. The incidence of rubella remains high in Europe and large rubella outbreaks have occurred recently in Romania and the Russian Federation (World Health Organization, 2005a). Nardone *et al.* (2008) reported that more than 10% of children of 2–14 years of age were susceptible to rubella in four countries, which therefore need to strengthen their vaccination programmes. Outbreaks of rubella and cases of congenital

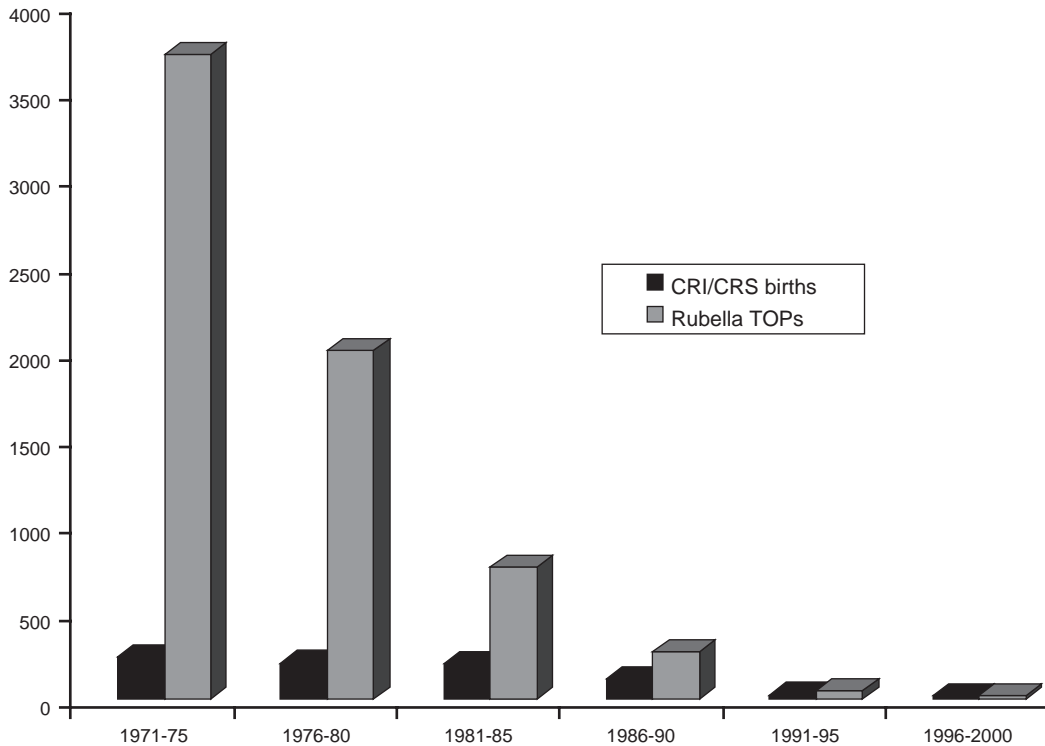


Figure 23.8 Congenital rubella births and rubella associated terminations of pregnancy (TOPs) in England and Wales, 1971–2000. CRI, congenital rubella infection; CRS, congenital rubella syndrome. (Source: From the UK National Congenital Rubella Surveillance Programme, 2003.)

rubella have also occurred in unvaccinated communities in the Netherlands in 2004–2005.

Developing Countries

Rubella vaccination strategies are unsatisfactory in many developing countries (Robertson *et al.*, 1997, 2003), and there is a considerable burden of CRS in many countries. In 2000 WHO proposed strategies for rubella elimination (World Health Organization, 2000), which are summarized

in Table 23.7. The major thrust of the proposal was to ‘piggy back’ rubella with measles (MR) or measles and mumps (MMR) vaccines. By 2006 rubella vaccination was included in national immunization programmes of 123 (64%) WHO member states, an increase of 58% since 1996 (Figure 23.9), with the most impressive coverage seen in the Pan American Health Organization (PAHO) region. By 2004, 43 of 44 countries in the PAHO region had incorporated MR or MMR into their childhood immunization

Table 23.7 Summary of recommendations from WHO for elimination of rubella and congenital rubella

<p>Countries undertaking measles elimination should take the opportunity to eliminate rubella through the use of measles–rubella or MMR vaccine in their childhood immunization programmes, as well as through campaigns. All countries undertaking rubella elimination should ensure that women of child-bearing age are immune and that routine coverage in children is sustained at 80% or above</p> <p>Countries that currently include rubella in their childhood immunization programmes should ensure that women of child-bearing age are immune and should move towards rubella elimination</p> <p>If a global measles eradication goal is established, rubella should be included</p> <p>Rubella vaccine should be considered as a priority for initiatives to introduce new or underutilized vaccines in developing countries.</p>
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Source: Reproduced from Hinman *et al.* (2002) with permission from the World Health Organization.

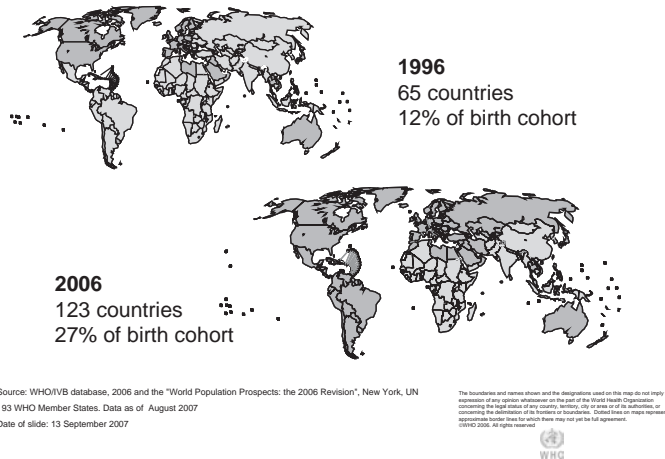


Figure 23.9 Countries using rubella vaccine in their national immunization systems. (Source: Reproduced with permission from the World Health Organization, 2003c.)

programmes. This has been combined with mass immunization of adult women or men and women in some countries, in order to avoid shifting the age of infection to susceptible adults (Badilla *et al.*, 2007; Best *et al.*, 2005; Minussi *et al.*, 2008; World Health Organization, 2003b). WHO have published guidelines for surveillance of rubella and congenital rubella (World Health Organization, 1999) and established a measles and rubella laboratory network to aid this work (World Health Organization, 2005b).

Careful consideration is needed before the introduction of rubella vaccination in countries with relatively low measles uptake rates, for example, parts of Africa with uptake rates of about 60%. Intermediate rubella vaccination rates may increase the average age of infection and consequently lead to more cases in young adults, including pregnant women, which would lead to an increase in the incidence of CRS. It is now recognized that children of both sexes and susceptible adult women should be vaccinated in order to eliminate rubella and avoid an increase in incidence of congenital rubella (Hinman *et al.*, 2002; Robertson *et al.*, 1997). The danger of low rubella or MMR vaccination coverage of children was illustrated by an epidemic of rubella in Greece in 1993, with a resulting incidence of CRS of 24.6/100 000 live births (Panagiotopoulos *et al.*, 1999).

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Mumps

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INTRODUCTION

Mumps is an acute infectious disease of children and young adults caused by a paramyxovirus. The description by Hippocrates in the fifth century BC of an epidemic disease with swelling near the ear and painful enlargement of the testis is usually cited as the first description of mumps. Over the last two centuries the disease has been reported from most countries of the world. Outbreaks in military personnel have received special attention, and mumps has been a considerable health problem for the armed forces until recently. In 1918, during the First World War, the mortality rate among the US and French armies was as high as 75/1000 men, causing as serious a problem as caused by the opposing army. Johnson and Goodpasture (1934) were able to show that the disease could be transmitted to rhesus monkeys by means of a filterable agent. Habel (1945) cultured the virus in chick embryo, and as early as in the mid 1940s both live attenuated and inactivated vaccines were tried in experimental animals and human volunteers (Enders *et al.*, 1946; Henle *et al.*, 1951).

THE VIRUS

Mumps virus is taxonomically located in the family *Paramyxoviridae* where, along with Newcastle disease virus and simian virus 5, it forms a separate genus called *Rubulavirus* (Bellini *et al.*, 1998).

Paramyxovirus virions are enveloped spherical particles with surface spikes projecting from the envelope (Figure 24.1). Inside, there is a large, helically arranged nucleocapsid. The virus is sensitive to lipid solvents and

is labile, 90–99% of infectivity being lost in 2 hours at 48 °C in protein-free medium.

Comprehensive details of the structure of mumps virus have been given by Kelen and McLeod (1977) and Strauss and Strauss (1983). The size of the virion shows considerable variation. Usually the diameter is 150–200 nm but bigger virions are occasionally seen (up to 340 nm). A 220-nm filter, which is commonly used in virological laboratories to remove bacteria from biological fluids, may, in certain conditions, retain a large proportion of viral activity. A few virions contain multiple copies of nucleocapsid.

The negative-stranded RNA is a single molecule with about 16 000 nucleotides with seven open reading frames coding for the nucleocapsid (N), the V protein and, after editing, the matrix (M), phosphoprotein (P), the large (L), the fusion (F), the haemagglutinin-neuraminidase (HN) and the small hydrophobic (SH) proteins. At least four nonstructural proteins, designated C, V, W and I, are encoded by the viral genome (Bellini *et al.*, 1998). The gene order (from the 3' end) is NP–P/V–M–F–SH–HN–L (Elango *et al.*, 1988).

NP plays a central role in the encapsidation, transcription and replication of viral RNA. It is the most abundant protein of the virion and of the infected cells. P proteins serve an important role in RNA synthesis. They are components of viral polymerase complexes and probably bind both to NP and L proteins. The M protein forms the structure which underlies the viral envelope. It is important for the assembly of virions during the replicative cycle. The HN glycoproteins agglutinate red cells of many species. Haemagglutination of avian cells is widely used as a diagnostic method for mumps. A separate part of the molecule carries neuraminidase activity, which mediates the attach-

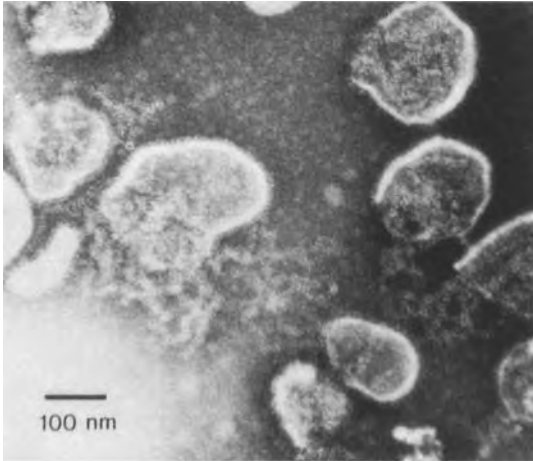


Figure 24.1 Electron micrograph of disrupted mumps virions, revealing the enveloped structure and unfolded nucleocapsid.

ment of the virion to target cells via receptor molecules of the cell which contain sialic acid. The protein also plays a role in the fusion of plasma membranes and viral envelope working together with the F protein.

F protein is actually made of two subunits, F1 and F2, which are formed as a result of proteolytic cleavage of a larger precursor molecule called F0. The F proteins play an essential role in the fusion of membranes. Antibodies reacting with F proteins inhibit haemolysis caused by mumps virus and neutralize it. The F proteins are probably crucial in determining the spread of the virus in a cell population and also in the body. The proteolytic cleavage of F0 to subunits is a host cell function. Failure of this cleavage due either to host cell malfunction or to change in the amino acid composition of F0 results in the production of non-infectious viruses.

The function of SH protein is still unknown. The SH region of the genome has been used for genetic distinction of mumps virus strains. Geographically restricted lineages have been identified which seem to be stable over time but which do not show similar progressive strain evolution as seen in influenza (Afzal *et al.*, 1997).

Antigenic and Genomic Structure of the Virus

Only a single serotype of mumps virus is known (Hopps and Parkman, 1979). It cross-reacts considerably with other members of the genus *Paramyxovirus*. In serum samples derived from mumps patients, significant rises in antibody titres are often seen against heterologous viruses, most often against parainfluenza type 1. The viral glycoproteins HN and F are responsible for the cross-reactivity, while NP and M proteins elicit a more type-specific

response. Two different antigenic preparations are used in the diagnostic laboratory. Mumps V antigen consists predominantly of HN glycoprotein, while mumps S antigen is largely NP. In mumps infections, antibodies develop first against S antigen and only later against V antigen. By measuring antibodies to the two antigens it is possible to make a specific laboratory diagnosis quite early in the course of infection (Figure 24.2).

Antibodies against HN and F proteins neutralize infectious virus but immunoblot analysis reveals that all components of the virion are immunogenic.

Though mumps virus is antigenically monotypic, genetic variation between strains has been described. In particular, the SH gene seems to contain strain-specific variability that can be used for epidemiological and diagnostic purposes. Molecular characterization can help identify transmission pathways of the virus and distinguish between wild-type and vaccine strains.

Replicative Cycle of Mumps

The virion attaches to its specific cellular receptor followed by fusion of the viral envelope with the plasma membrane. Replication occurs exclusively in the cytoplasm. The genome is arranged in a single linear sequence from where the transcription proceeds, so that the polymerase stops and reinitiates mRNA synthesis at each gene junction. Accumulation of viral proteins leads to a switch from transcription to virion maturation. Regulation of the various steps of transcription is a complex process involving different viral proteins. For viral assembly, complete nucleocapsids must be formed that are engulfed into the viral envelope by a budding process whose details are poorly understood (Bellini *et al.*, 1998).

Several details of the molecular biology of mumps still await experimental confirmation. Understanding these events at a molecular level should help us to understand the mechanisms whereby mumps virus can produce acute or chronic infection and which components are crucial for establishing protective immunity.

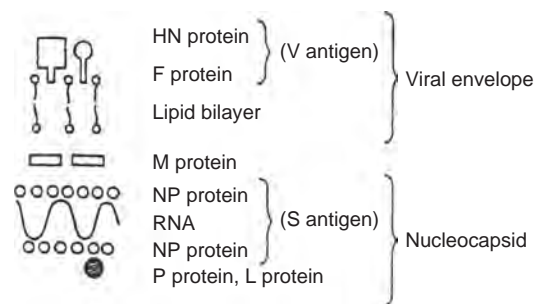


Figure 24.2 Structural components of the mumps virion.

PATHOGENESIS

Mumps is transmitted by droplet spread or by direct contact, and the primary site of replication for the virus is the epithelium of the upper respiratory tract or eye (Feldman, 1982). The first infected cells form the primary focus, from which the virus spreads to local lymphoid tissues. Further multiplication within this restricted area results eventually in primary viraemia, during which the virus is seeded to distant sites. The parotid gland is usually involved but so may be the central nervous system (CNS), testis or epididymis, pancreas or ovary. A few days after the onset of clinical parotitis, virus can again be isolated from blood, indicating that virus multiplication in target organs leads to a secondary viraemia (Figure 24.3). As a result, virus may again spread to various target organs. The clinical course of mumps virus infection is quite variable: meningitis may precede parotitis by a week, the disease may manifest with orchitis only, with a combination of pancreatitis and orchitis, and so on. Nevertheless, parotitis is the most frequent presentation, occurring in 95% of those with clinical symptoms.

Virus is excreted in the urine in infectious form starting at any time during the two weeks following the onset of clinical disease. It is not known whether virus actually multiplies in renal tissues or whether the virus is of haematogenous origin, but in most cases slight abnormalities can be detected in renal function, such as microscopic haematuria and proteinuria. Virus is also excreted in breast

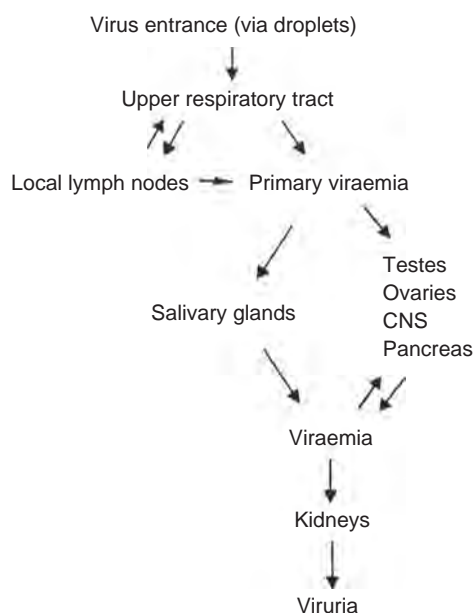


Figure 24.3 Pathogenesis of mumps infection.

milk, and it has been suggested that the virus multiplies in the lactating breast.

Interferon probably plays an important role in the pathogenesis, with the highest concentrations detected within the first few days of illness. Interferon can also be found in saliva and cerebrospinal fluid (CSF). Mumps infection results in a marked increase of specific antibodies of immunoglobulin G (IgG), IgM and IgA classes. It also stimulates a cell-mediated immune reaction which can be demonstrated *in vitro* for a few months after the infection. Which of these responses is most important for protective immunity is not known. Mumps is not a clinical problem in immunocompromised children.

Pathological changes induced by the virus in various organs are nonspecific. In the parotid glands they include serofibrinous exudate and polymorphonuclear cells in the connective tissue and within the ducts. The ductal cells show degeneration. In infected testes the changes may be more pronounced, with marked congestion and punctate haemorrhages. Similar changes have been described in pancreatic tissue.

In meningitis, electroencephalograms usually show little alteration, suggesting the lesions are located predominantly in the meninges. In the rare cases where encephalitis occurs as a complication of mumps, lesions are found in the spinal cord and brain tissue. It seems likely that the lesions are produced from the combined effect of direct viral cytopathic effect and immunopathological destruction of CNS cells. Involvement of the CNS may be associated with symptoms compatible with paralytic disease and deafness but the exact pathogenesis is not known.

On several occasions prolonged involvement of the CNS after clinically manifested mumps meningoencephalitis has been described (Julkunen *et al.*, 1985). Whether this reflects a true replication of virus within the brain tissue over an unusually long period or whether the clinical condition is a result of prolonged immunological damage is not known.

Mumps virus can multiply *in vitro* in human pancreatic islet cells (Parkkonen *et al.*, 1992). The virus can also grow in cell cultures from human joint tissues, causing chronic infection associated with an incomplete replicative cycle (Huppertz and Chantler, 1991).

CLINICAL PICTURE

Classic Parotitis

The incubation period is 16–18 days but may vary from 14 to 25 days. Parotid swelling appears in 95% of those who develop clinical disease (Figure 24.4). The rate of subclinical infections varies somewhat with age but is, on



Figure 24.4 A patient with mumps parotitis.

average, 30%. In a small proportion the symptoms may resemble only mild respiratory tract illness. Patients with classic mumps develop enlargement of one parotid gland which, in 75% of cases, is followed one to five days later by enlargement of the contralateral gland. For one to two days prodromal symptoms may dominate; these include malaise, myalgia, headache and low-grade fever.

Vaccination status of the affected population may influence the clinical presentation of the disease by shifting the prevalent age distribution or affecting the course of the disease by pre-existing immunity. In an outbreak affecting both vaccinated and unvaccinated individuals at all age groups, 57% of the clinically affected people were males and 43% were females, 58% had fever, 57% general malaise, 39% headache, 1% rash. Meningitis was seen in 1%, and orchitis in 7% of the male cases. Complications seem to be less common in vaccinated children (6% vs. 1%; Visser *et al.*, 1998).

The parotid involvement initially causes local tenderness and sometimes earache, and a few days later clinically apparent swelling. This involves the entire gland, including the area in front of the ear lobe. The orifice of the glandular duct becomes red and oedematous, and direct pressure on the glands yields only clear fluid, if anything. Submandibular and sublingual glands are occasionally also involved but practically never without parotid infection.

The parotid swelling starts to subside after four to seven days. Virus shedding into saliva begins a couple of days before clinical parotitis and ends seven to eight days later. Glandular swelling is followed by constitutional

symptoms such as fever, which seldom exceeds 40°C, and pain. These diminish when the swelling has reached its maximum and the general feeling of the patient starts improving.

Recovery is usually rapid. Sometimes presternal oedema and sublingual and laryngeal swelling may alter the classic picture. This is thought to result from lymphatic obstruction in the cervical and mandibular areas. Severe anorexia and photophobia have been described.

Complications

The course of mumps infection may be extremely variable, as described in the section on pathogenesis. Diseases such as orchitis or pancreatitis could therefore be regarded as systemic manifestations of mumps rather than true complications. However, since classic parotitis is so characteristic and clinically dominating, all other clinical features are often regarded as complications. For a further description of the clinical aspects of mumps virus infection see Marcy and Kibrick (1983) and Christie (1974).

Meningitis

Aseptic meningitis is a common complication of mumps patients. As many as 50% may show pleocytosis of the CSF and clinical signs are seen in 1–10%. Mumps meningitis can be associated with parotid swelling but can also occur in its absence, thus causing problems in differential diagnosis. Mumps virus used to be the most common virologically confirmed causative agent of aseptic meningitis before general vaccinations were started. It is two to three times more frequent in male patients than in female and the age distribution is the same as for mumps parotitis.

Symptoms are indistinguishable from other types of aseptic meningitis and they can start from one week before parotid swelling to three weeks after it. Sometimes the clinical picture is more severe, 6–18% of hospitalized patients having symptoms resembling encephalitis (see below).

Laboratory examination of CSF reveals pleocytosis (usually <500 lymphocytes/ml, sometimes up to 1000), normal or slightly decreased sugar. Virus can be isolated from the CSF during the first two to three days after the onset. Later, intrathecal synthesis of mumps antibodies can be demonstrated. Symptoms of meningitis subside 3–10 days after onset, and recovery is complete.

Encephalitis

Clinical features suggesting encephalitis are convulsions, focal neurological signs, movement disorders and changes in sensory perception. Sometimes polio-like paralysis

ensues and even fatalities have been reported. Probably both direct viral invasion and allergic inflammatory reactions lie behind the nervous tissue damage. The incidence of encephalitis is about 1/6000 cases of mumps.

Other Neurological Manifestations

Before vaccination, mumps used to be one of the leading causes of hearing loss in children and young adults. According to a study from military forces in Finland, 4% of mumps cases among military recruits were associated with deafness or significant hearing loss (Vuori *et al.*, 1962). In most cases the condition was transient but permanent dysfunction ensued in a few. Hearing problems did not correlate with meningitis. The incidence of permanent hearing loss has been estimated to be 1/15000 cases of mumps. Sometimes Ménière's disease is a late complication.

Orchitis and Oophoritis

After puberty the incidence of orchitis is 20–30%, and in 20–40% of cases there is bilateral involvement. Symptoms are acute, with pain and tenderness accompanied by fever, nausea and vomiting. The testis may enlarge three- to fourfold within four to five days. There is associated epididymitis in 85% of cases. Late sequelae include atrophy of testicular tissue, which may lead, in cases with bilateral disease, to infertility. Late sequelae of pre-pubertal orchitis are not known.

In females oophoritis is evident in approximately 5% of post-pubertal cases. No correlation with infertility has been recorded. The condition may present with pelvic tenderness and cause problems of differential diagnosis from acute appendicitis.

Pancreatitis

The exact frequency of pancreatitis is difficult to determine, but figures as high as 5% have been proposed. Clinical signs, such as epigastric pain and tenderness accompanied by vomiting, may lead to the diagnosis, which can be confirmed by serum amylase determination. Although the clinical picture may be quite dramatic, the outcome is almost invariably good.

Other Manifestations

Sometimes the lacrimal, thyroid or prostate glands become affected by mumps virus, producing unusual clinical presentations.

Arthralgia or monoarthritis, involving a large joint, are complications of mumps which may develop about two weeks after parotitis. They are most frequent in young male adults.

Myocarditis can usually be found only upon electrocardiographic examination. In 10–15% of all mumps patients typical changes are seen, with no late sequelae.

Transient renal dysfunction is a frequent feature of clinical mumps. In very rare instances severe nephritis and thrombocytopenia may ensue, with a fatal outcome on occasion.

If a pregnant woman contracts mumps she has an increased risk of abortion. This is thought to be due to hormonal imbalance caused by viral infection. No evidence of an increased risk of congenital malformations has been documented in humans, although paramyxoviruses cause CNS abnormalities of the developing fetus in monkeys and mumps virus can cause abnormalities in the developing chick lens.

Endocardial fibroelastosis, a rare condition affecting the inner lining of the heart, is associated with a positive skin test for mumps antigen, but a link between mumps virus and the condition is doubtful.

LABORATORY DIAGNOSIS

Virus Antibody Assays

Different serological tests have been used to demonstrate a rise in the specific antibody titres or the presence of IgM class antibodies. Complement fixation test employing S and V antigens prepared from the virions was earlier frequently used as the most common test. However, due to problems in reagent stability, this technique has been replaced in many laboratories by enzyme immunoassay (EIA) tests. EIA antibody levels correlate with levels of neutralizing antibodies, suggesting that EIA could also reflect the immune status of the individual (Leinikki *et al.*, 1979). However, the extent of cross-reactivity from exposure to closely related paramyxoviruses may cause problems in interpretation of the results in individual cases. The specificity of IgM antibody assays is greatly enhanced by using IgM capture techniques, where IgM class antibodies are separated from other serum constituents in an additional incubation step (Morgan-Capner, 1983).

Haemagglutination inhibition and neutralization antibody assays can be used as supplemental tests for both diagnostic cases and in immunity studies. Other tests, such as haemolysis in gel, can also be applied.

Virus Detection

Mumps virus can be isolated from clinical samples using cell cultures or embryonated eggs. Both saliva and urine can be used as samples for diagnostic or epidemiological investigations. Mumps antigen has also been demonstrated from saliva samples by immunofluorescence

or EIA techniques, allowing easy processing of large numbers of samples. Viral RNA can be detected by polymerase chain reaction (PCR) from oral fluid and used for diagnostic and epidemiological investigations.

EPIDEMIOLOGY AND CONTROL

Mumps is a disease of childhood. In unvaccinated populations the highest incidence is in children between five and nine years of age. The disease is somewhat less contagious than other childhood diseases, such as measles and varicella, and quite a number of children seem to escape the infection before puberty and even beyond. The proportion of seronegatives among medical students was less than 10% in a study of an unvaccinated population. According to a survey in the United States, one-tenth of the population had mumps during each of the first five years of life, 74% had had it by the age of 10 and 95% by 20 years of age (Feldman, 1982).

General vaccinations have changed the epidemiological pattern profoundly. Prior to vaccination, mumps used to be common in most urban areas, while in some less densely populated areas it caused widespread epidemics at two to four year intervals. A few descriptions of outbreaks in isolated areas have been published where the affected population had no pre-existing immunity. As can be expected, age as such did not protect; however, the proportion of subclinical infections increased with age, with the exception of the youngest children (two to three years of age), in whom subclinical infections were also very frequent. Up to 90% of infections at the age of 10–14 years were associated with clinical symptoms, while practically all infections were subclinical beyond 60 years of age. Although the clinical symptoms are usually more severe in adolescents and young adults than in children, the frequency of complications follows closely the frequency of mumps in general. Two well-known exceptions are orchitis and oophoritis, which have a much higher frequency after puberty than before, and meningoencephalitis, which is two to three times more common in males than in females.

In temperate zones of the northern hemisphere a clear-cut seasonal variation is evident. From June to September the number of reported cases is on the average less than one-third of the figures from January to May, according to a survey of 12 years and more than 150 000 cases. The peak months were February and March. No such seasonal variation is reported from tropical countries.

Only one serotype of mumps virus exists and one would expect a life-long immunity after natural infection, yet reinfections have been reported in up to 1–2% of cases. This could indicate that mumps infection is

not able to induce a lifelong protective immunity. Another explanation is that other microbes cause similar clinical pictures: in a recent study of clinically suspected mumps cases in a low prevalence population, a virus other than mumps was the causative agent in about half of the cases. Viruses such as Epstein–Barr virus (EBV), adenovirus, parainfluenza and even enteroviruses were found (Davidkin *et al.*, 2002, personal communication). A third explanation may come from divergent genomic lineages of mumps virus that have been identified by comparing RNA sequences of strains from different outbreaks (Ströhle *et al.*, 1996).

Vaccinations Against Mumps

Control of mumps by immunization can be very successful. In the United States, the number of cases notified to the Centers for Disease Control and Prevention has dropped from over 150 000 annual cases to around 1500 cases in 20 years (Centers for Disease Control and Prevention, 1995). In Finland, with a very high childhood vaccination coverage, the endemic disease has been eradicated (National Public Health Institute, 2001). Large outbreaks of mumps in the neighbouring Russian territories have not led to local outbreaks in Finland in spite of frequent travel across the border.

However, vaccinated populations have also encountered outbreaks of mumps in recent years (Briss *et al.*, 1994; Dias *et al.*, 1996; Visser *et al.*, 1998). These outbreaks have affected mostly young adults in several European countries and the United States. Victims have been unvaccinated or received only one dose of measles, mumps and rubella (MMR) vaccine (Savage *et al.*, 2005). Analysis of the genomic sequence has shown that multiple lineages of virus have co-circulated, suggesting lack of immunity due to either vaccine failures or low vaccination coverage.

A strain called Jeryl Lynn has been used in most vaccinations worldwide since the 1970s. Vaccines containing this strain have proved to be effective in protecting against clinical disease and have even led to eradication in some countries (Peltola *et al.*, 1994). Serious complications are extremely rare: less than one child/million vaccines is affected by aseptic meningitis (Patja *et al.*, 2000).

Another strain called Urabe was introduced as a vaccine in the 1980s. Unfortunately, it proved to be associated with increased frequency of CNS complications and has been withdrawn in most countries. Another vaccine strain, called Leningrad–Zagreb attenuated mumps virus strain, was recently found to be associated with an increase of aseptic meningitis and clinical mumps after mass vaccination campaigns in Brazil (da Cunha *et al.*, 2001). The risk for aseptic meningitis was approximately one case/10 000 doses of vaccine; for mumps it was one case/300 doses.

Table 24.1 Likely problems eliminated by mumps vaccine (Finland 1971 vs. 1997).

	Cases/year 1997	Cases/year 1971 ^a
Number of notified cases	0	22 980
Absent from school or work (person years)	0	650
Meningitis	0	6 400
Encephalitis	0	160
Orchitis	0	1 600
Bilateral	0	500
Male sterility	0	125
Mastitis	0	1 800
Temporary hearing problems	0	920
Permanent hearing problems	0	25
Thyroiditis	0	250
Pancreatitis	0	80
Myocarditis	0	30

^aThe year 1971 was an epidemic year. Finland has a population of about 5 million.
Source: Reproduced by permission from Peltola *et al.* (1999).

Leningrad strain has also been detected in cases of aseptic meningitis in St Petersburg recently (Davidkin *et al.*, personal communication).

A strain used widely in vaccines, the Rubini strain, has been found to give insufficient protection against wild mumps virus and outbreaks of mumps in previously vaccinated populations have been described (Germann *et al.*, 1996; Tabin *et al.*, 1993).

Even though general vaccination of children against mumps virus has led to significant public health achievements (Table 24.1), mumps remains a challenge to clinical virology. With lowering vaccine coverage among children and variations in the efficacy of the vaccine strains, individual cases and even outbreaks are probable. In particular, individual cases without an obvious epidemiological link may pose significant diagnostic problems and emphasize the importance of virological laboratory diagnosis.

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Enteroviruses

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INTRODUCTION

The enteroviruses belong to a genus of the family *Picornaviridae*. At least 66 enterovirus serotypes have been isolated from humans and around 30 new candidate enterovirus types have been identified in recent years. Their normal site of replication is the intestinal tract, where infection may often be clinically inapparent, or result in a mild, nonspecific illness. However, in a proportion of cases the virus spreads to other organs, causing severe illnesses, the characteristics of which are often typical of individual enterovirus types. Poliomyelitis is one of the most severe diseases caused by an enterovirus, but members of the genus are also implicated in aseptic meningitis, encephalitis, myocarditis, rashes and conjunctivitis. They may cause particularly severe disease in neonates. This chapter describes the virological properties of enteroviruses, and the pathogenesis and clinical aspects of the diseases that they cause. Prevention is, so far, limited to poliomyelitis. Although numerous agents with anti-picornaviral activity have been described, none has thus far become available as chemotherapeutic agents.

THE VIRUSES

Viruses of the *Picornaviridae* show similar morphological, structural and molecular properties and replication strategies. They are currently divided into nine genera by the International Committee for the Taxonomy of Viruses (ICTV): the rhinoviruses, which are one of the causative agents of the common cold (Chapter 20); the enteroviruses, which are closely related to the rhi-

noviruses; the parechoviruses, a recently recognized group which currently consists of four types, two of which were formerly classified as enteroviruses but are now known to be genetically and biologically distinct; the cardioviruses, which cause diseases of mice and other animals; the aphthoviruses, which are causative agents of foot-and-mouth disease of ruminants; the hepatoviruses, a genus encompassing hepatitis A viruses (Chapter 12); the teschoviruses (formerly porcine enteroviruses) and two genera which currently contain a single species representative: the kobuviruses and erboviruses (Minor *et al.*, 1995). The human enteroviruses include the polioviruses (PVs), group A coxsackieviruses (CVA) and group B coxsackieviruses (CVB), enterocytopathic human orphan (echo) viruses (ECVs) and additional numbered enterovirus serotypes, designated EV68–71. These serotypes were originally defined and differentiated on the basis of homologous seroneutralization. Approximately 30 additional enterovirus types have been identified in recent years, defined on the basis of nucleotide sequence similarity rather than serological neutralization. These types have been provisionally designated EV73–101, although they have yet to be formally recognized by the ICTV.

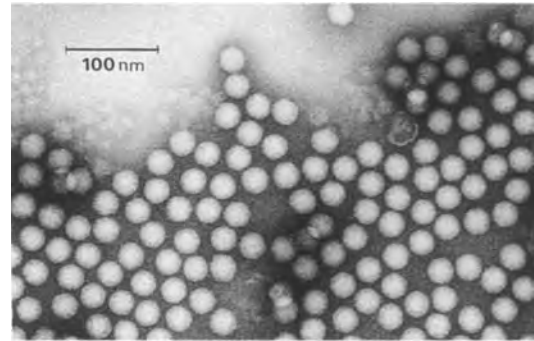
The phylogenetic approach to classification allows the human enteroviruses to be grouped into four major groups or species as described later. Enterovirus types differ in their cultural characteristics, antigenic properties and certain features of their replication cycle, such as the receptor sites by which they gain access to the host cell. In all cases the normal habitat and primary site of replication of the virus is thought to be the intestinal tract.

Physical Characteristics of the Virus Particle

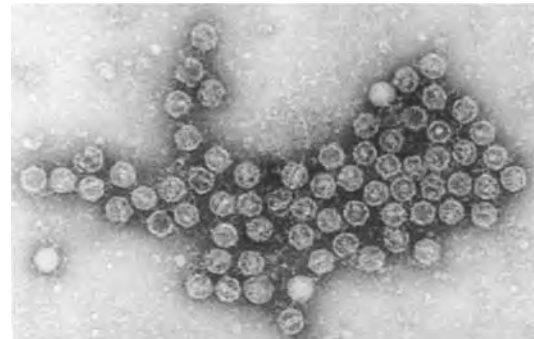
The enterovirus virion is a largely featureless, symmetrical particle, approximately 27 nm in diameter when examined electron microscopically (Figure 25.1a). Non-infectious empty capsids, unlike virions, are penetrated by stain, revealing the virion shell to be approximately 2.5 nm in thickness (Figure 25.1b). The essentially spherical appearance of the particle is consistent with icosahedral symmetry, and this has been broadly confirmed by particle shadowing, which produces pointed and blunt shadows (Figure 25.1c,d). The sedimentation coefficient of the intact virus on centrifugation is 155–160S, while that of the empty capsid is 70–80S. The buoyant density of the virion in caesium chloride gradients is 1.34 g ml^{-1} . A particle with the chemical composition of an enterovirus virion would be expected to have a density of 1.47 g ml^{-1} if freely permeable to caesium ions; the low density of the enteroviruses implies that the virion is essentially impermeable, consistent with the failure of electron microscopic stains to penetrate infectious particles (Figure 25.1a). In the past, the behaviour of the viruses on isopycnic centrifugation has been used as a criterion for differentiating enteroviruses from rhinoviruses and aphthoviruses, both of which have a higher buoyant density in caesium chloride. The enteroviruses are stable to acid pH, also unlike the rhinovirus and aphthoviruses, which are inactivated by brief exposure to pH 2. This property has also been used as a criterion for the identification of a picornavirus as an enterovirus.

Biochemical Structure of the Virus Particle

The infectious particle consists of a capsid made up of 60 copies of each of four viral proteins (VP1–4), arranged with icosahedral symmetry around a single-stranded positive (messenger)-sense RNA genome of approximately 7300 bases. Like many eukaryotic mRNA molecules, the genomic RNA terminates in a sequence of 40–100 adenylic acid residues at the 3' end (the poly(A) tract). At the 5' end, however, it bears a small virally encoded protein (VPg), approximately 22 amino acids long, covalently linked to the RNA by the hydroxyl group of a tyrosine residue. The virion is believed to be entirely devoid of carbohydrate and lacks a lipid membrane, although, as described below, lipid molecules form an integral part of the structure. The particle has a relative molecular mass of 8×10^{-6} , of which the RNA provides 32% and the protein 68% by weight. The four principal structural proteins of the virion are of molecular mass approximately 30 000 (VP1), 27 000 (VP2), 24 000 (VP3) and 7000 (VP4). VP2 and VP4 are formed by the cleavage of a precursor protein, VP0, as the last stage in the maturation of a virus



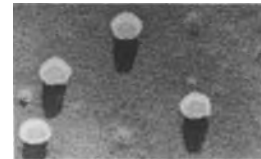
(a)



(b)



(c)



(d)

Figure 25.1 Electron micrograph of poliovirus, negatively stained with 4% sodium silicotungstate: (a) whole virus particles (D antigen) approximately 30 nm in diameter; (b) empty capsids showing penetration of stain; (c) shadowed virus (print reversed to give black shadows) producing pointed shadows; (d) shadowed virus (print reversed to give black shadows) producing blunt-ended shadows.

particle, probably in an autocatalytic process resulting from the encapsidation of the nucleic acid genome.

The absence of a lipid layer has made it possible to obtain crystals suitable for X-ray diffraction studies, and the molecular structures of poliovirus (Hogle *et al.*, 1985) and other picornaviruses have been resolved, such that the interactions involved in maintaining the structural features of the infectious virion are now clear. The three

largest virion proteins, VP1, VP2 and VP3, have a very similar core structure in which the peptide backbone of the protein loops back on itself to form a barrel of eight strands held together by hydrogen bonds (the β -barrel). This core forms a wedge shape, and the amino acid sequences between the sequences forming the β -barrel and the sequences at the N- and C-terminal portions of the protein contain elaborations which include the main antigenic sites involved in the neutralization of viral infectivity.

The proteins are arranged with icosahedral symmetry, with VP1 molecules at the pentameric apices of the icosahedron, orientated such that the pointed end of the wedge-shaped protein points towards the apex. The other two proteins, VP2 and VP3, alternate about the centre of the triangular face of the icosahedron (the threefold axis of symmetry). There are extensive interactions between the three large proteins, and also with the fourth protein, VP4, which is internal. In particular, the N-terminus of VP1 lies under VP3, and the N-terminus of VP3 under VP1. The apical structure formed by VP1 is separated from the plateau formed by VP2 and VP3 by a cleft or canyon; the peak of the VP1 structure is 16.5 nm from the virion centre, while the plateau made up of VP2 and VP3 is approximately 15 nm from the centre. The base of the cleft is of the order of 11 nm from the centre, and it has been postulated that this cleft may contain the regions of the virus to which the cellular receptor attaches. The interactions between the component proteins that form the most stable unit of the structure is a pentamer made up of five copies each of VP1, VP2, VP3 and VP4; it seems likely that this is the basic unit from which the virus is assembled. A diagrammatic representation of the three-dimensional structure of the capsid proteins of poliovirus type 1 is shown in Figure 25.2.

In addition to the protein components of the virus, lipid molecules are also found in the structure. Myristic acid is found covalently attached to the N-terminus of the smallest capsid protein, VP4. The aliphatic chain penetrates the apex of the icosahedron and it is possible that this forms a structural support for the virion. Myristic acid has been found attached to virion proteins in other viruses, including retroviruses and rotaviruses. A second lipid moiety, not yet identified, is present in the structures of PV1 and 3. It has a 16-carbon aliphatic chain which lies in the pocket formed by the β -barrel of VP1. The other end of the molecule has not been observed. Rhinovirus 14 has no such insert, and it is not yet clear how general a feature of enteroviruses this lipid molecule is. However, certain antiviral compounds insert into the corresponding region of rhinovirus 14 and, in so doing, prevent uncoating. It is therefore possible that the lipid plays a part in uncoating or assembly of enteroviruses.

Antigenic Structure of the Virus

The antigenic properties of the enteroviruses provide one of the classical methods of differentiation. In most cases serotype-specific antisera neutralize only virus isolates or strains of the homologous serotype, but this is not always so. For every picornavirus studied in detail, it has been shown that empty capsids prepared under appropriate conditions present major antigenic determinants differently from those of the infectious virus. Moreover, the infectious virus can be readily denatured by relatively mild conditions, such as treatment with ultraviolet light, heating to 56°C for 10 minutes or attachment to a plastic surface in solid-phase enzyme immunoassays (EIAs). The infectious particle is said to express D or N antigenic character, and the empty capsid or denatured virus C or H antigenic character. C antigen is less specific to a particular virus than is D antigen. Thus, there is evidence for cross-reaction of C antigen-specific monoclonal antibodies with PV1, 2 and 3, whereas cross-reaction by neutralizing monoclonal antibodies or those reacting with D antigen is rare. Monoclonal antibodies able to neutralize both PV1 and 2 have been reported (Uhlir *et al.*, 1990) and priming for a secondary response to PV1 or 3 by previous infection with PV2 is documented. Similarly, there is evidence for cross-reaction of ECV 11, 16 and parechovirus 1 with poliovirus with appropriate C-specific antibodies. In addition, some epitopes present on VP1 appear to be conserved among most or all human enteroviruses, and monoclonal antibodies to such epitopes have been used as pan-enterovirus detection reagents (Trabelsi *et al.*, 1995). The difference between particles expressing D and C antigen is due to the conformation of the proteins, rather than to the loss of a protein; cross-reactive epitopes are internalized in the infective virion, but become surface-exposed and thus able to bind antibody following denaturation. Thus, methods based on virus neutralization by antibody are required to determine serotype-specific humoral immunity, while solid-phase EIA or immunoblot methods detect both type-specific and cross-reactive antibody (Muir and Banatvala, 1990).

The antigenic structures involved in the neutralization of picornavirus infectivity have been the subject of much study and have been clarified by the resolution of the molecular structures of PV and rhinovirus and representatives of the other genera outlined above. Neutralizing epitopes have been identified by the isolation of antigenic variants resistant to monoclonal antibodies which are able to neutralize the parental strain, and then characterized by sequencing the genomic RNA. This has revealed four major sites to which neutralizing antibodies bind, formed from a number of exposed surface loops. For PV3 they include: (i) a sequence of VP1 about one-third of the way in from the N-terminus, involving residues 89–100, together

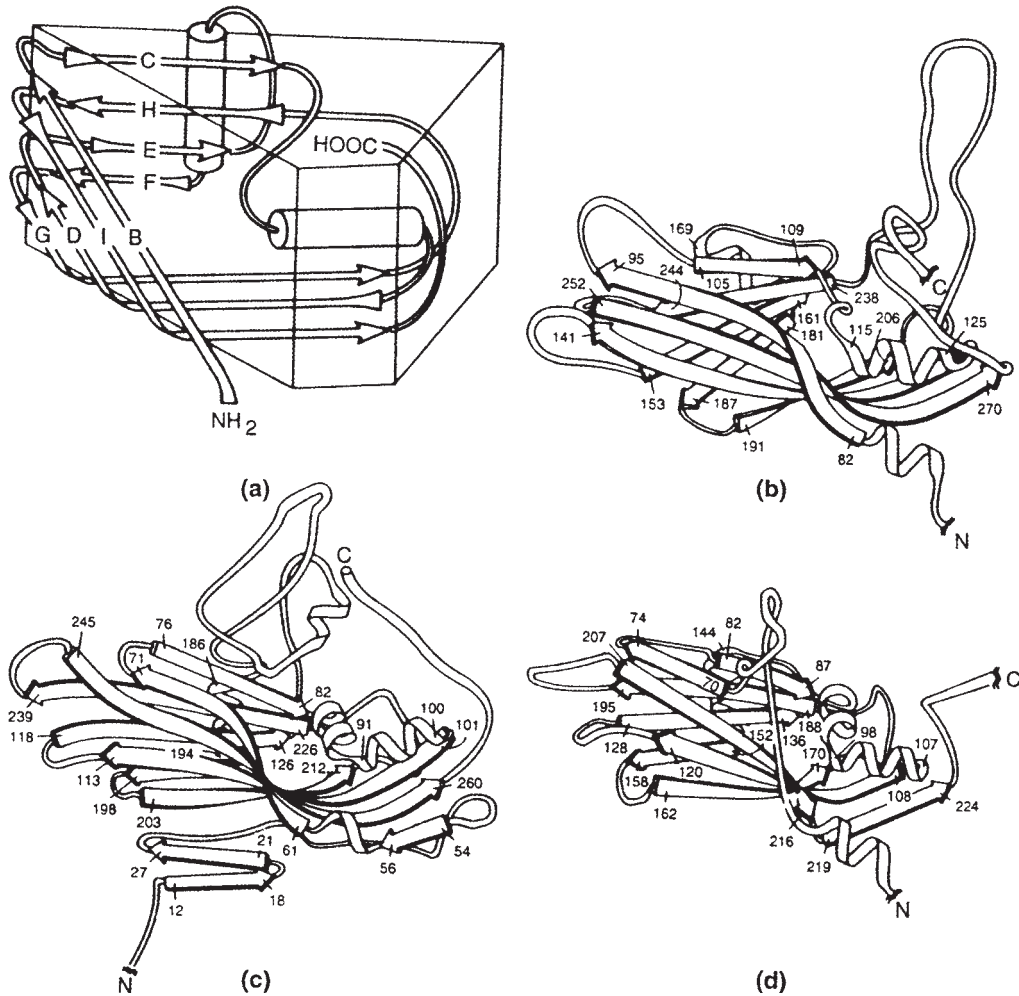


Figure 25.2 Three-dimensional structure of the capsid proteins of poliovirus. The numbers indicate positions of amino acid residues: (a) core structure common to capsid proteins; (b) VP1; (c) VP2; (d) VP3. Reprinted with permission from Hogle *et al.* (1985) The three dimensional structure of poliovirus at 2.9 Å resolution. *Science*, **229**, 1358. American Association for the Advancement of Science.

with sequences from three other loops from VP1, including amino acids 142, 166 and 253; (ii) a complex site involving residues 220–222 of VP1 and 160–170 of VP2 and the C-terminus of VP2; (iii) a complex site involving residues 286–290 of VP1 and 58–60 of VP3 (Minor, 1990). The fourth site involves residues in different pentamers and is therefore only found in the intact virion, while the other sites are also found in some viral subunits. For PV1, the site located at residues 89–100 of VP1 has been implicated in virus neutralization, although to a far lesser extent than for PV3, where the homologous region is the principal target for neutralizing antibodies in sera from immunized animals. The site involving VP3 is the next most common target of antibodies, including

most of the antibodies showing extreme strain specificity for poliovirus, while the site involving VP2 is the least common.

The antigenic structure of PV2 has been less well characterized. The site to which cross-reactive antibodies bind appears to involve sequences around 200–210 of VP3 and 239–245 of VP2, and may be linked to the strain-specific site involving VP1 and VP3 (Uhlrig *et al.*, 1990).

Cellular Receptor Sites

Enteroviruses attach to and enter cells by specific cell surface receptors. This was first shown for poliovirus by

the observation that purified genomic RNA was infectious for rabbit cells, whereas the virus itself was not. Competition experiments between different viruses have shown, for example, that all three poliovirus serotypes utilize the same cellular receptor, which is distinct from that for CVB. This phenomenon has been confirmed in a number of cases by the isolation of monoclonal antibodies which specifically block the attachment of certain types of virus by reacting with the host cell rather than with the virus (Minor *et al.*, 1984).

The enteroviruses and picornaviruses in general are responsible for a range of diseases with different target organs. These include the heart (CVB), nerve tissue (PV), liver (hepatitis A virus) and others, as well as the intestinal tract. It is possible that the expression of the receptor sites in specific tissues plays a part in the tropism of the virus, although it is unlikely to be the only factor. This may suggest a novel chemotherapeutic approach for enterovirus infections using synthetic receptor analogues.

The cellular gene encoding the poliovirus receptor has been identified as a previously unknown three-domain protein of the immunoglobulin superfamily termed CD155 (Mendelsohn *et al.*, 1989). The terminal domain is required for activity. Transgenic mice carrying the human *CD155* gene (Koike *et al.*, 1991; Ren *et al.*, 1990) are susceptible to poliovirus infection, with pathology similar to that observed in primates; the animals are not infectable by the oral route. Expression of the receptor occurs in all tissues unless the gene is inserted under the control of a tissue specific promoter, yet replication of the virus is highly restricted. There is evidence that the innate immune response plays a role in the tissue distribution and pathogenesis as when the transgenic mice are crossed onto a strain of mouse lacking an alpha/beta interferon (IFN- α/β), response replication is more general (Ida-Hosonuma *et al.*, 2005).

Mouse cells in culture are not normally susceptible to infection with poliovirus or most other human enteroviruses. When stably transfected with human CD155, however, they are rendered sensitive, and can thus be used to identify poliovirus in the presence of other enteroviruses. This is of value in clinical studies related to the programme to eradicate poliomyelitis, as described later.

Two major CVB receptors have been identified; one is decay-accelerating factor (DAF; CD55), a cell surface protein involved in the complement pathway (Shafren *et al.*, 1995). The second CVB receptor is also used by some adenoviruses and is termed the coxsackie-adenovirus receptor (CAR), a transmembrane cellular adhesion molecule localized to epithelial tight junctions which contains two immunoglobulin-like domains, and is expressed preferentially in brain, heart

and pancreas (Bergelson *et al.*, 1997). Binding of CVB to DAF triggers DAF-mediated cell signalling pathways resulting in actin rearrangements which permit virus movement to tight junctions. Here virus-CAR interaction leads to conformational changes in the viral capsid which precede viral entry and RNA release. Sequential interaction with DAF and CAR thus results in cellular changes even before the virus enters the cell, and ultimately allows CVB to cross the epithelial barrier. (Coyne and Bergelson, 2006).

A number of receptors for other enteroviruses have also been identified. The receptor for ECV1 has been identified as the integrin VLA-2, a known surface molecule (Bergelson *et al.*, 1992). DAF is also a receptor site for many ECVs (Bergelson *et al.*, 1994) and EV70 (Karnauchow *et al.*, 1996). CVA10, 15, 18 and 21 employ ICAM-1, as does the major rhinovirus group. CVB3 also uses nucleolin, and CVA9, $\alpha_v\beta_3$ -integrin.

Strategy of Replication

The genome of the virus is a single positive-strand RNA molecule which acts as a messenger RNA for the synthesis of the viral proteins found in infected cells. The genome can be divided into three regions, a 5' nontranslated region (NTR) of about 750 bases, followed by a large open reading frame encoding a polyprotein which comprises most of the genome, then a second short 3' NTR terminated by a poly(A) tail. The open reading frame can be divided into three regions, P1-P3, which correspond to the primary cleavage products of the polyprotein (see below). The P1 region encodes VP1-4, while the P2 and P3 regions encode nonstructural proteins necessary for virus replication, as detailed below. The genome arrangement is summarized in Figure 25.3. The NTRs are highly conserved, reflecting their importance in virus replication.

The 5' NTR of eukaryotic messenger RNA usually terminates in a methylated guanosine cap, which is important in binding ribosomes and thus translation of the RNA into protein. Thus, most eukaryotic messenger RNA molecules require a free 5' terminus, and initiation of translation internally is not possible. However, it has been shown that the 5' NTRs of poliovirus and other picornaviruses contain an internal ribosomal entry site (IRES), which enables ribosomal binding in the absence of a free 5' capped terminal structure (Pelletier and Sonnenberg, 1988). In fact, poliovirus infection results in the cessation of cap-dependent translation. The mechanism of internal initiation involves binding of cellular proteins, including the eukaryotic translation initiation factors, a pyrimidine tract-binding protein and poly(rC)-binding proteins to the IRES (Hellen *et al.*, 1993). There is evidence that additional cell type-specific proteins also interact with the IRES, and the ability of a given IRES to interact with such

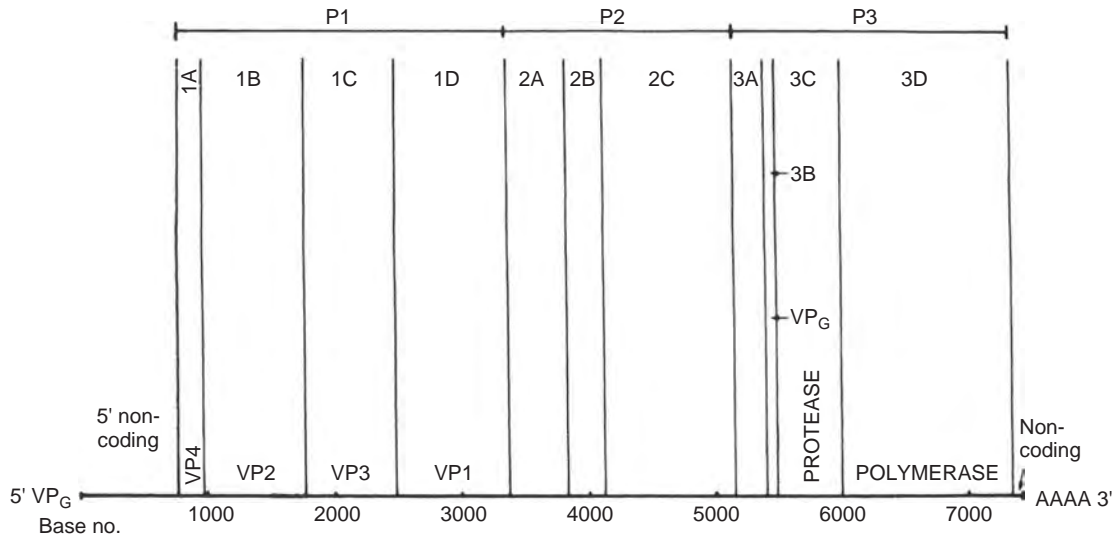


Figure 25.3 Organization of the genome of poliovirus, a typical enterovirus showing the two noncoding regions and the location of the regions coding for the various virus proteins. In the formal nomenclature (Rueckert and Wimmer, 1984), the P1 region encodes the structural capsid proteins, and the P2 and P3 regions encode nonstructural proteins found only in infected cells.

noncanonical translation initiation factors may in part determine the susceptibility of a given cell type to infection with a particular enterovirus (Malnou *et al.*, 2002), since it is known that the availability of cellular receptor proteins is not the sole determinant of tissue tropism.

The viral proteins are translated as a single polyprotein. This precursor protein includes two sequences which act as proteolytic enzymes, and these digest the polyprotein at specific positions during the course of translation. The first (P2A) acts to cleave the structural (P1) from the nonstructural (P2–3) proteins (Figure 25.3), while the second completes all the other processing of the protein, except the final maturational cleavage of VP0 to VP2 and VP4. The nonstructural proteins include proteases (2A, 3C and the intermediate cleavage product 3CD), a polymerase (3D), nucleotide triphosphatase (2C) and the small protein VPg (3B), which is covalently attached to the 5' terminus of both positive- and negative-sense viral RNA. Proteins 2B and 2BC induce extensive rearrangement of intracellular membranes into vesicles, which are a required component of viral replication complexes. The 2C protein-coding sequence also contains a highly conserved stem-loop RNA structure which is a cis-acting replication element (CRE) required for viral RNA synthesis.

In the early stages of the infection process the genome is used predominantly for translation of viral proteins. However as the pool of viral proteins increases, protease 3CD binds to the 5' NTR, repressing translation and favouring RNA synthesis. The positive-sense ge-

nomic RNA acts as a template for the synthesis of a negative-sense copy. Negative-strand synthesis requires circularization of the genome, in which a ribonucleoprotein complex formed at the 5' NTR interacts with poly(A)-binding protein bound to the 3' poly(A) tail. The viral polymerase (3D) then catalyses the uridylation of the VPg, using the poly(A) tail of the viral genome as template. The resulting VPg-poly(U) primes the synthesis of negative-sense RNA. This gives rise to a double-stranded replicative intermediate, which associates with membranous vesicles to form replication complexes. The replicative intermediate acts as a template for further positive-sense strand synthesis. Positive-strand synthesis also requires a uridylated VPg primer, but in this instance adenosine residues within the loop of the CRE RNA structure serve as the template for uridylation, in a complex between the 5' and 3' NTRs, the CRE and the 3C and 3CD proteins. The resulting uridylated protein (VPgpUpU(OH)) presumably associates with the 3' terminus of the negative-sense RNA and primes positive-strand synthesis. It is likely that the increasing excess of VPgpUpU(OH) over VPg during the course of infection favours synthesis of the positive over the negative strand. Thus, the different mechanisms involved in uridylation of VPg and the initiation of transcription for positive- and negative-strand synthesis leads to asymmetrical replication of viral RNA. Positive-sense RNA is the major species made after the early stages of infection and is used both for translation and for packaging into nascent virus.

The assembly of the virus particle involves the synthesis of VP0, VP1 and VP3 as a single unit, which is then assembled into pentamers, followed by the association of 12 pentamers into the virion, a process which involves host factors. The X-ray crystallographic structure of the virus implies that the final maturation cleavage of VP0 to VP2 and VP4 is autocatalytic. Thus, a serine residue is found close to the N-terminus of VP2. A number of serine proteases are known, and it may be concluded that as soon as the proton donor, such as the genomic RNA, is brought into the vicinity of the serine residue the final cleavage will take place. The virus is released from the cell by lysis.

Enterovirus Diversity and Classification

This historical classification of the enteroviruses closely followed the history of enterovirus discovery. Poliovirus was first identified in 1909 by inoculation of monkeys with specimens from cases of paralytic poliomyelitis. In 1948 Dalldorf and Sickles (1948) recovered a new group of agents by inoculation into newborn mice of faecal extracts from two children with paralytic disease. These agents were named coxsackieviruses after the town in

New York State where the isolations were made. CVA and CVB were subsequently differentiated on the basis of their pathology in newborn mice and their capacity to grow in cell cultures, as described below. Following the discovery by Enders *et al.* (1949) that poliovirus could be grown in cell cultures, a third group of enteroviruses, the ECVs, were identified as a cause of human disease. ECVs were nonpathogenic for subhuman primates and newborn mice, but produced cytopathic changes in cell cultures. Within a few years it was found that all three groups of virus were antigenically distinct and consisted of several and, in some cases, numerous serotypes. The virus types comprising each group are summarized in Table 25.1, together with the major diseases with which they are associated and their cultural characteristics.

Since 1970, new enterovirus types have been allocated sequential numbers (68–71) following those allocated to previously identified enteroviruses. Enterovirus types identified more recently using nucleic acid-based methods have also been named provisionally according to this numerical classification, pending ratification by the ICTV.

Complete genomic sequences for representative strains of all human enterovirus types and many other picornaviruses have now been determined. Comparison of such

Table 25.1 Types and characteristics of human enteroviruses

Group	Virus types	Cytopathic effects in cell cultures		Pathology in newborn mice	Major disease associations
		Monkey kidney	Human cell culture		
Poliovirus	3 types (poliovirus 1–3)	+	+	–	Paralytic poliomyelitis; aseptic meningitis; febrile illness
Coxsackie virus group A	23 types (A1–A22, A24)	– or ±	– or ±	+	Aseptic meningitis; herpangina; febrile illness; conjunctivitis (A24); hand, foot and mouth disease
Coxsackie virus group B	6 types (B1–B6)	+	+	+	Aseptic meningitis; severe generalised neonatal disease; myopericarditis; encephalitis; pleurodynia (Bornholm disease); febrile illness
Echovirus	31 types (types 1–9, 11–27, 29–33)	+	±	–	Aseptic meningitis, rash, febrile illness; conjunctivitis; severe generalised neonatal disease
Enteroviruses	4 types (types 68–71)	+	+	–	Polio-like illness (E71); aseptic meningitis (E71); hand, foot and mouth disease (E71); epidemic conjunctivitis (E70)

Table 25.2 Phylogenetic classification of human enteroviruses.

Human enterovirus species	Historical grouping	Types
HEV-A	CVA EV	2, 3, 5, 7, 8, 10, 12, 14, 16 71, 76, 89–91
HEV-B	CVA CVB ECV	9 1–6 1–9, 11–21, 24–27, 29–33
HEV-C	EV CVA	69, 73–75, 77–88, 93, 97, 100, 101 1, 11, 13, 15, 17–22, 24
HEV-D	EV	96
Poliovirus	PV	68, 70, 94 1–3

sequences reveals a surprisingly close relationship between the enteroviruses and rhinoviruses, the genomes of which show a high degree of homology (Stanway, 1990). In contrast, hepatitis A has no significant homology with enteroviruses at the nucleic acid level. Similarly, parechovirus 1 (formerly ECV 22) is also unique in the sequence, showing no homology with other enteroviruses.

The human enteroviruses can be divided into two genetic clusters on the basis of sequence comparisons of the 5' NTR (Hyypä *et al.*, 1997; Pöyry *et al.*, 1996). One includes polioviruses, CVA21 and A24 and EV70, while CVB, ECV, CVA9 CVA16 and EV71 form the second cluster. Similar sequence comparisons of the coding region and 3' NTR reveal four major genetic clusters of the human enteroviruses, and phylogenetic analysis of VP1 sequences now forms the basis of a new classification system in which human enteroviruses are assigned to one of four species (HEV-A to HEV-D; Oberste *et al.*, 1999; Table 25.2). Although the polioviruses fall within the same genetic cluster as HEV-C, they are currently assigned to a distinct species in recognition of their biological uniqueness as agents of poliomyelitis (Minor *et al.*, 1995). Enteroviruses showing >75% nucleotide homology in the VP1-coding region are considered to belong to the same enterovirus type, while those with <70% nucleotide homology are considered distinct types.

Sequenced animal enteroviruses, such as bovine enteroviruses, form groups distinct from the human enteroviruses when either the 5' NTR or the remainder of the genome is considered. Different regions of the genome vary in their homology between enteroviruses and other picornaviruses. Certain sections in the 5' NTR of the genome are near identical in all enteroviruses examined, implying some definite functional constraint; this identity may carry over into the rhinoviruses, but it is possible to identify sequences which are apparently enterovirus-specific. It is further possible to identify regions such as the 3' NTR which are strongly conserved

for a particular species, such as a poliovirus. Finally, certain regions, including those areas encoding the capsid protein VP1, are extremely strain-specific. In principle, as described later, it is possible to exploit these various types of region to devise probes or polymerase chain reaction (PCR) primers that are specific either for enteroviruses in general, for a particular species of enterovirus, for a particular enterovirus (sero)type or even for a particular strain. Such probes are valuable in clinical and epidemiological studies.

Although there is little documented evidence of progressive antigenic variation among enterovirus isolates, antigenically unusual strains can be isolated; for example, PV3 isolated from patients with paralytic poliomyelitis in Finland in 1984 were antigenically clearly distinguishable from classic PV3 strains isolated in the 1950s (Magrath *et al.*, 1986). It may be significant that the cases of poliomyelitis from which the viruses were isolated occurred in people immunized with inactivated vaccine. There is good evidence for recombination between vaccine strains of PV and wild-type PV or other HEV-C viruses. Viruses within a particular human enterovirus species are apparently able to recombine readily. Consequently, the nonstructural protein-coding regions of the genome are not informative beyond the species level. Recombination between enteroviruses may result in strains with biological properties distinct from those of the progenitor strains, and these recombinants be ultimately become established as new enterovirus types.

PATHOBIOLOGICAL AND CLINICAL ASPECTS OF HUMAN ENTEROVIRUSES

Enteroviruses have a worldwide distribution and collectively cause a considerable impact in morbidity and mortality. They exhibit a wide range of biological and pathogenic properties, some of which are characteristic

of specific enterovirus groups or types. The extraordinarily wide range of diseases included in their clinical 'repertoire' contributes to their attraction as subjects for study by the molecular virologist, pathologist, clinician and epidemiologist. An extensive literature of reviews on the clinical, pathological and epidemiological properties of human enteroviruses is available (Pallansch and Roos, 2001; Rotbart, 1995), to which the reader is referred for further detailed information.

The host range of the enteroviruses varies between groups and types. Table 25.1 summarizes the ability of the viruses to replicate and produce cytopathogenic effects in cell cultures of monkey kidney and human origin and their pathogenesis for suckling mice. Poliovirus, unlike other enteroviruses, causes flaccid paralysis in monkeys and chimpanzees when administered into the brain or spinal cord. A feature of coxsackieviruses not shared by other enteroviruses is their pathogenicity for suckling mice. CVA produce widespread myositis in the skeletal muscles of newborn mice, resulting in flaccid paralysis without other obvious lesions. CVB also produce myositis but generally of more focal distribution than that caused by CVA. In addition they cause necrotizing lesions of the brown fat pads (intrascapular cervical and cephalic pads) and, in the case of certain strains, encephalitis with spastic paralysis, pancreatitis, myocarditis, endocarditis and hepatitis in both infant and adult mice. CVA, unlike CVB, replicate poorly if at all in monkey kidney cell cultures. As already mentioned, most ECVs are nonpathogenic in primates and mice.

The common portal of entry for enteroviruses is generally thought to be the alimentary tract via the mouth. Replication of virus in the cells lining the alimentary tract may be preceded by, or accompany, oropharyngeal replication.

A viraemic phase is followed by infection of target organs, for example spinal cord and brain, meninges, myocardium or skin. Incubation periods before onset of disease vary widely from 2 to 30–40 days depending on the clinical manifestation. The pathogenesis of poliomyelitis has been studied extensively (Minor, 1996) but is still not fully understood. According to Bodian, the virus, having entered via the alimentary tract, multiplies locally at the initial sites of virus colonization (tonsils, Peyer's patches) and associated lymph nodes. At this stage virus may be isolated from both throat and faeces. Secondary spread of virus apparently occurs via the blood to other susceptible tissues (e.g. other lymph nodes, brown fat pads and nervous tissue). On the other hand, Sabin held the view that the virus replicates chiefly in the mucosal layers of the intestine or oropharynx, seeding the associated lymphoid tissues, where it does

not necessarily replicate. After a low-level, possibly undetectable viraemia, replication at distant unidentified sites leads to a secondary viraemia, which may result in infection of either the peripheral or central nervous system (CNS) (for further details, see Minor, 1996).

Within the CNS the virus appears to spread along nerve fibres. If local multiplication is extensive, motor neurons are destroyed and paralysis results. The anterior horn cells of the spinal cord are most prominently involved but in severe poliomyelitis the posterior horn and dorsal root ganglia are affected. Shedding of virus occurs from the throat and in faeces and thus transmission of infection occurs independently of invasion of the nervous system.

The pathogenesis of CVB-induced myocarditis is also well studied in murine models, and is discussed below. The major clinical aspects of enterovirus infection are now summarized.

CNS Infections

Paralytic Poliomyelitis

The clinical features and epidemiology of this disease have been reviewed in detail by Minor (1996). Progress towards its eradication is described later.

Poliovirus infection may be asymptomatic or mild and only about 1% of infections result in illness with neurological involvement. Cases of abortive poliomyelitis, often accompanied by fever, malaise, headache, nausea or vomiting, are followed by uneventful recovery. Some patients develop aseptic meningitis with clinical features similar to those caused by other enteroviruses (non-paralytic poliomyelitis). In a small number of cases, paralytic poliomyelitis occurs.

Most cases occur without evidence of an earlier phase of illness. The most predominant sign is acute flaccid paralysis, resulting from lower motor neuron damage. Painful spasms and incoordination of non-paralysed muscles may also occur. Death may be due to respiratory paralysis. In those who survive, some recovery of muscular function is common but may take six months or longer. In epidemics in developed countries in the early 1950s, typically 5% of cases were fatal, 10% showed full recovery with no sequelae, while the remainder showed permanent paralysis to some degree. Isolation of PV from a patient with acute flaccid paralysis confirms the diagnosis of paralytic poliomyelitis in the absence of any other cause. Poliomyelitis, when epidemic, occurs primarily in the summer months in temperate zones. Non-polio enteroviruses, particularly CVA7 and EV71, occasionally cause polio-like illness, and in some regions where wild polioviruses have been eradicated, EV71 is emerging as an important cause of acute flaccid paralysis (Alexander *et al.*, 1994; da Silva *et al.*, 1996).

Many years after maximal recovery from the original attack of poliomyelitis, some patients develop further muscle weakness, the post-poliomyelitis syndrome. There have been suggestions that in some cases this may be due to persistent PV infection of neural cells, based on the detection of intrathecal PV antibody and the presence of PV RNA in cerebrospinal fluid (CSF) (Leparc-Goffart *et al.*, 1996), although there are alternative hypotheses, such as ageing (Munsat, 1991). Persistence of PV or other enterovirus infection of the CNS has also been implicated in the aetiology of motor neuron disease, and the presence of enterovirus RNA in cerebrospinal fluid or neural tissue has been reported in small numbers of patients (Leparc-Goffart *et al.*, 1996; Woodall *et al.*, 1994). However, two other studies found that, while enterovirus RNA can be detected in post-mortem brain and spinal cord tissue of patients with post-poliomyelitis syndrome or motor neuron disease, viral RNA was also detectable in patients with other neurological or non-neurological diseases (Giraud *et al.*, 2001; Muir *et al.*, 1996), and was therefore unlikely to be related to chronic neurological disease.

Aseptic Meningitis

Aseptic meningitis occurs most frequently in young children. Enteroviruses are the commonest cause of aseptic meningitis in countries where mumps has been controlled by vaccination. A febrile illness followed by meningeal signs, with stiffness of the neck or back, and muscle weakness, clinically reminiscent of mild poliomyelitis, may occur (Rotbart, 1995). However, in infections caused by non-polio enteroviruses there is almost always complete recovery from paresis. PV, CVA and CVB (especially types B1–B6, A7 and A9), many ECV (notably types 4, 6, 11, 14, 16, 25, 30 and 31) as well as EV71 have been associated with aseptic meningitis.

Patients with aseptic meningitis have a clear CSF which is usually under normal, or slightly increased, pressure. A pleocytosis, usually of the order of $10\text{--}500\text{mm}^{-3}$, mainly of lymphocytes is often, although not invariably present. During the first day or so after the onset of symptoms, polymorphonuclear leukocytes may predominate. The protein concentration is normal or slightly increased and the CSF glucose concentration is generally within normal limits. Thus, a normal CSF does not exclude enterovirus infection. Enteroviruses can be cultured from CSF, although viral RNA detection by PCR is more sensitive, as described below. In neonates and infants, PCR detection of viral RNA in serum or urine also provides useful evidence of systemic infection.

Enterovirus meningitis is common in neonates, children, and adults under 40 years of age. The frequency of cases in different age groups varies both geographically and temporally, possibly reflecting variations in the

prevalence of enterovirus types, and the prevalence of immunity to the prevailing enterovirus type among different age groups.

Encephalitis

Although herpes simplex virus (HSV) is the most commonly identified pathogen in patients with encephalitis (see Chapter 6), enteroviruses have been associated with encephalitis (Modlin *et al.*, 1991). Enteroviral encephalitis may be associated with aseptic meningitis, or may present with absent or minimal meningeal involvement. Children and young adults are most frequently affected. Although most patients recover uneventfully, a few have neurological sequelae or damage to the hypothalamic–pituitary axis, which causes endocrine disturbance. CSF findings are similar to those occurring in aseptic meningitis. Again, a normal CSF does not exclude enterovirus infection. Viral detection in CSF by PCR is considered proof of aetiology, although the sensitivity of PCR in this setting has not been established. Viral detection at peripheral sites, or viral serology may be required to provide evidence of recent enterovirus infection.

Patients with hypogammaglobulinaemia may develop persistent infections following enterovirus infection, associated with chronic meningeal irritation, encephalitis, insidious intellectual impairment or sometimes a dermatomyositis-like syndrome (Wilfert *et al.*, 1977). Although some patients recover, there is a high mortality rate. Many ECV and CVB have been shown to cause such persistent infections; occasionally, multiple serotypes may be involved. Patients with hypogammaglobulinaemia may also develop paralytic disease following administration of attenuated live polio vaccine. Virus may be recovered from the CSF, sometimes intermittently, although high titres are often present. Treatment with high-titre specific immunoglobulin has not been shown to be particularly effective in eradicating virus from the CNS.

Heart Disease

Myocarditis

A wide range of viruses may cause myocarditis, including ortho- and paramyxoviruses, togaviruses, herpesviruses and adenoviruses. However, CVB are the most commonly identified cause. In addition, current understanding of pathogenesis in viral myocarditis derives mainly from studies of CVB infection, including both clinical studies in humans and experimental studies of CVB3 virus-induced murine myocarditis. Other enteroviruses, including PV, CVA and ECV, may also cause myocarditis, and the relative importance of other non-polio enteroviruses may have been underestimated in recent years because of the wider availability of serological tests for CVB infection.

Acute myocarditis is an inflammatory disease of the heart muscle. Diagnosis therefore requires histological examination of endomyocardial biopsy or autopsy tissue. As this is often not available, it is likely that myocarditis is underdiagnosed. Clinical features are nonspecific and variable.

Neonatal enteroviral myocarditis can be rapidly fatal, and may be confused with congenital heart disease. Other systemic symptoms may be present. The pathological changes vary with the duration of illness. In infants who die early after infection (two to five days), left ventricular dilatation is present. The endocardium and valves are normal, even though the myocardium is pale. Late in infection (9–11 days of illness) the size of the heart is increased, largely due to dilatation of the left, and occasionally the right, ventricle. Microscopically, myofibre necrosis and inflammation are evident. Initially the inflammatory infiltrate is composed of polymorphonuclear leukocytes, but by day 5 or 6 mononuclear inflammatory cells are found.

In contrast to the abrupt, severe and often fatal disease seen in the neonatal period, viral myocarditis in adolescents and adults usually has a delayed onset, often following an upper respiratory (typically influenza-like) or gastrointestinal illness. Acute heart disease may become evident a week to 10 days later and has a presentation that can mimic myocardial infarction, coronary artery occlusion or progressive heart failure. Some patients do not present with heart disease but manifest nonspecific symptoms and signs, such as fever, myalgia and headache; often, cardiac involvement is suspected only because of typical electrocardiogram (ECG) changes. In a proportion the main presenting feature is pericarditis, characterized by acute, often severe, retrosternal chest pain. Other clinical findings include shortness of breath, tachycardia, arrhythmias, murmurs, rubs and cardiomegaly (due to ventricular dilatation or pericardial effusion). Death can supervene due to arrhythmias or congestive heart failure, but this is relatively uncommon. As in the neonatal infection, manifestations of systemic disease in adults may be noted. In CVB infections these may include pleurodynia, meningitis, hepatitis, orchitis, lymphadenopathy and splenomegaly. There may be recrudescences following the acute episode; up to 20% of patients in whom the major presenting feature is pericarditis will experience one or more relapsing episodes in the following months or years (Muir *et al.*, 1989). Acute myocarditis may also lead to chronic myocarditis or dilated cardiomyopathy (DCM), as described below.

Viral infection of the myocardium is believed to be a prerequisite to myocarditis, although antiviral cellular immune responses and autoreactive T cells activated following local tissue damage and release of sequestered

autoantigen are likely to be important effectors of myocardial pathology (Horwitz *et al.*, 2000). At present there is a lack of consensus on the most appropriate treatment for myocarditis. Thus, while steroid therapy has been used, evidence of efficacy is lacking (Mason *et al.*, 1995) and concerns remain that immunosuppressive therapy may result in increased viral replication and persistence (Heim *et al.*, 1994).

Virological investigations including virus isolation, PCR and serological studies, as described later, may assist in establishing a viral aetiology but often give negative or inconclusive findings. Even if cardiac biopsy samples are taken early or the patient dies during the acute phase, it is exceptionally rare to isolate virus or detect virus antigen in heart tissue, although enterovirus RNA may be detected. Because myocarditis may present some time after the initiating viral infection, specific antibody titres may have already peaked. Thus, serological investigation using paired acute and convalescent samples may fail to demonstrate a diagnostic rise in titre. Enterovirus immunoglobulin M (IgM) testing may be used in the absence of suitably paired sera or isolation samples, but IgM seroprevalence in the community can be significant, especially during high-activity seasons, reflecting asymptomatic infection. Thus, a positive result provides only circumstantial evidence of aetiology.

Dilated Cardiomyopathy

Dilated cardiomyopathy is a post-inflammatory disease which is a significant cause of heart failure and sudden cardiac death, and one of the commonest reasons for requiring cardiac transplantation. Enteroviruses are the most commonly identified agents of acute myocarditis, and there is now strong evidence that they also cause persistent infection associated with chronic myocarditis and chronic DCM (reviewed by Muir and Archard, 1994). There is evidence that viral persistence after the initial infection is directly involved in the evolution of DCM (Bowles *et al.*, 1986), although immunopathological mechanisms, including the development of cardiac autoimmunity, may also contribute to chronic disease (Liu and Mason, 2001).

Infectious virus cannot be detected in cardiomyopathic hearts, although a proportion of patients have elevated neutralizing antibody titres (Cambridge *et al.*, 1979) and persistent enterovirus-specific IgM responses (Muir *et al.*, 1989). Viral RNA can be detected in cardiac muscle of a proportion of cases by nucleic acid hybridization (Bowles *et al.*, 1986; Kandolf *et al.*, 1990) or reverse transcriptase-PCR (Archard *et al.*, 1998; Kammerer *et al.*, 1994; Schwaiger *et al.*, 1993). Small numbers of patients with myocarditis or DCM have been treated with IFN- α , either alone or in combination with IFN- β , with evidence of viral clearance and clinical improvement in some.

Murine CVB Disease

Experimentally, CVB replicate and produce inflammatory lesions in the heart, pancreas, liver, spleen and brain in several strains of mice. Studies in weanling and adult animals with CVB have shown that parenteral infection results in viraemia and then replication in target organs. Viraemia is detected within 24 hours and usually persists until day 3. The virus grows in various target organs, with maximum levels achieved by day 3 or 4. After maximum virus growth is established, virus titres decline and are usually undetectable by 7–10 days, presumably due to the induction of neutralizing antibody. Nevertheless, viral RNA may persist in heart and other organs for several weeks or months after clearance of viral infectivity (Reetoo *et al.*, 2000). Murine models may therefore be useful for understanding the molecular basis and pathogenetic consequence of viral persistence following acute myocarditis. The details of viral pathogenesis in murine models vary according to both viral strain and host physiology and immunogenetics. Different host–pathogen combinations are characterized by differences in disease severity, and in susceptibility to persistent infection and chronic disease. The relative contribution of direct virus-mediated pathology, immune-mediated pathology and autoimmunity also varies, as do the cellular and humoral immune effectors. It is likely that this pathogenetic diversity reflects that which occurs in human disease.

Neonatal Infection

CVB and ECV, particularly types 6, 7 and 11, may cause severe and often fatal infection in newborn infants (Abzug *et al.*, 1995; Modlin, 1988). Although there have been occasional reports of intrauterine death resulting from maternal enterovirus infection, there is no confirmed association with congenital abnormalities. Infection may be transmitted transplacentally in late pregnancy, the infant developing heart failure from a severe myocarditis as described above, or meningoencephalitis soon after delivery. More frequently, infection is transmitted perinatally from the mother, or postnatally from other virus-infected infants in nurseries for the newborn or special care baby units. Some infected neonates may be asymptomatic, but others will develop manifestations at three to seven days of age, ranging from a mild febrile illness to fulminating multisystem involvement and death. Myocarditis, pneumonia and meningoencephalitis may be present. A severe hepatitis with jaundice, which results in an increased prothrombin time, and profuse haemorrhage may also occur. These features are indicative of a generalized infection, and neonatal enterovirus infection may be confused with other causes of neonatal sepsis. Enterovirus may be detected in stool, urine, serum, brain, spinal cord and my-

ocardium. It is essential to establish a diagnosis as rapidly as possible in order to guide patient management and institute appropriate infection control measures. In outbreaks of neonatal infection there is some evidence that administration of human convalescent serum containing a high neutralizing antibody titre to the virus serotype identified may be of value in preventing or attenuating infection in susceptible neonates (Abzug *et al.*, 1995; Modlin, 1988).

Bornholm Disease (Epidemic Pleurodynia)

CVB are the commonest cause of this syndrome, but ECV serotypes including 1, 6, 9, 16 and 19, and CVA such as A4, 6, 9 and 10, have also been implicated. Outbreaks involving families are common; more extensive community-wide epidemics have also been reported. The disease usually presents abruptly with fever and chest pain due to involvement of the intercostal muscles, or abdominal pain which results from involvement of the muscles of the upper part of the abdomen. This may be sufficiently severe to mimic an acute surgical condition requiring laparotomy, or myocardial infarction. Some patients have pain localized to the limbs. There is usually muscle tenderness and in some patients swelling may be seen or palpated in affected muscles. Most patients recover within a week, although about 25% of patients may experience relapses, usually within a few days of being symptom-free. Enterovirus isolation from stool and strongly positive virus-specific IgM responses provide evidence of concurrent enterovirus infection.

Enteroviruses have also been implicated in various types of chronic inflammatory myopathies (Bowles *et al.*, 1987), but more recent studies using PCR have failed to demonstrate enterovirus RNA in muscle (Leff *et al.*, 1992).

Herpangina

CVA 1–6, 8, 10 and 22 are associated with herpangina. The illness affects primarily children aged 2–10 years and is characterized by fever, sore throat and pain on swallowing, often associated with vomiting and abdominal symptoms. Small vesicular lesions occur on the fauces, pharynx, palate, uvula and tonsils. Recovery is generally uneventful. Detection of enterovirus in vesicle fluid or other samples may help differentiate this condition from primary HSV infection.

Exanthemata

Hand, Foot and Mouth Disease

An ulcerative exanthem of the buccal mucosa accompanied by mild fever is followed by painful vesicular lesions on the hands or feet. Less commonly, lesions

may be present on the buttocks and genitalia. Family outbreaks are common. This disease has been associated with several enteroviruses, but most commonly with CVA16 and, less frequently, with A4, 5 and 9, and CVB2 and 5. EV71 also causes outbreaks of hand, foot and mouth disease (HFMD). In recent years large epidemics of EV71-associated HFMD have occurred in the Asian and Pacific regions, with complications including aseptic meningitis, acute flaccid paralysis, brainstem encephalitis associated with pulmonary oedema or haemorrhage, myocarditis and herpangina (Chang *et al.*, 1999; Ho *et al.*, 1999). Case fatality rates of up to 0.1% in infants have been reported in these epidemics and significant numbers of survivors have residual neurological sequelae. Enteroviruses can be detected in vesicle fluid or stool, although suckling mouse inoculation or PCR may be required to detect noncultivable CVA.

Rubelliform Rashes

A fine rubella-like maculopapular rash is often a feature of some CVA and ECV infections. Enterovirus infection should thus be considered in the investigation of women exposed to or presenting with rubelliform rash during pregnancy, after exclusion of other causes (see Chapter 23). Most frequently, ECV9 is implicated but often other ECV serotypes and CVA9 may also be involved. Summer outbreaks, most frequently affecting children, are common. Fever, malaise and cervical lymphadenopathy may also occur in patients with rash. Patients generally make an uneventful recovery. Enterovirus detection in stool or throat swab, or the presence of virus-specific IgM, provides evidence of aetiology in the absence of another cause.

Respiratory Infections

Several enteroviruses have been associated with mild illness of the upper respiratory tract, including rhinitis, particularly during the summer and autumn. These include CVA2, 10, 21 (Coe virus), 24 and CVB2 and 5. Coe virus has caused epidemics of pharyngitis in military recruits. Among the ECV which have been isolated from cases of respiratory illness are included types 1, 11, 19 and 20. These viruses most commonly cause outbreaks in young children, in whom pneumonia and bronchiolitis may sometimes occur. Enteroviruses can be detected in nasopharyngeal aspirates, although the interpretation of a positive result must take account of the possibility of a coincidental infection, since enteroviruses can also be detected in respiratory samples from healthy infants.

Conjunctivitis

Several enterovirus serotypes are associated with conjunctivitis. ECV7 and 11 and CVB2 have been isolated from

conjunctiva in sporadic cases. Since the early 1970s, major epidemics of acute haemorrhagic conjunctivitis have been described in Africa, the Americas and the East Asia. Some epidemics are caused by a variant of CVA24 or adenovirus 11, but many are caused by EV70, a virus first identified in 1969.

Unlike most other enterovirus infections, conjunctivitis may result from direct inoculation of virus as a result of hand-to-eye contact, without passage through the intestinal tract. Subconjunctival haemorrhage is more common with EV70 than with CVA24. There is a high attack rate among family members and a short incubation period of one to two days. Recovery is usually complete within one to two weeks and, for CVA24, sequelae are rare. CVA24 and EV70 can be isolated from conjunctival scrapings, and neutralizing antibody tests may be helpful.

Neurological complications may, in a few cases, accompany conjunctivitis due to EV70, and occasionally a polio-like paralytic illness ensues. One in 10 000 patients, mainly adult men, may suffer a residual paralysis. The neurological involvement may develop two or more weeks after the onset of conjunctivitis. The potential for neurovirulence is a worrying feature of EV70 and this calls for vigilance in the investigation of epidemics of conjunctivitis.

Diabetes and Pancreatitis

Type 1 diabetes is an autoimmune disorder in which the insulin-secreting pancreatic islet cells are progressively destroyed. The disease has an extended preclinical incubation period in which islet cell autoantibodies (ICAs) can be detected. Type 1 diabetes occurs more commonly in genetically susceptible individuals, but environmental 'triggers' are also believed to play a role. Although definitive proof is lacking, seroepidemiological evidence, animal model studies and anecdotal case reports all support the hypothesis that enterovirus infections may act as such a trigger. Prospective studies of siblings of cases, who themselves have an increased risk of developing type 1 diabetes, have found that individuals who subsequently develop disease have a higher incidence of enterovirus infections in the pre-diabetic phase than those who remain asymptomatic (Hyöty *et al.*, 1995). These studies also documented a temporal association between ICA seroconversion and preceding enterovirus infection, and enterovirus infections were particularly frequent during the six-month period preceding the first detection of autoantibodies (Hiltunen *et al.*, 1997; Salmiinen *et al.*, 2003). This suggests that enterovirus infection may be involved in the initiation and progression of islet cell damage. Retrospective serological studies of maternal blood samples collected at delivery have reported a higher prevalence of enterovirus-specific IgM in mothers whose

infants subsequently developed type 1 diabetes (Hyöty *et al.*, 1995), suggesting that disease initiation may occur *in utero*. However, enterovirus infection may also precipitate disease onset, as suggested by the presence of enterovirus-specific IgM or enterovirus RNA in serum in a proportion of patients at the time of clinical onset of type 1 diabetes (Banatvala *et al.*, 1985; Clements *et al.*, 1995a; King *et al.*, 1983).

Autoimmunity may arise as a result of molecular mimicry between viral and host antigens, and molecular mimicry between an epitope of the 2C nonstructural enterovirus protein and the islet cell antigen glutamic acid decarboxylase has been demonstrated. However, recent studies in susceptible mouse strains suggest that this may be insufficient in itself to induce type 1 diabetes and that destruction of exocrine pancreas by a pancreatotropic enterovirus infection may release sequestered islet cell antigens, which restimulate autoreactive memory T cells (Horwitz *et al.*, 1998). Thus, successive enterovirus infections may result in frequent restimulation of islet cell-autoreactive T cells, resulting in cumulative loss of islet cells, which eventually culminates in clinical type 1 diabetes.

Although an acute pancreatitis is a predominant feature of CVB infections in mice, there is comparatively little evidence linking this disease with CVB infection in humans. However, the pancreas is often involved in generalized neonatal infection, and CVB4 has occasionally been implicated as a cause of pancreatitis in adults; sub-clinical involvement of the pancreas has been reported in 31 and 23% of CVB5 virus and CVA infections.

Chronic Fatigue Syndrome

Chronic fatigue syndrome (CFS) has a number of alternative names: myalgic encephalomyelitis (ME), Royal Free disease, Iceland disease, postviral fatigue syndrome and neuromyasthenia. It occurs as both sporadic and epidemic cases. Although it is a poorly characterized illness, the cardinal feature is excess fatigability of skeletal muscle, which may be accompanied by muscle pain. Many other symptoms may be present, including headaches, inability to concentrate, paraesthesiae, impairment of short-term memory and poor visual accommodation. Focal neurological signs are rare. Evidence of myopericarditis may be present occasionally.

The clinical spectrum of CFS is broad and diagnosis may be difficult. Physical examination is usually not helpful, although there may be some lymphadenopathy. A history of an initiating nonspecific 'virus' illness may be elicited, and such a corroborated history defines postviral fatigue syndrome. Routine laboratory investigations are usually noncontributory. Some groups, however, have described abnormalities of T cell function and muscle

structure (necrosis and increase in size and number of type II fibres) and function (abnormal jitter potentials and early intracellular acidosis on exercise), although these findings are not consistent. Recovery within a few weeks or months is usual, but in some patients the syndrome persists and may be relapsing.

There is a continuing debate on the aetiologies. Much attention has focused on a viral aetiology, although it is likely that those ascribed to be suffering from the syndrome are a heterogeneous group with organic and functional components contributing in varying degree (Straus, 1996). In Europe, most attention has focused on a possible enteroviral aetiology, while in the United States, various herpesviruses have been implicated. CFS occasionally follows confirmed virus infections, such as varicella zoster, influenza A and infectious mononucleosis, and rarely bacterial and protozoal infections such as *Toxoplasma gondii*, *Leptospira hardjo* and Q fever. Chronic fatigue may also be a prominent feature of chronic hepatitis C, and CFS may be diagnosed if hepatitis C is not suspected. In the majority of cases, however, the initiating viral illness cannot be diagnosed.

Viruses have been implicated not only as a trigger but also as a persistent active infection, although no evidence to support this hypothesis has been forthcoming in recent years. Although some cases may follow infectious mononucleosis, a significant role for chronic Epstein-Barr virus (EBV) infection is no longer considered likely (Straus, 1996). There is some evidence supporting a persistent enterovirus infection. In some studies, but not all, patients with CFS have a higher prevalence of elevated CVB neutralizing antibody titres and specific IgM compared with controls (Behan *et al.*, 1985; Mawle *et al.*, 1995). In one study, approximately 50% of patients had circulating immune complexes containing an enterovirus VP1 antigen detectable with a group-reactive monoclonal antibody. Enteroviruses were isolated only occasionally from faeces by routine methods, but were isolated from approximately 20% of patients when the faeces were acidified to disrupt virus-antibody complexes prior to cell culture (Yousef *et al.*, 1988). However others have been unable to reproduce these findings. Skeletal muscle biopsies from 96 patients have been examined with an enterovirus-specific DNA probe and 20 were positive (Archard *et al.*, 1988), suggesting enterovirus persistence.

Enterovirus RNA has also been detected in serum of patients with CFS (Clements *et al.*, 1995b), and the detection of near-identical viral sequences in sequential samples collected several months apart provides further evidence for persistence (Galbraith *et al.*, 1995). However, others have been unable to detect enterovirus sequences in serum from patients with CFS (Lindh *et al.*, 1996; McArdle *et al.*, 1996).

Thus, there is some evidence that in those cases of CFS with an organic origin enteroviruses may be implicated not only as a trigger but as a persisting infection. Although many treatments have been tried, such as human normal immunoglobulin, plasmapheresis and inosine pranobex, no evidence of efficacy has been presented.

LABORATORY DIAGNOSIS OF ENTEROVIRUS INFECTIONS

Diagnosis of enterovirus infections is useful where exclusion of serious or treatable conditions with overlapping clinical presentation is required. Thus, demonstration of an enteroviral aetiology in patients with meningitis or encephalitis may be helpful in excluding bacterial meningitis or herpes simplex encephalitis, while diagnosis of neonatal enterovirus infection may exclude other causes of sepsis-like illness, such as neonatal herpes simplex or group B streptococcal infection. Diagnosis may also play a role in infection control and epidemiological surveillance. If enterovirus-active antiviral agents become available, viral diagnosis will be necessary to guide appropriate therapy and for assessing therapeutic response. A combination of virus isolation and serological tests has traditionally been used to diagnose enterovirus infections, although molecular diagnosis now provides more rapid and sensitive viral detection in most settings. Timely submission of appropriate isolation samples and paired serum samples maximizes diagnostic yield. In general, demonstration of virus in samples taken from the site of infection provides the most conclusive proof of aetiology. Such samples are not always available, for example, in patients with myocarditis, and in such circumstances demonstration of concurrent viral excretion at peripheral sites, or serological diagnosis, can be used as circumstantial evidence of aetiology. However, it should be remembered that healthy infants and children frequently excrete enteroviruses: indeed, in tropical countries up to 40% of children up to the age of two years may be excreting enteroviruses at any one time. Where live attenuated polio vaccine is still in use, those recently immunized are likely to excrete virus from the upper respiratory tract and faeces for some time. Thus, the possible significance of isolating PV can only be assessed by knowing the vaccine history and clinical features.

Virus Isolation

Enteroviruses can be cultured from solid tissue, blood, CSF, urine, stool and respiratory samples, although the diagnostic significance of enterovirus detection at a normally virologically sterile site is considerably greater than

merely detecting virus in faeces or nasopharyngeal secretions, as discussed above. The most sensitive isolation specimens are faecal samples or rectal swabs. Virus excretion is often intermittent and more than one specimen should be collected, with an interval of 24–48 hours. Faecal excretion of virus commences within a few days of infection and may continue for weeks, especially with PV and Coxsackieviruses, although it rarely exceeds one month with the ECV. Concentrations of virus of $10^5 - 10^6$ tissue culture infectious doses per gramme of faeces are not uncommon.

Isolation from the pharynx is possible during the acute phase of the illness, especially in cases with respiratory symptoms (e.g. ECV9, CVA21 and EV71). The period with the highest rate of isolation is some five days before to five days after the onset of symptoms.

Viral culture of CSF generally gives a low diagnostic yield, and has largely been replaced by PCR testing, as described below, although during certain ECV epidemics up to 80% of CSF specimens have yielded virus.

In fatal cases, autopsy specimens of brain, spinal cord, heart and spleen, or other lymphoid tissue, are useful, especially where there is a recent history of oral polio vaccination, where investigations of the origin of the strain may be required.

PV, CVB, ECV and some CVA (e.g. A9 and A16) are readily isolated in cell cultures prepared from the kidneys of rhesus, cynomolgus or cercopithecus monkeys. Other useful cultures include human embryo kidney and amnion, primary liver carcinoma, rhabdomyosarcoma cells, or strains of diploid cells from human fetal lung. Possibly the most generally useful regimen is the inoculation of cell cultures of primary monkey kidney and human embryo lung fibroblasts. Enteroviruses cannot be readily differentiated by their growth in cell cultures, as all produce a similar cytopathic effect (CPE). Infected cells become rounded, refractile and ultimately shrink before detaching from the cell surface. Once a cytopathic agent has been isolated in cell culture, serological tests or nucleic acid sequencing must be performed to identify the type. Because of the number of potential strains, neutralization tests using individual type-specific sera are not satisfactory, and it is usual to employ either a single-stage procedure using antiserum pools containing selected antisera or a two-step method, first identifying the isolate as belonging to one of four groups, followed by a second test to identify the type. Because of the labour-intensive nature of serotyping procedures, many laboratories now confirm enterovirus isolates by direct immunofluorescence using enterovirus group-reactive monoclonal antibodies (Trabelsi *et al.*, 1995). Further identification is often not performed, but may also be achieved by immunofluorescent staining with serotype-specific monoclonal anti-

bodies. Some enteroviruses, notably most CVA, are not readily detected in cell cultures: if a CVA infection is suspected, specimens can be injected into two or more litters of mice by intracerebral, intraperitoneal and subcutaneous routes. However suckling mouse inoculation is no longer widely available. Neither PV nor ECV can be isolated in mice.

Twelve of the ECV possess the ability to agglutinate human group O erythrocytes. This activity is integral to the viral particle; spontaneous elution may occur at 37°C but this does not destroy the cell receptors, a property which differentiates the ECV from the ortho- and paramyxoviruses. The presence of haemagglutination activity helps reduce the number of possibilities when attempting to identify an enterovirus strain, although this is rarely employed nowadays.

Serological Diagnosis

Neutralization tests have generally been employed for such seroepidemiological purposes as determining the exposure and immunity of a population group to different enteroviruses, including responses to polio vaccination. These tests are labour-intensive and the results are seldom available in less than three to four days. They are no longer widely available, although PV-specific testing may be employed in investigating possible poliomyelitis cases. Antibody titres are compared in paired sera, the first being collected within five days of onset of symptoms, and the second some days later. Significant rises in antibody titre are detected only occasionally and this has led to significance being attached to elevated neutralizing antibody titres. However, elevated titres frequently occur in normal individuals, and so do not confirm recent infection. Significant antibody rises are particularly rare in cardiac disease, probably because cardiac events are a relatively late consequence of enterovirus infection. Serological diagnosis of recent enterovirus infection has also been achieved by detecting virus-specific IgM using M-antibody capture techniques (Bell *et al.*, 1986). IgM responses are frequently directed against group-reactive determinants, thus allowing an assay employing antigens from a limited range of serotypes to be used to detect responses elicited by a wide range of serotypes. The older the patient, the more likely that such heterotypic responses will occur. Enterovirus-specific IgM responses generally last for 8–12 weeks, but in some patients may persist for much longer, occasionally for some years. It has been suggested that such a prolonged response in, for instance, cases of recurrent pericarditis, may indicate persistence of the infecting enterovirus. Approximately 30–40% of patients with myocarditis, 60–70% of patients with aseptic meningitis and 30% of patients with postviral fatigue syndrome give positive results for CVB-specific IgM.

However, approximately 10% of normal adults also give a positive result, presumably reflecting recent asymptomatic infection.

Complement fixation tests and EIAs have also been employed to detect enterovirus-specific IgG, but the high prevalence of antibody in the general population, as measured using these group-reactive tests, and the difficulty in obtaining paired sera to demonstrate rising titres, limits diagnostic utility and these tests are no longer widely available. A rising titre of IgG antibody to PV must be treated with caution, as type 1 or type 3 infections may produce a significant boost to type 2 antibody in individuals previously primed to this type.

Molecular Diagnosis

Complementary DNA probes made by reverse transcription of purified CVB genomic RNA have been used to detect enterovirus RNA in infected cell cultures, infected mice and human tissue biopsies. Although prepared against a single CVB serotype, the probes included RNA sequences which are highly conserved amongst enteroviruses and are thus group-specific. They have been used to detect enterovirus RNA, both in RNA extracts from tissue and by *in situ* hybridization, although infectious virus or viral antigen is rarely detected in these tissues. PCR is technically more straightforward and quicker than nucleic acid hybridization methods, and is therefore more suitable for viral diagnosis. Identification of extremely conserved sequences within the 5' NTR of the enterovirus genome has allowed the design of PCR primers which allow detection of most enteroviruses (Rotbart, 1990) and numerous studies have shown that enterovirus PCR is more sensitive than viral culture for detection of enteroviruses in diverse clinical specimens. In most diagnostic laboratories examination of CSF by enterovirus PCR has replaced virus isolation for diagnosis of enteroviral meningitis, raising the possibility of more rapid diagnosis, which in turn may have greater impact on patient treatment (Schlesinger *et al.*, 1994). Enterovirus PCR is also useful for diagnosis of neonatal infections, where testing of blood, urine, CSF and stool is useful, and enterovirus myocarditis (Martin *et al.*, 1994; Nicholson *et al.*, 1995) if biopsy or autopsy tissue is available.

These enterovirus PCR protocols do not allow serotype or type-specific identification. Although serotypic identification is usually of less clinical urgency, the ability to differentiate polio and non-polio enteroviruses is important for investigation of suspected poliomyelitis cases, for diagnosis of non-polio enterovirus infection in patients recently vaccinated with live polio vaccine, for monitoring wild PV circulation in endemic regions, and for maintaining the polio eradication status of countries and regions which have been declared as polio-free by World

Health Organization (WHO). The ability to differentiate between wild-type and vaccine strains of PV, and between attenuated and neurorevertant vaccine virus is also crucial to the investigation of suspected vaccine-associated paralytic poliomyelitis. A number of PCR methods have been described for group or serotypic identification of PV (Kilpatrick *et al.*, 1996), or intratypic differentiation of wild and vaccine-strain PV (Yang *et al.*, 1991). These assays are available at WHO PV reference laboratories. Similar serotype-specific assays have also been described for other enteroviruses, and these may be of particular use in outbreak investigation once a particular outbreak strain has been identified. More commonly however the 'molecular serotyping' system based on nucleotide sequence analysis and comparison of VP1 sequences already described is used to type enterovirus isolates.

PREVENTION AND TREATMENT OF ENTEROVIRUS INFECTIONS

Vaccination

Experimental immunization with recombinant viruses expressing CVB3 capsid proteins protects mice against CVB3-induced myocarditis. However vaccination against non-polio enteroviruses is not currently available. The multiplicity of antigenic types and the usually mild nature of the diseases make the production of vaccines impractical. The only effective measures for their control are high standards of personal and community hygiene. Quarantine is not effective because of the high frequency of inapparent infections.

The observation that there are only three PV types and the discovery that they will grow in cell cultures of non-nervous tissue from monkeys made possible the development of vaccines against poliomyelitis. Natural infection with PV is by the oral route and results in viral replication in the mucosa of the pharynx and alimentary tract, causing viraemia and stimulating virus-specific IgA and IgG. The route by which the virus gains access to the CNS is not clear; although there is some experimental evidence that it can do so by travelling along autonomic nerve pathways from the site of replication, the greater body of evidence suggests that invasion of the CNS occurs via the bloodstream and may be prevented by circulating antibody. The aim of vaccination is to induce local or systemic immunity to prevent systemic spread and neuroinvasion.

Inactivated Poliovirus Vaccine

The first successful vaccine against poliomyelitis was developed by Salk and took the form of a virus preparation

carefully treated with a low concentration of formalin to inactivate it while retaining its antigenic properties. The very first batches of the vaccine used in the United States contained live virus and caused poliomyelitis in recipients; this was the Cutter incident (Nathanson and Langmuir, 1963) and was attributed to the presence of aggregates of virus which were not penetrated by the formalin. Subsequently better controlled filtration steps were introduced to remove aggregates and the vaccine has a good safety record. The initial vaccine was of relatively low potency because of the difficulty of preparing virus on the required scale, particularly the type 1 component. However the inactivated vaccine for parenteral injection (IPV) was used exclusively in Sweden, Finland, Iceland and the Netherlands and, with acceptance rates of 90% or better, virtually eliminated poliomyelitis. Surveys also show that the circulation of PV in the community was dramatically reduced, despite the fact that the vaccine does not induce detectable levels of secretory IgA and, in theory, would not be expected to prevent alimentary tract infections. In the 1980s supply of vaccine of far higher potency was ensured by the development of large-scale cell culture methods.

Sabin argued that the absence of PV in countries using IPV was due to the use of oral poliovirus vaccine (OPV) in neighbouring countries (Sabin, 1982) but studies in the Netherlands and Sweden have shown that during outbreaks of infection within religious sects refusing vaccination there was little virus circulation in the surrounding community with which they were in contact.

It has been observed that the pattern of virus shedding following virus infection of children previously vaccinated with IPV was related to the antibody titre at the time of infection. The presence of detectable IgG reduced pharyngeal shedding from 75 to 33% of children, but faecal shedding was reduced only when the titre of antibody was high, in excess of 1 : 128. The high standards of hygiene in the Netherlands and Scandinavia, where pharyngeal shedding of PV is probably the major source of infection, may explain the success of IPV in these countries. In 1984 a small outbreak of poliomyelitis with wide circulation of the type 3 strains occurred in Finland, a country which had relied exclusively on the older lower potency IPV but in which poliomyelitis had been unknown for 20 years. The level of type 3 antibody in the community was known to be low and the absence of poliomyelitis was attributed to persisting immunological memory, since revaccination of seronegative individuals induced rapid booster-like responses. The PV strains isolated in the outbreak were antigenically unusual and, in comparison with other PV3 strains, less well neutralized by antisera to reference strains of type 3 virus (Magrath *et al.*, 1986).

Oral Poliovirus Vaccine

The availability in the early 1960s of the live attenuated OPV, which was cheaper to produce and administer, easier to manufacture in quantity and which had some theoretical advantages over inactivated vaccine, led to its rapid adoption worldwide. The vaccine, administered by the oral route, parallels the natural infection and stimulates both local secretory IgA in the pharynx and alimentary tract and circulating IgG. Virus is excreted in the faeces for several weeks and possibly for several days in pharyngeal secretions. During this period the vaccine may spread to close contacts, inducing or boosting immunity in them but also, rarely, causing vaccine-associated paralytic poliomyelitis in recipients or non-immune contacts (see below).

Oral poliovirus vaccine has been used as the main tool of the Global Polio Eradication Initiative of WHO and is still used as part of childhood immunization programmes in many countries, such that individuals receive vaccine as they reach specific ages, typically at two, four and six months. This strategy was extremely effective in reducing the incidence of paralytic poliomyelitis to essentially zero in developed countries, but in other countries it had very little impact.

The Global Eradication Programme

In 1988 the WHO committed itself to the eradication of poliomyelitis by the year 2000. This ambitious goal was stimulated by the success of programmes in South America, where tropical countries had controlled polio by the use of OPV.

The strategy was to use national immunization days (NIDs), in which the aim is to immunize all children under the age of five in a country or region within a short period, usually a few days, and then to repeat the process a few weeks later. This results in a high coverage with fresh vaccine, and the colonization of susceptible individuals with vaccine virus, so breaking transmission. With modern approaches to NIDs two rounds have been enough to control the disease in most countries. The strategy is usually run in parallel with the programme strategy used elsewhere and has resulted in the eradication of poliomyelitis in the Americas, declared free of indigenous PV in 1994, and where the last case due to wild-type PV was seen in 1992, in Peru. The western Pacific region was declared PV-free in 2000 and the European region in 2002 (Centers for Disease Control and Prevention, 2003). At the time of writing, poliomyelitis was endemic in only four countries in the world: India, Pakistan, Afghanistan and Nigeria, although re-importations have been recorded in several other countries. Progress towards eradication of wild-type PV can be followed at the polio eradication website of

WHO: www.polioeradication.org. One index of success in controlling transmission comes from molecular analysis of isolates, which can be classified into different genotypes, which disappear one by one as transmission is broken and they die out. PV2 is completely eliminated first, followed by type 1 and then type 3. In fact the last case of poliomyelitis anywhere in the world attributable to naturally occurring wild-type 2 PV occurred in October 1999; thus complete eradication is theoretically possible.

Difficulties in the Eradication Programme

Most infections with poliovirus are silent, in contrast to smallpox, the only other disease to be eradicated by vaccination. This raises particular problems and makes strategies such as quarantine largely ineffective. Thus if one country in the world continues to support polio circulation, all countries are at risk. This was illustrated by events in Nigeria in 2004. Resistance to vaccination against polio began to develop in response to claims that the vaccine contained steroids which would sterilize recipients, the view that the programme was imposed by Western countries and the fact that the country had other more significant diseases, including malaria, human immunodeficiency virus (HIV) and tuberculosis. As a result the northern provinces ceased vaccination, and within a short period polio had been reintroduced and was circulating in many countries in Central Africa. Moreover the pilgrimage to Mecca resulted in the introduction of polio into Yemen and Indonesia from Nigeria. Resistance to vaccination is thus a major issue.

A second issue arose in northern India, where polio cases were disproportionately found in the Muslim minority population, who for whatever reason were not caught by vaccination activities. The response has included supplementary immunization activities (SIAs) which include house to house vaccine delivery. The scale of the programme is colossal and there continue to be problems in financing it adequately. Delivery of vaccine in areas of conflict, including Pakistan and Afghanistan, is also obviously difficult, and plays a part in the persistence of polio in these two countries.

Polio continues to circulate in northern India and the reasons are not clear. Some children are reported to have received 16 doses of vaccine before the age of five yet have still developed the disease and the usual view is that for unknown reasons oral polio vaccine is less effective in northern India than in any other part of the world.

A recent strategy to attempt to deal with the poor efficacy of OPV in certain areas has been to introduce monovalent polio vaccine. The usual vaccine contains all three serotypes including type 2 but in some areas only one serotype is a problem. Thus the use of all three types may reduce efficacy by competition between the relevant

type and the other two. The use of monovalent type 1 and type 3 vaccines has increased the efficacy of vaccination in certain problem areas. It may be that this could be partly attributed to its being a new strategy, so energizing the vaccine delivery teams, but there is a reasonable scientific explanation for it as well.

The difficulties in finally eradicating the virus from the few remaining countries where it is endemic have led some to call the entire programme into question and doubt its feasibility. On the other hand the success of the programme to date cannot be questioned. Increased usage of IPV has been advocated, and it is clear that the nonreplicating vaccine will play a bigger part in the immediate future as the last pockets are cleared and attention turns to how vaccination against polio could be stopped safely.

The End Game

The success of the eradication programme has focused attention on cases of poliomyelitis caused by the oral vaccine or strains derived from it. For vaccine recipients and their immediate contacts, Nkowane *et al.* (1987) estimated the risk of poliomyelitis in the United States as 1/530 000 primary vaccinees and $1/2 \times 10^6$ overall. Cases associated with the type 1 strain are 10-fold less than with the type 2 and type 3 strains taken together. This incidence should be taken in the context of more than 10 000 cases/year in the United States before the use of vaccines. The rate is the same in India, at the time of writing, to within a factor of 2. Thus there is a low but real incidence of vaccine-associated paralytic poliomyelitis (VAPP), and the virus can clearly spread to others, causing contact cases; this is actually an advantage where wild polio is endemic as it increases effective coverage.

However, a time is rapidly approaching when poliomyelitis will only be caused by the vaccine, raising the question of how vaccination can be safely stopped. Vaccine-associated cases have been a major driver in convincing developed countries to use IPV and the whole of North America and most of the countries of the European Union now use IPV exclusively. The switch has been made easier by the use of combinations including the other childhood vaccines such as diphtheria, tetanus and pertussis, so that one vaccination now immunizes against many diseases.

However there are other reasons for wishing to stop the use of OPV if possible. The ability of the vaccine virus to spread from recipients is an advantage when the virus is endemic as more are vaccinated than the recipient, but becomes a major problem as the wild-type virus is eliminated. In countries where the routine vaccine coverage is poor, immunized and unimmunized children mix freely and transmissible strains can be selected. Outbreaks in

Hispaniola, Egypt, Madagascar and the Philippines and several other countries have been caused by such viruses (Adu *et al.*, 2007; Kew *et al.*, 2005; Shimizu *et al.*, 2004; Vinje *et al.*, 2004). They have included representatives of all serotypes, but most commonly type 1. In most cases the viruses were recombinant strains whose genome included a large sequence unrelated to the vaccine, probably derived from a non-polio enterovirus of group C. At the time of writing there is such an outbreak in Nigeria which is one of the few countries that has not yet eradicated polio. It may be possible to deal with circulating vaccine-derived strains of PV by stopping routine vaccination campaigns of poor coverage with a final NID. This depends on the occurrence of circulating vaccine-derived polioviruses (cVDPV) being relatively rare.

A second vaccine-associated problem is the occurrence of prolonged virus excretion in a proportion of hypogammaglobulinaemic individuals, who may shed virus for several years. Approximately 20 cases of long-term excretion are known at present and the incidence of long-term excretion in patients inadvertently or deliberately given the vaccine is probably of the order of 1% (Halsey *et al.*, 2004). Although most spontaneously cease excreting virus after a period of a few years some continue for very long periods, of which the longest is an individual who has been excreting type 2 PV for over 20 years so far. Such individuals, who are more common and long lived in developed countries, pose a hazard to themselves and to all unimmunized individuals around them. Treatments to stop virus excretion have not been identified (MacLennan and MacLennan 2005; MacLennan *et al.*, 2004).

In view of these considerations, there is a developing interest in replacing OPV with IPV globally, in order to limit excretion of vaccine-strain virus. However it is not known whether IPV can break transmission in tropical settings, and as the vaccine must be given by injection there may also be problems in achieving high coverage.

Containment of Polioviruses After Eradication

When PV as well as poliomyelitis is eradicated there is the possibility that it will re-emerge. Polio has been a laboratory virus for many years and is present in many clinical samples such as faecal specimens taken for reasons not related to polio. Efforts have been made wherever polio has been declared eradicated to identify and contain or destroy such laboratory stocks, and great progress has been made (Dowdle *et al.*, 2006).

However, it will be necessary to manufacture vaccine for some time after eradication, and the most likely kind will be IPV to avoid seeding the world with virus. IPV is prepared from wild-type strains which are grown in very high quantities and there are isolated examples of escape of virus from production facilities in the past. Such

facilities are operated under conditions specified by WHO to ensure that the virus will not escape. It is likely that OPV will be stockpiled as a way of interrupting epidemics should they arise and consideration has been given to the use of the Sabin strains to manufacture IPV. This is in fact extremely difficult from a number of points of view, including the need for clinical trials to demonstrate effectiveness, but it could provide a potential source of OPV should it be needed. Alternative polio strains for production may also be developed (Burns *et al.*, 2006; Macadam *et al.*, 2006)

Re-emergence of Polio After Eradication

Poliovirus could re-emerge after eradication by escape from manufacturing plants, laboratory or other unrecognized reservoirs including long-term excretors of the virus, or by deliberate release by bioterrorists. It is conceivable that a CVA could evolve to fill the vacated niche. It is thus necessary to maintain stocks of polio vaccine to deal with future emergencies. How this is to be done and how the stock is to be used have not been fully worked out as yet.

Antiviral Therapy

It has long been recognized that picornavirus infection in cell culture could be inhibited by agents which bind stably to the virion, preventing cellular attachment, uncoating and intracellular delivery of the viral genome. Although a series of such compounds has been developed, to date only one agent, pleconaril, has sufficiently low toxicity to be a promising therapeutic agent (Romero, 2001). Pleconaril (3-[3,5-dimethyl-4[3-(3-methyl-5-isoxazolyl)propyl]oxy]phenyl]-5-(trifluoro-methyl)-1,2,4-oxadiazole; Picovir) reduces viral replication and morbidity in animal models of enterovirus disease and, in a phase II clinical trial, reduced the duration and severity of picornavirus-related respiratory infections. Pleconaril is also effective in children and adults with enteroviral meningitis, although no benefit was observable in a study of infants under one year with meningitis. Pleconaril was available on a compassionate use basis for life-threatening infections, and reports of such treatments suggested a measure of efficacy in immunocompromised patients with persistent infection, and in neonates with fulminant infection. Although drug-resistant viruses were isolated in cell culture, they appeared to be attenuated with respect to virulence in animal models. However pleconaril has now been withdrawn.

FUTURE PROSPECTS

Poliomyelitis is the most significant disease caused by a human enterovirus, and enormous progress has been

made towards its eradication. It is anticipated that complete eradication of disease caused by the wild-type virus will be achieved in the near future. Other enteroviruses remain and contribute significantly to human disease. New research methods, including the use of genetically cloned and modified viruses and transgenic and genetic knockout animal models, will increase our understanding of viral pathogenesis and may suggest novel therapeutic strategies. The advent of molecular diagnosis will enable the diagnostic laboratory to issue results within a time frame that influences patient management in a way not previously possible using slower methods. The case for developing new vaccines against non-polio enteroviruses continues to be made, particularly where a particular serotype is associated with severe disease or public health risk, although the costs involved are a major obstacle. In the meantime, it is likely that additional antiviral agents will be developed, targeting viral enzymes involved in the replication process as well the structural components of virus particles. Other antiviral strategies under investigation include the use of soluble receptor proteins to block virus uptake, and antisense oligodeoxynucleotides to interfere with viral RNA replication. Here too, though, the costs of development, licensing and marketing may prove a significant barrier to affordable, widely available treatment for enterovirus infections.

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Poxviruses

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INTRODUCTION

Viruses classified as members of the *Poxviridae* family include significant human pathogens including variola, the agent of smallpox. During the twentieth century alone, smallpox is estimated to have caused over 500 million human deaths (Tucker, 2001). Yet the disease and the naturally occurring virus itself were eradicated by means of the World Health Organization global eradication campaign (Fenner *et al.*, 1988). This programme of intensively vaccinating all humans in a ring surrounding every suspected case of smallpox was successful in part because variola is a human-only disease; there are no animal reservoirs to reintroduce the virus into the human population. Despite the eradication of naturally occurring smallpox, variola virus remains a concern because of the possibility that clandestine stocks of the virus may be in the hands of bioterrorists (Henderson *et al.*, 1999). The impact of a reintroduction of smallpox virus into the human population now would be even more catastrophic than during the last century; vaccination programmes were abandoned worldwide around 1976, the prevalence of immunosuppressed populations has grown, and mobility, including intercontinental air travel, has accelerated the pace of viral spread worldwide. As an example, monkeypox virus, which persists in endemic foci in West and Central Africa, was recently imported inadvertently into the United States via a shipment of rodents originating in Ghana (Centers for Disease Control and Prevention, 2003a; Charatan, 2003; Reed *et al.*, 2004). More than 50 human infections were documented as a consequence of close contact with infected animals traced back to this shipment. This chapter deals only with the poxviruses that cause human infection.

VIRUS CHARACTERISTICS

Classification

Poxviruses infect most vertebrates and invertebrates, causing a variety of diseases of veterinary and medical importance. The poxvirus family is divided into two main subfamilies, the *Chordopoxvirinae*, members of which infect vertebrates, and the *Entomopoxvirinae*, members of which infect insects. The *Chordopoxvirinae* subfamily is divided into eight genera, one of which is *Orthopoxvirus*, which includes the human pathogens variola, monkeypox virus, and other species which infect humans including cowpox and vaccinia viruses. Members of the poxvirus family are mostly zoonotic pathogens, and, with the exception of the orthopoxviruses, only a minority produce disease in humans (Table 26.1). Poxviruses of domestic animals, such as sheeppox, camelpox and avipox, can cause considerable problems for communities dependent on these animals (Baxby, 1988, 1998).

Morphology

Orthopoxviruses are oval, brick-shaped particles with geometrically corrugated outer surface. The particles sizes range from 220 to 450 nm in length and 140 to 260 nm in width. The outer envelope consists of a lipoprotein layer embedding surface tubules and enclosing a core historically described as bi-concave, due to an electron microscopy fixation artefact. The core contains the viral DNA and core fibrils, and is surrounded by the core envelope and a tightly arranged layer of rod-shaped structures known as the palisade layer. Between the palisade layer and the outer envelope exist two oval masses known as the lateral bodies. (Figure 26.1). Two infectious forms of

Table 26.1 Poxviruses pathogenic for humans

Genus	Virus	Reservoir host	Animal naturally infected	Geographical distribution	Comment
Orthopoxvirus	Smallpox	Humans	—	Formerly worldwide	Last endemic case 1977
	Monkeypox	Squirrels?	Squirrels African rodents Monkeys	West and Central Africa	Eradication confirmed 1979 Rare zoonosis. Overall mortality 10%. Limited case-to-case spread. Recent importation to non-endemic area (USA)
	Cowpox	Rodents	Cats	Europe, western Commonwealth of Independent States	Rare zoonosis. Contact with cattle unusual
	Vaccinia	Buffalo?	Cattle Buffalo	India	Variant of vaccinia. Established in nature
Parapoxvirus	Orf	Sheep	Sheep	Worldwide	Common trivial zoonoses Occupational hazards
	Pseudocowpox	Goats Cattle	Goats Cattle		
Molluscipoxvirus	Molluscum	Humans	—	Worldwide	Trivial infections. Often sexually transmitted
Yattapoxvirus	Tanapox	Monkeys	Monkeys	Kenya, DRC	Rare trivial zoonosis. Obtain travel history

orthopoxviruses (described below) result from the replication cycle.

Phylogenetic Relationships

The evolutionary relationships among the poxviruses have been elucidated by the recent availability of complete DNA sequences for over 30 species. Phylogenetic analysis reveals that variola and camelpox virus are more closely related to each other than any other members of the genus, while vaccinia is most closely related to cowpox virus strain GRI-90 (Gubser and Smith, 2002; Gubser *et al.*, 2004). Interestingly, cowpox virus strain Brighton appears to be less closely related, indicating that there are at least two separate species currently contained under the name cowpox virus. Monkeypox virus does not group closely with any other orthopoxvirus, indicating it diverged from the rest of the members of the genus relatively long ago. Yet vaccination prevents the disease. Concern has been raised that minor modifications to the camelpox virus genome might conceivably result in a virus with the attributes of variola. Virulence or attenuation may hinge on a relatively few number of genetic determinants. For example, variola major (associated with a 30% fatality rate) and variola minor (<1% fatality rate) are >98% identical over the length of the 185 000 kb genome.

As anticipated from the genomic homologies, members of the *Orthopoxvirus* genus are antigenically related. Cross-reacting and species-specific neutralizing antigens have been identified by serum absorption and monoclonal antibody studies (Baxby, 1982). Nine neutralizing epitopes have been identified among the intracellular mature viral (IMV) particles of different species of orthopoxviruses (Czerny *et al.*, 1994), and additional epitopes, believed to be critical in protection against infection *in vivo*, exist on extracellular enveloped viral (EEV) particles (Vanderplasschen *et al.*, 1997; Viner and Isaacs, 2005). Viral antigens contained in the viral envelope are of pre-eminent importance with regard to protective antibody responses: envelope antigens were absent from virion suspensions used for inactivated smallpox vaccines, which proved to be ineffective (Kaplan *et al.*, 1965; Payne, 1980).

Replication

Orthopoxvirus genomes are linear, double-stranded DNA ~200 kb. The genomes encode about 176–266 proteins, including a number of enzymes and factors that are necessary for self-replication and maturation. The central region of the genome contains highly conserved genes that are essential for viral replication, and the

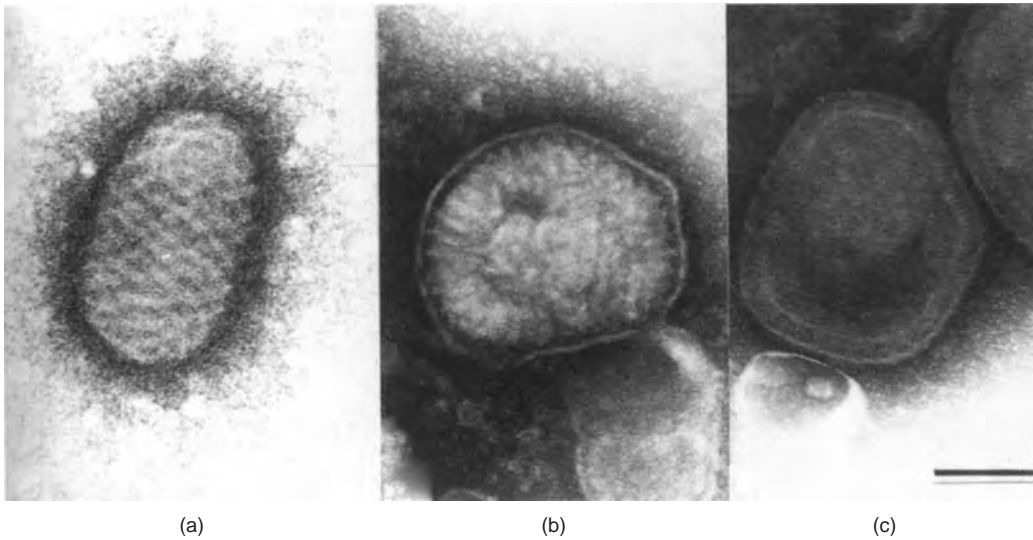


Figure 26.1 (a) Mulberry (M) form of non-enveloped parapox virion; (b) naturally relaxed (extracellular) M form of vaccinia virus and (c) capsule (c) form of vaccinia virus (negative stain; bar = 150 nm).

terminal regions contain less conserved genes that are important for virus–host interactions. The virus contains a number of virus-encoded enzymes, in particular a DNA-dependent RNA polymerase, which transcribes the viral genome (Moss, 2001). Replication takes place in cytoplasmic factories referred to as B-type inclusions, in which virions at various stages of assembly are seen. It is not clear whether or not host cell nuclear factors are involved in viral replication or maturation. Cells infected with some poxviruses (e.g. cowpox, avian poxviruses) also contain electron-dense A-type inclusions, usually containing mature virions; A-type inclusions are easily seen by light microscopy.

Viral replication begins with attachment of viral particles to host cell surface, most likely through cell receptors, and involves expression of early, intermediate and late genes (Moss, 2001). Initial uncoating occurs during entry, followed by synthesis of early mRNAs which are translated to facilitate further uncoating and transcription of intermediate mRNAs. Intermediate mRNAs, in turn, are translated to allow transcription of the late mRNAs. The late mRNAs are translated into structural and enzymatic components of the virions. These components, along with DNA concatemers that are formed during the early phase of replication, are assembled into genomic DNA and packaged into immature virions, which then evolve into brick-shaped infectious IMV particles. IMV particles are infectious only when they are released by cell lysis. They can acquire a second membrane from an early endosomal component to form the intracellular enveloped virion (IEV). IEVs migrate to the cell surface via

microtubules and fuse with the cell membrane to form cell-associated virions (CEV). Finally, CEV induce polymerization of actin to form filaments which effect the direct transfer of CEV to adjacent cells. If CEV become dissociated from the cell membranes they are termed extracellular enveloped virions. While IMV particles are produced in greatest abundance in cell culture, and are the most stable to environmental degradation, CEV and EEV particles are suspected to play a more critical role in cell-to-cell spread in the intact animal (Smith *et al.*, 2002).

Many of the orthopoxvirus gene products interact with and modulate essential functions of the host cells and immune processes (Moss, 2001; Johnston and McFadden, 2003). The limited host range of variola is thought to relate in large part to the unique association of viral gene products with the myriad of host signalling pathways. Therefore, strategies which block such key pathways in the replication and maturation of poxviruses provide potential targets for therapeutic intervention (McFadden, 2005).

CLINICAL ASPECTS OF ORTHOPOXVIRUS INFECTIONS

Smallpox

Variola virus is highly stable and retains its infectivity for long periods outside the host (Huq, 1976). It is infectious by aerosol (Henderson *et al.*, 1999); natural air-borne spread to other than close contacts is unusual, but does

occur (Meiklejohn *et al.*, 1961; Downie *et al.*, 1965). Approximately 30% of susceptible contacts became infected during the era of endemic smallpox (Foege *et al.*, 1975) and the World Health Organization (WHO) eradication campaign was predicated on close person-to-person proximity being required for transmission to occur reliably. Nevertheless, variola virus's potential in low relative humidity for air-borne dissemination was alarming in two hospital outbreaks (Wehrle *et al.*, 1970). Patients with smallpox were infectious from the time of onset of their eruptive exanthem, most commonly from days 3 to 6 after onset of fever. Infectivity was markedly enhanced if the patient manifested a cough. Indirect transmission via contaminated bedding or other fomites was infrequent (Maccallum and McDonald, 1957). Some close contacts harboured virus in their throats without developing disease, and hence might have served as a means of secondary transmission (Sarkar *et al.*, 1973a, 1973b).

After a patient is exposed to aerosolized virus, variola travels from the upper or the lower respiratory tract to regional lymph nodes, where it replicates and gives rise to viraemia, which is followed soon thereafter by a rash (Breman and Henderson, 2002). The incubation period of smallpox averages 12 days (range 9–14 days), and contacts are quarantined for a minimum of 16–17 days following exposure (Breman and Henderson, 2002). Following infection via the respiratory route and replication in local lymph nodes, variola virus disseminates systemically to other lymphoid tissues, spleen, liver, bone marrow and lung. During this asymptomatic, prodromal period, variola virus can be recovered from the blood, but the yield is much lower later in the illness. Clinical manifestations begin acutely with malaise, fever, rigours, vomiting, headache and backache; 15% of patients develop delirium. Approximately 10% of light-skinned patients exhibit an erythematous rash during this phase. Two to three days later an enanthem appears concomitantly with a discrete rash about the face, hands and forearms. Because of the lack of a keratin layer on mucous membranes, lesions there shed infected epithelial cells and give rise to infectious oropharyngeal secretions in the first few days of the eruptive illness, and occasionally 24 hours prior to eruption (Downie *et al.*, 1961). These respiratory secretions are the most important but not the sole means of virus transmission to contacts. Following subsequent eruptions on the lower extremities, the rash spreads centrally during the next week to the trunk. Lesions quickly progress from macules to papules and eventually to pustular vesicles.

Lesions are more abundant on the extremities and face, and this centrifugal distribution is an important diagnostic feature. In distinct contrast to the lesions seen in varicella, smallpox lesions on various segments of the body remain generally synchronous in their stage of development.

From 8 to 14 days after onset, the pustules form scabs, which leave depressed depigmented scars on healing. Although variola titres in the throat, conjunctiva and urine diminish with time (Sarkar *et al.*, 1973a) virus can readily be recovered from scabs throughout convalescence (Mitra *et al.*, 1974) Therefore, patients should be isolated and considered infectious until all scabs separate.

During the last century of smallpox occurrence, two distinct forms of smallpox came to be recognized: variola major and variola minor. The prototypical disease, variola major, was the highly virulent form of historical significance and was still prevalent in Asia and parts of Africa during the twentieth century. Variola minor, first described in Africa by Korte in 1904, was distinguished by its milder systemic toxicity and more diminutive pox lesions (Fenner *et al.*, 1988), although Dixon reported many cases that were indistinguishable from variola major in his extensive comparison of lesion types (Dixon, 1962). A similar mild form, called alastrim, was found by Chapin to have occurred in North America as early as 1896, and subsequently exported to South America, Europe and Australia. Variola minor and alastrim were apparently caused by two distinct viral strains of reduced virulence and both typically caused 1% mortality in unvaccinated patients (Fenner *et al.*, 1988).

According to the Rao classification there were five distinct clinical presentations of variola (Rao, 1972). Three-quarters of cases of variola major fell into the *classic, or ordinary, type*. After the development of prodromal fever and constitutional symptoms, patients developed the typical variola rash, centrifugal in distribution, with synchronous progression from macules to papules, to vesicles to pustules and then scabs. The fatality rate was 3% in vaccinated and 30% in unvaccinated patients. Less frequently other clinical presentations of smallpox occurred, probably as a consequence of difference in host immune response. *Flat-type smallpox*, noted in 2–5% of patients, was characterized by both severe systemic toxicity and the slow evolution of flat, soft, focal skin lesions which did not resemble the classical variola exanthem. This syndrome caused 66% mortality in vaccinated and 95% mortality in unvaccinated patients. *Haemorrhagic-type smallpox*, seen in fewer than 3% of smallpox patients, was heralded by the appearance of extensive petechiae, mucosal haemorrhage and intense toxemia; death usually intervened before the development of typical pox lesions (Downie *et al.*, 1969a). However, on occasion haemorrhagic smallpox also occurred in the classic type later in the disease. Both haemorrhagic and flat-type smallpox may have been heralds of underlying immunodeficiency, and haemorrhagic forms occurred more commonly in pregnant women and very young children (Rao *et al.*, 1963). The fourth presentation was the modified type

which occurred typically but not exclusively in previously vaccinated individuals and was characterized by moderation of constitutional symptoms, typically reduced numbers of lesions and rapid evolutions of lesions with scabs forming by the ninth day of the illness. The *variola sine eruptione* was characterized by occurrence of prodromal fever and constitutional symptoms but these patients, almost all of whom had a history of vaccination, never developed a rash (Rao, 1972). In actuality, the manifestations of variola infection fall along a spectrum, and classification is primarily for the purpose of prognosis.

Bacterial superinfection of pox lesions was relatively common in the days before antibiotics but especially so in tropical environments and in the absence of proper hygiene and medical care (Fenner *et al.*, 1988). Arthritis and osteomyelitis developed late in the course of disease in about 1–2% of patients, more frequently occurred in children, and was often manifested as bilateral joint involvement, particularly of the elbows (Gupta and Srivastava, 1973). Viral inclusion bodies could be demonstrated in the joint effusion and bone marrow of the involved extremity. Cough and bronchitis were occasionally reported as prominent manifestations of smallpox, with attendant implications for spread of contagion; however, pneumonia was unusual (Fenner *et al.*, 1988). Pulmonary oedema occurred frequently in haemorrhagic and flat-type smallpox. Orchitis was noted in approximately 0.1% of patients. Encephalitis developed in 1 in 500 cases of variola major, compared with 1 in 2000 cases of variola minor. Keratitis and corneal ulcers were important complications of smallpox, progressing to blindness in slightly fewer than 1% of cases. Disease during pregnancy precipitated high perinatal mortality, and congenital infection was also recognized.

Partial immunity from vaccination resulted in modified-type smallpox, in which sparse skin lesions evolved variably, often without pustules, and quickly, with crusting occurring as early as the seventh day of illness. Some fully immune individuals would develop fever, sore throat and conjunctivitis (called contact fever) on exposure to smallpox. This lasted several days, but did not give rise to the toxicity or minor skin lesions that signify *variola sine eruptione*.

Individuals who recovered from smallpox possessed long-lasting immunity, although a second attack could occur in 1 in 1000 after an intervening period of 15–20 years (Rao, 1964). Both humoral and cellular responses are important components of recovery from infection. Neutralizing antibodies peak two to three weeks following onset, and last longer than five years (Downie *et al.*, 1969b), and perhaps several decades in some individuals (Viner and Isaacs, 2005).

A recent review of all pathology reports published in English from the last 200 years (Martin, 2002) suggested that in general, otherwise healthy patients who died of smallpox usually succumbed to renal failure, shock secondary to volume depletion and difficulty with oxygenation and ventilation as a result of viral pneumonia and airway compromise, respectively. Degeneration of hepatocytes might have caused a degree of compromise, but liver failure was not usually the proximate cause of death.

Due to the limited tools that were available when smallpox was an endemic disease, much of the pathogenesis remains a mystery. Detailed analysis of the pathophysiology of the disease course using the monkeypox and variola primate models and in comparison with limited clinical and pathology data from human smallpox victims suggest a role for dysregulation of the immune response involving the production of pro-inflammatory cytokines, lymphocyte apoptosis and the development of coagulation abnormalities. In the animal models, high viral burdens were identified in numerous target tissues. It is likely that these viral burdens were associated with organ dysfunction and multisystem failure. Distribution of viral antigens by immunohistochemistry as well as replicating virus by electron microscopy correlated with pathology in the lymphoid tissues, skin, oral mucosa, gastrointestinal tract, reproductive system and liver. Apoptosis was a prominent observation in lymphoid tissues with a striking loss of T cells observed. The cause of this widespread apoptosis remains unknown. However, it is likely that strong production of proinflammatory cytokines at least in part contributes to the upregulation of various proapoptotic genes. The strong upregulation of cytokines may also have contributed to the development of a haemorrhagic diathesis. The detection of D-dimers, as well as other changes in haematological parameters in monkeys that developed classical or haemorrhagic smallpox suggests that activation of the coagulation cascade is a component of both disease syndromes, although in human populations the occurrence of haemorrhagic smallpox was only ~1–3% of the total cases observed.

From these recent studies of variola and monkeypox virus infection in primates, the 'toxaemia' described by clinicians for human smallpox (Fenner *et al.*, 1988) may be fundamentally related to the processes underlying septic shock (Levi *et al.*, 2003). Common denominators include lymphocyte apoptosis, pro-inflammatory cytokines (exuberant production of type I interferons, IL-6, tumour necrosis factor- α , and IFN- γ measurable in plasma); and disseminated intravascular coagulation. Aberrant activation of all of these pathways is a hallmark of pathological activation of the innate immune system and contributes to toxic shock.

To facilitate viral replication, orthopoxviruses generally have the ability to modulate their host's immune response to the pathogen's advantage. Poxviruses encode proteins that target or interrupt the natural inflammatory response, interfere with apoptosis, synthesis of steroids and initiation of the complement system. In general, these proteins block either extracellular immune signals (by mimicking or interfering with cytokine/chemokine proteins and/or receptors); or they work intracellularly by interfering with apoptosis, targeting by the immune system, or intracellular immune cell signalling. A combination of these mechanisms may allow the virus to overcome immunologic surveillance and establish clinical disease in the host (Moss and Shisler, 2001).

Monkeypox

Monkeypox virus was so named because it was first detected in captive Asiatic monkeys; however, the virus has only been found naturally in Africa and evidence points to squirrels (*Funisciurus* spp., *Heliosciurus* spp.) as important reservoir hosts. Recently, a Gambian rat (*Crictomys gambiensis*), rope squirrel (*Funisciurus* spp.) and doormouse (*Graphiurus* spp.) from the African shipment originating in Ghana and implicated in the US monkeypox outbreak were found to be infected with monkeypox by viral isolation and nucleic acid detection (polymerase chain reaction (PCR)) (Centers for Disease Control and Prevention, 2003a; Enserink, 2003). Initial surveys in Zaire (DRC) detected monkeypox-specific antibodies in 85 of 347 (25%) squirrels sampled but from none of 233 terrestrial rodents. Monkeypox-specific antibody has been detected in very few monkeys, which, like humans are probably only occasional hosts (Khodakevich *et al.*, 1988). Subsequent work (Hutin *et al.*, 2001) in the Democratic Republic of Congo (DRC) found evidence of orthopoxvirus seroreactivity in a some terrestrial rodents tested, including Gambian rats (*Crictomys emini*) and elephant shrews (*Petrodromus tetradactylus*).

Historically, as a consequence of smallpox surveillance activities beginning in the 1970s, human cases of monkeypox were recognized for the first time. Alarmingly, monkeypox cases were almost indistinguishable from smallpox. Only occasional human cases were reported from Central Africa following cessation of routine surveillance activities post-smallpox eradication, but there was a resurgence in 1996–1997 which has not yet been fully explained. Increased political unrest leads to population displacement and breakdown of routine control measures, and the levels of vaccine-induced immunity will decline with time. A potentially serious finding that requires clarification is the observation that case-to-case transmission appears to have occurred more frequently during 1996–1997 than earlier (Heymann *et al.*, 1998).

Monkeypox continues to be sporadically reported (Meyer *et al.*, 2002). Comparison of the genomes of smallpox virus and monkeypox virus strains isolated up to 1986 suggested they have evolved separately (Douglass and Dumbell, 1992; Chen *et al.*, 2005) and the results of complete genome analysis (Shchelkunov, 2001) confirms this observation.

Sporadic outbreaks continue to occur and cause concern (Di Giulio and Eckburg, 2004b; Heymann *et al.*, 1998; Rimoin *et al.*, 2007) but most information is available about cases that occurred prior to 1988 (Bremner *et al.*, 1980). More recently, monkeypox infection of humans was identified in the United States as a result of exposure to ill prairie dogs, which were probably infected after exposure to infected West African small mammals imported as exotic pets (Charatan, 2003).

The clinical features of human monkeypox are classically described as being similar to those of smallpox (Jezek *et al.*, 1983). Disease begins with a two- to four-day pre-eruptive phase with high fever and prostration. The rash develops and progresses synchronously over two to four weeks, evolving from macules to papules to vesicles and pustules to scabs. Lesions are usually umbilicated, have a centrifugal distribution and involve the palms and soles. Sore throat and frank tonsillitis are frequent during the eruptive phase of human monkeypox (Jezek *et al.*, 1983, 1986). Lymphadenopathy is a common finding that differentiates monkeypox from smallpox (Figure 26.2). Lymphadenopathy has been documented in up to 83% of unvaccinated patients with monkeypox, most frequently arising early in the course of infection, involving the submandibular and cervical nodes with somewhat less frequent involvement of axillary and inguinal nodes.

Clinical manifestations of human monkeypox are probably more diverse and not as stereotypical as those of smallpox. Mild infections were frequent in the first recognized African cases, with 14% of patients having fewer than 25 lesions and no incapacity (Jezek *et al.*, 1983). In a series of 282 patients, the exanthema first appeared somewhere other than the face in 18%, 31% of vaccinated patients had pleomorphic or 'cropping' appearance of rash lesions and 9.4% had what was described as centripetal distribution (Jezek *et al.*, 1987b). All of these features are inconsistent with a mimic of smallpox. Cases from the recent US outbreak tended to be mild with fewer lesions than most African cases. Only 19 of 78 suspected cases in the United States were hospitalized and only two had significant illness requiring some form of medical intervention (Centers for Disease Control and Prevention, 2003b; Sejvar *et al.*, 2004). None of the initial cases was suspected as a smallpox-like disease. A '*sine eruptione*' form of monkeypox has not been described, but the number of serologically diagnosed infections without history



Figure 26.2 Human monkeypox on the eighth day of rash. Note the submaxillary and inguinal lymphadenopathy. (Source: Reproduced from (Fenner *et al.*, 1988) by permission of WHO.)

of consistent rash illness suggest that this is a possibility (Jezek and Fenner, 1988). No haemorrhagic form of human monkeypox has been documented (Di Giulio and Eckburg, 2004a, 2004b).

Complications of monkeypox are more common in unvaccinated people and children (Jezek *et al.*, 1988). During intensive surveillance in the DRC between 1980 and 1986, secondary bacterial superinfection of the skin (19.2% unvaccinated) was the most common complication, followed by pulmonary distress/pneumonia (11.6% unvaccinated), vomiting/diarrhoea/dehydration (6.8% unvaccinated) and keratitis (4.4% unvaccinated). With the exception of keratitis, the incidence of these complications in vaccinated patients was at least threefold less. Alopecia has been noted in a number of cases (Hutin *et al.*, 2001). Encephalitis was detected in at least one case of monkeypox in the DRC and in one of the cases in the US outbreak of 2003 (Jezek *et al.*, 1987b; Sejvar *et al.*, 2004). As in smallpox, permanent pitted scars are often left after the scabs separate.

Severity of disease and death is related to age and vaccination status of patients, with younger unvaccinated children faring worse (Hutin *et al.*, 2001; Jezek *et al.*, 1983, 1987a; Meyer *et al.*, 2002). The case fatality rate in Africa has varied between different outbreaks and periods of increased surveillance. The fatality rate was 17% during 1970–1979, 10% from 1981–1986 and 1.5% from 1996–1997 (Heymann *et al.*, 1998). There were no fatalities among 72 suspected cases in the recent US outbreak (Centers for Disease Control and Prevention, 2003b). The presence of co-morbid illnesses such as measles, malaria or diarrhoeal disease may have a significant impact on mortality in children (Jezek *et al.*, 1988). Cause of death in monkeypox is not universally clear, although 19 of 33 fatalities in one series of patients had pulmonary distress or frank bronchopneumonia, suggesting superimposed bacterial pneumonia.

Other Orthopoxviruses Infecting Humans

Cowpox

Cowpox is a relatively unimportant zoonosis, of interest principally because of recent re-evaluation of its epidemiology. Despite its name, cowpox virus is not enzootic in cattle. The virus is maintained in a variety of European rodents, (Crouch *et al.*, 1995) and the most commonly reported victim is the domestic cat, from which source human infections are acquired (Baxby and Bennett, 1997).

Cowpox is primarily a localized, cutaneous disease (Esposito and Fenner, 2001). Baxby and co-workers reviewed 54 cases of cowpox infection with a detailed discussion of clinical manifestations (Baxby *et al.*, 1994). The disease usually consists single pock-like lesions on the hands or face, although multiple lesions are seen in roughly a quarter of cases. Typical lesions progress from macule to papule to vesicle to pustule to dark eschar, with a haemorrhagic base being common in the late vesicular stage. Progression from macule to eschar is slow, often evolving over two to three weeks. Local oedema, induration and inflammation is common and can be pronounced. Lesions are painful and are accompanied by regional lymphadenopathy. Complete healing and scab separation usually occurs within six to eight weeks of onset, but can take 12 weeks or longer. A majority of patients experience some constitutional symptoms prior to the eschar stage.

The majority of human cowpox infections are self-limited and without complication. Ocular involvement, including the cornea, can occur, but usually resolves without permanent damage. A small number of severe generalized cowpox infections have been reported, including one fatality (Baxby *et al.*, 1994; Pelkonen *et al.*, 2003). Three of these four described cases had a

history of atopic dermatitis, indicating a risk of increased severity of disease analogous to vaccinia.

Epidemiology As indicated above, cowpox is maintained in rodents; in Britain these are bank voles and woodmice (Baxby and Bennett, 1997; Crouch *et al.*, 1995). The domestic cat is the most common source of human infection and this probably explains the occurrence of cases in children; 26% of 54 cases were in children under 12 years. Most feline and human cases occur between July and October, with only occasional cases between January and June. Human cases sometimes occur in which no source is traced, but despite detailed enquiries only three human cases in Britain since 1968 have been traced to a bovine source, and no case of bovine cowpox has been detected since 1976.

Cowpox virus has a wide host range, and an interesting finding has been its occurrence in a variety of captive exotic species in European zoos. Victims have included cheetahs, lions, anteaters, rhinoceros, elephants and okapi, and infection has occasionally been transmitted to animal handlers (Baxby and Bennett, 1997; Pilaski and Roesen-Wolff, 1988).

Buffalopox

Buffalopox infection in humans has not been extensively described. The limited available data suggest that human infection usually occurs in the hands and consists of inflamed and painful pustular lesions progressing through a Jennerian evolution (Baxby and Hill, 1971; Lal and Singh, 1977; Wariyar, 1937). Regional lymphadenopathy and fever can accompany local disease (Wariyar, 1937).

DIAGNOSIS

Clinical Diagnosis

The clinical presentation of smallpox is very similar to that of many other vesicular and pustular rash illnesses, including varicella, herpes simplex, drug reactions and erythema multiforme. While the index of suspicion for an eradicated disease may be low, the failure to recognize a case of smallpox could result in the unwitting exposure of hospital contacts and the seeding of an outbreak. The 'Smallpox: Diagnosis/Evaluation' page (www.bt.cdc.gov/agent/smallpox/diagnosis) is an essential resource to assist the clinician in evaluating a febrile patient presenting with a rash. This site provides an algorithm to quickly determine the likelihood of clinical smallpox, and a standardized worksheet to classify the risk of smallpox using the Centers for Disease Control and Prevention criteria.

Laboratory Diagnosis

Collection of appropriate specimens is paramount for accurate laboratory diagnosis of orthopoxvirus infection. For virologic diagnosis, specimens from skin lesions are most important, because when viraemia does occur in orthopoxvirus infections, it is an early phenomenon (Fenner *et al.*, 1988). Ideally, cutaneous tissue and blood will be sent for diagnostic testing, with other samples being sent at the request of public health officials or experts in the field (Di Giulio and Eckburg, 2004). Detailed instructions for specimen collection can be obtained from the Centers for Disease Control and Prevention (www.cdc.gov/ncidod/monkeypox/diagspecimens.htm). Briefly, vesicles or pustules should be unroofed, with the detached vesicle skin sent placed in a dry tube and the base of the lesion scraped to make a touch-prep on a glass slide. Biopsy specimens should be split (if possible) and sent in formalin and in a dry tube. If scabs are collected, two scabs should be sent in a dry tube. For oropharyngeal swabs, dacron or polyester swabs should be used and transported in dry tubes. Blood should be collected in a marble-topped or yellow-topped serum separator tube (then centrifuged to separate serum) and in a purple-topped anticoagulant tube for whole blood. Clinical specimens potentially containing orthopoxviruses other than variola virus, including monkeypox virus, may be handled in a biosafety level 2 using BSL-3 procedures (Centers for Disease Control and Prevention, 2007).

Many phenotypic and genotypic methods involving virological, immunological and molecular approaches have been used to identify orthopoxviruses.

Phenotypic Diagnosis

In the past, a presumptive diagnosis of orthopoxviruses required a laboratory with capabilities and expertise in viral diagnostics. Microscopists with experience in poxvirus infections can often recognize the characteristic inclusion bodies (Guarnieri bodies, corresponding to B-type poxvirus inclusions in tissue samples under light microscopy. These cytoplasmic inclusions are haematoxylinophilic, stain reddish purple with Giemsa stain, and contain Feulgen-positive material (Kato and Cutting, 1959). Microscopy alone cannot differentiate members of the genus *Orthopoxvirus*; nevertheless, the epidemiological setting can suggest which species is involved. The orthopoxviruses with pathogenicity for humans can (with the exception of molluscum contagiosum) be grown on the chorioallantoic membranes of 12-day-old embryonated chicken eggs, where they form characteristic pocks. These viruses also grow readily in easily obtained cell cultures, including Vero cells, other monkey kidney cell lines, A549 and others. Variola could characteristically be differenti-

ated from other viruses by a strict temperature cut-off at 39°C. Methods for isolation and identification of individual virus species have recently been reviewed (Damon and Esposito, 2002; Meyer *et al.*, 2004). Electron microscopy will reveal the unmistakable brick-like morphology of orthopoxviruses in thin sections of infected materials. Immunogold stains will permit more precise identification to the species level.

Immunodiagnosis

Serologic testing for anti-orthopoxvirus antibodies is an old technique, and various assays were used extensively in the study of smallpox (Fenner *et al.*, 1988). However, significant serologic cross-reactivity exists between all the orthopoxvirus species, therefore, species differentiation is not possible with conventional serologic assays. Techniques developed in the 1980s to detect monkeypox-specific antibodies are complex and are considered unreliable by some experts (Jezek and Fenner, 1988; Karem *et al.*, 2005). Although complement-fixation tests detect antibodies that disappear within 12 months of infection, other traditional techniques such as immunofluorescence assay (IFA), radioimmunoassay, enzyme linked immunosorbant assay (ELISA), haemagglutination inhibition and neutralization assay detect IgG antibodies that are persistent. Thus differentiating antibodies due to acute infection from antibodies due to prior vaccination can be difficult with single specimens.

Recently, IFA and ELISA have been used to detect IgM in acute infection directed against cowpox and monkeypox, respectively (Karem *et al.*, 2005; Pelkonen *et al.*, 2003). Detection of IgM allows for diagnosis of infection after virus detection is impossible because lesions have healed and scabs have separated, but still allows for establishment of recent infection since IgM seems to disappear within six months. In the investigation of the 2003 US monkeypox outbreak, the Centers for Disease Control and Prevention relied on anti-orthopoxvirus IgG and IgM ELISAs for serologic diagnosis (Sejvar *et al.*, 2004). More recently, a combination of T cell measurements and a novel IgG ELISA was employed to enhance epidemiological follow-up studies to this outbreak (Hammarlund *et al.*, 2005; Slifka and Hammarlund, 2006).

Nucleic Acid Diagnosis

The molecular diagnostic approaches, including DNA sequencing, PCR, restriction fragment length polymorphism (RFLP), real-time PCR and microarrays, are more sensitive and specific than the conventional virological and immunological approaches. Of these techniques, sequencing provides the highest level of specificity for species or strain identification, but current sequencing techniques are

not yet practical as rapid diagnostic tools in most laboratories. RFLP analysis (Loparev *et al.*, 2001; Ibrahim and Mellott, 2005) and microarray genotyping (Lapa *et al.*, 2002) also provide high levels of specificity, and when combined with PCR, these approaches can offer high levels of sensitivity as well. Real-time PCR methods provide exquisite levels of sensitivity and specificity (Saijo *et al.*, 2008; Sofi Ibrahim *et al.*, 2003). The basic concept behind real-time PCR is the measurement, by fluorescence detection, of the amount of nucleic acids produced during every cycle of the PCR. Several detection chemistries, for example, intercalating dyes (SYBR Green), hydrolysis probes (5' nuclease or Taqman, minor groove binding proteins), hybridization probes (fluorescence resonance energy transfer (FRET)) and molecular beacons are used. Several instruments for real-time PCR, for example, the ABI 7700, Smart Cycler, LightCycler, RAPID, Opticon, Rotor-Gene and others are commercially available. When combined with portable analytical platforms such as the Smart Cycler or LightCycler, real-time PCR systems can be readily deployed to field sites for rapid testing.

Successful performance of PCR-based diagnostics requires purification of DNA from body fluid and tissue samples, careful design of oligonucleotide primers and probes and optimization of amplification and detection conditions. There are numerous commercial nucleic acid purification methods for a variety of sample types, which involve cell lysis and protein denaturation followed by DNA precipitation or fractionation by reversible binding to an affinity matrix. Selection of appropriate primers, probes and optimization of assay conditions requires knowledge of genome sequences and molecular biology techniques.

One of the basic techniques used in PCR-based diagnostics is gel analysis, where PCR-amplified regions of the genome are separated on agarose gels by electrophoresis, and the amplicon sizes are used to identify the sample. A number of PCR gel analysis assays have been used to identify cowpox, monkeypox, vaccinia or variola viruses from clinical specimens (Dhar *et al.*, 2004; Meyer *et al.*, 2004; Ropp *et al.*, 1995; Schupp *et al.*, 2001).

Long-distance Polymerase Chain Reaction (LPCR)-RFLP analysis requires the amplification of large DNA fragments with high-fidelity DNA polymerase enzymes. The amplified LPCR products are purified on agarose gels and digested with a restriction enzyme. The digested DNA fragments are then electrophoresed on polyacrylamide gels for a constant period of time at constant voltage, stained with ethidium bromide and the restriction pattern is visualized and photographed with a digital camera. The positions for all DNA fragments in each restriction pattern are determined and digitized by appropriate fingerprinting software. From this pattern a similarity coefficient is

calculated for every pair of restriction patterns and used as an index for species differentiation.

Recently developed real-time PCR assays can test clinical specimens for all orthopoxviruses or for specific species such as vaccinia, variola or monkeypox and can be performed in a matter of hours (Egan *et al.*, 2004; Espy *et al.*, 2002; Kulesh *et al.*, 2004; Saijo *et al.*, 2008; Sofi Ibrahim *et al.*, 2003). Real-time PCR was one of the diagnostic techniques used in the investigation of the 2003 US monkeypox outbreak (Sejvar *et al.*, 2004). Because of its sensitivity, rapidity and ease, real-time PCR will likely become the primary method of preliminary diagnosis of orthopoxvirus infection, with isolation and growth in a high-level containment laboratory reserved for confirmation.

MEDICAL MANAGEMENT

Vaccination

During the WHO Global Eradication Programme, most of the human population received vaccinia virus by scarification. While derivation of current vaccinia strains is uncertain, it is not a form of cowpox, and because Jenner lost his original material used for vaccination, the specific source of current vaccinia strains remains unknown (Dixon, 1962). Although there were multiple manufacturers worldwide, and vaccine lots varied with respect to potency and purity, almost all vaccinia administered was derived from one of two lineages, the New York Board of Health and Lister strains (Fenner *et al.*, 1988). Live vaccinia virus suspension is placed as a drop on the skin or drawn up by capillary action between the tines of a bifurcated needle; the nominal dose of live vaccinia was about 10^5 virions. Primary vaccination is usually uneventful; following introduction into the skin, the virus replicates in basal layer keratinocytes, spreading cell-to-cell, and leading to formation of discrete vesicles. Within a week, the vesicle evolves into a pustule surrounded by inflammatory tissue. This lesion scabs over within 10–14 days; eventually, the scab is shed. It was not uncommon for vaccinees to experience tender axillary lymph nodes, fever and malaise for brief periods. Occasionally, however, complications arose with varying degrees of severity. Accidental transfer of vaccinia from the inoculation site was fairly common, but of little consequence unless transferred to the eye. Generalized vaccinia, which involved systemic spread of the virus and eruption of multiple pocks at distant sites, was more serious; in individuals with eczema or atopic dermatitis, however, this could lead to extensive inflammation and secondary bacterial infection. More serious, life-threatening complications arose in vaccines with defects in cell-mediated immunity; the vaccination

site frequently enlarged to form an ulcer, secondary ulcers appeared, and the infection cleared slowly or not at all. The most serious event was post-vaccinal encephalitis. Although rare, this condition was frequently fatal. Historically, death occurred in approximately one in one million primary vaccinations (Fulginiti *et al.*, 2003; Lane and Goldstein, 2003). Concern is that these adverse event might be more frequent and severe if mass immunization were to be resumed in a unselected general population which now includes transplant recipients on immunosuppressive drugs, human immunodeficiency virus (HIV)-infected individuals and geriatric patients.

Protective immunity elicited by live vaccinia is thought to depend on a combination of humoral and cellular immune responses. Using a monkey model where animals are immunized with vaccinia and challenged with monkeypox, Edghill-Smith has shown that vaccinia-specific B cells are critical for protection (Edghill-Smith *et al.*, 2005). Antibody depletion of B cells, but not $CD4^+$ or $CD8^+$ T cells abrogated vaccinia-induced protection. Edghill-Smith has also shown that simian immunodeficiency virus (SIV)-compromised monkeys could withstand vaccinia if it was preceded by a dose of nonreplicating modified vaccinia ankara (MVA) strain vaccinia, but they were not protected against monkeypox challenge when their $CD4^+$ T cell counts were $<300 \text{ mm}^{-3}$.

Adverse events following vaccinia inoculation include generalized vaccinia, eczema vaccinatum, progressive vaccinia and encephalopathy. Generalized vaccinia is characterized by a disseminated maculopapular or vesicular rash, frequently on an erythematous base and typically occurring six to nine days after primary vaccination. Eczema vaccinatum occurs in individuals with a history of atopic dermatitis, regardless of current disease activity, and can be a papular, vesicular or pustular rash. This rash may be generalized or occur as a localized rash with involvement anywhere on the body but has a predilection for areas of previous atopic dermatitis lesions. Mortality ranges from 17 to 30%. Contact precautions should be used to prevent further transmission and nosocomial infection (Cono *et al.*, 2003).

MVA is an alternative vaccine that has promise as a nonreplicating immunogen. It was utilized in Germany in the later stages of global eradication and was shown to be safe and immunogenic, but its protective efficacy has not been established in humans. MVA was generated by more than 500 serial passages in chick embryo fibroblasts, which resulted in multiple deletions and mutations and an inability to replicate efficiently in human and most other mammalian cells (Blanchard *et al.*, 1998). Ultrastructural examination of purified MVA reveals that most of the particles are enveloped; the host restriction occurs at a late

stage of maturation. The presence of enveloped particles is believed to be important in the development of protective immunity. Experimentally, MVA was demonstrated to protect monkeys against a monkeypox virus challenge after one or two doses of MVA or MVA followed by Dryvax (Earl *et al.*, 2004). Surprisingly, a single dose of MVA also protected when challenge followed immunization by as little as 10 days, although protection was not absolute; a modest number of pocks and a low level viraemia occurred in the MVA recipients following challenge. Recently, rhesus monkeys were used in a similar intravenous challenge model to evaluate a DNA vaccine strategy using a combination of four genes (L1R, A27L, A33R and B5R), with promising results (Hooper *et al.*, 2004).

Treatment

Passive Immunization

Vaccinia immune globulin (VIG) is available from the Centers for Disease Control and Prevention as an investigational new drug (IND) in two formulations, intramuscular and intravenous (Hopkins *et al.*, 2004). VIG may be beneficial in treating some of the adverse events associated with vaccination. It has no proven benefit in treatment of smallpox, and its efficacy in treatment of monkeypox infections is unknown. Monoclonal antibodies have been shown to be beneficial in animal models under certain conditions, but this concept has not yet been sufficiently developed for efficacy testing in humans.

Antiviral Drugs

Antiviral drugs would be useful for treatment of orthopoxviral diseases including smallpox, monkeypox and adverse events associated with vaccination. Presently, the only available antiviral drug is cidofovir, which may be offered under emergency use protocols maintained by both the US Department of Health and Human Services and the Department of Defense. Oral formulations of cidofovir analogues with better bioavailability and lower toxicity are currently under development (Raulin, 2005).

The elaborate replication strategy of poxviruses offers a number of potential targets for therapeutic intervention (Harrison *et al.*, 2004), ST-246 (Sbrana *et al.*, 2007) and Gleevec (Reeves *et al.*, 2005) are drugs with potential to effectively treat systemic orthopoxvirus disease. Recently, a severe case of vaccinia vaccinatium occurred in a 28-month-old child who was a household contact of a recent vaccinee. The child was treated with VIG followed by the antiviral drug cidofovir but his condition continued to deteriorate. Finally, ST-246 was administered under an Emergency Use Authorization (EUA), and the child began to recover soon after (Vora *et al.*, 2008).

While inhibition of viral replication may be necessary to halt the pathogenic disease course, it may not be sufficient. It may also be necessary to reverse the effects of the mounting damage that increasingly appears to be the result of a cytokine storm, which accounts for the 'toxicity' of systemic orthopoxvirus infection (Jahrling *et al.*, 2004). In this regard, cytokine antagonists developed to treat bacterial sepsis and other conditions may play a role in the effective management of smallpox- and monkeypox-infected patients.

OTHER POXVIRUSES INFECTING HUMANS

Parapoxvirus

Parapoxvirus infections are widespread in sheep, goats and cattle. Human infections from these sources are a common occupational hazard for those in contact with infected animals.

Parapoxvirus infection in sheep and goats is usually referred to as contagious pustular dermatitis or orf, and the corresponding human infection as orf. Parapoxvirus infection of cattle is usually referred to as paravaccinia, pseudocowpox or ring sores, and the human equivalent as paravaccinia, pseudocowpox or milker's nodes.

Pathogenesis

Infection occurs via cuts and scratches and usually remains localized. Lesions are produced by hypertrophy and proliferation of epidermal cells, often marked, and leukocyte infiltration. Histological examination shows many small multilocular vesicles within the dermis; true macrovesicles rarely occur (Johannesson *et al.*, 1975; Yirrell and Vestey, 1994). Lymphadenopathy, malaise and generalized lesions are relatively uncommon and the immune response is poor (Leavell *et al.*, 1968; Yirrell and Vestey, 1994).

Clinical Features

The progressive stages of human infection have been described in detail (Johannesson *et al.*, 1975; Leavell *et al.*, 1968; Yirrell and Vestey, 1994) and illustrations provided (Diven, 2001). Lesions start as erythematous papules and progress to a 'target' stage. This, seen one to two weeks after infection, has a red centre surrounded by a white halo and an outer inflamed halo. This progresses to a nodular then papillomatous stage, which often has a 'weeping' surface. In some patients this may enlarge and persist for some weeks before resolving, and may cause some concern (see section on Diagnosis below). The lesion resolves via a crusting stage, which may last some weeks. Occasionally very large granulomatous

lesions occur which may need surgical removal (Pether and Guerrier, 1986).

Most patients have only one lesion, but multiple primary lesions may occur. Systemic reaction is relatively uncommon and the lesion is often not particularly painful. Attention has been drawn to erythema multiforme as a common complication of orf, but because most ordinary cases go unreported, the actual incidence of erythema is probably low. The immune response in natural human infection has been investigated (Yirrell and Vestey, 1994). There is a vigorous but short-lived cell-mediated response, and a relatively poor and short-lived humoral response. This is consistent with the occurrence of second attacks in 8–12% of individuals (Robinson and Petersen, 1983; Yirrell and Vestey, 1994).

Diagnosis

Clinical The viruses that cause orf and paravaccinia are closely related (Mercer *et al.*, 1997), and in the UK human cases are reported as ‘orf/paravaccinia’, whatever the animal source (Baxby and Bennett, 1997). Clinical diagnosis of uncomplicated cases in patients with a known animal contact should not cause difficulties. However, most farmworkers and others working with animals recognize the infection and tend not to seek medical attention for routine cases. Consequently a disproportionately large number of reported cases have no known contact with infected animals. Of approximately 500 cases surveyed during 1978–1995, some 45% had no such contact. Clinical diagnosis of such cases, particularly if severe or prolonged, may cause difficulties. In particular, large weeping granulomatous or papillomatous lesions may be misdiagnosed as malignancies, resulting in one case in unnecessary amputation (Johannesson *et al.*, 1975).

Laboratory Virions with the characteristic morphology of parapoxviruses are usually easily seen by electron microscopy in lesion extracts, and this provides a rapid, certain diagnosis. The virus can be grown in cell culture but this is not attempted routinely. Some nucleic acid detection techniques have been published (Torfason and Gunadottir, 2002).

Epidemiology

Human infection is an occupational hazard of farmworkers, abattoir workers, veterinary surgeons and students and others with frequent exposure to sheep, cattle or goats. It is most common in the lambing and calving seasons, and more commonly reported in sheepworkers than catteworkers; this probably reflects differences in animal husbandry. Of 191 cases with a known source surveyed during 1978–1995, 84% had an ovine source and 16%

were from cattle. During the same period 32 cases occurred in abattoir workers (Baxby and Bennett, 1997).

Control

Most workers at risk get infected at some stage and reinfection is not uncommon. The impact of human infection in the farming and meat industries occasionally causes concern, and has led to industrial disputes (Johannesson *et al.*, 1975; Robinson and Petersen, 1983). Individuals should take care not to spread infection by auto-inoculation or to contacts, including animals. The vaccine used to control orf in sheep is fully virulent and has caused human infection.

Molluscum Contagiosum

Although lesions resembling molluscum and containing poxvirions have been detected in, for example, horses, human molluscum contagiosum is regarded as a specifically human infection and there is no evidence of transmission between humans and other animals. Molluscum is a benign skin tumour which occurs worldwide. It has been the subject of a comprehensive recent review (Birthistle and Carrington, 1997).

Pathogenesis

After a variable, sometimes lengthy, incubation period, papules develop, formed by epidermal hypertrophy. This produces a nodule and also extends the dermis downwards, but the basement membrane usually remains intact. Characteristic inclusions (Henderson–Patterson bodies) are formed in the prickle cell layer and gradually enlarge as the cells age and migrate to the surface. These cells are replaced by hyperplasia of the basal cell layer. The inclusion is a well-defined sac packed with virions (Shelly and Burmeister, 1986). The lesion is circumscribed by a connective tissue capsule and the dermis, apart from distortion, remains essentially normal. Occasionally an inflammatory infiltration of the dermis may occur (Brown *et al.*, 1981).

Clinical Features

Infection is via trauma to the skin. The characteristic lesion begins as a small papule and, when mature, is a discrete, waxy, smooth, dome-shaped pearly or flesh-coloured nodule, often umbilicated. There are usually 1–20 lesions but occasionally there may be hundreds. They may become confluent along the line of a scratch and satellite lesions are occasionally seen.

In children, lesions occur mainly on the trunk and proximal extremities. In adults they tend to occur on the trunk, pubic area and thighs, but in all cases infection may be transmitted to other parts by auto-inoculation

(Brown *et al.*, 1981). Individual lesions last for about two months but the disease usually lasts six to nine months (Steffan and Markman, 1989). More severe and prolonged infection tends to occur in individuals with impaired cell-mediated immunity, including HIV infection (Birthingistle and Carrington, 1997).

Diagnosis

The appearance of lesions in normal cases is generally sufficiently characteristic to permit clinical diagnosis. Virions can usually be seen in large numbers if material expressed from the lesion is examined by electron microscopy. The lack of a marked inflammatory response and failure to isolate an agent in cell culture or chorioalantoic membranes (CAM) should eliminate other poxvirus infections.

Epidemiology

Restriction endonuclease analysis of molluscum virus DNA has detected three main subtypes. Their incidence varies from 80 to 90% for MCV-I to about 1% for MCV-III, but all subtypes cause similar lesions and infect the same anatomical sites (Porter *et al.*, 1992; Scholz *et al.*, 1989).

The virus occurs worldwide and tends to be more common in socially deprived areas. Traditional modes of transmission are associated with mild skin trauma such as contact sports and shared towels; however, there is increasing evidence that the disease is sexually transmitted and that genital lesions are more common (Birthingistle and Carrington, 1997).

Control

Infection is benign and recovery usually spontaneous, but treatment may be sought for cosmetic reasons, particularly for facial or multiple lesions. Various treatments have been tried (Birthingistle and Carrington, 1997). Chemical treatments include phenolics, silver nitrate, trichloroacetic and glacial acetic acid. Physical methods include curettage and cryotherapy. Mild trauma may induce a cure, which may be due to release of virus-infected cells accessible to the immune system.

Prevention is based on attention to personal hygiene and, in developing countries, to general improvements in living conditions. Although relatively unimportant *per se*, the possibility that molluscum may act as a marker for more serious conditions has been raised (Oriol, 1987).

Tanapox

Human infection with tanapox virus was first recognized in the Lake Tana area of Kenya in 1957, and particular attention was paid to it during post-eradication smallpox surveillance. An account of 264 laboratory-confirmed

cases from Zaire (DRC), with colour illustrations, is available (Jezek *et al.*, 1985), as is information on the virus itself (Knight *et al.*, 1989). Recent anecdotal reports of human disease outside Africa have been published and illustrate the need to consider poxvirus aetiologies of illness in travellers returning from and emigrants from endemic areas (Croitoru *et al.*, 2002; Stich *et al.*, 2002).

Pathogenesis and Clinical Features

Infection is via the skin. The lesion is characterized by pronounced epidermal hyperplasia with little involvement of the dermis. There is a short prodromal illness with fever and malaise. The lesion starts as a macule and progresses to a raised nodule, which becomes umbilicated. The lesions are relatively large (~10 mm) and usually break down to form ulcers. There is usually erythema and oedema and lymphadenopathy is common. The lesions generally disappear within six weeks. Most (78%) patients have only one lesion, and very few have more than two. They may occur on any exposed area but the head tends to be spared.

Diagnosis

For diagnosis of tanapox the limited geographical distribution should be considered, as well as travel history. The solid nodular/ulcerated lesions are larger and develop more slowly than those of monkeypox, but are smaller and develop more rapidly than those of tropical ulcers.

Virus can be detected by electron microscopy but this would not exclude morphologically similar viruses; nucleic acid tests (Stich *et al.*, 2002) or diagnostic serological tests on lesion extract would do this. Tanapox virus grows in a number of cell lines (e.g. owl monkey kidney, Vero, MRC-5, BSC-1) but not on CAM.

Epidemiology and Control

The virus probably has a simian reservoir and is restricted to Africa, principally Kenya and DRC. Human-to-human transmission does not occur naturally, and it is thought that transmission from monkeys occurs mainly due to overcrowding during flooding, unrest and so on. With the exception of vaccination, measures for the prevention of monkeypox would be applicable to tanapox; however, the mild and sporadic nature of the infection probably means that specific measures are unnecessary.

DIAGNOSIS

When considering a diagnosis of poxvirus infection restricted geographical distributions and potential animal

sources are important features to consider. In many cases the differential diagnosis is between a particular poxvirus infection and other infections, for example, herpes, tropical ulcers, anthrax, bacterial abscess, rather than between two different poxviruses.

Summary of Diagnostic Approaches

Electron microscopy is important in rapid diagnosis and will confirm parapox infection or exclude pox infection altogether if herpes virus is seen. Further information, if required, can be obtained by virus isolation in cell culture and/or CAM, often making use of efficiency of growth at elevated temperatures. Virus-specific antigens may be detected by a variety of techniques, and nucleic acid detection tests for virus-specific gene products by PCR are now widely used (Ibrahim *et al.*, 2003; Meyer *et al.*, 1997; Ropp *et al.*, 1995).

Poxviruses will remain infective at ambient temperatures, particularly if kept dry. If specimens cannot be tested 'on the spot', infectivity is retained during transport by first-class mail without the need for special transport medium. Vesicle fluid should be smeared on a slide and air-dried. On receipt the material can be reconstituted in buffer. Scrapings from molluscrum and parapox lesions can be treated similarly. The infectivity of virus in dried crusts is retained for long periods. Virus may be extracted from such material by freeze-thawing and ultrasonic treatment. However, if the differential diagnosis includes pathogens less resistant than poxviruses, greater care should be taken and appropriate transport media used.

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Alphaviruses

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INTRODUCTION

One of the original arbovirus definitions was based on biological criteria and described by the World Health Organization (1967) as:

Viruses which are maintained in nature principally, or to an important extent, through biological transmission between susceptible vertebrate hosts by haematophagous arthropods: they multiply and produce viraemia in the vertebrates, multiply in the tissues of arthropods, and are passed on to new vertebrates by the bites of arthropods after a period of extrinsic incubation.

The arboviruses were initially divided into group A (later known as alphaviruses) and group B (later known as flaviviruses), based on the recognition of further antigenically related groups of arboviruses. Advances in our understanding of alphavirus and flavivirus replication and nucleotide sequence data have placed both these groups of viruses as two separate genera in the family *Togaviridae* (Weaver *et al.*, 2005). Currently, 29 known alphaviruses are grouped together into eight complexes (Fauquet *et al.*, 2005). The prototypes of which are western equine encephalitis virus (WEEV), eastern equine encephalitis virus (EEEV), Venezuelan equine encephalitis virus (VEEV), Semliki Forest virus (SFV), Middelburg virus (MIDV), Ndumu virus (NDUV), Trocara virus (TROCV) and salmon pancreas disease virus (SPDV) (Table 27.1). More recently the flaviviruses have been classified as a separate family, the *Flaviviridae*.

Arthropod-borne (arboviruses) are identified as the causative agents for some of the most medically important emerging infectious diseases currently responsible for

significant global health problems (Gubler, 2001). Many of these vector-borne togaviruses are members of the genus *Alphavirus*. Alphaviruses are transmitted to their vertebrate hosts by arthropods (Figure 27.1) and have defined geographic distributions. The eastern equine encephalitis and Venezuelan equine encephalitis lineages are closely related and restricted to the New World, while the Sindbis-like (with the exception of Aura), Semliki Forest (with the exception of Mayaro and Una), Barmah Forest, Middelburg, and Ndumu lineages occur in the Old World. Natural vertebrate hosts include birds and rodents where infection is usually inapparent, but can, under certain circumstances, cause disease and death. When transmission occurs to domestic animals and humans the spectrum of disease varies from a clinically inapparent infection to a severe disease and even death.

THE VIRUS

Morphology

The alphavirus virions are essentially spherical, 60–70 nm in diameter (Figure 27.2) and sensitive to ether and detergent. The virion consists of three components: an outer glycoprotein shell, a lipid bilayer and an RNA-containing core or nucleocapsid (Peters and Dalrymple, 1990). The lipid bilayer is derived from the host cell plasma membrane. The viral-encoded glycoproteins, designated E1 and E2, combine forming glycoprotein spikes that are distributed on the outer surface of the virus and interact with cellular receptors and host-derived antibodies. The glycoproteins are arranged in an icosahedral surface lattice (Harrison, 1986). Complete sequence information available suggests that the viral genomes are 11–12 kb

Table 27.1 Antigenic classification of alphaviruses, global distribution, primary vertebrate hosts, primary and transmission vectors.

Antigenic complex	Virus species	Geographical distribution	Vertebrate host	Primary vector
Barmah Forest	Barmah Forest virus (BFV)	Australia	Birds	<i>Cx. annulirostris</i>
Eastern equine encephalitis	Eastern equine encephalitis virus (EEEV)	North and South America	Birds	Enzootic— <i>Culex</i> spp. Bridge vector— <i>Aedes</i> sp.
	Middelburg virus (MIDV)	South, West and Central America	?	
Ndumu	Ndumu virus (NDUV)	South, West and Central America	?	
Semliki Forest	Semliki Forest virus (SFV)	Africa and Asia	?	<i>Ae. abnormalis</i> and <i>Ae. africanus</i>
	Chikungunya virus (CHIKV)	Africa and Asia	Primates	<i>Ae. africanus</i> and <i>Ae. aegypti</i> <i>Cx. tritaeniorhynchus</i> <i>Ae. aegypti</i>
Western equine encephalitis	Getah virus (GETV)	Asia and Australasia	Mammals	
	Mayaro virus (MAYV)	South America	?	
	Bebaru virus (BEBV)	Asia	&	
	Ross River virus (RRV)	Australia and Oceania	Marsupials	
	Una virus (UNAV)	South America	&	
Venezuelan equine encephalitis	Western equine encephalitis virus (WEEV)	Americas	Birds and mammals	<i>Cx. tarsalis</i>
	Sindbis virus (SINV)	South America	?	<i>Ae. serratus</i>
	Aura virus (AURAV)	North America	Birds	
	Fort Morgan virus (FMV)	Western North America	Birds	<i>Cs. melanura</i>
	Highlands J virus (HJV)	Eastern North America	Birds	
Troara	Whataroa virus (WHAV)	New Zealand	?	
	Venezuelan equine encephalitis virus (VEEV)	The Americas	Mammals	Enzootic cycle— <i>Culex</i> sp. Epizootic cycle— <i>Aedes</i> sp. Epizootic cycle— <i>Aedes</i> sp.
Troara	Trocar virus (TROCV)	Atlantic Ocean and tributaries	Salmon	

in size and encodes four nonstructural proteins (nsP1, nsP2, nsP3, nsP4) at the 5' end and the five structural proteins (proteins C, E3, E2, 6K and E1) at the 3' end. The nonstructural proteins (nsPs) are translated from genomic RNA, and the structural proteins, from subgenomic RNA. Electron cryomicroscopy and image reconstruction of Sindbis virus (SINV), SFV, Ross River virus (RRV) and Aura virus (AURAV) show that the envelope glycoproteins form a $T = 4$ icosahedral lattice (Cheng *et al.*,

1995; Mancini *et al.*, 2000). The capsid proteins form a $T = 4$ icosahedral nucleocapsid arranged in distinct pentameric and hexameric capsomeres on the exterior of the nucleocapsid.

Biochemical and Biophysical Properties

The genome of the alphaviruses consists of a single-stranded (ss) positive-sense RNA, almost 12 000 nucleotides in length, which is capped at the 5' end

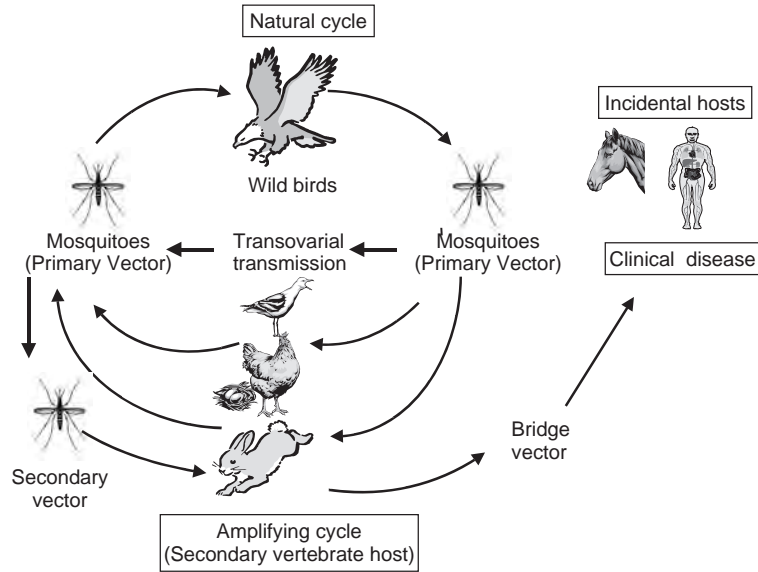


Figure 27.1 Schematic transmission and maintenance cycles.

and polyadenylated at the 3' end (Figure 27.3). The naked genome is infectious (Schlesinger and Schlesinger, 2001; Strauss and Strauss, 1994). The sedimentation

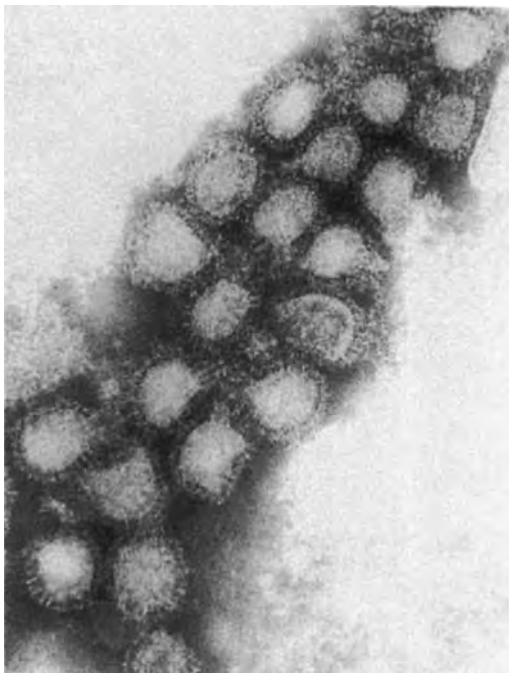


Figure 27.2 Electron micrograph of a preparation of Sindbis virus.

coefficient has been reported to be 42–49S. The genome is divided into two regions. The 5' two-thirds of the viral genome codes for the nonstructural proteins and the 3' third encodes the structural proteins (Strauss *et al.*, 1984). Both the structural and nonstructural proteins are translated as polyprotein precursors. The nonstructural polyprotein precursor is cleaved into four nonstructural proteins: nsP1, nsP2, nsP3 and nsP4. These function as the replicase; transcriptase of the virus (Strauss and Strauss, 1986). The structural polyprotein precursor is cleaved to form three major polypeptides. The capsid protein C (30–40 kDa) and the envelope glycoproteins E1 and E2 (45–59 kDa) are found in mature alphavirus particles. In addition, a small glycoprotein termed E3 has been demonstrated in SFV (Garoff *et al.*, 1974). A further small 6K polypeptide is also encoded, but has not been demonstrated in any alphavirus particles.

Replication

Virus attaches to host cell receptors via the E2 glycoprotein spikes on the viral surface. Alphaviruses are able to infect both vertebrate and arthropod hosts and enter the host cell by endocytosis or direct fusion with the plasma membrane. After endocytosis the capsid is thought to be released by low-pH catalysed membrane fusion initiated by the E1 glycoprotein (Wang *et al.*, 1992). Once released, the positive-sense genomic ss RNA is used as the messenger RNA for the translation of the nonstructural proteins (p270 and p230 polyproteins). A virus-encoded transcriptase transcribes the positive-sense RNA into

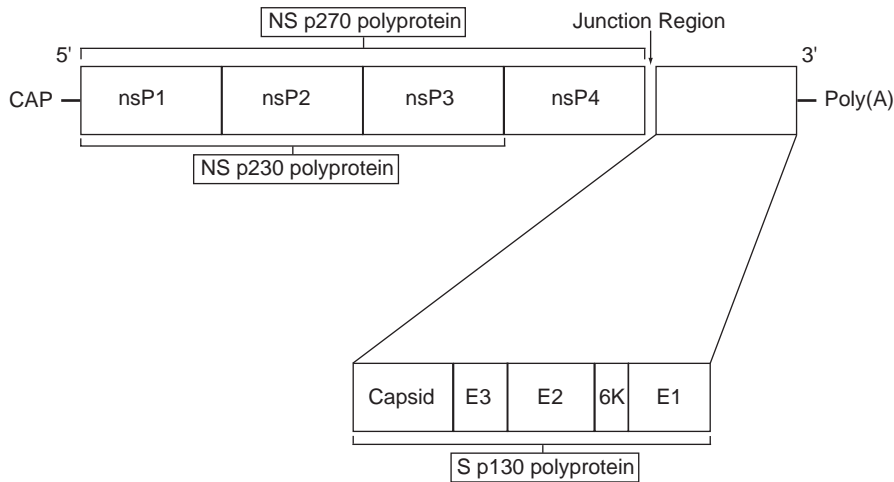


Figure 27.3 Organization of the alphavirus genome. The 5' two-thirds of the viral genome encode the nonstructural proteins, nsP1, nsP2, nsP3 and nsP4. The 3' third encodes the structural proteins. The capsid and envelope glycoprotein (E1 and E2) are located in the lipid bilayer, present in mature alphavirus particles. A third glycoprotein, E3, has been demonstrated in purified SFV. The 6K polyprotein is not known to be a structural protein.

minus-stranded RNA, which then forms a template for the synthesis of the positive-stranded progeny RNA. A subgenomic RNA (26S RNA) containing the last third of the genome is also synthesized. The structural proteins (p130 polyprotein) are translated from the 26S subgenomic protein. The capsid protein is cleaved from the p130 polyprotein; the remainder of the polyprotein moves into the endoplasmic reticulum, the N-terminal section of E3 serving as the signal sequence. Post-translational glycosylation and acylation and further cleavage occurs, yielding four separate proteins (E3, E2, 6K and E1) (Simizu *et al.*, 1984; Weaver *et al.*, 2005). The membrane-embedded proteins move towards the cell surface where virus assembly occurs. The C-terminal region of the E2 interacts with the nucleocapsid and the N-terminal region of the E2 interacts with E1 to form the heterodimer spike structure (Anthony and Brown, 1991; Paredes *et al.*, 1993). Nucleocapsids are assembled in the cytoplasm and maturation occurs by budding through host cell membranes (Garoff *et al.*, 1998).

An important advance in the study of alphaviruses has been the construction of full-length cDNA clones containing the complete viral genome. The production of *in vitro* transcribed RNA can be transfected into cells resulting in the generation of infectious virus. These clones have provided tools to further understand and analyse the factors that influence viral pathogenesis, cell tropism and synthesis and intracellular transport of membrane proteins.

Antigenic and Genotypic Properties

The alphaviruses have a group-reactive nucleoprotein. Complex and type-specific reactivity is determined by the envelope glycoproteins. This antigenic comparison of alphaviruses has been conducted using haemagglutination inhibition (HAI) and complement fixation tests as well as cross-protection tests in mice and neutralization tests in cell culture (Calisher *et al.*, 1980). The E1 glycoprotein of Sindbis contains the antigen responsible for haemagglutination (Chanas *et al.*, 1982). It is assumed that this is also the case for other members of the genus *Alphavirus*. The E2 glycoprotein induces virus-specific neutralizing antibody. On the basis of this, alphaviruses have been divided into six antigenic complexes: WEEV, VEEV, EEEV, SFV, MIDV and NDUV (Peters and Dalrymple, 1990). A further virus Barmah Forest virus (BFV) has been shown to be biochemically typical of the genus, but is not related serologically to members of the six antigenic complexes (Table 27.1). Each complex consists of either a single virus species having no known close relatives, for example EEEV, VEEV, MIDV and NDUV, or several species, subtypes and varieties that are more closely related to each other than the other members of the genus, for example SFV and WEEV. Plaque reduction or kinetic HAI tests can often only distinguish these variants. Kinetic HAI tests have also been used to differentiate between geographical variants of EEEV (Casals, 1964). Studies utilizing monoclonal antibodies have been able to define the alphavirus antigenic cross-reactivity more precisely and map the antigenic determinants.

Table 27.2 Principal alphaviruses of medical importance.

Clinical features	Virus
Fever, rash	Sindbis and Ockelbo
Fever, rash polyarthrits	Ross River and Barmah Forest
Fever, rash, myalgia, arthralgia	Chikungunya, o'nyong-nyong, Mayaro, Sindbis, Ockelbo, Ross River, Barmah Forest and Igo Ora
Fever, encephalitis	Eastern equine encephalitis Western equine encephalitis Venezuelan equine encephalitis Everglades, Semliki Forest

A recent study sequenced one strain of each alphavirus species and this allowed for the classification of alphaviruses into six genotypes (compared with seven antigenic complexes): SINV, NDUV, VEEV, WEEV, BFV and SFV. The authors included EEEV and WEEV in the same genotypic complex; this homology is to be expected as WEEV is thought to be a recombinant virus which derives its nonstructural genes from an EEEV ancestor (Pfeffer *et al.*, 1997).

SPECTRUM OF DISEASES CAUSED BY ALPHAVIRUSES

Alphaviruses cause a wide range of diseases in animals and humans, ranging from a clinically inapparent infection to a severe disease that may result in death. The main target organs are muscle, brain, reticuloendothelial system and the joints. This group of viruses may be clinically divided into those that are associated with fever, rash and polyarthrits found in the Old World or those that cause encephalitis, primarily found in the New World (Table 27.2). MIDV and NDUV are not known to cause disease in humans. Some alphaviruses are also responsible for disease in animals, particularly Equidae.

DIAGNOSIS OF ALPHAVIRUS INFECTIONS

The clinical features in a patient living or having recently travelled to an appropriate geographical region are the essential early components of establishing a differential diagnosis (Table 27.3). However, differentiation from other viral infections may be difficult. For example, it

is sometimes difficult to clinically differentiate infection with RRV from rickettsial, rubella, parvovirus B19 or enterovirus infections. Laboratory confirmation may therefore be required to identify the causative agent.

Laboratory diagnosis of alphavirus infections are determined on the basis of examining acute-phase serum for either immunoglobulin M (IgM) and IgG serological responses, isolation of causative virus or use of molecular techniques to detect evidence of the viral genome. In general, virus isolation is often only successful when acute-phase antibody-negative serum samples are used, particularly when the serum sample is taken in the first 48 hours of illness. Thereafter, the amount of virus in serum reduces rapidly and isolation becomes increasingly difficult. Virus identification is facilitated by the use of murine monoclonal alphavirus-specific antibodies. Virus may be isolated by intracerebral inoculation of suckling mice as well as in a variety of cell culture systems, including monkey kidney (Vero) and mosquito (C6-36) cell lines. Advances in the development of genomic detection methods have improved the early detection of infection. As these are RNA viruses any polymerase chain reaction (PCR)-based assay must first transcribe the RNA into DNA. A reverse transcription polymerase chain reaction (RT-PCR) has been developed for the genus-specific detection of alphaviruses. This utilizes degenerate primers localized within a conserved region of the nonstructural protein (NS1) and has become a routine sensitive and rapid alternative to virus isolation in the diagnosis of an acute infection (Pastorino *et al.*, 2005; Pfeffer *et al.*, 1997; Schuffenecker *et al.*, 2006).

Detection of an appropriate specific antibody response is frequently used to diagnose alphavirus infections. Class-specific IgM assays have been developed to diagnose an acute or recent infection with a particular alphavirus. However, care must be taken in the interpretation of the result, as cross-reactions may occur with other members of the same alphavirus antigenic complex (Table 27.1) (Calisher *et al.*, 1986). A serological diagnosis of a recent alphavirus infection is demonstrated by a rise in specific IgM or IgG antibodies, when acute and convalescent serum samples are tested in parallel. This is usually achieved through using enzyme-linked immunosorbent assay (ELISA), immunofluorescent antibody (IFA) or neutralization tests.

MANAGEMENT AND PREVENTION

Management of alphavirus infections is usually supportive and directed at symptomatic relief, for example the symptomatic relief of joint symptoms with nonsteroidal

Table 27.3 Generalized alphavirus case definition.

Clinical description	An illness with acute onset, with fever $>38^{\circ}\text{C}$, including variations of the following:
	Rash Arthralgia Polyarthritits Myalgia, headache, nausea and vomiting Malaise and weakness Disorientation and drowsiness
Laboratory criteria for diagnosis in one or more areas	Isolation of virus from blood, CSF or synovial joint fluids Positive IgM serology or IgG seroconversion in paired serum Demonstration of virus antigen in autopsy tissue by immunocytochemistry or in serum by ELISA Positive RT-PCR from serum, synovial joint fluid, CSF or autopsy tissue
Case classification	
Suspected	A case that meets the clinical case description
Probable	A case compatible with the clinical description with one of the following: supportive serology (comparable IgG) or positive IgM antibody test in an acute- or convalescent-phase serum
Confirmed	Occurrence at same location and time as other confirmed alphavirus cases A suspected case that is laboratory confirmed, that includes seroconversion and/or virus isolation or PCR positive or epidemiologically linked to a confirmed case or outbreak

anti-inflammatory agents and aspirin. Prevention of alphavirus infections has focused on vector control and the protection of the individual against mosquito bites. A detailed knowledge of the vector and vertebrate host range is therefore essential to allow for a coordinated strategy to prevent and control alphavirus infections. Vaccines are also available against some of the individual alphaviruses infections but not generally available (see below).

ALPHAVIRUSES ASSOCIATED WITH FEVERS AND POLYARTHRITIS

Sindbis Virus

SINV is considered the prototype of the alphaviruses and is the most widely distributed of all known arboviruses. It was originally isolated from *Culex pipiens* and *Culex univittatus* mosquitoes collected in the Egyptian village of Sindbis (Taylor *et al.*, 1955). In Sweden symptomatic disease resulting from infection with a Sindbis-like agent has been termed Ockelbo disease. A Sindbis-related virus has also been described as the cause of Pogosta disease in Finland and as Karelian fever in the Karelian region of the former USSR. Babanki virus is a Sindbis-like agent that occurs in West and Central Africa; the clinical significance of infection with this virus remains to be defined (Peters and Dalrymple, 1990).

Epidemiology and Host Range

SINV occurs in many parts of the world, including Europe, Asia, Africa and Australia. Despite its wide distribution, symptomatic infections in humans have been reported in only a few geographically restricted areas, such as northern Europe and occasionally in South Africa (Malherbe *et al.*, 1963), Australia (Boughton *et al.*, 1984; Doherty *et al.*, 1969) and China (Zhou *et al.*, 1999). Although serological evidence exists that SINV was the causative agent of Pogosta disease it was not till 2002 that the virus was isolated from acutely ill patients (Kurkela *et al.*, 2004). Similar disease is also found in Sweden (Ockelbo disease) and Russia (Karelian fever). The virus is known to infect humans, domestic animals and birds, with birds forming the principal reservoir. Grouse (*Tetraonidae*) and passerines (especially thrushes, *Turdidae*) have been suggested as the amplifying hosts in northern Europe. Humans are not considered to be essential to the survival of the virus in nature. SINV is transmitted among birds by the *Culex* mosquitoes. Infection in birds does not appear to result in disease. Where humans and birds exist in close proximity, for example in the Nile Valley, transmission to humans may occur (Peters and Dalrymple, 1990). Studies from South Africa have shown that the virus is distributed widely throughout the country. Migratory birds may also play a role in distributing SINV over long distances. Supporting evidence is provided by demonstrating that SINV strains obtained

from vast geographic areas of Australia share identical nucleotide sequence profiles (Sammels *et al.*, 1999). Further serological and phylogenetic studies have indicated that South African and northern European SINV strains are closely related, supporting the hypothesis that migratory birds have carried the virus to northern Europe (Buckley *et al.*, 2003; Shirako *et al.*, 1991). Transmission to the avian population and probably to humans occurs annually. Extensive human disease only occurs, however, during years of abundant rainfall and flooding (Jupp *et al.*, 1986; McIntosh *et al.*, 1964). In Europe, disease caused by SINV occurs after excursions into forested areas by, for example, lumberjacks or berry pickers. Here the virus has been isolated from *Culiseta*, *Aedes* and *Culex* mosquitoes.

Clinical Disease

SINV is among the least virulent of the alphaviruses. Serological surveys suggest that infection with SINV is relatively common, but clinically apparent disease is unusual. When symptomatic, the spectrum of disease in humans varies from a mild illness to one consisting of a rash and arthralgia. The illness is usually characterized by a sudden onset of the rash, but occasionally prodromal symptoms may be present. The rash is usually macular or papular but may become vesicular or haemorrhagic. Malaise, fatigue and headache are frequently present (Jupp *et al.*, 1986; Kurkela *et al.*, 2008; McIntosh *et al.*, 1964). The initial joint involvement is usually migratory, followed by a gradual resolution of symptoms.

Ockelbo disease was first described in the central part of Sweden in the 1960s in clusters of patients with fever, arthralgia and a rash (Niklasson and Vene, 1996). Some patients with Ockelbo disease have reported joint symptoms persisting for more than a year and recurrent joint problems were documented in one-third of a group of Swedish patients interviewed two or more years post infection (Niklasson *et al.*, 1988). By contrast, reports from South Africa have emphasized the joint involvement less and have suggested that the involvement is of tendon and periarticular tissue, rather than a true arthritis (McIntosh *et al.*, 1964).

Pogosta disease incidence during 1980–1996 was reported to be the highest in the provinces of North Karelia and Central Finland. In 2002, 600 cases were diagnosed serologically. The highest incidence moved to southern Ostrobothnia. SINV seroprevalence among hospitalized patients in Finland found 19% of the patients were aged < 10 years.

Pathogenesis

It is not clear whether the skin lesions are the result of a direct viral cytopathic effect or are immunopathological.

Evidence to support the former hypothesis comes from Malherbe *et al.* (1963), who demonstrated the isolation of SINV from a vesicle in the absence of a serum viraemia. This was associated with a subsequent rise in specific antibody. Other workers have, however, failed to isolate SINV from skin lesions (McIntosh *et al.*, 1964). The pathogenesis of the joint complications, and whether they are articular or periarticular, is also unclear.

Diagnosis

Isolation of SINV from human sera has been reported as being intermittently successful from the acute phase of the illness, even using sera taken early on in the illness. This is because the viraemia associated with SINV infection is often of low level and transient. In addition, medical attention is frequently sought relatively late in the course of the illness. As mentioned above, SINV has also been isolated from a skin lesion. When inoculated into three- to five-day-old mice, either intracerebrally or intraperitoneally, SINV causes a fatal infection, which is preceded by a short period of paralysis. The virus has also been isolated *in vitro* in fibroblastic cell lines. Antibodies appear 7–10 days after the onset of illness and may be detected by HAI, ELISA and immunofluorescence (Doherty *et al.*, 1969; Espmark and Niklasson, 1984; Kurkela *et al.*, 2008; McIntosh *et al.*, 1964). Although the detection of Sindbis-specific IgM may be of use in diagnosis an acute infection, this class of antibody may remain detectable three to four years after the initial infection. A positive IgM result must therefore be interpreted together with the available clinical information (Lane *et al.*, 2004).

Chikungunya Virus

This virus was first isolated from patients and mosquitoes in the Newala district of Tanzania in 1952–1953 during an epidemic of dengue-like illness (Robinson, 1955; Ross, 1956). The name is derived from a local term for the illness, meaning ‘that which bends up’, referring to the crippling arthralgia and arthritis associated with infection with this virus. Chikungunya virus (CHIKV) is found in the savannahs and forests of tropical Africa as well as in many parts of Asia, including, Cambodia, Vietnam, Burma, Sri Lanka and India (Table 27.4). Between the 1960s and 1990s, the virus was isolated frequently in a number of countries in central and southern Africa including Sudan, Uganda, Democratic Republic of Congo (DRC), the Central African Republic, Malawi, Zimbabwe, Kenya and South Africa. Chikungunya has also been cause of infections in western African countries including Senegal, Benin, Cote d’Ivoire and Nigeria.

In South East Asia, frequent outbreaks of chikungunya were reported from the early 1960s though to

Table 27.4 Origins and geographic distribution of the Semliki Forest virus complex.

Antigenic complex	Species	Origin	Main geographic distribution	Other features
Semliki Forest	Bebaru	Malaysia	Malaysia	Epidemics in East Africa, Indian Ocean Islands and Asia
	Chikungunya	Tanganyika, 1953	Africa, Asia, Philippines and Indian Ocean	
	O'nyong-nyong	Uganda, 1959	West and East Africa and Zimbabwe	Epidemics in East Africa and Zimbabwe
	Getah	Malaysia, 1955	Malaysia, Japan, Australia, Cambodia and Philippines	Epidemic in Central African Republic
	Ross River	Australia, 1959	Australasia and South Pacific	
Semliki Forest	Africa, 19??	Africa	One epizootic in Japan	
Sagiyama	Japan, 1956	Japan and Okinawa	Japan and Okinawa Trinidad and South America South America Panama, Surinam, Trinidad and French Guiana	
Mayaro	Trinidad, 1954	Trinidad and South America		
Una				

2003 in India, Pakistan, Malaysia, Indonesia, Cambodia, Vietnam and Thailand (Table 27.4). Numerous large cities in South East Asia including Calcutta and Bangkok have been identified as active sites of transmission and disease. Major disease clusters of chikungunya are increasingly being recorded in areas that have included Senegal 1986 and 1996–1997 (Diallo *et al.*, 1999), Ivory Coast in 1996–1997 (Thonnon *et al.*, 1999), DRC during 1998–2000 (Nur *et al.*, 1999), Indonesia in 2003 (Laras *et al.*, 2005; Porter *et al.*, 2004), Kenya in 2004, Comoros in 2005 (Sergon *et al.*, 2007), the Seychelles, Mauritius, Madagascar and Réunion Island during 2005–2006 and in India 2006–2007 (Ravi, 2006; Saxena *et al.*, 2006). Outbreaks have also occurred continuously with hundreds of thousands of reported cases involving new geographical areas in the Indian Ocean and India.

Associated with the Indian Ocean island outbreaks over 900 imported cases of chikungunya cases have been recorded in Europe, approximately 50 cases in United, mostly amongst returning tourists (Pfeffer, 2006; Warner *et al.*, 2006). In July 2007, the first indigenous transmission of chikungunya in Europe was reported from the rural area in Emilia-Romagna, Italy, where 254 suspected cases were recorded with 74 laboratory confirmed (Rezza *et al.*, 2007). The presumed index case was reported to have returned to the area from Kerala state, India, where an epidemic of the disease was ongoing at the time. By September approximately 300 suspected cases had been identified and the virus had also been isolated from local *Aedes albopictus* found in the local

area, which is widespread in Italy. The emergence of chikungunya in Italy illustrates that this situation could develop in other countries where *Ae. albopictus* is prevalent.

The CHIKV strains from Africa and Asia have been shown to be closely related, using a panel of monoclonal antibodies prepared against strains from Africa and Asia (Blackburn *et al.*, 1995). Sequence analysis of isolates from past outbreaks in Africa, India and South East Asia revealed the existence of geographically clustered lineages of the virus. Powers *et al.* (2000) reported that Asian outbreak strains originated in Africa, but still formed a distinct Asian genotype separate from West African and Central/East African genotypes. More recently the viruses that emerged from the major outbreak originating in the south-west Indian Ocean all align with the Central/East African genotype (Schuffenecker *et al.*, 2006). Gene sequences from recent isolates originating from India also belong to this Central/East African group (Yergolkar *et al.*, 2006).

Epidemiology and Host Range

The epidemiology of CHIKV infections differs in Africa and Asia. In Africa this virus is transmitted in savannahs and forests by *Aedes* mosquitoes, mainly members of the subgenera *Aedimorphus*, *Diceromyia* and *Stegomyia* (Diallo *et al.*, 1999; Jupp and McIntosh, 1990; McIntosh *et al.*, 1977). The most important vertebrates in the cycle of infection are the non-human primates, for example baboons and *Cercopithecus* monkeys. Bush babies and

certain species of bats may also be infected in nature, but their role in viral maintenance is likely to be of secondary importance. Humans may be infected in African villages and rural areas, particularly where *Aedes aegypti* is present in large numbers. In contrast to the situation in Africa, transmission in Asia is primarily from human to human by *Ae. aegypti*. While *Ae. aegypti* was considered as the usual vector, *Ae. albopictus* has been recently involved in the chikungunya outbreak in the Indian Ocean in 2005–2006 (Schuffenecker *et al.*, 2006). This species, native to South East Asia, has succeeded in colonizing both tropical and temperate regions including parts of Europe (Vazeille *et al.*, 2008).

Although the most important vector for CHIKV transmission to humans is the *Aedes* mosquito, transmission has also been described by *Mansonia African*. The active replication of alphaviruses in the mosquito is essential for perpetuation in nature, but the explosive nature of the chikungunya epidemics has led to speculation that mechanical transmission of the virus may also occur from a viraemic host by an uninfected mosquito (Peters and Dalrymple, 1990).

Recent molecular epidemiology studies of CHIKVs isolated from 2005 to 2007 outbreaks has led to the speculation that viral mutations have led to increasing virulence. Genetic studies of CHIKVs suggest three distinct lineages derived from West Africa, Asia and all South/Central/East African genotype which have included strains introduced into Asia.

Clinical Disease

The incubation period varies but is usually 2–4 days with a range of 1–12 days. Chikungunya illness typically begins with an abrupt onset of fever reaching as high as 40°C that may last for 10 days. The fever precedes the rash and joint pains. The most significant manifestation of chikungunya illness is the severe joint pains affecting mainly the small joints of the hands, wrist and feet (Deller and Russell, 1968). This may be associated with an erythematous flush over the face and upper chest in approximately 80% of patients. A maculopapular rash together with a generalized lymphadenopathy appears three to four days later. The arthralgia is most commonly symmetrical and peripheral, being noted in the ankles, toes, fingers, elbow, wrists and knees. Although the arthralgia may resolve within a few weeks, pain, swelling and morning stiffness may continue for months and even years after infection. In rare instances, the rheumatic condition results in joint destruction before resolution after 15 years (Brighton and Simpson, 1984). Petechiae and bleeding from the gums does occur, but there are no significant haemorrhagic manifestations.

During many of the recent outbreaks a number of atypical clinical presentations have been noted. A study from Bangkok concentrating on a paediatric outpatient department showed that the most frequent presenting symptom of CHIKV infection in children was vomiting, which was present in 35% of patients. In addition, 18% had abdominal pain or anorexia. None of the patients in this study were noted to have joint symptoms; arthritis and arthralgia therefore appear to be less prominent features in children. On clinical examination of this paediatric population the most frequent sign was a pharyngitis, which was present in 71% of patients, followed by facial flushing (24% of patients). None of these children had a rash. This study illustrates very clearly that the clinical presentation of chikungunya infections in adults and children differ (Halstead *et al.*, 1969).

In Asia, several virus isolations have been made from severely ill children diagnosed as having haemorrhagic fever, similar to dengue haemorrhagic fever. Haemorrhagic forms of CHIKV infection have been described in India and South East Asia, but these are seldom as severe as dengue haemorrhagic fever. In a hospital-based study in Bangkok about 8% of children with suspected haemorrhagic fever had CHIKV infection (Nimmannitya *et al.*, 1969).

Atypical clinical features have been a particular feature of the 2005–2007 Indian Ocean outbreaks where descriptions of cases have included neurological involvement in adults, fetuses and neonates (Robillard *et al.*, 2006; Schuffenecker *et al.*, 2006). In addition, the first cases of mother-to-child transmission have been reported. It was concluded that mother-to-child CHIKV transmission was frequently a feature in the context of intrapartum maternal viraemia, and often led to severe encephalitic neonatal infection. All infected neonates were found asymptomatic at birth and the median onset of disease was three to seven days. Pain, prostration and fever, together with thrombocytopenia, was observed in all cases studied (Gérardin *et al.*, 2008; Ramful *et al.*, 2007). Finally, a recent study in India has documented a chikungunya case in a 15-year-old female who had developed a sudden unilateral sensorineural hearing loss following an episode of fever, arthralgia and rashes one month earlier (Bhavava *et al.*, 2008)

Pathogenesis

CHIKV may be isolated from the blood early in the course of disease. As the disappearance of the viraemia correlates with the appearance of IgM and IgG antibodies, it would appear that recovery from infection correlates with the development of viral-specific antibody rather than cell-mediated immunity. The haemostatic abnormality

seen in both children and adult patients is possibly the result of an acquired platelet defect as it has been shown that the association of CHIKV with human platelets *in vitro* promotes platelet clumping (Chernesky and Larke, 1977).

Diagnosis

The clinical features described above in a patient who has recently returned from, or is living in, sub-Saharan Africa, Indian Ocean or Asia enables a presumptive clinical diagnosis to be made. In addition, a minority of patients will have a leukopenia with a relative lymphocytosis. In most, however, the white cell count will be normal. The platelet count may be decreased, but this is usually not significant.

CHIKV can be isolated by the intracerebral inoculation of suckling mice or by viral culture in either mosquito or mammalian cell culture systems (Peters and Dalrymple, 1990). Viraemia will be present in most patients during the first 48 hours of disease and may be detected in sera as late as day 4 post onset. Thereafter the amount of virus in serum drops rapidly and recovery becomes increasingly difficult. Early serological techniques found both HAI, IFA and neutralizing antibodies appeared on days 5–7 of the illness. The appearance of these antibodies is associated with a decrease in viraemia (Calisher *et al.*, 1986; Carey *et al.*, 1969; Grivard *et al.*, 2007; Litzba *et al.*, 2008). IgG antibodies rise rapidly and peak in the second week and are detectable by HAI, capture ELISA or IFA methods. Virus-specific IgM antibodies are readily detected by capture ELISA both in patients in the acute phase of illness and those recovering from infection as they persist in excess of six months. However, cross-reaction with other members of the same alphavirus antigenic complex must be considered, so the results obtained must be interpreted with caution (Table 27.2). The use of a specific IgM assay may be especially relevant in an epidemic situation as illustrated during the recent Indian Ocean outbreak. Utilization of a simple indirect ELISA or IFA proves helpful in achieving an acute-phase diagnosis and results showed a strong concordance with early HAI tests (90% agreement) as still used in many parts of the world, although ELISA and IF is able to identify twice as many patients at initial hospital admission (Thein *et al.*, 1992).

The increased use of real-time RT-PCR assays targeting the E1 gene have been developed and proved extremely effective in the early detection of illness both during endemic episodes and as part of the differential diagnosis in returning travellers (Carletti *et al.*, 2007; Edwards *et al.*, 2007; Grivard *et al.*, 2007). The differential diagnosis within the laboratory should include Ross River, dengue, Sindbis and West Nile virus infections.

Vaccine

Currently, no vaccine is available. A formalin-inactivated CHIKV vaccine prepared in monkey kidney tissue culture has been described. Despite being apparently safe and immunogenic, its use has been limited to initial safety studies and laboratory workers (Harrison *et al.*, 1971). More recently a live-attenuated vaccine has been produced and currently undergoing safety and immunogenicity studies.

O’Nyong-nyong Virus

O’nyong-nyong literally means ‘weakening of the joints’. It was first described as an epidemic viral disease in East Africa (Haddow *et al.*, 1960). Antigenically, o’nyong-nyong virus is a subtype of the species chikungunya (Table 27.3).

Epidemiology and Host Range

The initial epidemic began in 1959 in north-western Uganda and spread from there to Kenya, Tanzania, Mozambique, Malawi and Zaire. It involved some 2 million people. Within an infected area spread was rapid, with an extremely high attack rate. In an affected village a high proportion of the population, regardless of age or sex, were incapacitated. This disease was also apparently unknown to the tribes who were affected. The origin of o’nyong-nyong virus is not clear. It might have existed unrecognized prior to the initial epidemic described above, or, conversely, the epidemic may well have resulted from a mutant or recombinant virus. The vectors of this virus are *Anopheles funestus* and *Anopheles gambiae*. It is not known whether a non-human vertebrate host exists. After this initial epidemic very little further information on this virus was obtained until 1978 when it was isolated from *A. funestus* mosquitoes in the Kao Plain in Kenya (Johnson *et al.*, 1981). The re-emergence of epidemic o’nyong-nyong fever in south-western Uganda after an absence of 35 years has recently been described (Rwaguma *et al.*, 1997).

Clinical Disease

The clinical features are similar to those of CHIKV infections, with a sudden onset of a five-day fever often accompanied by a rigour. Characteristic features of the disease include joint pains followed by the development of a rash. Joint pain primarily affects the knees, although ankles, elbows, wrists also can be involved.

The morbilliform macupapular rash erupts on the face extending to the trunk and extremities, four to seven days post onset of infection and is similar to chikungunya. Some fatalities have been described, but morbidity is substantial (Kiwanuka *et al.*, 1999).

Diagnosis

Virus may be isolated in one-day-old mice from serum samples taken during the acute stage of the infection. However, mouse passage or subinoculation into chick fibroblasts may be necessary. The mice demonstrate alopecia and runting. The virus may then be further identified using plaque inhibition, cross-neutralization assays or HAI (Williams and Woodall, 1961). Antibodies to o'nyong-nyong virus may be detected by complement fixation, HAI, ELISA, immunofluorescence and neutralization assays. Interpretation may be difficult due to cross-reaction with CHIKV. In general, however, the antibody titre to the homologous virus will be higher than that to the heterologous virus (Peters and Dalrymple, 1990).

Ross River Virus

RRV is an important arbovirus that is endemic and enzootic in Australia and Papua New Guinea. Also known as epidemic polyarthritis, it is the most common arboviral infection of humans in Australia, being responsible for over an average of 5000 notifications per year (Jacups *et al.*, 2008). In the late 1970s and early 1980s RRV also became endemic in the Pacific Islands (Table 27.4). In Australia approximately 60% of cases arise in tropical and central Queensland (most commonly between January and May). In recent years it has increasingly been identified in travellers returning to Europe and North America (Klupsing *et al.*, 2005).

Epidemiology and Host Range

Until 1979 the distribution of RRV infections was thought to be limited to Australia, Papua New Guinea and the Solomon Islands, all in the western half of the Pacific basin. In Australia both epidemic and sporadic transmission of RRV occurs. Epidemics are characteristically preceded by heavy rainfall or high tides affecting salt marshes, river valleys and irrigated lands in coastal areas. This leads to increase in mosquito numbers (Kelly-Hope *et al.*, 2004) However, other factors, such as temperature, are important—a sudden period of cold can delay the build-up of the vector and so abort an epidemic (Hawkes *et al.*, 1985). The virus was first isolated from *Aedes vigilax*, which is accepted as being the major vector in the coastal regions of Australia. Other mosquito species, including the salt marsh arthropod *Aedes comptorhynchus* found in coastal regions of northern and southern coastal regions and *Culex annulirostris*, which breeds in freshwater habitats, are also considered important vectors (Peters and Dalrymple, 1990). Besides humans, other possible vertebrate hosts include marsupials, domestic animals and rodents (Doherty, 1977).

In 1979 an explosive epidemic of RRV infection occurred in Fiji (Aaskov *et al.*, 1981). The virus spread to American Samoa and then to the Wallis and Futuna Islands and the Cook Islands. The mosquito vector in this region is unknown, although the virus has been isolated from *Aedes polynesiensis*, a mosquito that is found in many parts of the eastern Pacific (Rosen *et al.*, 1981). Evidence suggests that humans are the most important vertebrate host in this region. A study from the Cook Islands showed that the incubation period could be as short as three days and the explosive nature of this epidemic led to speculation that mechanical transmission of RRV may occur. This theory has been supported by laboratory evidence of mechanical transmission of RRV from viraemic donors to uninfected recipient suckling mice. In addition, it has also been suggested that transovarial transmission in mosquitoes is a potential survival mechanism for this virus (Kay, 1982).

The number of annual reported cases of RRV disease has increased during the past decade to between 4 and 6000. However, these figures represent a combination of improved laboratory diagnostic reagents, greater awareness by clinicians, housing developments on salt marshes and migration northwards to warmer climates in retirement (Mackenzie *et al.*, 2001).

Clinical Disease

Infection with RRV does not necessarily result in disease in humans and it has been estimated that 50 subclinical infections occur for each clinically recognized case (Table 27.3). The disease is not fatal. Adults are more likely to be symptomatic than children. The onset of clinical symptoms is characterized by joint pains (epidemic polyarthritis) accompanied by a rash and fever. In a recent postal survey of general practitioners in South Australia the complaint of 'pain in the joints' was the most important symptom for suspecting an infection with RRV, with joint effusion, rash and pyrexia being important signs (Flexman *et al.*, 1998; Stocks *et al.*, 1997). The rash may occur simultaneously with the joint pains or may follow one to two days later. Although the rash is usually macular, maculopapular or papular, vesiculation and petechiae have been described. The rash and joint pains are commonly accompanied by some degree of constitutional upset, including myalgia, headache, anorexia and nausea. The joint involvement is usually asymmetrical and migratory and most often affects the small joints of the hands and feet together with the knees. In addition, there may also be periarticular swelling and a tenosynovitis (Clarke *et al.*, 1973; Hawkes *et al.*, 1985). The arthralgia and arthritis tend to run a relapsing course but with an overall gradual improvement. The symptoms of epidemic polyarthritis may last for 30–40 weeks, with some

patients having symptoms for more than a year. While joint pain, rash and fever are the most common clinical features, glomerulonephritis has also been reported in the acute phase of RRV disease (Fraser *et al.*, 1988). This case report describes a patient presenting with haematuria and proteinuria coincident with an acute RRV infection; the frequency of this complication has yet to be defined.

Pathogenesis

The pathogenesis of disease is not completely understood. While virus has been isolated from the blood, it has not been isolated from either the skin or joints. However, RRV antigen has been detected by immunofluorescence at both of these sites. Histological examination of the skin lesions shows a mononuclear cell infiltration (Fraser *et al.*, 1983). It has also been shown that RRV is capable of infecting human synovial cells (Cunningham and Fraser, 1985). Serum complement is normal and circulating immune complexes do not usually exceed normal levels. It has therefore been suggested that RRV plays a direct role at these sites of inflammation.

Diagnosis

Diagnosis is greatly assisted with knowledge of travel history. A differential diagnosis would also include rubella, other alphavirus-induced arthritis, dengue, rheumatoid arthritis, Lyme disease, parvovirus B19 and other chronic rheumatic diseases (Fraser, 1986).

Isolation of RRV is only successful using acute-phase antibody-negative serum samples. Both mosquitoes and mosquito cell lines have been used. The current method of choice for RRV isolation is the inoculation of the mosquito cell line C6/36. As this virus does not display a cytopathic effect in C6/36 cells, viral antigen is detected using immunofluorescence (Rosen *et al.*, 1981). RRV may also be isolated in Vero (E6) cells. More recently methods have been developed to detect RRV RNA using RT-PCR. This methodology has potential application to virus surveillance programmes in mosquito populations (Sellner *et al.*, 1994).

The most sensitive and established serological assay involves the use of an IgM capture and epitopic blocking enzyme-linked immunosorbent assay (Oliveira *et al.*, 2006). However, there is a complication in that the assay can remain positive for up to two years after the acute infection (Carter *et al.*, 1985). It should be noted that IgM antibody cross-reacts with Mayaro virus (MAYV), CHIKV and SFV occurs with sera from patients with RRV infections (Table 27.4). The presence of RRV-specific IgA or low-avidity IgG can assist the diagnosis of a recent infection (Carter *et al.*, 1987). A more precise diagnosis

can be made using neutralization tests (Kanamitsu *et al.*, 1979).

Vaccine

Preliminary studies into the efficacy of a formaldehyde-inactivated RRV vaccine demonstrated that this candidate vaccine neutralized all strains of RRV tested, but to varying degrees (Aaskov *et al.*, 1997; Kistner *et al.*, 2007). Further studies are awaited with interest as, while RRV does not usually cause a severe disease, it may result in considerable morbidity with an associated loss of productivity.

Barmah Forest Virus

This alphavirus has been recently reported to cause both clinical and subclinical infections in Australia. Clinical disease resulting from BFV infection was first reported in 1986. The first epidemic of human disease occurred in 1992 in the Northern Territory (Mackenzie *et al.*, 1994). To date, Australia is the only country in which this virus has been detected (Hills, 1996).

BFV is the sole member of the seventh alphavirus serocomplex (Table 27.4). The nucleotide sequence of the BFV genome has recently been described and, from amino acid sequence comparisons with sequenced alphaviruses, BFV has been shown to be most closely related to RRV and SFV (Lee *et al.*, 1997). In recent years there has been an apparent increase in incidence of polyarthritis caused by BFV. This prompted a study on the molecular epidemiology of this alphavirus which showed a high degree of sequence homology between BFV isolates with no evidence of geographical or temporal divergence (Poidinger *et al.*, 1997).

Epidemiology and Host Range

BFV is an alphavirus that is enzootic in Australia and has been isolated from the two most common vectors of RRV—*Cx. annulirostris* and *Ae. vigilax*. It has been shown to circulate amongst mosquitoes and terrestrial animals, most noticeably marsupial species. Occupational and recreational exposure to these vectors is therefore an important risk factor for infection with this virus. A higher incidence of antibodies to BFV in RRV antibody-positive blood donors compared with RRV antibody-negative blood donors suggests a common ecology. Further information is required on the duration of viraemia in humans to determine whether other vertebrate hosts are of importance in viral maintenance. Currently, human disease caused by BFV has not been recognized in southern Australia or Tasmania.

Clinical Disease

Human infections have been recognized since 1986 and its incidence has increased through greater clinical awareness and improved laboratory diagnostic methods. Currently, approximately 500 cases are recorded each year (Lindsey *et al.*, 1995). As mentioned above, both clinical and subclinical infections occur. The most common symptoms noted in a study from Queensland were arthritis; arthralgia, myalgia and fever, which were present in approximately 75% of patients (Table 27.3). In addition, half the patients in this study had a rash (Phillips *et al.*, 1990). A more recent study from New South Wales reported lethargy (89%), joint pain (82%) and rash (68%) as the most common symptoms. In the latter study just over half of the patients reported an illness lasting for more than six months. These authors also reported a possible association between the presence of a rash and an improved prognosis (Beard *et al.*, 1997). In general, the symptoms are similar to those associated with RRV infection; laboratory confirmation is therefore necessary to achieve a precise diagnosis. The wider availability of serological testing for BFV infections has also enabled their more unusual features to be described. Recently the first case of glomerulonephritis after BFV infection was diagnosed. The authors suggest that BFV infection should be considered as a possible aetiological agent for glomerulonephritis (Katz *et al.*, 1997).

Diagnosis

Clinical differentiation from RRV infection in patients living in endemic areas is difficult. Laboratory confirmation is therefore necessary for a precise diagnosis to be made. BFV has been isolated from a patient in the mosquito cell line (C6/36). The serological response to infection with this virus in humans remains to be defined precisely. However, in contrast to RRV infections, where the antibody response may be delayed for seven days, past studies demonstrated the presence of both HAI and neutralizing antibody titres in two patients with BFV infection at the onset of symptoms (Phillips *et al.*, 1990). This could either be due to a different serological response to these two viruses or, alternatively, BFV-associated disease may have a less clearly defined onset than that caused by RRV infections.

Igbo Ora Virus

Igbo Ora virus is closely related to both chikungunya and o'nyong-nyong viruses. Human disease associated with infection with this virus is characterized by fever, arthritis and the development of a rash ('the disease that breaks your wings'). The virus was isolated from human

sera from patients in Africa (Nigeria, Central African Republic) in the latter part of the 1960s (Moore *et al.*, 1975) and was responsible for an epidemic that involved four villages in the Ivory Coast in 1984 (Peters and Dalrymple, 1990).

Mayaro Virus

Although infection with MAYV was first recognized in Trinidad in 1954, it was an epidemic in Brazil in 1978 that permitted a detailed evaluation of human infections (LeDuc *et al.*, 1981; Pinheiro *et al.*, 1981; Torres *et al.*, 2004).

Epidemiology and Host Range

MAYV is enzootic in South America, where the suspected vectors are forest-dwelling *Haemagogus* mosquitoes and the vertebrate hosts are marmosets and other non-human primates (Pinheiro and LeDuc, 1998). During the extensive epidemic described by LeDuc *et al.* (1981), 20% of the entire population of approximately 4000 people in the rural village of Belterra in Brazil were infected. The numbers infected were higher among those who lived near a plantation forest. The epidemic began with the onset of the wet season and ended with the onset of the dry season. This correlated with the abundance of the mosquito vector, *Haemagogus janthinomys*. In addition to humans, marmosets and other primates are susceptible to infection (Hoch *et al.*, 1981). It has therefore been suggested that non-human primates play an important role in the maintenance of this virus, as experimental infection of marmosets with MAYV showed that a significant viraemia develops. The role of other vertebrates, for example birds, remains to be defined. Although MAYV is enzootic in several South American countries, the first human cases of Mayaro fever were described in Venezuela by Torres *et al.* (2004). In this recent study the red howler monkey (*Alouatta seniculus*) is the suspected host of MAYV in nature. The associated vectors in this region suggest the involvement of other *Haemagogus* species of that genus, such as *Haemagogus celestae*, *Haemagogus equinus* and *Haemagogus lucifer*.

Clinical Disease

A detailed evaluation of the clinical features of infection with this virus in humans is available from studies done during the 1978 epidemic in Brazil. Mayaro fever is described as a dengue-like, three to five day acute febrile illness accompanied by headache, retro-orbital pain, arthralgia, arthritis, vomiting and maculopapular rash (Pinheiro *et al.*, 1981; Torres *et al.*, 2004). Joint involvement in Mayaro fever may persist for several months

and has been described in some cases as preceding the fever.

Diagnosis

Leukopenia, sometimes accompanied by a modest lymphocytosis, is a frequent finding. A mild thrombocytopenia may also be present. MAYV may be isolated from acute-phase serum samples in an *in vitro* cell culture system using Vero cells. The virus may then be identified using HAI, ELISA or plaque reduction neutralization tests. In addition, MAYV is also pathogenic for infant mice on intracerebral inoculation. In the Belterra epidemic, virus was isolated from the serum in 97% of the patients bled in the first 24 hours after the onset of symptoms. Thereafter viral recovery rates decreased to only 7% on day 4. Serological evidence of a MAYV infection can be achieved by demonstrating a seroconversion using HAI or ELISA in paired serum samples taken from a patient during the acute and convalescent phase of the illness (Pinheiro *et al.*, 1981; Torres *et al.*, 2004). In addition, the use of a MAYV-specific IgM assay has been described as viraemia subsides, which is usually four to five days after the onset of symptoms. Among the case studies of patients identified in Venezuela, IgM antibody was reported to persist for three to six months (Torres *et al.*, 2004). In addition, convalescent sera collected from self-limited febrile disease with polyarthritides similar to that described in patients having MAYV infection showed extremely high IgG antibody levels. This provided further evidence that MAYV was enzootic in the area (Torres *et al.*, 2004). Cross-reactivity of IgM antibody occurs with alphavirus infections by viruses belonging to the same antigenic complex (Table 27.1). However, the ratios of the homologous to heterologous IgM titres are high, allowing a reasonably specific and rapid diagnosis to be made. The IgM assay is therefore a useful diagnostic tool in an epidemic situation (Calisher *et al.*, 1986; Torres *et al.*, 2004).

ALPHAVIRUSES ASSOCIATED WITH ENCEPHALITIS

The alphavirus genus contains four viruses that produce encephalitis in humans: EEEV, WEEV, VEEV and Ev-erglades virus (EVEV). EEEV, WEEV and VEEV were first isolated from the brains of dead horses in the 1930s. The severity of disease associated with infection with this group of viruses differs: EEEV is the most neurovirulent, while infection with VEEV is associated with a febrile illness, with encephalitis occurring infrequently. Focal epidemics of EEEV have periodically occurred in

eastern United States, WEEV is endemic in western United States and Canada and VEEV in Central and South America and occasionally in North America. EVEV is related to EEEV but is restricted to the state of Florida in the United States and only five cases of encephalitis associated with it have been reported, despite a high seroprevalence (27%) in south Florida.

Eastern Equine Encephalomyelitis Virus

Eastern equine encephalomyelitis virus is the most severe of the arboviral encephalitides and an important cause of human disease in the eastern and north central states of the United States and Canada. Its distribution extends through Central America to Trinidad, Brazil, Guyana and as far south as Argentina (Gibbs and Tsai, 1994). This virus is among the most virulent of the alphaviruses. It has been reported that there are two distinct antigenic variants (North American and South American), the former being genetically quite stable (Roehrig *et al.*, 1990). It causes severe disease in humans, certain species of birds such as the pheasant, as well as horses, all of whom have high fatality rates after infection.

Epidemiology and Host Range

The physical, biological and ecological factors associated with epizootic transmission are complex, but the abundance of EEEV circulating in the enzootic cycle and the various characteristics of the epizootic vectors are important determinants of risk. In endemic areas birds are the most important vertebrate host and the natural cycle is between birds and the ornithophilic mosquito, *Culiseta melanura*. Birds vary in their susceptibility, with some, for example the pheasant, developing severe disease, while other avian species suffer no appreciable morbidity or mortality (Peters and Dalrymple, 1990). *Cs. melanura* is thought to be the main endemic vector (Howard and Wallis, 1974). However, because of its ornithophilic nature this mosquito is unlikely to play a major role in the transmission of EEEV to horses or humans. However, the virus escapes from enzootic foci located in swamp areas through bridge vectors such as *Aedes sollicitans* and *Cophilatidia perturbans*, which are thought to be important in the transmission to vertebrates. These species also feed on both mammals and birds and can transmit the virus to humans, horses and other hosts. Both humans and horses are 'dead-end' hosts for EEEV infections; infected birds are therefore necessary for the epidemic spread to these species. In South and Central America the enzootic cycle is maintained in most forests where *Culex (Melanocoon)* spp. are considered the primary vectors (Scott and

Weaver, 1989) Disease in humans often follows an epizootic course in horses. Fewer than five cases of EEEV are reported annually in the United States. Case fatality rates as high as 30% in past epidemics are indicative of the severity of EEEV infection in humans. Even during a major equine epizootic, EEEV-associated disease in humans is rare. In addition to being limited to certain geographical regions, the prevailing climatic conditions, in the form of an unusually hot, wet summer, also play an important role, for this allows for an abundance of the vector *Cs. melanura*. This, together with the presence of a susceptible bird population and an additional vector that can transmit virus from viraemic birds to susceptible horses and humans, are important predisposing factors for the occurrence of human and equine cases. Although the transmission cycles have not been well established, additional reservoirs of infection have been reported in forest-dwelling rodents, bats and marsupials (Ubico and McLean, 1995), reptiles, amphibians (Morris, 1988), white-tailed deer (Schmitt *et al.*, 2007) and African penguins maintained in an aquarium (Tuttle *et al.*, 2005).

Clinical Disease

EEEV infections may produce severe, often fatal encephalitis in humans (Clarke, 1961; Deresiewicz *et al.*, 1997; Farber *et al.*, 1940). The ratio of inapparent to apparent infections is approximately 23 : 1 in adults and 8 : 1 in children under the age of four (Goldfield *et al.*, 1968). The encephalitis associated with EEEV infection tends to be fulminant; a recent study, which reviewed all cases of EEEV infection in the United States between 1988 and 1994, showed a mortality of 36%, with 35% of patients being left moderately or severely disabled. The remaining patients were left mildly disabled, with only one patient recovering fully. The most common clinical features were fever (83%), headache (75%), nausea and vomiting (61%) and malaise and weakness (58%). The incubation period for EEEV is 4–10 days. However, the onset of neurological symptoms was associated with a rapid deterioration of the patient. Eighty-nine per cent (32 of 36 patients) were or became stuporous or comatose, with about one-quarter having seizures. Interestingly, abdominal pain was present in 22% of cases, with two patients presenting with acute abdominal pain in the prodromal period (Deresiewicz *et al.*, 1997). Although it has been suggested that an age of over 40 and a long prodromal course correlates with a good functional recovery (Przelomski *et al.*, 1988), others have found that these parameters do not significantly predict outcome (Deresiewicz *et al.*, 1997). A biphasic course in children is common, beginning with fever, vomiting and headache for one to two days, followed by an apparent recovery,

finally taking the form of a fulminant encephalitis. In children under five years of age who survive the disease, neurological sequelae such as mental retardation, convulsions and paralysis are frequent observations. Deresiewicz and colleagues did, however, demonstrate that high initial cerebrospinal fluid (CSF) white cell counts and the development of severe hyponatraemia were poor prognostic signs, possibly because both could be markers of intense cerebral inflammation (Deresiewicz *et al.*, 1997).

Pathogenesis and Pathology

Post-mortem histopathological studies have demonstrated necrotic foci together with arteriolar and venular inflammation and perivascular cuffing. An inflammatory meningeal infiltrate may be present. Although the distribution of the focal lesions may vary, involvement of the basal ganglia and thalami appears to be prominent (Deresiewicz *et al.*, 1997). Viral particles have been visualized in the oligodendroglial cells by electron microscopy (Bastian *et al.*, 1975). The neurological damage is probably due to a combination of a direct viral cytopathic effect, inflammatory damage and a vasculitis (Peters and Dalrymple, 1990).

Diagnosis

A diagnostic clue may be obtained from the prevailing climatic conditions (a hot wet summer), together with associated illness in the horse and pheasant populations. However, the symptoms are nonspecific and confirmation needs to be obtained serologically or by demonstration of virus in CSF or both. Neuroradiological imaging may be helpful, with focal lesions visible in the basal ganglia, thalami and brainstem (Deresiewicz *et al.*, 1997).

Laboratory diagnosis is based on molecular detection of viral antigen, virus isolation or antibody detection. Samples for isolation can be CSF, blood or central nervous system (CNS) tissue by inoculation into newborn mice or tissue culture using Vero cells or MRC-5 (Sotomayor and Josephson, 1999). As with all alphavirus infections, an acute serum sample should be taken as soon as possible after the onset of illness. EEEV has been isolated from a laboratory worker on the second day of illness (Clarke, 1961). In this case 4 of 25 one-day-old mice died after intracerebral inoculation with a serum sample taken from this patient. Further identification was done serologically. In addition, virus has also been isolated from post-mortem brain tissue taken from a fatal case of EEE. More recently an RT-PCR based assay has been developed to detect EEEV RNA (Voskin *et al.*, 1993).

Serology is an important diagnostic tool. Paired serum samples may be tested in parallel using ELISA (Scott and Weaver, 1989) or plague-reduction neutralization test

(PRNT). A class-specific anti-EEEV IgM antibody in CSF or elevated anti-EEEV IgM antibody detected by IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) has been described as a rapid and specific diagnostic tool (Centers for Disease Control and Prevention, 2006; Sudhirs *et al.*, 1994). As EEEV is the only species of virus belonging to the eastern equine encephalitis complex (Table 27.1), cross-reaction with heterologous viruses belonging to the same antigenic complex does not pose a diagnostic problem (Calisher *et al.*, 1986).

Prevention and Control

Protection against mosquito bites and control of the vector is important. An effective equine vaccine is licensed in the United States and is recommended for livestock in areas where EEEV transmission is known to occur. Specific control measures to prevent human disease include a formalin-inactivated vaccine derived from a North American strain of EEEV (PE-6) available for horses. No specific antiviral treatment is available and no human EEEV vaccine is currently licensed for human use. The vaccine has been shown not to induce significant levels of neutralization or anti-E2 antibody responses to South American strains of EEEV (Strizki and Repik, 1995). However, as this human disease is rare, even during an equine epizootic, this vaccine is only issued to those individuals engage in an occupation that places them at risk of exposure. Prompt notification of suspected cases of arboviral encephalitis to the local public health authority is also essential. Although vaccination protects equines against disease, it does not reduce the risk of exposure to humans.

Western Equine Encephalitis Virus

The WEEV was first isolated in California in 1930 from the brain of a horse and remains an important cause of encephalomyelitis in horses and humans. The western equine encephalitis complex in the New World contains three additional viruses in addition to WEEV named: Highlands J (HJ), Fort Morgan (FM) and Aura. Only WEEV is recognized as causing human disease (Calisher, 1994). Viruses in the western equine encephalitis complex found in the Old World include Sindbis, Whataroa and Kyzlagach. WEEV causes more human infections than EEEV, but the illness is less severe and mortality seldom exceeds 10%. Its distribution covers the Pacific coast of the United States, but also includes the Great Plains of the United States and Canada and extends into Central America and the northern parts of South America (Chamberlain, 1987; Hardy, 1987; Iverson, 1994; Reeves, 1987). The western equine encephalitis antigenic complex consists of six species (Table 27.5). It has been

suggested that WEEV arose through recombination between a EEEV-like virus and a Sindbis-like virus (Hahn *et al.*, 1988).

Epidemiology and Host Range

The enzootic cycle of WEEV involves passerine birds and culicine mosquitoes. WEEV is transmitted in the western United States by the mosquito *Culex tarsalis*. Birds are the most important vertebrate host. Once infected, the birds develop viraemia with sufficiently high titre to infect the mosquito vectors. Endemic transmission results in a few human cases. In addition, major equine epidemics may result in a significant number of human cases. As with other arboviruses, climatic factors exert a major influence on the epidemiology and distribution of WEEV.

In the eastern United States, WEEV is replaced by Highlands J virus (HJV), whose primary vector is *Cs. melanura*, which is also the primary vector of EEEV. The strictly ornithophilic nature of this vector explains the absence of significant disease outbreaks in the eastern United States. WEEV and HJV are closely related both serologically and at a molecular level.

Clinical Disease

Most WEEV infections of adults are asymptomatic, patients presenting with an incubation period of 5–10 days. In adults there is a sudden onset of fever, headache, nausea, vomiting, anorexia and malaise. In children, fever, headache and malaise occur a few days before the neurological symptoms. Flaccid and spastic paralysis and abnormal reflexes are seen more frequently in children than adults. The estimated case : infection ratio in adults is approximately 1 : 1000. This decreases to approximately 1 : 60 in children and 1 : 1 in babies (Peters and Dalrymple, 1990). Permanent neurological damage is rare in adults but frequent in children who may suffer from mental retardation, spastic paralysis and recurrent convulsions. Drowsiness, headache and mental confusion, sometimes leading to coma, may be seen in adults. *In utero* infection followed by an acute encephalitis has been documented (Shinefield and Townsend, 1953). Recovery from the acute illness may be slow and symptoms such as fatigability, irritability and headache may persist for up to two years (Earnest *et al.*, 1971).

Pathogenesis and Pathology

WEEV is less neurovirulent than EEEV both in humans and horses. Post-mortem examination of the brain of fatal cases shows a perivascular mononuclear and polymorphonuclear infiltrate together with parenchymal necrosis.

Table 27.5 Origins and geographical distribution of the western equine encephalitis virus complex.

Antigenic complex	Species	Origins	Main geographic distribution	Other features
Western equine encephalitis	Aura	Brazil, 1959	North America, Mexico, Guyana, Brazil and Argentina Brazil and Argentina Western United States	Epizootics: North America only
	Fort Morgan	Colorado, 1973		
	Highlands J	Florida, 1964		
Sindbis-like virus	Sindbis	Egypt, 1953	Florida, eastern United States	
	Ockelbo	Sweden, 1982	Africa and Australia	
	Babinki	Cameroon, 1969	Mediterranean and Europe	
	Kyzylgach	Azerbaijan, 1969	Africa	
	Whataroa	New Zealand, 1962	Azerbaijan and China	
			New Zealand	

Diagnosis

During the acute phase of the illness viral isolation may be attempted from blood, throat swabs or a CSF samples using suckling mice, but this is frequently not successful, since by the time it is recognized clinically the viraemic period is over. Virus may usually be isolated from brain biopsy material or post-mortem brain tissue. However, serology, together with the appropriate clinical manifestations in a patient living in or who has recently travelled to an endemic area, forms the most important method of diagnosing of disease associated with infection with WEEV. Classical serological techniques, for example HAI, ELISA, immunofluorescence test and serum neutralization can be used to demonstrate a rise in antibody titre between acute and convalescent serum samples. In addition, the detection of WEEV-specific antigen capture or IgM-capture ELISA can provide a rapid acute-phase diagnosis. However, cross-reaction may occur with other viruses within the complex (Table 27.1). Knowledge of the geographical distribution of the viruses within the complex allows interpretation of a positive IgM result together with the appropriate patient details (Calisher *et al.*, 1986). Antigen detection using nucleic acid sequence-based amplification (NASBA), standard RT-PCR and TaqMan nucleic acid amplification assays with patient serum or CSF is well established (Lambert *et al.*, 2003; Pfeffer *et al.*, 1997).

Prevention and Control

Vector control is an important aspect of the control of all mosquito-borne encephalitides. Outbreaks in humans and horses are associated with unusually high densities of mosquitoes. There is therefore a correlation between *Cx. tarsalis* density and human cases of WEEV infection. Besides vector control, changing behaviour patterns, for example, air-conditioning houses and promoting indoor activities may also protect from vector-borne diseases

and are complementary to mosquito control programmes (Gahlinger *et al.*, 1986). An inactivated vaccine is available to those at risk. An inactivated vaccine is available for horses.

Venezuelan Equine Encephalitis Virus

As the name suggests, VEEV was first isolated in Venezuela in 1938, where it causes important epizootics in horses, but also infects humans. The geographical distribution includes Central and South America. VEEV caused epidemics; epizootics among people and horses in Latin America from the 1920s to the 1970s. It reached the United States in 1971, but no further activity was reported until 1992–1993, when a small outbreak in Venezuela confirmed the re-emergence of VEEV from its enzootic habitat to humans. This was followed by a major outbreak occurring in 1995, involving an estimated 75 000–100 000 people (Rico-Hesse *et al.*, 1995; Weaver *et al.*, 1996). A variant of the virus, known as Everglades virus, is endemic in South-Central Florida and another variant, Mucambo virus, is found in Brazil, Trinidad and Surinam (Table 27.6).

Epidemiology and Host Range

Both epizootic and enzootic strains of VEEV occur. Serotypes IAB and IC are equine virulent, while ID, IE and II, III and IV are equine avirulent (Table 16.6) (Powers *et al.*, 1997; Weaver *et al.*, 1996). The principal vertebrate host for enzootic transmission is the rodent, although birds and other species may play a less important role. Horses may be infected by several subtypes or variants of VEEV (Table 16.1) but they do not play an important role in enzootic transmission which occurs primarily between small mammals and the *Culex* mosquitoes of the subgenus *Melanoconion* (Cupp *et al.*, 1986). In contrast, during equine epizootics horses do play an important role in viral spread. It has been suggested that

Table 27.6 Origins and geographical distribution of the Venezuelan equine encephalitis virus complex.

Antigenic complex	Species	Subtype	Origin	Main geographic distribution
Venezuelan equine encephalitis	I	1 A-B	Trinidad, 1948	
		1C	Venezuela, 1963	Venezuela, Colombia, South and Central America
		1D	Panama, 1961	
		1E	Guatemala, 1968	
		1F	Brazil, 1978	
		II	Everglades	Florida, 1963
	IIIA	Mucambo	Brazil, 1954; French Guinea, 1968	South America and Trinidad
			Colorado, 1974	French Guinea
	IIIB	Tonte		Western North America
		Bijou Bridge		
	IV	Pixuna	Brazil, 1961	South America
V	Cabassou	French Guiana, 1968	French Guiana	
VI	AG80-663	Argentina, 1980	Argentina	

epizootic strains of VEEV are maintained in other mammalian hosts and birds until the conditions are favourable for the development of an equine epizootic. A number of mosquito species have been implicated as likely vectors during epizootics. Human disease usually follows equine disease, but humans are not thought to be important in the maintenance of the epidemic due to low-titre viraemia.

The major outbreaks of Venezuelan equine encephalitis that occurred in Venezuela and Colombia in 1995, involving an estimated 100 000 human cases, were remarkably similar to an outbreak occurring in 1962–1964 (Rico-Hesse *et al.*, 1995; Rivas *et al.*, 1997). Further outbreaks recorded in 1999/2000 and 2003 in Venezuela suggested the persistence of VEEV subtype IC (Navarro *et al.*, 2005). Both outbreaks followed unusually heavy rains which led to an increase in the mosquito vector, and both occurred in the same geographic region. Phylogenetic analysis showed that isolates from the 1995 epidemic of Venezuelan equine encephalitis were closely related to the serotype IC viruses isolated during the 1962–1964 epidemic. As a similar virus was identified in mosquitoes in Venezuela in 1983, the authors raise the possibility of a serotype IC enzootic transmission cycle in northern Venezuela (Weaver *et al.*, 1996).

Epizootic transmission involves horses, mules and donkeys as amplification hosts and the mosquito vectors in the genera *Ochlerotatus*, *Psorophora* and others. Most human cases of Venezuelan equine encephalitis occur during equine epizootics but endemic transmission has been recorded in communities in close proximity to sylvatic enzootic cycles in the absence of equine disease.

Clinical Disease

In horses, enzootic strains of VEEV usually produce a fever and mild leukopenia. In contrast to this, horses infected with epizootic strains exhibit high fevers, severe leukopenia and signs of encephalitis. Only VEEV and EVEV (genetic variant of VEEV in subtype ID) are associated with encephalitis in humans, while the others such as Mucambo cause febrile illness. Natural human epidemics in 1952 and 1995 in Colombia and Venezuela and Panama during 1962–1964, resulted in over 100 000 cases with 500 deaths, demonstrating the highly infectious nature and severity of the disease.

Infection may be subclinical (usually caused by enzootic strains) or result in significant disease caused by epizootic strains of VEEV (Table 27.3). The incubation period lasts for two to five days. Symptoms and signs in patients seeking medical care include fever, chills, severe headache, myalgia, vomiting and diarrhoea. Most patients will have an acute and self-limiting febrile illness; however, convulsions, disorientation and drowsiness may also be present (Weaver *et al.*, 1996). The proportion of cases that develop neurological sequelae appears to vary from outbreak to outbreak. A review of the medical records of patients with convulsions in the 1995 epidemic showed that, although the age varied greatly (from 2 months to 48 years), most were children; however, only approximately 20% of these were under three years of age. Of interest is that about half of these patients had their first seizure between the 5th and 10th day after the onset of their illness and when their temperature had returned to normal (Weaver *et al.*, 1996). A further prospective study which included a total of 13 patients showed that six patients had a severe incapacitating febrile illness lasting three to five

days, two others had a 'flu-like' illness, and the remaining five (38%) were asymptomatic (Martin *et al.*, 1972). The overall mortality is thought to be less than 1%. Fetal loss may occur in pregnant women infected with VEEV.

Transmission can occur by the respiratory route as well as by mosquitoes. Accidental laboratory aerosol infections with epizootic strains of VEEV has been reported to cause a febrile illness with abrupt onset of chills, headache, myalgia, vomiting and pharyngitis two to five days after exposure without evidence of encephalitis (Erenkranz and Ventura, 1974).

Pathogenesis and Pathology

The disease caused by VEEV in humans is relatively mild compared to that caused by either WEEV or EEEV. In horses, as well as other species, there is a clear difference in the pathogenicity of the epizootic and the enzootic strains of VEEV. Epizootic strains are generally more virulent, with infection in horses resulting in viral replication in the lymphoid tissue and bone marrow. This is associated with lymphoid necrosis and a lymphopenia and is accompanied by a high-titre viraemia. Spread to the CNS probably occurs in the bloodstream, resulting in fatal encephalitis in horses, as well as rodents and some primates (Peters and Dalrymple, 1990).

Diagnosis

The diagnosis of VEEV-associated disease should be suspected in patients presenting with a 'flu-like' illness in an appropriate geographical region when there is a concurrent equine epizootic. Material obtained from sick horses confirming an epizootic of VEEV may therefore be an important indicator of human disease. Isolation of virus may be attempted from acute-phase serum and throat swabs as well as from brain tissue obtained from aborted and stillborn fetuses (Weaver *et al.*, 1996). Culture of VEEV has been achieved by inoculation into Vero or mosquito cells or suckling mice (Dietz *et al.*, 1979). Intracerebral inoculation of suckling mice may result in death within 72 hours. Isolates identified in cell culture may be characterized further using VEEV serotype-specific polyclonal sera. Alternatively, detection and genetic characterization may be done using the more recently developed standard RT-PCR, and TaqMan nucleic acid amplification assays with sequencing of the products generated from the E3 and E4 genes (Brightwell *et al.*, 1998; Weaver *et al.*, 1996).

A number of serological assays (IFA, ELISA, HAI) have been described. Acute and convalescent serum samples may be tested in parallel by HAI. This may provide diagnostically useful information. More recently attention has been focused on the use of an IgG ELISA based on

an antigen prepared from the attenuated VEEV vaccine strain of virus. In experimentally infected guinea pigs, VEEV-specific IgG could be detected at 6 days post inoculation, compared with 10 days for HAI antibodies. However RRV-, EEEV- and WEEV-specific IgG exhibit a weak cross-reaction on the IgG ELISA, so results must be interpreted with caution. The appearance of IgG antibodies detectable by ELISA coincides with the development of antibodies detectable by plaque reduction neutralization (PRN) assay. While less sensitive than the IgG ELISA, PRN is more specific and therefore eliminates some of the problems of cross-reaction discussed above. It may therefore be used as a confirmatory test. VEE-specific IgM antibody has been detected as early as four days post inoculation in experimentally infected guinea pigs (at a time at which infectious virus was present in the serum). In summary, suspected cases of VEEV infection may be investigated serologically using a VEEV-specific IgM and IgG ELISA, together with the more specific PRN as a confirmatory test (Coates *et al.*, 1992)

Prevention and Control

Personal protection against mosquito bites together with vector control, as for other alphavirus infections, is important. Control of an equine epizootic by immunizing horses is also important in the prevention of subsequent human disease. Two types of vaccine are currently available for the prevention of Venezuelan equine encephalitis in humans and horses. The first is a live-attenuated vaccine, TC-83, produced by serial passage of wild virus in guinea-pig fetal heart cell culture. While this vaccine has been proven to be efficacious and relatively safe, 25% of individuals immunized develop a clinical illness with a low-grade viraemia. A formalin-inactivated vaccine, C-84, which is derived from the TC-83 strain of virus, has been shown to be safe and provides effective protection in experimental animals injected with virulent VEEV but only limited protection from aerosol challenge.

OTHER ALPHAVIRUSES

Semliki Forest virus

Semliki Forest virus is found in sub-Saharan Africa and has been used as a model virus for research. Although the disease potential for this virus remains to be elucidated, asymptomatic seroconversions have been described. However, the clinical features of infection with SFV have not been well defined. One scientist working with SFV developed an encephalitis and SFV was isolated from both CSF and post-mortem brain tissue (Peters and Dalrymple, 1990). This highlights the necessity for caution

when working with any infectious agents of unknown pathogenic potential. No overt disease was recognized until a large outbreak of a mild febrile illness was reported in Bangui, Central African Republic in 1987. SFV is considered to be maintained in a non-human primate; an *Aedes* mosquito cycle and ticks are also thought to be involved (Pfeffer, 2001).

Getah virus

Getah virus (GETV) was originally isolated from *Culex gelidus* and *Culex tritaeniorhynchus* in Malaysia in 1955. The viruses Sagiyama, Bebaru and RRV are considered to be subtypes of Getah (Kumanomido, 2001; Powers *et al.*, 2001). The virus has been isolated from a wide variety of mosquito species, *Culex* spp. in South East Asia, Japan, the Philippines, China and Australia, and from *Aedes* spp. in Serbia and has the potential to be amplified in domestic pigs (Wen *et al.*, 2007). GETV has been linked to severe encephalitis in horses, producing fever, rash and limb oedema (Fukunaga *et al.*, 2000) Although GETV has not been implicated in human disease, serological studies have demonstrated antibodies to GETV in sera taken from individuals from the Pacific Basin, Japan and China (Johnson and Peters, 1996). Serological cross-reactivity with RRV makes interpretation of serosurvey data difficult.

Una virus

Una virus (UNAV) was first isolated from *Psorophora ferox* mosquitoes originating from the Amazonian region of Brazil in 1959. The virus has also been isolated in Trinidad. Considered to part of the SFV complex of viruses, as yet this virus has not been recognized as causing human disease.

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Flaviviruses

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INTRODUCTION

The *Flaviviridae* are a family of viruses comprising some 94 members and are responsible for a major portion of disease and death in humans and animals. The family is subdivided into three genera: *Flavivirus*, *Pestivirus* and *Hepacivirus*.

Flavivirus

The genus *Flavivirus* consists of 82 confirmed or provisional members. It was formerly termed 'group B arboviruses' and prior to 1984 was placed together with the 'group A arboviruses' within the family *Togaviridae*. However, as knowledge of the flaviviruses increased, it became apparent that their features and properties, for example, their replication strategies, structure and biochemistry, were sufficiently distinctive that they would need to be placed in a separate family, the *Flaviviridae*, with the two additional genera, *Pestivirus* and *Hepacivirus*, being added subsequently. More recently, GB virus C (GBV-C), GB virus B (GBV-B) and GB virus A (GBV-A), which are closely related to hepacivirus, have been tentatively added to the family *Flaviviridae*, although their taxonomic designation remain to be defined.

Most members of the genus *Flavivirus* are arboviruses, which are transmitted between a vertebrate host and an invertebrate (mosquito or tick) host. Replication and amplification of virus occurs in both the vertebrate and invertebrate hosts. With some of the flaviviruses humans may be the major vertebrate host, for example, in the viruses causing dengue and urban yellow fever, and the

virus is maintained by being alternately transmitted from humans to mosquito to humans. In most of the flavivirus infections, the virus is maintained in nature by alternate infection of a variety of mammals or birds, or occasionally other vertebrate hosts, and their respective mosquito or tick vectors. Humans are infected as an accidental event when people intrude into this natural ecosystem. In these situations humans are a 'dead-end' host of no biological significance to the life cycle of the virus. The non-arboviruses that are members of the genus *Flavivirus* may be found in either arthropods or in vertebrates, but not in both, and are of little medical importance.

Whether flaviviruses have a narrow or wide host range, their distribution is dependent on the ecology of their specific vertebrate and invertebrate hosts. Some flaviviruses have very restricted geographic distributions, such as Kyasanur Forest virus, found to date only in Karnataka (previously Mysore) State in India, Omsk haemorrhagic fever virus (OHFV), found in the Omsk district of western Siberia, or Rocio virus in the Santista lowlands and the Ribeira valley of Sao Paulo state in south-east Brazil. By contrast, flaviviruses such as West Nile virus (WNV) are distributed widely through Africa, Asia, large parts of Europe and, more recently, the United States and Canada. Japanese encephalitis virus (JEV) is found in at least 16 countries in a wide belt stretching from eastern to south-eastern to southern Asia, affecting a combined population in excess of two billion. Dengue virus has moved over the past 20 years from South East Asia into the Pacific, the Americas, Africa and Australia, progressively establishing itself in local populations of the widespread human-biting mosquito *Aedes aegypti*.

The clinical manifestations of the majority of flavivirus infections are relatively nonspecific. In endemic areas, the diagnosis of infection is often made on clinical suspicion, especially if the physician is alerted by epidemic activity in the region. Occasionally outbreaks of flavivirus infection may follow climatic events, for example, heavy rains after a period of drought, which result in the formation of numerous stagnant pools, facilitating the breeding of mosquito vectors and also attracting bird life. Outbreaks may follow uncontrolled urbanization and the breakdown of vector control measures, as has occurred in recent yellow fever outbreaks in West Africa. In some situations human cases may follow an extensive zoonosis in the vertebrate host, especially where birds or livestock are involved. Surveillance and epidemiological monitoring, which involve, among other things, the continual and sustained sampling of arthropod vectors and vertebrate hosts for arbovirus activity, is of critical importance in the prevention and management of outbreaks of flavivirus infections. Recently calls have been made for mobile units to be established and sent into the field to conduct clinical diagnosis and therapeutic research, as well as for epidemiological surveillance in endemic areas.

The diagnosis of infection due to flaviviruses becomes particularly difficult in individuals who have travelled from an endemic area and present themselves for medical management many thousands of miles away from their source of infection. In the modern era of jet travel, a history of travel and also a knowledge of the prevalent infections in different parts of the world, are now an important component of infectious diseases medicine. In addition, travellers should be educated to alert their attending physician should they become ill on returning home.

Pestivirus

The second genus in the family *Flaviviridae*, *Pestivirus*, is also referred to as the mucosal disease virus group. The viruses of this genus are the causes of important veterinary diseases worldwide, such as bovine viral diarrhoea, border disease of sheep and hog cholera. They are not known to be spread by arthropod vectors, transmission taking place by direct contact and also via faeces, urine and nasal secretions, as well as vertically. The pestiviruses are not able to infect humans, therefore will not be discussed further.

Hepacivirus

The third and newest genus of the family *Flaviviridae* is *Hepacivirus*. The biochemical and biophysical characteristics of the virus have been determined and have been shown to be characteristic of the family *Flaviviridae*. Hepaciviruses are not arboviruses and are not known to infect

any non-human host in nature. Experimentally, the only animal able to be infected is the chimpanzee. Infection is transmitted by blood transfusion, penetrating injuries with blood-contaminated needles and instruments, sexually, and from mother to child during birth. Since the implementation of widespread screening for hepatitis B virus in blood, hepacivirus has become the major cause of post-transfusion hepatitis. Current screening programmes for hepacivirus have, in turn, reduced significantly the incidence of post-transfusion hepatitis due to this virus.

The most recent member of the family *Flaviviridae* is GBV-C virus. The virus was first detected in 1995 in laboratory tamarins experimentally inoculated with blood from a surgeon with clinical hepatitis. The GBVs, although closely related to hepacivirus, are distinct having only 29% amino acid homology with it. The pathological role, if any, of GBV-C has not yet been established. (GBV-B is a true hepatitis virus of primates, while GBV-A, also an agent of primates, is not known to cause disease.) Hepacivirus and GBVs are dealt with fully in Chapters 12 and 13.

PROPERTIES OF THE VIRUS

The type species of the genus *Flavivirus*, yellow fever virus, has been the most extensively studied member of the family. Recent interest in especially WNV, dengue virus and Japanese encephalitis has increased the knowledge of flavivirus molecular biology substantially.

Morphology and Morphogenesis

The virus particles are spherical, with a diameter of some 40–50 nm. The envelope is tightly applied to a 25–30 nm spherical core and surface peplomers are often visible. Replication of virus takes place in the cytoplasm of the cell in association with the rough and smooth endoplasmic reticulum. Accumulations of viral particles are seen within the lamellae and vesicles and replication is characteristically associated with the proliferation of intracellular membranes.

Biophysical and Biochemical Properties

The S₂₀W of flaviviruses is 70–210, that of pestiviruses is 140 and that of hepacivirus is ≥ 150 . The buoyant density of the flaviviruses in CsCl is 1.22–1.24 g cm⁻³ and 1.15–1.20 g cm⁻³ in sucrose.

The nucleic acid of flaviviruses consists of a single molecule of positive-sense ssRNA of approximately 11 000 nucleotides in length. A single open reading frame (ORF) on the genomic RNA is translated directly into a polyprotein, which is further processed into 10 major

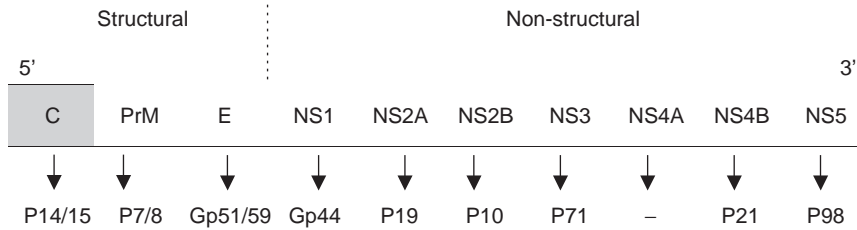


Figure 28.1 Schematic representation of the organization of the flavivirus genome.

proteins—three structural and seven nonstructural proteins. Each of the individual proteins is derived by co- and post-translational cleavage by host cell signalases and the virus-specific proteases encoded by NS2B–NS3. Flanking the ORF, 5' and 3' noncoding regions with conserved secondary structures are found, which may play a role in genome replication and as translation enhancers. In order from the N-terminal, these are the internal RNA-associated C protein and then the two envelope proteins, prM and E, followed by the nonstructural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Figure 28.1). The lipid bilayer is derived from the host cell membrane. The E and M proteins are inserted into the envelope. The glycoprotein E is rich in mannose and complex glycans. The C protein exists as two forms: a membrane-anchored form (C_{anch}) and the mature virion associated form (C_{vir}) (14–16Da) which remain associated with the membranes of the endoplasmic reticulum and the viral nucleic acid. WNV C proteins have been associated with the induction of apoptosis in infected cells. The prM protein is a glycosylated precursor protein. Co-synthesis of prM and E proteins is essential for correct folding of E suggesting that prM may act as a chaperone and may protect the E protein from degradation during transport to the cell surface in acidic post-Golgi vesicles. Subviral particles are formed when prM and E are expressed together *in vitro*, although their structure differs from the native virus. When virus release from the host cell takes place, prM is cleaved by host cell furin-like proteases, resulting in a smaller nonglycosylated M protein (molecular weight 7–9 kDa) and dimerization of the E protein on the virion surface. The E protein is the most conserved of the flavivirus structural proteins and has an important role in virus attachment and fusion. It is also the major neutralizing antigen. X-ray crystallographic structures of truncated soluble E proteins from tick-borne encephalitis virus (TBEV), dengue 2 and dengue 3 suggests that the E protein exists as a dimer. The individual monomers consist of three distinct structural domains: a central β -barrel (domain I), an elongated dimerization domain (domain II) and an immunoglobulin-like C-terminal domain (domain III). This third domain is the putative

receptor-binding domain for flaviviruses and the target for virus-specific neutralizing antibodies.

The nonstructural proteins play important roles in viral replication and may interact directly with host cell proteins to modulate the immune response to viral infection. The best characterized of the nonstructural proteins are NS1, 3 and 5. NS1 appears to have a role in early replication and signal transduction events. The NS3 protein encodes the viral proteinase that mediates cleavage of the translated polyprotein and is involved in viral RNA replication nucleotide triphosphatase, RNA 5' triphosphatase and helicase activities. The NS5 protein encodes a methyltransferase and a viral RNA-dependent DNA polymerase. The NS2A, NS2NB, NS4A and NS4B proteins are all small hydrophobic proteins with roles in viral replication. In addition they seem to have a function as interferon antagonists, potentially by blocking Stat1 and/or Stat2 phosphorylation (reviewed in Beasley, 2005).

Antigenic and Genetic Properties

The antigenic properties of the genus *Flavivirus* are defined by serological tests, such as the highly cross-reactive pH-dependent haemagglutination inhibition (HAI) test, the less cross-reactive complement fixation (CF) test, and the much more specific neutralization test (NT) or plaque reduction neutralization test (PRNT). Other techniques mainly used for diagnostic serology are IgG and IgM detection by enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assays, for viral RNA detection the polymerase chain reaction (PCR) or related molecular techniques are used. The antigenic features of the virus are characterized by reactivity with antigenic domains and epitopes on the membrane E protein of the virus.

The genus *Flavivirus* is subdivided further into subgroups on the basis of cross-neutralization tests, using a polyclonal hyperimmune mouse ascitic fluid prepared against each member virus. The members of each subgroup give significant cross-neutralization results against each other, whereas the 'unassigned' subgroup contains a miscellany of flaviviruses which will display only limited cross-reactivity with each other or with at least one other virus in any of the established subgroups.

Phylogenetic analysis using partial gene sequences of the NS5 or the envelope gene suggest that the flaviviruses form an extensive series of clades that are defined by their epidemiology and disease associations. These genetic clusters are grouped into mosquito-borne, tick-borne and no-known-vector virus clades, which could be further subdivided into clades defined by their principal vertebrate host. Two distinct epidemiological groups were evident amongst the mosquito-borne flaviviruses, namely (i) the neurotropic viruses, often associated with encephalitic disease in humans or livestock which correlated with the *Culex* mosquito vector and bird reservoirs such as WNV, JEV, Usutu, Murray Valley encephalitis virus (MVEV) and St Louis encephalitis virus (SLEV) and (ii) non-neurotropic viruses, which are associated with haemorrhagic disease in humans, and correlates with the *Aedes* mosquito vector and primate hosts, such as dengue 1–4 and yellow fever. This suggests that the phylogenetic topology may reflect differences in the feeding behaviour and hosts between *Aedes* and *Culex* mosquitoes.

The tick-borne flaviviruses also clustered into two distinct groups. The first group is associated with seabirds while the second, the tick-borne encephalitis complex viruses, is primarily associated with rodents, and includes Kyasanur Forest disease, Langat, Omsk haemorrhagic fever, Powassan and Karshi.

The 'no known vector' flaviviruses form three distinct groups, the first of which are closely associated with bats and genetically closely related to the mosquito-borne viruses (Sepik, Entebbe bat, Sokoluk, Yokose), a second group, also related to bats but genetically more distant to the mosquito-associated viruses (Rio Bravo, Bukalasa Carey Island, Dakar Bat), and a third which includes viruses associated with rodents (Apoi, Cowbone Ridge, Jutiapa, Modoc, Sal Vieja, San Perlita). Geographical clusters are also evident, clustering into Old World or the New World viruses, where mosquito-borne viruses appear to have originated in the Old World (Gaunt *et al.*, 2001).

The antigenic classification of members of the genus *Flavivirus* of medical importance together with their respective vectors, disease manifestations, geographic distribution, vaccine availability and frequency of reported cases, is given in Table 28.1. The flaviviruses that either cause no human disease or have only rarely been reported in association with illness will not be dealt with further here.

YELLOW FEVER

The yellow fever virus is the type species of the genus *Flavivirus* and also the source of its name (Latin: *flavus* = 'yellow'). Yellow fever was one of the classical diseases of antiquity, instilling dread and terror in the

Americas, Europe and Africa from the seventeenth to the twentieth century. It was romanticized in classic works such as Samuel Taylor Coleridge's *Rime of the Ancient Mariner* and Wagner's opera *The Flying Dutchman*. At the turn of the century, the disease almost put paid to the construction of the Panama Canal, and in West Africa yellow fever more than any other disease was responsible for the appellation 'the white man's grave'. As late as 1988, an estimated 44 000 cases with 25 000 deaths were estimated to have occurred in Nigeria in the three years 1986–1988.

History

The disease entity of yellow fever was first described in 1667 in Barbados, and in the following two centuries devastating epidemics raged on the continents of America and Africa and also, to some extent, in Europe. The panic and chaos was matched by the extravagance of the speculations as to the cause. The confusion was further aggravated by the difficulty in distinguishing it from the other tropical plagues of malaria and dengue. In 1848, Nott proposed that yellow fever was transmitted by the bite of a mosquito, a suggestion supported by Beauprethuy in 1854 and Carlos Finlay in 1881. In 1900 the US Army Yellow Fever Commission, under Major Walter Reed, demonstrated in historical experiments on human volunteers that the infection was indeed transmitted by mosquitoes and the following year, Reed demonstrated that yellow fever was due to a filterable agent. Indeed yellow fever was the first human disease shown to be caused by a filterable agent. The discovery of the aetiological agent, however, had to wait a further 26 years when workers of the Rockefeller Foundation's West Africa Yellow Fever Commission demonstrated the transmission of infection from a Ghanaian patient called Asibi to a rhesus monkey, and the subsequent passaging between monkeys. The Asibi strain was later to be the parent strain of the 17D vaccine, developed by Nobel Laureate Max Theiler at the Rockefeller Foundation in New York in 1937.

The 17D vaccine strain was developed by serial passage through mouse embryo culture to chick embryo culture and then through minced chick embryo devoid of nervous tissue. The final vaccine virus, propagated through fertilized chicken eggs, is remarkably attenuated yet immunogenic, and the vaccine has proved to be very safe and effective, probably producing lifelong immunity after a single injection (World Health Organisation 2005a).

Epidemiology

Yellow fever is endemic in the tropics on both sides of the Atlantic. The endemic zone stretches between latitudes 10°N and 40°S in the Americas and 16°N to 10°S in

Table 28.1 Antigenic classification of flaviviruses of medical importance and their clinical and epidemiological features in humans

Subgroup	Virus	Vector	Disease manifestation	Geographic distribution	Vaccine	Frequency of reported cases ^b
Unassigned	Yellow fever	M	Fever, HF, jaundice, etc.	Tropical Africa and South America	Yes	Numerous
	Rocio	M	Encephalitis	South-eastern Brazil	No	Uncommon
	Bussuquara ^a	M	Fever, arthralgia	South America	No	Rare
	Ilheus ^a	M	Fever, encephalitis	South America	No	Rare
	Sepik ^a	M	Fever	New Guinea	No	Rare
	Spondweni ^a	M	Fever	Africa	No	Rare
	Wesselsbron	M	Fever	Africa, Thailand	No	Uncommon
	Zika	M	Fever, rash	Africa, Malaysia, Indonesia, Micronesia	No	Uncommon
	Alkhumra ^a	Unknown	Fever, hepatitis, HF, encephalitis	Saudi Arabia	No	Rare
	Dengue	Dengue types 1–4	M	Fever, rash, myalgia, HF	Asia, Pacific, Americas, Africa	No
Japanese encephalitis (mosquito-borne encephalitis)	Japanese encephalitis	M	Encephalitis	East Asia, Australasia	Yes	Numerous
	St Louis encephalitis	M	Encephalitis	North America, Jamaica, Haiti, South America	No	Numerous
	West Nile	M	Fever, rash, encephalitis	Africa, Asia, Europe, USA, Canada	No	Numerous
	Murray Valley encephalitis	M	Encephalitis	Australia, New Guinea	No	Uncommon
	Kokobera ^a	M	Arthritis	Australia	No	Rare
Tick-borne encephalitis	Kunjin ^a	M	Fever, encephalitis	Australia, Borneo, Indonesia, Malaysia	No	Rare
	Usutu ^a	M	Fever, rash	Africa, Austria	No	Rare
	TBE-European subtype (CEE)	T	Encephalitis	Central and Western Europe	Yes	Numerous
	TBE-Far Eastern subtype (RSSE)	T	Encephalitis	Asiatic Russia	Developmental	Numerous
	Omsk HF	T	Fever, HF	Western Siberia	CEE?	Numerous
	Kyasanur Forest disease	T	Fever, encephalitis	Kanataka State, India	Yes	Uncommon
	Powassan	T	Encephalitis	North America	No	Uncommon
	Louping III ^a	T	Encephalitis	UK	No	Rare
	Negishi ^a	?	Encephalitis	Japan, Russia	No	Rare
	Kumlinge ^a	T	Encephalitis	Australasia	No	Rare

(continued overleaf)

Table 28.1 (Continued)

Subgroup	Virus	Vector	Disease manifestation	Geographic distribution	Vaccine	Frequency of reported cases ^b
Rio Bravo	Rio Bravo ^a	?	Fever	USA, Mexico	No	Rare
	Dakar Bat ^a	?	Fever	Africa	No	Rare
Uganda S	Uganda S ^a	M	—	Africa, ? Far East	No	Rare
	Banzi ^a	M	Fever	Africa	No	Rare

^aThese viruses are of very little importance to clinical medicine and will not be discussed further.

^bNumerous, >1000 cases reported, usually many thousands to millions; uncommon, 10–1000 cases reported; rare, <10 cases reported. M, mosquito; T, tick; HF, haemorrhagic fever; TBE, tick-borne encephalitis; CEE, Central European encephalitis; RSSE, Russian spring–summer encephalitis.

Africa, involving 44 countries, 33 in Africa and 11 in South America (Figure 28.2).

In the Americas, effective control of *A. aegypti* has resulted in the virtual disappearance of urban yellow fever from the Western Hemisphere, and the last epidemic of urban yellow fever occurred in Trinidad in 1954. However, jungle fever remains endemic in Bolivia, Brazil, Colombia, Ecuador, Peru, Panama, Venezuela and the Guyanas. The annual incidence by notification is approximately 100 cases, although this is probably a substantially underestimated number. Remarkably, the infection has not extended to the heavy concentrations of *A. aegypti* which have built up in urban centres. Although over 80% of all cases are reported from Africa, the case fatality rate in South America was 47% compared with 11% in Africa in 2004 (World Health Organization, 2005b).

In Africa, recent epidemics of yellow fever have been far more extensive than those in the Americas. The features of jungle yellow fever differ in East and West Africa. In East Africa, endemic yellow fever activity is relatively quiet with few notified cases. However, two vast epidemics took place in 1940 in the Nuba mountains of Sudan, causing 40 000 infections with over 15 000 clinical cases and 1500 deaths. The second epidemic in 1960–1962, the largest epidemic of yellow fever ever recorded, took place in south-western Ethiopia, causing 30 000 deaths in 100 000 clinical cases in a rural population of some 1 million. In contrast to this, in West Africa, frequent outbreaks have occurred, usually on a considerably smaller scale than the two East African epidemics, with the exception of the Nigerian epidemic of 1986–1988, when an estimated 44 000 cases and 25 000 deaths may have occurred (World Health Organization, 1991). Over the last two decades significant epidemics have been reported from several West African countries, both preceding the Nigerian epidemic, in the Ivory Coast (1982) and Burkina Faso (1983), as well as after it, Mali (1987), Angola (1988), Cameroon (1990) and Niger (1990). Yellow fever has expanded into Gabon,

Liberia and Kenya, which reported their first cases since 1950 between 1992 and 1995 (World Health Organization, 2005b).

The transmission cycles of yellow fever and the ecological interrelationships of its vectors and reservoir hosts are complex. Essentially, three cycles of transmission are recognized:

1. The 'enzootic forest cycle' represents the predominant maintenance of infection in its major vertebrate host, the monkey. In South America, *Alouatta*, *Cebus* and *Ateles* monkeys are the major primate reservoir hosts and they are infected by tree hole-breeding *Haemagogus* mosquitoes in the forest canopy. The monkey is, however, only a transient host because of the short-lived viraemia. The major amplification host is the mosquito, which remains infected for life and is also able to pass infection on transovarially. Occasionally a single or a few human cases may occur when people venture into the forests. The cycle in Africa is similar and involves *Cercopithecus* and *Colobus* monkeys, with the *Aedes filfricanus* mosquito as the principal vector.
2. The 'jungle yellow fever cycle' represents the most important epidemiological form of yellow fever with respect to human infection. Epizootic upsurges of yellow fever are frequent, both on the fringes of the rain forests and in the surrounding riverine gallery forests. Human infection occurs when forest mosquitoes invade adjacent plantations, clearings and villages. Once infection has been introduced into the human host, human-to-human transmission sustains the epidemics, resulting in the dramatic outbreaks reported in recent years. In South America, *Haemagogus* mosquitoes and possibly other mosquito species may establish epidemics in humans. In East and Central Africa east of Cameroon, *Aedes simpsoni* will readily bite monkeys and humans. In the East African epidemics in Sudan and Ethiopia, the anthropophilic *A. aegypti* was the most frequently

Yellow Fever epidemiology in Africa at country level, 1950–2004



Yellow Fever epidemiology in America at country level, 1950–2004



Figure 28.2 Yellow fever endemic zones in Africa and America. (a) Africa 1950–2004. (b) Central and South America, 1950–2004. (Source: Courtesy WHO—reproduced from www.who.int/csr/disease/yellowfev/impact1/en.)

responsible mosquito, as well as a number of 'wild' mosquitoes. In West Africa and Central Africa west of Cameroon, *A. simpsoni* does not bite humans and *Aedes fuscifer* is the major vector. In the Nigerian epidemic the major vector was *Aedes africanus*.

3. The 'urban yellow fever cycle' is maintained by *A. aegypti* and was the dominant form of yellow fever before extensive mosquito control eliminated the disease from South American towns. Periodic reinfestations with *A. aegypti* have been observed in many South American countries and have revived fears of a resurgence of urban yellow fever. In West Africa, *A. aegypti*-transmitted yellow fever is still responsible for outbreaks in towns and rural villages.

It is thought that yellow fever emerged in Africa before it was exported to the Americas, most likely through the slave trade. Nucleotide-sequencing analysis of the prM and E proteins identified a total of seven genotypes that have greater than 9% nucleotide variation and 7% amino acid variation. Five of these were identified in Africa, each genotype circulating in different geographic regions. In West Africa, two genotypes were present, West Africa genotype I was found from eastern Ivory Coast and Burkina Faso to Cameroon, as well as Nigeria. The second genotype, West Africa genotype II, was found in western Ivory Coast and Mali to Senegal. Two genotypes were identified in East/Central Africa: the East/Central African genotype which includes strains from the Central African Republic, Central Sudan, Ethiopia and the Democratic Republic of Congo, and the East African genotype, which includes strains from Kenya, southern Sudan, and Uganda. One further genotype was identified based on a single isolate from Angola (Angola genotype). In South America, two genotypes exist. The major genotype, South America genotype I, is found in Brazil, Panama, Columbia, Ecuador, Venezuela and Trinidad, while South American genotype II is mainly found in Peru, and a few isolates from Brazil and Trinidad (reviewed in Barrett and Higgs, 2007).

Clinical Features

The clinical presentation of yellow fever follows the general pattern of arbovirus disease—a short incubation period of three to six days followed by an acute biphasic illness. It is the severity and extent of the second phase of the acute illness which has imparted to this infection its classical notoriety.

The initial phase of illness is characterized by a viraemia that renders the patient infectious to biting mosquitoes and is also responsible for the acute constitutional symptoms. These symptoms last about three days and are generally those of a nonspecific febrile illness: headache, malaise, nausea, lassitude and widespread

muscle pain, especially in the back. The differential diagnosis is wide and includes malaria, other arboviral infections including dengue, typhoid, rickettsial infections, influenza, enterovirus infections and acute human immunodeficiency virus (HIV). With more intensive and careful physical examination of patients, signs and symptoms more suggestive of yellow fever may be revealed, such as flushing of the head and neck, conjunctival injection, strawberry tongue and a relative bradycardia. Probably the majority of clinically manifest yellow fever infections are aborted at this phase of the illness, accounting for the underestimation of the true numbers of cases by up to 500-fold (World Health Organization, 1991).

In cases of severe yellow fever, the early acute illness is followed by a brief period of remission before the onset of the haemorrhagic, hepatic and renal disease. The latter is heralded in by a return of fever, vomiting, abdominal pain, dehydration and prostration. The onset of the haemorrhagic diathesis is usually marked by coffee ground haematemesis, the classic 'black vomit', and bleeding from puncture sites where injections or drip needles have been inserted. This is accompanied by jaundice, albuminuria and oliguria. Deepening jaundice, massive haematemesis or haemoptysis or intra-abdominal bleeding, progressive renal failure, hypotension and shock may occur, followed by stupor, coma and death by the seventh to tenth day. Occasionally the illness may run a rapid fulminant course with death within a few days of onset. Mortality has been estimated to be of the order of 20–50% of cases entering the second phase of illness, although case fatality rates of up to 83% have been reported (World Health Organization, 1990). These figures probably represent a marked overestimate of the fatality rate in the more severe cases which are likely to come to the attention of the health authorities. The differential diagnosis of severe yellow fever is usually that of the causes of viral haemorrhagic fever (HF): Congo–Crimean haemorrhagic fever, Rift Valley fever, meningococcal septicaemia, Marburg and Ebola, generalized herpes simplex, as well as hepatitis B virus infection, leptospirosis and toxic hepatitis. During the severe second phase of illness, virus is usually absent from the blood and antibodies are present, suggesting that autoimmunity may well play a major role in the pathogenesis of severe yellow fever.

Patients who survive generally recover completely, although a chronic phase of illness lasting weeks or sometimes even months may occur in some individuals. This is characterized by prolonged jaundice and disturbances of liver function as well as prolonged renal failure. Occasionally sudden death may occur in the chronic phase as the result of myocardial damage or cardiac arrhythmias.

A number of host factors may affect the clinical expression and severity of yellow fever virus infection.

Age has played a significant role in South American epidemics, the majority of infections occurring in young adults, especially between the ages of 20 and 25. Age distribution has not, however, been a significant characteristic of African epidemics. In some epidemics gender has played a significant role in the distribution of cases, for example, in the 1972–1973 Brazil epidemic there was a marked predominance of males affected—a 9 : 1 ratio, whereas in others there was only a slight male preponderance (e.g. 53% males in the 1986 Nigerian epidemic). More controversial has been the purported association of race and susceptibility to yellow fever, especially in the classical literature, which frequently made reference to the mildness of the disease in the indigenous inhabitants of the African jungle. There is, however, no evidence confirming any difference in susceptibility between races.

There are no known viral factors affecting either transmissibility or virulence. Although genomic heterogeneity has been demonstrated by fingerprinting and sequencing studies and also some antigenic heterogeneity has been observed between isolates, there has been no demonstrable clinical or epidemiological differences between American and African isolates.

Diagnosis

The development of reliable rapid tests for the urgent diagnosis of yellow fever has become a major public health priority in the management of the viral haemorrhagic fevers. The IgM antibody capture ELISA has become the test of choice for rapid serological diagnosis of yellow fever virus infection. Cross-reactions may occur but IgM antibody levels will normally be higher against yellow fever. Immunofluorescence tests using infected cells spotted onto microscope slides and then acetone-fixed can be used for detecting IgG and IgM in patients' sera. In the immunofluorescence test, however, the rheumatoid factor may be a much greater problem than in the IgM antibody capture ELISA, and pre-treatment to remove IgG prior to IgM testing is necessary. The classical techniques, such as HAI, CF and NT tests still have a place in yellow fever surveillance and diagnosis but a fourfold or greater rise in titre would have to be demonstrated before a definitive diagnosis could be made. Specific diagnosis still may be difficult, particularly if the patient has had previous experience of flavivirus infection. In these instances higher titres may appear against heterologous viruses, in keeping with the doctrine of original antigenic sin.

Isolation of virus may be performed in specialized reference laboratories, either by intracerebral or intraperitoneal inoculation of suckling mice or by the intrathoracic inoculation of male *A. aegypti* or *Toxorhynchites* mosquitoes. These techniques are particularly sensitive

and are essential for epidemiological monitoring and research, but unfortunately take up to three weeks to provide an answer. More rapid viral isolation techniques using mosquito cell lines, such as the lines from *Aedes albopictus* (C6–36) and *Aedes pseudoscutellaris* (MOS 61), which are sensitive to infection and, combined with the immunofluorescence test using monoclonal antibodies, may give a diagnosis within three to four days. It is recommended that early antibody is dissociated from viral antigen using dithiothreitol prior to isolation attempts in mosquito cell cultures. An antigen-capture ELISA technique is available which is slightly less sensitive than virus isolation but is able to produce a specific result with the use of monoclonal antibodies in less than 24 hours. A sensitive method for detecting and quantifying viral RNA has been developed using real-time reverse transcription polymerase chain reaction (RT-PCR).

Liver biopsy is contraindicated in acute yellow fever. However, liver tissue from post-mortems may provide useful histopathological information. The classical liver pathology is that of a coagulative mid-zonal necrosis. The inclusion bodies which have been held to be pathognomonic of yellow fever are those in the cytoplasm due to eosinophilic degeneration (Councilman bodies) and intranuclear eosinophilic inclusion bodies (Torres bodies). Many of these features are, however, also found in fatal cases of viral haemorrhagic fever due to other viruses, and the histopathology is now no longer regarded as being diagnostic of yellow fever.

Control

The worldwide control of yellow fever has been achieved by immunization and effective vector control, which have largely eliminated the urban yellow fever cycle due to *A. aegypti*. However, in recent times, poverty, war and competing health priorities have led to a reduction in immunization and surveillance efforts, resulting in a resurgence of disease, especially in the endemic zone of Africa. In addition, reinfestation of towns and villages adjacent to forests with *A. aegypti*, aggravated by the massive uncontrolled urbanization and population migrations to the towns of developing countries, also exacerbated particularly by severe drought, has renewed the spectre of urban yellow fever in Africa.

Prior to 2001 surveillance for yellow fever infection was generally poor. However, since then a network of diagnostic laboratories has been established usually on pre-existing measles networks. A WHO recommended case definition has been drawn up—any clinical presentation of acute onset of fever with jaundice within 14 days of onset of illness.

It is over 50 years since the development of the first yellow fever vaccine by Theiler, and the original method

for the production of chick embryo-passaged 17D vaccine has undergone little modification over the years. The French neurotropic vaccine, developed by passage in mouse brain, is now no longer used because of the prohibitive danger of encephalitic complications. The vaccine is a live attenuated purified product produced by growing vaccine virus in chick embryos and is supplied as a lyophilized preparation, which should be stored frozen or at least kept at temperatures of not more than 5 °C. After reconstitution it should be used immediately and any remnants discarded within an hour. A single dose of 0.5 ml given subcutaneously provides excellent, long-lasting immunity to 99% of vaccinees. Although international health regulations demand booster doses every 10 years, neutralizing antibodies have been shown to persist for over 30 years and immunity is probably life long.

Side effects to immunization occur in less than 5% of recipients and are generally mild, low-grade fever, myalgia and headache. Occasionally, hypersensitivity reactions have been reported, especially in individuals allergic to egg protein. The most serious side reaction, encephalitis, has been reported in 18 cases out of more than 35 million doses of vaccine which have been administered to date. Only two cases have occurred in children over the age of seven months, although the only fatality was in a three-year-old child. Regarding the latter case, it was postulated recently that a change identified in amino acid position 303 of the isolate P-16065 compared with the parent vaccine virus 17D-204 USA may have affected the virulence of the vaccine virus (Jennings *et al.*, 1994).

The contraindications to immunization are those determined by age, pregnancy, history of egg allergy and immunosuppression (Centers for Disease Control and Prevention, 1990). The vaccine should not be given to children under the age of nine months unless travel to an endemic area cannot be avoided but should, at any rate, never be given to infants less than four months of age. The effect of immunization in pregnancy has not been determined; however, being a live attenuated vaccine, it should not routinely be given to pregnant women. However, if travel to an area with ongoing yellow fever cannot be avoided, the danger of infection would far outweigh the theoretical risk to a pregnant mother and her fetus. The vaccine should also be avoided in people with a history of egg allergy or in immunosuppressed individuals, for example, due to HIV infection, malignancy or immunosuppressive treatment. However, as in the case of pregnancy, the relative risks of travel to an endemic area vs. the slight or even theoretical risk of the vaccine would need to be evaluated on an individual basis. Yellow fever vaccine should be administered at least 14 days before departure. Booster doses are required for international travel regulations every 10 years.

Universal immunization of infants with yellow fever vaccine was recommended by WHO and UNICEF in 1988 for the 44 endemic countries in Africa and South America (World Health Organization, 1996a). The vaccine has been incorporated into the routine schedule to be given at nine months of age together with measles vaccine. By the year 2000, however, only 12 African countries had the vaccine in their routine schedule and in these the coverage was disappointingly low, lagging far behind measles. Recognizing the problem of vaccine funding to be a major obstacle, the Global Alliance for Vaccines and Immunization (GAVI) agreed, in 2000, to financially support the provision of vaccine to all eligible countries and brought the total for Africa up to 22 countries. In addition to routine immunization, 'one-off' mass immunization programmes have been carried out in several countries to rapidly reduce susceptibility.

The current worldwide production of yellow fever vaccine is estimated to be of the order of 70 million doses per year. In 2003 the GAVI Board approved the purchase of a stockpile of 6 million doses of vaccine to be used for preventive vaccination campaigns as well as for outbreak response.

Vector control strategies such as aerial spraying, domiciliary spraying and the enforcement of public health regulations to reduce collections of stagnant water and other breeding sites, have had successes in the past in eliminating *A. aegypti* and controlling urban yellow fever. Present-day socio-economic difficulties have, however, hampered recent efforts to again control the reinfestation of villages and towns in Africa. Control of vectors responsible for the jungle yellow fever cycle is impractical.

There still remain many gaps in our knowledge of the epidemiology of the infection and the permanent control of the disease will probably need not only resources to implement what is already known, but also the elucidation of the remaining enigmas regarding the infection.

OTHER MEMBERS OF THE 'UNASSIGNED' SUBGROUP OF FLAVIVIRUSES

Rocio

Rocio virus is an arboviral cause of encephalitis localized to the Ribeira valley and Santista lowlands in the southern coastal region of Sao Paulo state in south-eastern Brazil. The virus was first isolated in 1975 from the brain of a fatal case of encephalitis diagnosed during an unusual epidemic of encephalitis in 1975–1976. No further cases have occurred since 1980, although two children from the Ribeira valley tested positive for IgM antibodies in 1989. A total of 821 cases were diagnosed during 1975–1978, twice as many in males and usually aged 15–30 years.

The virus has been isolated from pools of mosquitoes, such as *Psorophora ferox*, and also from wild birds. The transmission cycle of the virus has not, however, been established. Clinical and epidemiological features of the disease have been reviewed in 821 cases between 1975 and 1978 (Iversson, 1980). The disease commences with nonspecific signs of pyrexia, headache and vomiting. This may then be followed by disturbances of consciousness and signs of encephalitis, including localizing signs. Death may follow a prolonged coma or there may be a sudden fulminant course. Serious neurological sequelae occur in some 20% of clinical cases and the overall case fatality is found to be 10%. The IgM capture ELISA has been used to diagnose Rocio virus infection in children and is preferable to the HAI test for identifying recent infection.

Wesselsbron

Wesselsbron virus was first isolated from a lamb in the village of Wesselsbron in South Africa in 1955. Virus has been isolated from 23 human cases of infection, 11 of them resulting from laboratory infection or infection of laboratory field workers. The vertebrate host is chiefly livestock, especially sheep, and isolations have been made from throughout sub-Saharan Africa, especially South Africa where it is causes a febrile illness in sheep associated with hepatitis, haemorrhage, abortion and mortality in newborn lambs. Isolations have also been made from ostriches. It has also been isolated from pools of wild-caught *Aedes* mosquitoes, such as *Aedes (neo) lineatopennis*. The major vector in sheep is *Aedes caballus-juppi*. Humans are infected by mosquito bite or by direct contact in handling carcasses or tissues of animals that have died of the disease.

Human infection is characterized by a sudden onset of pyrexia, severe headache and retro-orbital pain associated with photophobia and hyperaesthesia of the skin, with an evanescent skin rash frequently present. Muscle and joint pains are also commonly seen. In severe cases signs of encephalitis, such as blurred vision and some mental impairment, may occur. Patients recover after a few days to a week and no permanent sequelae have been reported.

Diagnosis of infection may be achieved by isolation of the virus from blood or serological tests. The HAI test is only useful in individuals with no previous flavivirus infection history, because of the extensive cross-reaction between Wesselsbron and other flaviviruses. The capture IgM assay is the test of choice for diagnosis of recent Wesselsbron infection.

DENGUE

Dengue is, at present, the most important arboviral cause of death and disease in humans (Gubler and Costa-Valez,

1991). The infection has spread widely from South East Asia to the Americas, the Pacific and Africa, now involving several million people annually. In all major tropical areas of the world the incidence of dengue fever (DF) and dengue haemorrhagic fever (DHF)/dengue shock syndrome (DSS) has increased dramatically over the past few years, with an ever-increasing frequency and extent of epidemics and a greater severity of cases. The spectre of the introduction of infection to *A. aegypti* populations in non-endemic countries is of great international concern.

History

Outbreaks of 'break bone fever', in which patients presented with symptoms that included fever, headache severe muscle pain and joint pain, nausea, vomiting and rash, haemorrhagic manifestations and asthenia in convalescence, clinically resembling dengue fever have been recorded since 1779 and 1780 in Java (Indonesia), Cairo and Philadelphia. The name dengue, which means 'affectation' in Spanish, was given following an outbreak of a similar syndrome in Madrid in 1801. Epidemics of dengue-like illnesses occurred at 10- to 30-year intervals throughout tropical and subtropical regions of the world. In 1953, DHF was first recognized in children in Manila, and subsequently detected throughout South East Asia. Although the precise aetiology could not be established and infections such as chikungunya are clinically and epidemiologically very similar, the majority of these epidemics were probably dengue. A dengue pandemic started in South East Asia following the Second World War, spreading to tropical areas across the world including Africa, Oceania and the Americas (Gubler, 2004; Rigau-Perez, 2006). The spread of the disease has been markedly accelerated by the advent of widespread air travel over the last three decades. Dengue fever was generally regarded as a relatively benign illness, affecting predominantly colonial expatriates living in tropical countries and, although responsible for severe and often incapacitating muscle pain, it was seldom lethal. However, the gravity of the dengue pandemic was really confirmed by the recognition in the 1950s of the severe complications of infection, viz. DHF/DSS affecting mainly children in the endemic areas. Epidemics of DHF/DSS ravaged South East Asia and in the following 30 years was responsible for over 700 000 children being hospitalized and over 20 000 fatalities (Halstead, 1984).

In the Western Hemisphere, the first major epidemic of DHF/DSS in Cuba in 1981 was responsible for 24 000 cases of DHF and 10 000 of DSS (sporadic cases of suspected DHF had been reported from Central America since 1968). Only the energetic response of the Cuban health authorities, with intensive education and mass

hospitalization, kept the mortality down to only 158. Following on this epidemic, confirmed or suspected cases of DHF have been reported in the Americas almost every year, with the most severe outbreaks of dengue fever occurring in Peru in 1990, involving over 76 000 cases (Centers for Disease Control and Prevention, 1991a). Dengue returned to Cuba in 1997. Eradication of the vector *A. aegypti* as part of an effort to eradicate urban yellow fever from the American region in the 1950s and 1960s reduced the occurrence of epidemic dengue dramatically in this region, however following the discontinuation of this programme the geographic distribution of this vector is much wider than before it began. By 2003 DHF has been reported from 24 countries of the American region, and DHF is now considered endemic in these countries. At present it is estimated that 2.5 billion people live in high-risk areas, and more than 100 million infections occur per year, including 250 000–500 000 cases of DHF with a average fatality rate of 5% (CDC epidemiological data, www.cdc.gov/ncidod/dvbid/dengue/index.htm). Imported cases of dengue in travellers returning to temperate countries are reported in the United States annually and DHF was reported in two cases in the United Kingdom in 1991 (Jacobs *et al.*, 1991). Blood donations collected during a dengue outbreak in Hong Kong were thought to have resulted in two cases of dengue from infected transfusion blood.

Virus Properties and Host Range

The dengue viruses form a subgroup of the genus *Flavivirus* and although extensive cross-reactivity is seen with serological tests such as HAI which cannot reliably distinguish dengue from many other flaviviruses, NT tests are able to define dengue virus as a distinct antigenic subgroup. There are four serotypes of dengue based on NT tests. However, with all serological tests there is extensive cross-reactivity between the four serotypes, although they are distinguishable with the highly specific PRNT test. Following natural infection, all four genotypes induce specific but not cross-protective long-term immunity. Dengue serotypes 1, 3 and 4 show a closer antigenic and genetic relationship to each other than dengue 2. However, within each of the serotypes, considerable heterogeneity and strain variation is demonstrable by sequence analysis as well as antigen signature analysis using monoclonal antibodies. Severe disease may be caused by all four types. DHF is associated with reinfection with a heterologous secondary dengue virus infection. Dengue viruses exhibit 65–70% sequence homology, but although serotype cross-reactive protective immunity exist early after primary infection, this response wanes after six months and the host becomes susceptible to the remaining three

heterologous serotypes (Green and Rothman, 2006; Halstead, 2003). The only vertebrate hosts of dengue virus in nature are humans and several species of Asian and African sub-human primates. Other vertebrates can be infected experimentally only with difficulty, including baby mice, which usually require several blind passages of patient material to obtain an isolate. The invertebrate hosts of dengue are members of the genus *Aedes*, especially the subgenus *Stegomyia*. The most important mosquito hosts as far as human infection is concerned are *A. aegypti*, *A. albopictus* and *A. polynesiensis*. Other *Aedes* species, including *A. africanus*, *A. leuteocephalus* and the *A. furcifer* group, are involved in the maintenance of the forest cycle of dengue in Africa, whereas in Asia the mosquitoes concerned belong to the *A. niveus* complex.

Epidemiology

Dengue displays many epidemiological similarities to yellow fever and chikungunya viruses and there is considerable overlap in the ecologies of these three virus infections. Essentially there are three transmission cycles of dengue: a forest cycle in primates and involving forest species of *Aedes*; a rural or semi-rural cycle in humans, with the peri-domestic *Aedes* species being the vectors; and an urban cycle in humans involving domesticated *Aedes* species. By far the most important of these three for both endemic and epidemic human dengue is the urban cycle. It is, in fact, doubtful whether the forest cycle plays any significant direct role in human dengue.

The two major mosquito vectors of urban dengue are *A. aegypti* and *A. albopictus*. Although *A. albopictus* is considered more sensitive to oral infection with dengue viruses, *A. aegypti* is a more important vector for human disease and is especially responsible for explosive epidemics. The anthropophilic *A. aegypti* usually preferentially feeds on humans and occasionally also on domestic animals, while the purely domestic *A. albopictus* randomly feeds on humans, domestic animals and feral animals and birds. To establish infection, *A. aegypti* would need to feed on individuals with high levels of viraemia, and this may select for viral strains of higher virulence, which are more likely to produce the severe epidemics associated with *A. aegypti*. In addition, the biting habits of *A. aegypti*, which characteristically takes interrupted blood meals and will thus feed on a number of individuals before becoming engorged, enhances its ability to spread the virus. It is thus not unusual for a single mosquito to spread infection among several individuals at a single feeding. In both mosquitoes, biting activities are maximal soon after daybreak and in late afternoon, and are related to human activities and movements. Outbreaks associated with both vectors are climatically influenced by heavy rainfall and high temperature.

The recent upsurge of dengue in existing endemic zones and the spread to new areas has been attributed to modern phenomena of human and social behaviour. Infestations of *A. aegypti* in tropical towns and villages, which were controlled by extensive spraying and other vector control measures in the 1950s and 1960s, have now re-emerged due to uncontrolled urbanization and the mass migration of rural populations into informal housing settlements and squatter camps on the peripheries of cities and towns. These population movements have been accentuated by famine, poverty and war. Stagnant water pools and lack of reticulated water supplies have provided ample opportunities for mosquito breeding, coupled with the breakdown of vector control in many tropical countries. Overcrowding and grossly inadequate housing, which is so characteristic of the sprawling slums of the tropical world, greatly facilitate the spread of vector-borne diseases and is especially conducive to dengue transmission resulting from the interrupted feeding habits of *A. aegypti*. The spread of infection has also been enhanced by the modern era of jet travel, which facilitates the transportation of infected individuals to non-infected areas, creating the threat of the introduction of infection if infestations of *A. aegypti* are sufficiently high. Modern air travel has also been responsible for increasing the number of imported cases of dengue into countries where physicians are, in the main, ignorant to the infection, resulting in perplexing diagnostic difficulties until a history of travel to endemic zones is elicited.

More recently, the influence of international trade in the spread of dengue has been observed. Motor vehicle tyres and casings from South East Asia containing remnant pools of water where infected *A. albopictus* mosquito larvae have been found, have been shipped to various non-endemic areas of the world, posing a serious threat of introducing both the virus as well as its vector.

An increase in frequency of epidemic dengue/DHF has been observed over the past 30 years as all four serotypes spread throughout the tropical parts of the world. Today the tropical world has become hyperendemic with multiple dengue virus serotypes co-circulating. This may have contributed to the emergence of DHF in the Pacific and the Americas. In Asia major DHF epidemics have been reported in Sri Lanka, India, Pakistan, Taiwan, China and the Maldives islands since the 1980s caused by all four serotypes. Reintroduction of dengue viruses in the early 1970s in the Pacific has resulted in DHF epidemics caused by all four serotypes.

Although surveillance in Africa has been poor, epidemic dengue fever caused by all four serotypes has been reported in mainly West and East Africa. Major epidemics have been reported in the Seychelles (1977), Kenya (1982, dengue 2), Mozambique (1985, dengue 3), Djibouti

(1991–1992, dengue 2), Somalia (1994, dengue 2). Prior to 1980, major dengue epidemics were last reported in South Africa in 1920, in the Durban area and in Senegal. The outbreak in South Africa was probably imported and the virus did not establish in the local mosquito population. An enzootic forest cycle that involves non-human primates and a number of mosquito species has been reported in West Africa involving dengue 2 viruses. DHF has not been reported in Africa and the Middle East although sporadic cases of clinically compatible DHF were reported in Mozambique, Djibouti and Saudi Arabia.

Dengue/DHF has emerged dramatically in the American region. Only dengue 2 was found in 1970 in the Americas although dengue 3 may have been present in areas of Colombia and Puerto Rico. Dengue 1 was introduced in 1977, causing major epidemics over a period of 16 years. This was followed by introduction of dengue 4 and a new strain of dengue 2 from South East Asia in 1981. The latter resulted in the first major DHF epidemic in the Americas in Cuba, spreading rapidly and causing outbreaks of DHF in Venezuela, Colombia, Brazil, French Guiana, Surinam and Puerto Rico. Today DHF is endemic in various countries across the American region. A new strain of dengue 3 also caused a dengue/DHF epidemic in Nicaragua, Panama and Costa Rica. Sequencing data suggest that this strain was imported from Sri Lanka and India and was distinct from strains previously found in the region. (Information from the Centers for Disease Control and Prevention website: www.cdc.gov/ncidod/dvbid/dengue/index.htm; reviewed by Gubler, 2004.)

Clinical Features

The majority of infections with dengue virus, based on the extent of population seropositivity, are asymptomatic. Clinical manifestations of dengue occur in two forms—classical dengue fever and DHF/DSS. Classical dengue fever is an acute disease characterized by a sudden onset of fever, severe headache which is typically frontal in distribution, together with retro-orbital pain, nausea and vomiting. Severe muscle and bone pain and arthralgia are characteristic of dengue and usually more pronounced in the back, which led to it being termed ‘break bone fever’ by Dr Benjamin Rush in 1778. He also aptly described the associated severe depression as ‘break heart fever’. There is frequently a diffuse, discrete maculopapular rash which usually heralds the recovery phase of the illness. In spite of the severity of symptoms, which may be incapacitating, the disease is temporary and full recovery takes place.

The grave complication of DHF/DSS is governed by two factors, prior infection and age. Thus, DHF and DSS occurs in approximately 0.18 and 0.007%, respectively, of cases of primary DF compared with 2.0 and 1.1%,

respectively, of dengue fever due to secondary infection (Halstead, 1981). It is also rare in individuals over the age of 15 years. DHF/DSS resembles yellow fever in its biphasic presentation. The first phase is not unlike uncomplicated dengue fever. This is followed by a brief remission of symptoms, when the fever subsides to normal or close to normal. There is then a sudden deterioration in the patient's condition, marked by profound prostration, hypotension, circulatory collapse and manifestations of bleeding and shock. Bleeding is seen commonly as petechiae in the skin and mucous membranes, especially in the oral cavity, ecchymosis, bleeding at injection and skin puncture sites, gastrointestinal bleeding and haemorrhagic pneumonia.

The cause of the haemorrhagic diathesis in DHF is complex. There is evidence of vascular injury with increased permeability and extravasation of fluid from the vascular into the interstitial fluid compartment, producing hypotension and DSS. Bleeding due to vascular damage is suggested by the presence of petechiae and a positive tourniquet test. In addition there is a marked thrombocytopenia, although it is not yet clear what the relative contributions of impaired platelet formation due to direct suppression of megakaryocyte production in the bone marrow or excess destruction due to endothelial damage. Evidence exists for both. Third, there is haematological evidence of a consumptive coagulopathy with a moderate increase in fibrin degradation products which, however, rarely reaches the stage of disseminated intravascular coagulation.

The definition of DHF is controversial. The WHO criteria for DHF are an acute onset of fever, haemorrhagic manifestations, which include at least a positive tourniquet test, a thrombocytopenia of 100 000 per ml or less and a haemoconcentration with a haematocrit increase of 20% or more. Manifestations of haemorrhagic fever, however, do differ from country to country and amendments to the criteria have been proposed to include, amongst others, references to the age of the patient (≤ 16 years). The severity of DHF/DSS has been graded by the WHO from I to IV (Technical Advisory Committee on DHF for the South East Asian and Western Pacific Regions, 1980):

- Grade I—Fever with nonspecific constitutional symptoms, the only haemorrhagic manifestations being a positive tourniquet test.
- Grade II—As for grade I, but with specific haemorrhagic manifestations.
- Grade III—Signs of circulatory failure or hypotension.
- Grade IV—Profound shock with pulse and blood pressure undetectable

Since the emergence of DHF among the severe dengue syndromes, cases of 'unusual manifestations' with bleak

prognosis have been identified that do not fit into the above grades. These include encephalopathy and less frequently encephalitis, hepatic failure, cardiomyopathy as well as dengue fever with severe haemorrhage, and more recently acute respiratory distress syndrome and acalculous cholecystitis. These cases do not fit into the WHO definition of severe DHF and are not well defined. Their frequency differs between populations and outbreaks (Rigau-Perez, 2006).

Pathogenesis of DHF/DSS

The pathogenesis of DHF/DSS has been studied intensively for a number of decades. Although the pathways to the development of severe dengue have not, as yet, been definitively established, significant advances in the immunopathogenesis has brought us closer to understanding the mechanism behind DHF in recent years. DHF is characterized by a short-lived plasma leakage that is thought to be immune mediated. Heterologous secondary dengue virus infection has been linked in epidemiological investigations to plasma leakage syndrome and bleeding diathesis/DHF. Ninety per cent of DHF cases occur in secondary heterologous dengue virus infection whereas only 10% occur in primary infection most often in infants in the second half of their first year of life. In the latter group maternal transmission of non-neutralizing dengue virus antibodies are thought to be involved in antibody-dependent enhancement in which pre-existing dengue antibodies bind to heterologous dengue virus, thereby enabling enhanced viral entry through monocytes and other FcR-presenting target cells. Dengue virus target cells are mainly cells of the reticuloendothelial system (spleen, liver, bone marrow) and include monocytes, lymphocytes, Kupffer cells as well as alveolar macrophages. Infection of these cells by dengue virus results in the production of immune mediators that will shape the adaptive and cellular immune responses.

Several mechanisms may work together to result in plasma leakage. Complement activation and plasma leakage may be triggered by the circulation of high levels of secreted NS1 in the presence of pre-existing heterologous non-neutralizing anti NS1 antibody. The role of antibody-mediated enhancement is not yet fully understood. It is thought that upon primary infection, antibodies are generated against the cell surface expressed proteins E and NS1. The E protein contains serotype-specific and serotype cross-reactive neutralizing epitopes. The sequence of dengue virus infection plays a role in the beneficiary or disease enhanced effect of cross-reactive memory humeral immune response. For example, serum containing neutralizing cross-reactive antibodies against dengue 3 was beneficial upon secondary infection, but the same did not hold true for secondary infection with dengue 1 or 2. The effect of antibody-dependent enhancement is

believed to be a result of an increase in viral entry that results in an increased viral burden in the host upon heterologous secondary infection. *In vitro* studies have shown that anti-E antibodies enhance infection through FcR2, while anti-prM antibodies were able to enhance infection of both FcR2 and non-FcR2-bearing cells (reviewed in Green and Rothman, 2006).

Recent reports have suggested that low-avidity cross-reactive T cells raised to the first infecting dengue virus serotype predominate during a secondary heterologous infection which may lead to immunopathology by inducing an altered profile of cytokines that could lead to plasma leakage. Evidence suggests that host factors may be a key issue in susceptibility to DHF since only limited numbers of secondary infections result in plasma leakage syndrome. Both protective and pathogenic roles have been assigned to different human leukocyte antigen (HLA) alleles but with disparate results. Protective roles has been assigned to HLA A33 in ethnic Vietnamese and A*0203 in ethnic Thai populations while a pathogenic role has been associated with HLA A24 in Vietnamese and A*0207 in Thai populations. Other genes that have been implicated in protection include the vitamin D cell receptor which may be associated with a stronger T helper cell response, FcγR2 A which is associated with reduced opsonization by IgG2, and reduced antibody enhancement, the CD209 promoter, DCSIGN1-336 that through decreased expression reduces the susceptibility of dendritic cells for dengue virus infection. A variant of TNF-308 which is associated with high levels of tumour necrosis factor-α (TNF-α) production has been associated with more severe disease (reviewed in Green and Rothman, 2006).

The alternative hypothesis of Rosen (1986) holds that the severe complications of DHF/DSS are the direct results of properties of the virus, that is, the consequence of infection with unusually virulent strains of dengue circulating in a particular area and giving rise to outbreaks of DHF/DSS. However, strain variations exist within serotypes and no consistent relationship between strain variation and either virulence or heightened infectivity has been reliably demonstrated.

Diagnosis

The clinical diagnosis of dengue, both the uncomplicated dengue fever form and DHF/DSS, is often unreliable. Dengue fever may resemble clinically a variety of acute febrile illnesses, although the severe muscle and bone pain is suggestive of dengue. Similarly, DHF may resemble other causes of haemorrhagic fever, although thrombocytopenia with haemoconcentration and signs of a moderate consumptive coagulopathy is suggestive of dengue.

The most widely used serological test is the HAI test, detecting antibodies as early as four days post onset. A

specific diagnosis of dengue can be made early in primary infections, but cross-reactions with other flaviviruses occur in late primary or secondary infections. The IgM antibody capture ELISA (MAC ELISA) is now used during outbreaks of dengue, and there are rapid assays available for the detection of dengue IgG and IgM, although cross-reaction may occur with the IgG assay. The IgM antibodies may persist for over three months, so the test is also useful for retrospective studies, but this persistence may cause diagnostic problems in areas where dengue is endemic. A combined IgG and IgM assay, detecting high levels of IgG indicating secondary infection, is useful in dengue endemic areas. Immunofluorescence assays have been used successfully to detect dengue IgG and IgM antibodies. The CF test is more specific than the HAI test, but the antibodies detected by this assay appear later and disappear earlier. The NT and PRNT tests are the most specific and sensitive but are difficult to perform and thus tend to be used only for specific purposes.

Virus isolation is difficult and if mice are used, a number of blind passages are usually required. Intracerebral inoculation of adult or larval *Toxorhynchites* spp. is a sensitive and rapid method for the isolation of dengue virus, giving results in two to three days. The use of a rapid centrifugation method may increase sensitivity and reduce time scales. Intrathoracic inoculation of mosquitoes is easier and just as sensitive, but head squashes cannot be made or tested for specific dengue antigen for at least seven days post inoculation. The most commonly used system of virus isolation is the inoculation of mosquito cell lines, viz. *A. albopictus* (C6-36), *A. pseudoscutellaris* (AP-61) and *Toxorhynchites ambionensis* (TRA-248), which are almost as sensitive as mosquito inoculation, which allows specific results to be obtained within two to three days using fluorescent-labelled monoclonal antibodies. A combination of MAC-ELISA and RT-PCR on peripheral blood leukocytes has been shown to give high levels of sensitivity and specificity.

Several RT-PCR assays have been developed for diagnosis, quantification and typing of dengue virus in acute-phase serum and post-mortem tissues (Lanciotti, 2003). These methods are the most sensitive for detection of dengue virus and eliminate problems of cross-reactivity, although the virus nucleic acids can only be detected during the acute phase. Real-time RT-PCR assays that make use of TaqMan probes have also recently been reported for these purposes, which further improves the sensitivity and time to diagnosis and typing (Kong *et al.*, 2006).

Control

There is, at present, no licensed dengue vaccine. Current strategy is to develop a vaccine against all four serotypes.

Several candidate vaccines have been developed, including live attenuated mono- and tetravalent formulations, inactivated whole virus vaccines and recombinant sub-unit vaccines. Although some of these vaccine candidates have reached phase 1 or 2 clinical trials, to date none of them has been licensed for human use mainly because of high reactogenicity. Evaluation of further live attenuated tetravalent vaccine candidates in cynomolgus macaques is ongoing (Edelman *et al.*, 2003; Kitchener *et al.*, 2006; Koraka *et al.*, 2007; Sun *et al.*, 2003).

Because of the lack of availability of a vaccine, control of dengue depends on (i) surveillance to obtain early warning of epidemics or preferably to be able to predict impending epidemics and (ii) effective vector control.

Epidemiological surveillance may be of two types: first, proactive surveillance in interepidemic periods in endemic areas or in countries that are not yet infected but are vulnerable to the introduction of new infections because of high infestations of *A. aegypti*; second, reactive surveillance, where monitoring is instituted once suspected or confirmed cases of dengue have already occurred. This form of surveillance is very insensitive as numerous cases may occur that are not suspected to be dengue before authorities are alerted to the outbreak. In the Comoros Islands, dengue type 1 was diagnosed in 62/116 clinical cases in 1993. However, an investigation to determine the prevalence of dengue infection in over five year-olds, using a dengue IgM assay, estimated that at least 60 000 recent cases had occurred at that time.

A number of instruments of surveillance may be used for proactive monitoring. Disease surveillance requires intensive educational efforts, especially at peripheral primary health care level, to alert health care workers to the possibility of dengue. It is, however, very difficult to sustain interest, especially if no cases materialize. Viral and serological surveillance involves the active recruitment of specimens, for example, as carried out in Puerto Rico, where blood samples are collected by collaborating physicians on a regular basis and sent for analysis. Interest and cooperation is also difficult to sustain. The use of blood samples sent to laboratories for testing for acute febrile illnesses or 'viral syndromes' may be an easier way of recruiting specimens for serological testing. Surveillance may also utilize the routine investigation of all viral haemorrhagic fever cases, which should include tests for dengue. Vector surveillance may be of benefit to demonstrate low infestations (house index of <1%) or to look out for the introduction of exotic mosquito species, for example, *A. albopictus*.

Vector control aims at the elimination of the main mosquito vector, *A. aegypti*. While this is technically feasible, with both adulticide campaigns using ultra-low-volume spraying with malathion and also

larvicide treatment of stagnant water, the costs are high and the effects are temporary. In many tropical countries where *A. aegypti* eradication had been achieved, reinfestations have taken place to levels equalling, or even exceeding, those in pre-control times. However, long-term community-based programmes need to be implemented in endemic countries. In non-endemic countries there should be a vigilant monitoring for the possible importation of dengue, for example, through motor car tyres, or travellers or migrants from endemic areas.

ZIKA

Zika virus is closely related to dengue virus and antigenically cross-reacts with it and may give false-positive diagnostic test results for dengue. 'Zika fever' also resembles dengue fever in its clinical presentation although in a milder form.

The virus was first isolated in the Zika forest of Uganda in 1947. It has been described in West, East and Central Africa and, subsequently, in Malaysia, Indonesia and most recently (2007) in the Yap island of Micronesia. Like dengue, the vector is *Aedes* spp. mosquitoes and its epidemiology follows that of other aedine-borne viral infections.

Clinically zika fever is characterized by a low-grade pyrexia, maculo-papular rash, which is often pruritic, starting on the face and spreading to the trunk and lower extremities. Arthralgia involving mainly the small joints of the hands and feet and occasionally also large joints, is a common feature. Less commonly there may be conjunctivitis with retro-orbital pain, headache, myalgia, ankle oedema, lymphadenopathy and diarrhoea. Symptoms generally last for two to seven days and clear without sequelae. Limited observations of infections in pregnancy have not demonstrated any adverse effects on the infant—making it important to differentiate from rubella which it may resemble clinically.

JAPANESE ENCEPHALITIS

Japanese encephalitis is the major arbovirus cause of encephalitis in the world. First described as a clinical entity in Japan in 1870, the virus was isolated in 1935. It is now thought to be responsible for about 50 000 cases annually, half of whom are left with permanent neurological or psychological handicap, and in a further quarter it is rapidly lethal. Infection has been demonstrated in 16 countries in South East Asia, stretching to India in the west and to southern Russia in the north—a population of over 2 billion people.

Viral Features and Host Range

Japanese encephalitis virus shows some cross-reactivity with SLEV, WNV and MVEV, and together they form the mosquito-borne encephalitis complex. The virus is antigenically relatively homogeneous and recovery from infection results in solid protection. Nucleic acid analysis, however, has revealed some significant heterogeneity. Using PCR sequencing, isolates of JEV could be separated into four distinct genotypic groups, each group found in separate geographic divisions. Antigenically there is cross-protection across all groups and the epidemiological significance remains to be established. In the early part of the year 2000, Japanese encephalitis reappeared in the Australasian region and isolates were identified as genotype I, whereas all previous isolates were genotype II.

The vertebrate hosts of JEV are humans and domestic animals with aquatic birds playing a major amplifying role. The major mosquito vector of the virus is *Culex tritaeniorhynchus*, although a number of other species of the genera *Culex*, *Monsonia*, *Aedes* and *Anopheles* have yielded isolates of JEV.

Epidemiology

Nestling birds, particularly of the heron family, play an important epidemiological role in the dissemination of JEV, with a second cycle involving domestic animals, particularly the pig. In seroprevalence studies, high NT antibodies were found in several other animal species, for example, cattle, horses, dogs, monkeys and bats. As with the wild bird population, the high turnover of the domestic pig population resulting in a continuous supply of susceptible animals is a contributing factor to the pig being a major amplifying host for JEV. The virus rarely causes disease in domestic animals, although fatal encephalitis in horses and abortions in sows have been recorded. Studies on birds have implicated other species, whose main habitat is the rice paddies, as vertebrate hosts of JEV—these water birds include herons and bitterns. In India ardeid birds such as cattle egrets are implicated.

JEV has been isolated from many species of mosquito but the principal vector in many areas is *C. tritaeniorhynchus*. Other mosquitoes, mainly the *Culex* spp., are considered to be important in specific regions, for example, *Culex gelidus* in South East Asia, for the transmission of JEV.

Humans are a 'dead-end' host and play little role in the amplification of the virus. Japanese encephalitis is thus predominantly a rural problem, with disease closely related to rainfall and irrigation.

Sequence analysis of the C/PrM and E genes have identified four virus genotypes while a single strain of JEV isolated in Singapore in 1952 from a patient who

originated in Muar, Malaysia (Muar strain), may represent a fifth genotype. Genotype I consists of isolates from northern Thailand, Cambodia and Korea, genotype II contains isolates from southern Thailand, Malaysia, Indonesia and northern Australia, genotype III includes mainly isolates from temperate regions of Asia, (Japan, China, Taiwan, the Philippines, and the Asian subcontinent, while genotype IV includes isolates from Indonesia. Although genotypes I and III occur in epidemic regions, while II and IV are associated with endemic disease, differences in clinical epidemiology may not be linked to the genotypes as initially postulated. Several strains have been identified that do not fit this paradigm. Examples include Vietnam epidemic disease that occurs in the north and endemic disease that occurs in the south, although genotype III strains have been isolated in both areas, and a genotype I strain that has recently been found in northern Australia. No significant differences were observed in mouse neuro-invasiveness between genotypes. Phylogenetic analysis suggests that genotypes IV and V are the oldest and most divergent from the other genotypes while genotypes I–III evolved more recently. Since all genotypes are present in Indonesia (excluding New Guinea) and Malaysia it is postulated that JEV originated here, from where individual genotypes spread to Australia and New Guinea (genotype I and II); Taiwan and the Philippines (II and III); Thailand, Cambodia and Vietnam (I, II III); Japan, Korea and China (I and III) and India, Sri Lanka, and Nepal (genotype III) (reviewed in Solomon *et al.*, 2003).

Clinical and Pathological Features

Infection with JEV is considerably more widespread than the incidence of encephalitis would indicate. Infection may present nonspecifically as a mild febrile illness or as an aseptic meningitis, or, rarely, as a variety of inflammatory manifestations in the viscera. The typical disease manifestations of acute meningomyeloencephalitis have been widely estimated to occur at between 1/20 (Rodrigues, 1984) to 1/600–800 persons infected (Halstead, 1981).

The major target cells for JEV are the T lymphocyte and the peripheral blood mononuclear cells. In fatal cases of encephalitis, viral antigen is also demonstrable in the neurons. Factors determining the neuro-invasiveness of JEV involve both viral and host factors. Nucleotide sequencing of non-neurovirulent mutants of JEV have demonstrated single base changes in the coding region for E protein (Cecilia and Gould, 1991). Age is an important host factor determining neurovirulence, encephalitis being more common and more severe in the young as well as in elderly individuals. Experimental work in rats has shown a relationship between neurotropism and

neuronal maturity in that the virus selectively infects immature neurons (Ogata *et al.*, 1991). The reason for greater neuro-invasiveness in the elderly is unknown, but is consistent with the features of the related St Louis encephalitis and WNV, which similarly display greater neuro-invasiveness in the elderly.

The typical case of Japanese encephalitis commences after an incubation period of one to two weeks, with fever and headache, followed rapidly by depression of the level of consciousness, progressing from stupor to coma. Localizing cranial nerve and other neurological signs occur in about 30% of cases and in children generalized seizures are common—the frequency of seizures in patients increases with the severity of the encephalitis. A quarter of cases of clinical encephalitis will recover with no permanent sequelae and a quarter will die rapidly. The remaining half will recover with varying degrees of permanent neuropsychiatry sequelae. In addition, especially in children, the virus may persist in lymphocytes and reactivate to give recurrent disease after recovery (Sharma *et al.*, 1991).

Grave prognostic signs include a short prodromal period, deep coma, decerebrate posture, breathing abnormalities and the ability to isolate virus from the cerebrospinal fluid (CSF). Recently a reduction of serum iron levels has been demonstrated in patients due to the sequestering of iron in the spleen, and a direct relationship between low serum iron and prognosis has been shown (Bharadwaj *et al.*, 1991).

The pathology of the brain in fatal cases has revealed micro foci of necrosis scattered throughout the central nervous system, but especially involving the thalamus, the basal ganglia and the deep cerebral nuclei.

Diagnosis

Routine diagnosis is usually carried out by serology using HAI, immunofluorescence, CF or ELISA techniques. Test results should, however, be treated with caution because of extensive cross-reactivity with other flaviviruses and because up to a quarter of patients fail to demonstrate a serological rise in titre due to their late presentation to medical attention. A preferable serological test is the MAC-ELISA on serum or CSF, or a more recently developed particle agglutination assay, which detects JEV-specific IgM. The detection of specific IgM antibodies in CSF is diagnostic of acute Japanese encephalitis.

Virus isolation from blood is rarely successful during the acute illness because the viraemic phase is probably over by the time central nervous system symptoms appear. Virus isolation from the CSF is usually also unsuccessful and, if positive, indicates a poor prognosis, as mentioned above. A variety of isolation techniques are very sensitive

to JEV, including intracerebral inoculation of suckling mice, intrathoracic inoculation of live mosquitoes, the use of common mammalian cell lines such as Vero and LLC-MK2 and mosquito cell lines, especially those of *A. albopictus* and *A. pseudoscutellaris*.

Control

The major component of the control of Japanese encephalitis is widespread immunization of both humans and domestic animals, especially pigs. A number of human vaccines have been developed. A formalin-inactivated lyophilized vaccine of mouse brain origin, derived from the Nakayima-NIH strain of JEV, is prepared by Biken, Japan, and widely used in Japan and Korea and also by travellers to endemic areas. In China, a BHK-prepared inactivated vaccine is used. Both vaccines are highly immunogenic and protection rates of over 90% are achieved. Experimental live attenuated vaccines are being developed and Chinese workers have successfully attenuated the SA14 strain by 100 passages in BHK cell culture (Stephenson, 1988). The live attenuated vaccine (SA14-14-2) is now widely used in China. Recombinant DNA technology also holds promise for the future development of safe, effective Japanese encephalitis vaccines (Konishi *et al.*, 1991). ChimeriVax-JE using YF 17D as a live vector for the envelope genes of SA14-14-2 is in the trial stage. Similarly, a number of veterinary vaccines have been used in pigs and horses. Vaccination thus holds the potential to eliminate Japanese encephalitis. Reports of allergic reactions to the Biken vaccine have resulted in a cautionary warning being issued against routine administration of Japanese encephalitis vaccine to travellers, except to those who are likely to be at high risk in endemic areas, particularly rural areas, for a month or longer during the season of vector activity (Nothdurft *et al.*, 1996).

Because humans are only an incidental host, interruption of transmission of the virus would be independent of the extent of immunity in human populations, and eradication of infection would need to aim for the elimination of virus circulating in the vertebrate reservoir of domestic animals. At present, economic realities preclude anything approaching a sufficiently widespread programme of immunization in domesticated pigs.

Vector control is difficult because of community resistance to mosquito control programmes, which need to include and may significantly tamper with subsistence agriculture of rural populations. Thus, methods which have been attempted include short-term drainage of rice fields and attempts to introduce more drought-resistant rice in order to reduce potential mosquito-breeding sites. Ultra-low-volume insecticide spraying has had limited success, with increasing insecticide resistance, especially

in *C. tritaeniorhynchus*. Measures to prevent biting by mosquitoes, such as the use of insect repellents, mosquito netting, and so on, should be encouraged. Most biting activity is concentrated at nightfall.

ST LOUIS ENCEPHALITIS

St Louis encephalitis virus was isolated in 1933 from human brain following an outbreak of encephalitis in St Louis, Missouri. It was the major cause of epidemic viral encephalitis in the United States before the appearance of WNV in the United States in 1999. Although the distribution of the virus ranges from southern Canada to Argentina and a few sporadic cases have been reported in South and Central America, virtually all cases of human disease have occurred in the United States.

Viral Features and Host Range

Antigenically, SLEV is closely related to the other members of the Japanese encephalitis subgroup of flaviviruses. In addition, cross-immunity to other flaviviruses has also been demonstrated, including dengue virus type 2. Antigenic heterogeneity has been shown with monoclonal antibodies, although there is no evidence that this has any epidemiological implications. Phylogenetic analysis of the E protein suggests that SLEV isolates form a monophyletic group in which isolates are generally clustered according to geographic origin. Only 10% variation exists between the strains from different regions. Isolates from Panama and South America predominantly fall into two large groupings, while isolates from the United States form two other major groups. Several strains from South and Central America were identified that were more closely related to strains isolated in the United States. This suggested that SLEV is predominantly maintained locally although strains are occasionally transported between regions (Kramer and Chandler, 2001).

The major vertebrate hosts of SLEV are birds, especially sparrows (*Passer domesticus*), which act as the main amplifying host (Centers for Disease Control and Prevention, 1991b). In addition, small mammals such as racoons, opossums and rodents are also infected, as well as a variety of domestic animals. However, other than birds, there is no evidence that animals play any role as maintenance or amplifying hosts.

Invertebrate vectors of SLEV are various species of *Culex* mosquitoes, depending on location. In the rural west it is *Culex tarsalis*, in the northern and southern regions of the central United States it is mainly *Culex pipiens* and *Culex quinquefasciatus*, and in Florida *Culex nigripalpus*.

Epidemiology

In all parts of the United States the transmission cycle of SLEV involves birds and mosquitoes. Humans and probably domestic animals are incidental and dead-end hosts. Epidemiological characteristics, however, differ in the three regions described above. Thus, in the central United States, where *C. pipiens* and *C. quinquefasciatus* are the major vectors, epidemic outbreaks result from the build up of virus in house sparrows and mosquito larvae breeding in discarded containers and open house foundations, characteristic of older housing construction. Regular outbreaks have occurred at approximately 10-year intervals until 1977, followed by irregular, unpredictable outbreaks observed in large urban localities. In the western United States, SLEV occurs as low-grade endemic activity transmitted by *C. tarsalis* and associated with agricultural irrigation, although there are exceptions, such as the large focal outbreak in Los Angeles in 1984, which was probably due to *C. pipiens* acting as an accessory vector.

Clinical Features

The majority (over 90%) of infections with SLEV are asymptomatic. In clinically apparent cases the disease is characterized by an abrupt onset of a febrile illness accompanied by constitutional symptoms of malaise, nausea, vomiting and headache. Occasionally there may be a more slow insidious onset. Central nervous system involvement may be in the form of aseptic meningitis or focal encephalitis. Encephalitis signs may be manifested as neck stiffness, dizziness, ataxia, mental confusion and disorientation. Cranial nerve palsies may occur in about 20% of cases, but the absence of focal findings or seizures may be useful differential features to distinguish St Louis encephalitis from cases of focal encephalitis, such as herpes simplex. In more severe cases there may be midbrain involvement and progressively severe coma. Overall case fatality is approximately 9% of symptomatic cases, although in outbreaks it may reach 20%. The likelihood of encephalitis, and also its severity, is related directly to age, with case fatalities in the elderly during outbreaks reaching 30% (Monath, 1980).

Diagnosis

For rapid serological diagnosis of SLEV infection, the MAC-ELISA is the method of choice, using either serum or CSF. Cross-reactions may occur if other flaviviruses are active in the area, but this is not a major factor in North America. The immunofluorescence test on SLEV-infected cells is a useful alternative to the IgM ELISA for testing sera, and the HAI test may be used for surveys or diagnosis if acute and convalescent sera are available.

In regions where flavivirus activity is confined mainly to one virus, the HAI test would be a useful screening test, followed by the SLEV MAC-ELISA test to determine recent SLEV infection.

Control

Although Sabin developed an inactivated mouse-brain vaccine during the Second World War, no licensed vaccine is available at present because of the low priority accorded to the infection. Similarly, specific vector control programmes directed at SLEV are either impractical or thought not to be of sufficient urgency. Secondary measures to protect against mosquito bites, such as insect repellent and screening, may be of some benefit during reported outbreaks.

WEST NILE VIRUS

West Nile virus is one of the most ubiquitous of human arbovirus infections. The virus was first isolated from a febrile patient in the West Nile province of Uganda in 1937. Since then it was shown to be widely distributed throughout Africa, the Middle East, Asia, parts of Europe and Australia and since its invasion of the Western Hemisphere in 1999 has spread throughout the United States, southern Canada, South America and the Caribbean.

Viral Features and Host Range

According to NT tests, WNV is related to the other members of the JE serogroup of flaviviruses. At least two distinct lineages, lineage I and II, exist although a further three lineages have been proposed. Lineage I has initially been divided into three different sublineages. Lineage 1a consists of viruses from North Africa, the Middle East, Europe, and North and South America, 1b consists of Kunjin virus strains from Australia and 1c consists of strains from India. Lineage II consist mainly of viruses from Southern Africa and Madagascar although a single lineage II isolate has been associated with encephalitis in a goshawk fledgling in Hungary in 2004, suggesting that lineage II strains may be carried outside of this region by migratory birds (Bakonyi *et al.*, 2006; Beasley *et al.*, 2002; Burt *et al.*, 2002; Lanciotti *et al.*, 2002). Recently two new variants of WNV have been identified in *C. pipiens* mosquitoes in the Czech Republic (Rabensburg (97–103) and in *Dermacentro marginatus* ticks in the North-West Caucasus Mountain Valley of Russia (LEIV-Krnd88–190). Based on their distance to lineage I and II strains these two isolates have been proposed to represent two new lineages (lineages III and IV) (Bakonyi *et al.*, 2005; Lvov *et al.*, 2004). This led

to the re-evaluation of WNV phylogenetic clusters using both full genome and partial genomic sequences (921 nucleotides of the C-prM-E). Five distinct lineages that differ by 20–26% at the complete genome level have been proposed. Lineage I includes the lineage 1a and 1b sublineages, but the Indian strains that formerly formed sublineage 1c were reclassified as a separate cluster (lineage V); lineage II includes the Southern African strains and the strain from Hungary; lineage III includes the Rabensburg (97–103) strain as prototype and lineage IV includes strain LEIV-Krnd88–190 from Russia (Bondre *et al.*, 2007).

Since the late 1990s an increase in neurological disease has been noted in humans, horses and birds as a result of WNV infections in North Africa, Europe and the Middle East and since its emergence in the Western hemisphere in 1999 has become a major public health problem in the United States. Although initial speculation suggested that lineage I strains were more pathogenic than lineage II strains, phylogenetic analysis and mouse neuro-invasive experiments have shown that highly neuro-invasive and mild strains exist in both lineages and that the neuro-invasive phenotype is related to genotype not to lineage. The perceived virulence of WNV in recent epidemics probably reflects high medical alertness, active surveillance programmes and the emergence and re-emergence of existing strains of WNV in geographic locations with immunologically naive populations. The lineage I strain that was imported into North America in 1999/2000 has been demonstrated to be highly neuro-invasive in mice and its uniform spread across the continent may also account for a higher incidence of neuro-invasive disease (Beasley *et al.*, 2001; Burt *et al.*, 2002; van der Poel, 1999). Phylogenetic analysis suggests that the virus has remained highly conserved since its emergence in the United States; although two distinct populations have emerged, the maximum divergence of recent strains compared with the NY99 prototype is still only 0.4–0.5%. A small number of WNV isolates from the United States (Texas) and Mexico have been obtained that have an attenuated phenotype in mice which was associated with mutations in the NS proteins or loss of E protein glycosylation respectively (reviewed in Beasley, 2005).

The maintenance and amplification of WNV in nature is between birds, which is the major vertebrate host, and mosquitoes. In endemic countries the majority of bird species tested appears to be resistant to WNV disease. Susceptible birds sustain infectious viraemia for one to four days after exposure before developing lifelong immunity. In South Africa high titres of WNV antibodies were detected in 27 species of wild birds tested without isolating virus from any. Inoculation experiments demonstrated

high levels of viraemia that lasted three to four days in 13 common avian species found near Johannesburg with no illness or mortality (Jupp, 2001). Death has however been reported in young ostrich chicks in South Africa (Burt *et al.*, 2002). WNV-associated bird mortality has rarely been reported from Europe, although recent reports from Central Europe of an encephalitis outbreak in a flock of geese that resulted in a 14% death rate in six-week-old geese as well as a goshawk fledgling suggest susceptibility in young or non-endemic migratory birds (Bakonyi *et al.*, 2006).

It has been suggested that migratory birds are central in the transmission between continents. Various outbreaks have also been reported in storks and geese in Israel (Malkinson *et al.*, 2002; Steinman *et al.*, 2002). In contrast to endemic countries, major bird deaths have been recorded in the United States since 1999. Around 284 species of birds have been reported in the CDC mortality database. Vector competency indices suggested that the blue jay, common crackle, house finch and American crow and sparrow are the most important species involved in WNV amplification (www.cdc.gov/ncidod/dvbid/westnile/birdspecies.htm).

In addition to birds, the virus is able to infect a variety of domesticated animals (particularly equines) and wild animals, as well as humans and sub-human primates. The most significant outbreaks in animals have occurred in horses in particular in Europe, the Middle East and the United States (Steinman *et al.*, 2002; Trock *et al.*, 2001). Approximately 15 000 equine cases occurred in the United States during the 2002 season; however the introduction of an inactivated vaccine has dramatically decreased the number of cases in subsequent seasons. These are generally considered as dead-end transmissions due to a lack of significant viraemia in these hosts to facilitate further transmission. Isolations from a wide range of vertebrate hosts, including dogs, sheep, alpacas, a number of wild mammals, as well as crocodiles and the death of a harbour seal demonstrates the wide susceptible host range (reviewed in Beasley *et al.*, 2004).

The maintenance vectors of WNV consist of a variety of mosquitoes, especially of the genus *Culex*. The major *Culex* mosquito vector in Africa and the Middle East is *Culex univittatus*; in South East Asia, *C. tritaeniorhynchus*; and in France, *Culex modestus*. Although WNV has been identified in as many as 59 different mosquito species in North America, only 10 of these are considered to be the principal vectors. During the 1999 outbreak 57% of positive mosquito pools were *C. pipiens*, however as the virus spread westward, *C. tarsalis* became more common in 2003, while *C. quinquefasciatus* comprised 51% of positive pools in 2004 as the major outbreaks occurred in the south-west.

Epidemiology

The transmission cycle of WNV consists mainly of wild birds as the vertebrate host and ornithophilic *Culex* mosquitoes as the maintenance vector. The intensity of transmission appears to be determined by the abundance of competent vectors as well as the infection rate of these mosquitoes (reviewed in Hayes *et al.*, 2005).

Major epidemics of WNV have been reported in Israel during the 1950s, in France in 1962 and the largest epidemic ever recorded took place in South Africa in 1974, which involved tens of thousands of individuals. Epidemics have occurred characteristically in relatively localized areas, for example, close to Tel Aviv in the Israeli epidemic, in the Rhone delta in France and the semi-desert Karoo region and in and around major cities of the highveld of South Africa. The distribution of WNV in South Africa is predominantly in the inland plateau region, where it shares the same geographical distribution and ecology as Sindbis virus, which also produces a disease that is usually clinically indistinguishable from WNV. The presence of WNV in the United States was first recognized in 1999, and then spread extensively across the United States and the Caribbean as well as southern provinces of Canada, Mexico and South America. From 1999 to May 2007, 23 974 cases have been reported in the United States of which approximately 9849 were neuro-invasive disease with 962 fatalities (data from the USA CDC website, as of May 2007). Despite this large number of cases the infection rate in epidemics in the United States, Europe and the Middle East has been <5%. Such low infection rates will not reduce the frequency of epidemics through protective immunity (Hayes and Gubler, 2006). During major outbreaks in South Africa, high attack rates in humans have been observed, for example, in some of the worst-affected towns in South Africa, 50–80% of the human population were infected due to the high rate of feeding by *C. univittatus*. This mosquito is probably also responsible for sporadic cases in inter-epidemic periods. Seroprevalence in the 1950s to 1970s in South Africa ranged from 17% in the Karoo to 8% in the highveld and 2% in Natal outside of epidemics (McIntosh *et al.*, 1976). No recent serological data are available.

Outbreaks of WNV, as with many other arboviruses, have been governed by climatic conditions, such as heavy rainfall, particularly in early and late summer, and high summer temperatures. In the early stages of the epidemic in the United States, WNV infection was recognized in mid to late summer. In 2002 the virus was detected as early as May. Because viraemia is low in humans, epidemic activity is directly due to infection of mosquitoes from viraemic birds and human outbreaks are merely the 'spill-over' of extensive epizootic activity in birds—an

important factor which facilitates epidemiological surveillance. Furthermore, no human-to-human transmission occurs, although transmission reports of transfusion and organ donor infection has necessitated the screening of blood from donors in countries affected in the Western Hemisphere. A case of intrauterine transmission has also been reported. As is the case with JEV, human population immunity has no bearing on the suppression of epidemic activity itself, and susceptible individuals remain vulnerable irrespective of the proportion of immune individuals in the population.

Clinical Features

The majority of WNV infections are asymptomatic. Symptoms will develop in 20–40% of people infected with the virus, and most of these will present with a flu-like mild febrile illness (West Nile fever). The onset of disease is characteristically sudden, following a short incubation period of three to five days. Fever is usually the first sign, followed by headache, nausea and vomiting. Ocular pain is frequently reported, as is pharyngitis. Muscle pain occurs diffusely and there may be arthralgia.

During the first few days after onset, a maculopapular rash usually appears which is discrete, with each of the rash elements demarcated by a sharp halo. The rash usually first appears on the trunk and then spreads to the face and extremities, and may persist for a week. Unlike measles, there is no desquamation.

Convalescence is rapid in children but may be somewhat more prolonged in adults and characterized by weakness and malaise. The illness not infrequently recrudesces during the convalescent period. Although WNV is classified virologically within the JEV group of mosquito-borne encephalitis, involvement of the central nervous system was believed to be very rare, with 1% of cases resulting in meningoencephalitis. Since the mid-1990s an increase in cases of neuro-invasive disease and death was noted in the Middle East and Europe, and in the United States up to 41% of reported clinical cases showed some evidence of meningoencephalitis. Acute flaccid paralysis (AFP) attributed to Guillain–Barré syndrome has been associated with WNV infection, several cases having been reported in the United States (Centers for Disease Control and Prevention, 2002). Up to May 2007, approximately 4% of reported cases in the United States were fatalities. Although all ages may be affected by WNV, the frequency of neuro-invasive disease is significantly higher in elderly and immunocompromised patients. Although initial reports suggested that WNV neuro-invasive disease rarely occurs in children, from 2002 to 2004 more than a thousand cases have been reported in children younger than 19 years in the United States, and 30% of these were neuro-invasive (Davis *et al.*, 2006).

Rarely, cases of visceral involvement including severe hepatitis, occasionally with a haemorrhagic presentation, have been reported.

Diagnosis

The HAI, CF or NT tests can be used for serological diagnosis of WNV infection. The HAI test detects antibodies within a few days after onset, but the MAC-ELISA is recommended as the diagnostic test of choice for WNV infection. Cross-reactivity is not a problem in patients with no previous exposure to flavivirus infection or vaccination. The NT assay would be used to identify specific flavivirus antibodies.

Virus isolation may be readily achieved from blood from infected individuals, despite lower levels of viraemia. Suckling mice are particularly sensitive to intracerebral inoculation and, in addition, cell culture of mammalian origin as well as insect cell lines are commonly used for virus isolation. RT-PCR is a rapid method for the detection of WNV viral RNA. Because of the short viraemic period this is not the test of choice and will usually be combined with serological tests. Screening of blood donations for viraemic WNV patients is achieved by RT-PCR. Real-time RT-PCR that can reliably detect all lineages has been reported (Tang *et al.*, 2006).

Control

With the impact of WNV on human health in the United States, there has been increasing interest in the development of a vaccine: During outbreaks, vector control by insecticide spraying may be applied. Epidemiological surveillance makes use of sentinel animals, such as hamsters, goats, guinea-pigs or pigeons, to detect early warning signs of impending outbreaks. The introduction of an inactivated vaccine for use in horses has dramatically reduced the number of neuro-invasive cases in these animals since 2004.

MURRAY VALLEY ENCEPHALITIS

MVEV is a relatively uncommon cause of human disease—less than 1000 cases have been reported—all of them confined to Australia and New Guinea.

The disease was first recognized during two epidemics of a virulent encephalitis in Queensland and in the Murray Valley in 1917 and 1918. A number of epidemics were subsequently described until 1925, when there was an inexplicable gap until the 1950s. The last substantial epidemic took place in 1974 and since then only a few individual sporadic cases have occurred.

The infection is found in a patchy distribution from New Guinea through Darwin to the northern parts of Western Australia and down the east coast as far south as Brisbane, and in the basin of the Murray Darling River. The virus was reintroduced to central Australia in 2000 following a period of unusually high rainfall.

Viral Features and Host Range

The virus displays a close relationship with Kunjin virus and also JEV. Four distinct lineages can be demonstrated based on E protein sequencing: lineages I, II, III and IV. Lineage I contains mostly strains from Western Australia, as well as some strains from Queensland and Papua New Guinea. Strains from Papua New Guinea and Australia are the most divergent within this lineage with 94% nucleotide identity, while strains from Australia have a sequence identity of 96%. Lineage II consists only of strains isolated in Western Australia in 1971 and in 1995, and is more closely related to lineage III and IV strains (87% nucleotide identity) from Papua New Guinea than to lineage I strains (84% nucleotide identity). Lineages III and IV consist exclusively of two strains identified in Papua New Guinea in 1956 and 1966 respectively. Recent isolates of MVEV from the Western Province in Papua New Guinea were closer to Australian isolates of MVEV than to older isolates from Papua New Guinea in 1956 and 1966, providing evidence for the movement of flaviviruses between Papua New Guinea and Australia (Johansen *et al.*, 2007).

Epidemiology

The major maintenance vector is *Culex annulirostris* and a number of vertebrate hosts, chiefly wild birds, are involved in the transmission cycle. Domestic animals are infected, but they probably do not play any significant role as amplifying hosts. Outbreaks have characteristically occurred in the summer months.

Clinical Features

As with most other arboviruses, the vast majority of infections are asymptomatic, only about 1/800–1000 infections being clinically manifest (Anderson, 1954). Once clinical disease occurs, however, high case fatality rates of 18–42% have characterized the various outbreaks. The clinical features in symptomatic patients are a sudden onset of high fever (up to 40.6 °C), nausea, vomiting and severe frontal headache. Signs of encephalitis vary from mild neurological involvement with disturbances of consciousness and neck stiffness to rapid onset of coma with respiratory failure. Patients with severe neurological involvement and coma, who were kept alive on life-support

systems and subsequently survived, have all had remaining permanent and severe sequelae.

Diagnosis

Serology is generally carried out by HAI or ELISA tests. The RT-PCR assay may detect MVEV early after onset of illness, providing a rapid and specific diagnosis.

TICK-BORNE ENCEPHALITIS

Genetic analysis of the tick-borne flaviviruses suggests that these viruses can be assigned into three major groups (mammalian, seabird and Kadam tick-borne flavivirus groups). The mammalian tick-borne flavivirus group includes six human and animal pathogenic viruses, formerly known as the 'tick-borne encephalitis serocomplex'. These include louping ill virus (LIV), TBEV, OHFV, Langat virus (LGTV), Kyasanur Forest disease virus (KFDV) and Powassan virus (POWV). All of these are encephalitic viruses, with the exception of OHFV and KFDV species, which cause haemorrhagic fever in humans. The recently identified haemorrhagic virus that appeared in Saudi Arabia in 1992, identified as Alkhurma haemorrhagic fever virus (AHFV), has been recommended for inclusion as a subtype of KFDV. No genetic link could yet be identified to viruses associated with haemorrhagic fevers (Grard *et al.*, 2007).

Although serological tests such as HAI and CF give considerable cross-reactivity with members of the flavivirus group, the tick-borne encephalitis subgroup of flaviviruses are far more closely genetically and antigenically related to each other. In contrast to many of their mosquito-borne arbovirus counterparts, the tick-borne encephalitis subgroup is found almost exclusively (with some exceptions, such as louping ill in the United Kingdom and Powassan in North America) in Asia and Eastern and Central and Western Europe. Within the subgroup is the entity of TBEV, which consists of three subtypes: TBE-Western European subtype, TBE-Far Eastern subtype and TBE-Siberian subtype, all transmitted mainly by *Ixodes* ticks. Other viruses within the tick-borne encephalitis subgroup are OHFV, KFDV and POWV, which will be considered separately.

Virological Features

On the basis of the recent availability of the complete genome sequences of all of the members of the mammalian tick-borne flaviviruses, certain changes have been proposed to the taxonomy of the TBEV. Grard *et al.* (2007) proposed that a single species of virus could be created namely TBEV, with four different types:

LIV, which includes Spanish, British and Irish subtypes and although infectious for humans, mainly causes encephalitis in sheep; western tick-borne encephalitis virus (W-TBEV); eastern tick-borne encephalitis virus including Far Eastern tick-borne encephalitis virus (FE-TBEV) and Siberian tick-borne encephalitis virus (S-TBEV) subtypes which are important human pathogens; and Turkish sheep encephalitis virus, including the Greek goat encephalitis virus subtype. They supported this taxonomic proposal by cut-off distances for species demarcation at 0.09 based on complete amino acid sequences and at 0.063 based on the complete NS3 protein (Grard *et al.*, 2007).

All subtypes of TBEV are transmitted by ticks in the *Ixodes* genus. The W-TBEV subtype are transmitted by *Ixodes ricinus*, while the FE-TBEV and the S-TBEV are transmitted by *Ixodes persulcatus*. The amplifying host are thought to be small rodents while wild animals (goat and deer) contribute indirectly through feeding of adult ticks thereby maintaining the tick population. *I. ricinus* species are distributed through Europe, Central Asia and parts of northern Africa, while *I. persulcatus* are found in Russia as well as northern Japan. Since both tick species are present in the Baltic countries, Latvia and Estonia, all three TBEV subtypes have been shown to co-circulate in both (Golovljova *et al.*, 2004; Lundkvist *et al.*, 2001). The western (European) subtype includes viruses isolated from Russia in the east through Finland and Sweden in the north, through Germany to France in the west and down to Italy, Greece and Yugoslavia in the south, encompassing the Central European countries in between. The eastern subtype includes the Far Eastern viruses isolated in northern Japan and the Siberian subtype isolated in the taiga region (the coniferous forest belt on the edge of the steppes and the tundra region of Siberia) and in western Siberia (reviewed in Suss and Klaus, 2006).

The TBEV are closely related to each other and are distinguishable serologically only by monoclonal antibodies or specialized techniques such as antibody adsorption tests. However, monoclonal antibody studies of the W-TBEV, especially those directed against glycoprotein domains, demonstrate a degree of antigenic complexity. Also, studies of different isolates of Far Eastern subtypes from different geographical locations have shown a heterogeneity of biological characteristics, such as mouse pathogenicity and plaque size. The viruses display clearly distinguishable biological differences from each other. The distribution of the infections closely follows that of their arthropod vectors—*I. ricinus* in the case of western subtypes and *I. persulcatus* in the case of Far Eastern and Siberian subtypes, although in countries in the Baltic region where both types of ticks occur, the western subtypes have been isolated from *I. persulcatus*. Recently reports of

isolations of Siberian and Far Eastern subtypes of TBEV have also been made in Europe.

The pathogenic potentials of the W-TBEV, S-TBEV and FE-TBEV subtypes also differ. Infection with the western subtype causes a biphasic febrile illness, characterized by a viraemic phase with fever, malaise, headache, myalgia, leukocytopenia, thrombocytopenia and elevated liver enzymes; this is followed by a one-week latent period, after which 25% of patients develop neurological disease, which may include meningitis or meningoencephalitis and poliomyelitis in a small number of cases. Although residual sequelae are observed in 25–50% of patients, less than 2% of these are fatal. W-TBEV has also been shown to induce a very much milder disease in experimentally infected sheep and monkeys than the Siberian and Far Eastern subtypes. In contrast, the Siberian and Far Eastern subtypes present as monophasic syndromes. Human infections reported in the East Asia are usually severe and are often associated with neurological signs with a fatality rate of 5–35%, although chronic cases are rare. In contrast, TBEV infections in Siberia causes less severe disease with fatality rates that range between 1 and 3%, although chronic forms are often reported. Recently eight fatal cases were reported in the Novosibirsk region of Siberia caused by Far Eastern strains that were associated with a pronounced haemorrhagic syndrome with massive gastrointestinal bleeding and multiple haemorrhages in mucosa and internal organs. Experimental data suggest that the Far Eastern strains have a clear tropism for neurons, which may account for the degenerative manifestations that are frequently associated with infection (reviewed in Charrel *et al.*, 2004).

Domestic animals, such as sheep, goats and cows, infected with TBEV excrete virus in their milk. The virus is also relatively stable to low pH and, experimentally, animals can rarely be infected by oral inoculation. In humans, milk-borne transmission of TBEV through ingestion of goat, sheep or cow's milk or dairy products made from them, such as cheese, is an important route of acquisition.

Epidemiology

W-TBEV, S-TBEV and FE-TBEV are endemic diseases with an increased seasonal incidence in the warmer months, April to November, related to climatic conditions of temperature and humidity, which affect tick activity. It is estimated that approximately 3000 cases of tick-borne encephalitis occur in Europe which translates to less than four cases per 100 000. The highest number of cases are reported in Latvia, where up to 199 cases per 100 000 population have been reported, although rates of 50–100 per 100 000 are more common. The incidence of tick-borne encephalitis has increased during the past

20 years, and the virus is now detected in previously unaffected areas. Changes in climate and vector distribution may account for the changes in tick-borne encephalitis incidence, although the decrease in disease in Sweden from 1984 onwards seemed to occur independently of the increase in recorded temperatures from 1989 (reviewed in Charrel *et al.*, 2004). In Russia an average of 12 cases per 100 000 population is reported. In China, TBEV is frequently detected in the Hunchun area, Jilin province and has been isolated from *Hymenocallis concinna* ticks. Antibody prevalence in humans in this region is as high as 10.9%. In the subtropical region of western Yunnan near the Burmese border, TBEV isolated from *Ixodes ovatus* was closely related to Russian spring summer encephalitis virus (RSSEV). Although data on tick-borne encephalitis morbidity are scarce, 3500 cases of the disease were reported in 1994 (reviewed in Suss and Klaus, 2006).

Infections occur predominantly in rural populations, especially farmers and forest workers. In addition, some 10–20% of W-TBEV infections are transmitted through ingestion of goat, sheep and cow's milk and dairy products. Seroprevalence studies of RSSEV have shown population prevalences of up to 50% in inhabitants of the taiga. Seroprevalence to W-TBEV depends on rural residence, occupation and age. Thus, in various studies in Central Europe, seroprevalence figures of 11–20% have been found in hunters, 5% in farmers and 1% in children (Gresikova *et al.*, 1973).

Infections with LIV are usually occupational, for example in sheep farmers, veterinarians and abattoir workers. A seroprevalence of up to 8% has been found in abattoir workers, suggesting asymptomatic infection. A large number of laboratory-acquired infections have been reported, which may indicate transmission by direct mucous or respiratory pathways. Tick-transmitted cases in humans are scarce (Charrel *et al.*, 2004).

Clinical Features

Disease caused by W-TBEV is relatively mild, with a low fatality of less than 5%. The incubation period is one to two weeks, followed by symptoms and signs of a non-specific febrile illness, headache, nausea, vomiting and lassitude; occasionally some signs of neurological disorders may appear, especially related to visual disturbances: blurring of vision and diplopia. This initial viraemic phase of the illness usually lasts some four to six days and is followed by a brief remission period.

The majority of individuals infected probably only experience a monophasic illness, which is rarely diagnosed specifically.

The second phase of the biphasic illness commences after a brief remission period, and is heralded in by a recrudescence of fever and signs of meningitis. The

most important signs of encephalitis are extrapyramidal and cerebellar syndromes, which may often persist for months after recovery. Localizing neurological signs, such as cranial nerve involvement, occur uncommonly and are usually mild. The mortality in various outbreaks that have been studied has varied (1–5%).

A more severe degree of encephalitis is usually seen with E-TBEV. The incubation period is similar but this is usually followed by sudden onset of fever and constitutional symptoms and, in the second phase of illness, a more intense meningitis. In some individuals an aseptic meningitis picture may be the sole clinical manifestation of disease. In others encephalitic signs and symptoms, such as disturbed consciousness, may lead to stupor, coma and death. A characteristic feature is lower motor neuron paralysis, which may resemble poliomyelitis but usually predominantly affects the upper limbs, spreading to the neck, and which may be followed by bulbar paralysis and death. The reported case fatality rates have varied (8–54%). In addition, residual paresis and atrophy of muscles of the upper limbs and neck may persist for long periods. Post-recovery epileptiform seizures may reflect permanent neurological damage.

The clinical symptoms of LIV in humans are similar to that of the biphasic meningitis typical of Western European TBEV. Following an incubation period of four to seven days, patients present with influenza-like illness with fever, headache, dizziness, retro-orbital pain, articular pain and myalgia. Patients usually then improve although in 50% of cases an encephalitic phase follows. A petechial rash is sometimes visible, with leukopenia in the first stage and leukocytosis in the second (Charrel *et al.*, 2004).

Diagnosis

MAC-ELISA on serum or CSF is the serological test of choice for diagnosis of TBEV infection. The HAI test is useful but a significant rise in antibody level must be demonstrated before a diagnosis can be made. Virus can be isolated from blood or CSF but specimens must be taken early after onset of symptoms. The virus may also be readily isolated from post-mortem tissues such as brain and also infected tick pools. The virus is readily isolated by intracerebral inoculation of suckling mice or cell cultures, such as Vero or chick embryo. RT-PCR assays are available, however because of the short viraemia period and low level of RNA present, virus isolation and RT-PCR is not often successful.

Control

Two commercially available vaccines are currently available and used widely in Central and Eastern Europe, namely new versions of Encepur (Chiron Vaccines,

Marburg, Germany) and FSME-IMMUN (Baxter Vaccines, Vienna, Austria). For paediatric applications, half the antigen dose in a 0.25 ml volume is available (FSME-IMMUN Junior). Both manufacturers' TBEV vaccines are considered safe and highly protective. The conventional vaccination schedule for both vaccines consist of three doses at day 0, months 1–3 and months 9–12 (or 5–12 months after the second dose). Rapid immunization schedules consisting of three doses at days 0, 7 and 21 are licensed for the historical and current versions of Encepur adults and Encepur children. First booster doses are given every 12–18 months then every five years in individuals ≤ 60 years and every three years in individuals > 60 years (reviewed in Beran, 2005).

Two licensed vaccines have been prepared from formaldehyde-inactivated, purified and concentrated Far Eastern subtype and Sofjin strains of TBEV, one called EnceVir (Virion, Tomsk, Russia), which has been available since 2001, and another produced at the Institute of Poliomyelitis and Viral Encephalitis (Moscow, Russia) since 1984. Both are certified for use in children. Up to 7 million doses have been used in Russia. The primary course of vaccination consists of three doses for both vaccines. Prevention has also been achieved though specific immunoglobulins, administered to tick-bitten people within three days of the tick bite. This therapy has a reported efficiency of 98% in a curative context and 100% in a prophylactic context (Charrel *et al.*, 2004).

OMSK HAEMORRHAGIC FEVER

Viral Features and Host Range

Omsk haemorrhagic fever virus was isolated in 1967 from the blood of a patient in western Siberia. It is closely related to TBEV and cannot be differentiated using polyclonal hyperimmune sera. They are differentiated on gel precipitation using cross-absorbed monospecific sera. The infection is conveyed by ixodid ticks, *Dermacentro reticulatus* and *D. marginatus*. A number of animal species are susceptible and the virus has been isolated from wild rodents. However, the most important vertebrate host is the muskrat (*Ondatra zibethica*), which is highly susceptible to infection, the virus usually producing a rapidly fatal haemorrhagic disease. The virus is excreted in the urine and faeces of sick animals and horizontal infection as well as arthropod infection is thought to play a role in transmission. The majority of human infections (60%) have occurred in hunters of muskrats, with transmission occurring as a result of direct contact during the skinning of animals. A further 28% of infections occur in adult family contacts of hunters.

Epidemiology

Infection has been limited to the Omsk region in the forest-steppe landscape of western Siberia, adjacent to tick-borne encephalitis endemic zones. The majority of cases were recorded between 1945 and 1949. Between 1945 and 1958, a total of 1488 cases were recorded. Occasional laboratory-acquired infections have been reported (Jelinkova-Skalova *et al.*, 1974). In recent years, most human cases have been related to direct contact with muskrats (*O. zibethica*), during the hunting season from September to October. A total of 165 cases of OHFV were recorded between 1988 and 1997. Of these, only 10 were transmission through tick bite while the rest were associated with exposure to muskrats. Muskrats were introduced into Siberia from Canada in 1928 and are therefore a relatively new host for the OHFV. Although the reason remains unknown, the OHFV endemic area is much smaller than the areas in which dermacentor ticks and muskrats are distributed (Charrel *et al.*, 2004).

Clinical Features

Clinically the disease presents with a sudden onset of fever, headache and myalgia. There is a recrudescence of fever followed by haemorrhagic manifestations, especially epistaxis, but also gastrointestinal bleeding and bleeding at other sites. Bronchopneumonia is a frequent complication and occasionally meningitis may occur with long-term complications, such as psychomotor retardation and depression.

Diagnosis and Control

Infection may be diagnosed by isolation of virus from patients' blood and intracerebral inoculation into suckling mice. Serological testing by ELISA, CF and NT are also available. Control measures to prevent infection involve the avoidance of ticks and care in handling muskrat carcasses in endemic areas, as well as laboratory safety measures. Because of extensive cross-reactivity, Central European encephalitis (CEE) vaccine would probably impart good protection. OHFV is classified as a biological safety level (BSL)-4 pathogen in most countries.

KYASANUR FOREST DISEASE

Viral Features and Host Range

The first isolation of KFDV was made in 1957 from a dead monkey found near the Kyasanur State forest in Kamataka (formerly Mysore) State in India. A few months previously a lethal epizootic amongst monkeys had been reported in the adjacent forested areas, with

human cases termed 'monkey disease' by the villagers. So far all human cases (on average, about 500 per year) have been limited to Karnataka State, in addition to a number of laboratory-acquired infections in both India and the United States. There is no evidence of the disease having existed prior to 1957.

Epidemiology

In addition to monkeys, a number of rodents are known to be infected, such as rats and shrews, as well as bats and other animals. The main tick vector is *Haemaphysalis spinigera*. Infection occurs predominantly in poor villagers working in forests.

Clinical Features

The disease is characterized by a sudden onset of fever after an incubation period of three to eight days. The fever may rise rapidly to 40 °C, associated with headache and severe myalgia reminiscent of dengue fever. Muscle pain is also predominantly found in the back and neck regions. A regular finding in patients in the acute stage is papulovesicular lesions on the soft palate. There is usually a cervical and axillary lymphadenopathy but occasionally this is generalized. Earlier reports of the disease laid great emphasis on the haemorrhagic manifestations, which occurred as early as the third day of illness. These consist of bleeding from the nose, gums and gastrointestinal tract. Associated with this there is a marked thrombocytopenia and neutropenia, but no evidence of bone marrow suppression or capillary damage. The cause of the haemorrhagic diathesis has more recently been thought to be autoimmune in nature (Pavri, 1989). Many of these earlier cases were reported in poor villagers, who were often infected with bacteria and parasites, and the associated raised interferon levels and IgE antibodies may also have contributed to the original clinical picture. Later studies of the disease, including those of laboratory-acquired infections, have rather put emphasis on neurological complications, such as severe headache, neck stiffness, coarse tremors, abnormal reflexes and mental disturbances. Mortality in Kysanur Forest disease is approximately 5–10%.

Diagnosis and Control

The virus can be readily isolated from patients' blood using mice or cell culture and antibodies can be detected by HAI, CF, NT or ELISA tests. Vector control programmes have been carried out in the forest, especially spraying in the vicinity of dead monkeys when these are encountered. A formalin-inactivated vaccine has been prepared in India and immunization programmes of villagers in the affected areas have been carried out.

POWASSAN VIRUS

Viral Features and Host Range

Powassan virus is named after the town of Powassan in northern Ontario, where the first human virus isolate was made from a fatal encephalitis case in a five-year-old boy in 1958. The disease is rare, less than 50 cases having been reported worldwide. Case reports have come from Canada and the United States as well as Russia.

Epidemiology

A number of arthropod vectors have yielded isolates of the virus. In North America the major vector has been *Ixodes cookei*, with *Dermacentor andersoni*, *Ixodes marxi* and *I. spinipalpus* and, in Russia, *Haemaphysalis neu-manni*, *I. persulcatus* and *Dermacentor silvarum* occasionally being infected. In addition, infected mosquito species (*Aedes togoi* and *Anopheles hryanus*) have also been reported. Vertebrates infected with POWV have occurred mainly in mammals, but also birds, amphibians and reptiles.

Clinical Features

Clinical cases of POWV infection have presented with encephalitis, meningoencephalitis and aseptic meningitis. In some cases focal encephalitic signs have occurred and in one case from Russia the patient died following bulbar paralysis. In a series of 19 cases in North America, two deaths occurred in the acute illness phase. Serological diagnosis of POWV infection can be achieved by specific IgM antibody detection in acute serum or spinal fluid, or by IgG seroconversion. Because of cross-reactions with other flaviviruses, NT assays are required for confirmation.

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Bunyaviridae

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INTRODUCTION

At present, the family *Bunyaviridae* comprises approximately 300 animal viruses assigned to four genera: *Orthobunyavirus* (named after Bunyamwera virus), *Hantavirus* (named after Hantaan virus), *Phlebovirus* (named after phlebotomus/sandfly fever) and *Nairovirus* (named after Nairobi sheep disease virus). A further 41 inadequately characterized animal viruses are considered possible members of the family and there is a genus of plant viruses (Büchen-Osmond, 2006; Calisher, 1991; Calisher and Karabatsos, 1989; Karabatsos, 1985; Murphy *et al.*, 1995; Peters and LeDuc, 1991). Most of the animal viruses were discovered in the course of surveys on haematophagous arthropods or wild vertebrates, and the fact that new members of the family are constantly being encountered has been interpreted to indicate that many remain to be discovered (Peters and LeDuc, 1991). Some of the viruses are important pathogens of humans or livestock, but the majority have no known medical or veterinary significance. Sometimes a pathogenic role is discovered for a virus years after its initial isolation. Although most members of the family are thought to be arthropod-borne (i.e. arboviruses), transmission by vectors has been demonstrated conclusively in comparatively few instances. Members of the rodent-associated *Hantavirus* genus are not considered to be arthropod-borne.

The origins of the family can be traced to the initial detection of close antigenic relationships within certain groups of viruses, including one containing Bunyamwera virus (named for a place in Uganda) (Casals, 1957, 1961; Casals and Whitman, 1960, 1961). Subsequently

the demonstration of weak serological cross-reactions between the groups resulted in the viruses being included in a Bunyamwera supergroup (World Health Organization, 1967). The family *Bunyaviridae*, containing a single genus *Bunyavirus*, was erected when members of the supergroup and certain ungrouped viruses were found to have similar morphology (Fenner, 1976; Murphy *et al.*, 1973; Porterfield *et al.*, 1974), and subsequently *Phlebovirus*, *Uukuvirus*, *Nairovirus* and *Hantavirus* genera were added to the family as morphological and biochemical affinities between the viruses became evident (Bishop *et al.*, 1980; Matthews, 1981; Schmaljohn *et al.*, 1985). Later, the uukuviruses were included in the *Phlebovirus* genus since they were found to share coding and replication strategies with the phleboviruses, while the genus *Bunyavirus* was renamed as the genus *Orthobunyavirus* (Calisher, 1991; Murphy *et al.*, 1995; Simons *et al.*, 1990). Within genera, members were classified on the basis of antigenic affinities and arranged into serogroups, antigenic complexes, viruses (or serotypes), subtypes and varieties, in order of increasing relatedness, with serotypes being separable by definition only in cross-neutralization tests. However, classification of viruses by serotype is progressively being replaced by the definition of genotypes through nucleotide sequencing of the genome. This was initially applied particularly to agents which proved difficult to grow in laboratory culture systems, such as recently discovered hantaviruses, but is finding increasing application in phylogenetic studies of orthobunyaviruses, phleboviruses and nairoviruses (Ahmed *et al.*, 2005; Armstrong and Andreadis, 2006; Bird *et al.*, 2007; Briese *et al.*, 2004; Chamberlain *et al.*, 2005; Deyde *et al.*, 2006; Monroe

et al., 1999; Liu *et al.*, 2003; Xu *et al.*, 2007; Yandoko *et al.*, 2007).

THE VIRUS

Structure

The viruses of the family are spherical, 80–120 nm in diameter, and have a host cell-derived bilipid-layer envelope through which virus-coded glycoprotein spikes or peplomers project. The virions contain three major structural proteins: two envelope glycoproteins, G1 (G_n) and G2 (G_c), and a nucleocapsid protein N, plus minor quantities of a large or L protein (145–259 kDa) believed to be the viral transcriptase, an RNA-dependent RNA polymerase (Table 29.1) (Bishop, 1990; Calisher, 1991; Peters and LeDuc, 1991; Schmaljohn and Patterson, 1990). (Hazara virus of the genus *Nairovirus* is believed to have three glycoproteins.) Members of the family have a three-segmented, single-stranded RNA genome and each of the segments, L (large), M (medium) and S (small), is contained in a separate nucleocapsid within the virion. The sizes of the structural proteins and RNA segments vary with genus (Table 29.1). The genomic RNA is in the negative-sense (complementary to mRNA), but the S segment of the phlebovirus genome consists of ambisense RNA, that is, has bi-directional coding, a property which is shared only with the RNA of viruses of the family *Arenaviridae*. The first 8–13 nucleotide bases at the 3' ends of the RNA segments tend to have a sequence that is conserved within the viruses of each genus, with a complementary (palindromic) consensus sequence occurring at the 5' end; and the ends of the segments are noncovalently linked so that the RNA occurs in a loosely bound circular configuration within the nucleocapsids. The segmented nature of the genome suggests that the potential exists for reassortment to occur in co-infections, and it is thought that this mechanism may have contributed to the evolution of diversity in the family, but experimental evidence indicates that there are genetic restraints and

that reassortment occurs with facility only between members of genotypes (Bowen *et al.*, 2001; Briese *et al.*, 2006; Gerrard *et al.*, 2004; Peters and LeDuc, 1991).

The L RNA segment of the genome codes for the viral transcriptase, and the M segment for the G proteins, as well as a nonstructural protein NS_m in the *Orthobunyavirus* and *Phlebovirus* genera. The S segment RNA codes for the N protein, as well as a nonstructural protein NS_s in the bunyaviruses and phleboviruses. Nonstructural proteins have not been demonstrated in the nairoviruses or hantaviruses. The viral glycoproteins are responsible for recognition of receptor sites on susceptible cells, manifestation of viral haemagglutinating ability and for inducing protective immune response in the host. The N protein induces production of and reacts with complement-fixing antibody.

Biological Characteristics

Viruses that attach to receptors on susceptible cells are internalized by endocytosis, and replication occurs in the cytoplasm. Virions mature primarily by budding through endoplasmic reticulum into cytoplasmic vesicles which are presumed to fuse with the plasma membrane to release virus, but it appears that virus can also bud directly from the plasma membrane (Anderson and Smith, 1987). Most of the viruses have been isolated and propagated by intracerebral inoculation of suckling mice, but the hantaviruses produce chronic and inapparent infection of laboratory rodents. Members of the family can be grown in a variety of cell cultures (Vero cells have been used most commonly), but some of the viruses are noncytolytic so that their presence has to be demonstrated by immunofluorescence or similar means. Hantaviruses are difficult to grow *in vitro*, and some of the more recently discovered members of the genus have not yet been adapted successfully to cell cultures (Klempa *et al.*, 2006, 2007; Monroe *et al.*, 1999).

An abridged classification of the family, showing selected members known to cause infection of humans and livestock in relation to their vectors and distribution, is

Table 29.1 Major biochemical properties of members of the *Bunyaviridae*. Information derived from sources cited in the text

Viruses	Molecular weights of major structural proteins ($\times 10^3$)			Molecular weights of RNA species ($\times 10^6$)		
	G1	G2	N	L	M	S
Bunyaviruses	108–120	29–41	19–26	2.7–3.1	1.8–2.3	0.3–0.5
Sandfly fever group	55–70	50–60	20–30	2.6–2.8	1.8–2.2	0.7–0.8
Uukuniemi group	70–75	65–70	20–25	2.0–2.5	1.0–1.3	0.4–0.7
Nairoviruses	72–84	30–40	48–54	4.1–4.9	1.5–2.3	0.6–0.7
Hantaviruses	64–76	52–58	48–54	2.2–2.9	1.2–2.9	0.6–0.75

presented in Tables 29.2–29.4 (Büchen-Osmond, 2006; Calisher, 1991; Calisher and Karabatsos, 1989; Karabatsos, 1985; Peters and LeDuc, 1991). In addition, high prevalences of antibody to many other viruses have been found in particular human populations, but conclusive evidence of infection or disease association is lacking: antigenic cross-reactivity between viruses can complicate the interpretation of survey findings or render it difficult to arrive at a serological diagnosis in individual cases of disease.

There is a broad tendency for antigenic grouping and phylogenetic classification to correlate with geographic distribution and with the type of vector involved in transmission (Tables 29.2–29.4). Although a greater variety of arthropod-borne members occurs in tropical and subtropical countries of Latin America and Africa, many viruses including several important pathogens occur in temperate countries and the distribution of the family extends to the arctic region. Moreover, there are many instances on record of residents of temperate countries which lack indigenous disease, acquiring infection during travels abroad. Most of the viruses appear to be transmitted by culicine mosquitoes including aedines, but some are transmitted by anopheline mosquitoes. Simbu serogroup viruses are associated particularly with ceratopogonid midges (*Culicoides* spp.), while the sandfly fever serogroup of phleboviruses (apart from Rift Valley fever and a few other mosquito-borne viruses), are associated with phlebotomids (sandflies). The Tete serogroup of bunyaviruses, Uukuniemi serogroup of phleboviruses and the nairoviruses are associated with ixodid and argasid ticks. Some viruses have been isolated from more than one type of vector.

Transmission

It is characteristic of arthropod-borne viruses that they produce viraemia in at least one species of vertebrate to allow the infection to be acquired by biological vectors which take blood meals. During a so-called *extrinsic incubation period*, commonly lasting one to two weeks in dipterid vectors (mosquitoes, midges and sandflies), the virus replicates in the vector and spreads to produce infection of the salivary glands, thereby permitting transmission to occur to a second vertebrate host. Virus is thus maintained by circulation between the vector and a vertebrate host. The maintenance cycle may be cryptic, involving wild vertebrates which develop inapparent infection, with incidental spread of infection to susceptible domestic animals or humans which impinge on the cycle. It has been postulated that through selection pressure brought about by long association with the virus, natural maintenance hosts often develop transient viraemic infection without displaying susceptibility

to the pathogenic effects of the virus concerned. Small mammals and birds that occur in large numbers, breed prolifically to ensure a constant supply of non-immune individuals and are subject to periodic population explosions constitute ideal maintenance hosts for arboviruses. Species susceptible to disease may themselves serve to amplify circulation of virus through infecting vectors, but humans serve this purpose for members of the *Bunyaviridae* in few instances only (Oropouche, sandfly fever), and are usually ‘dead-end’ hosts. Domestic animals which develop disease or undergo inapparent infection may serve as link hosts between the natural cycle and humans, which in turn gain infection from contact with infected tissues of livestock or products such as milk, or from vectors infected by feeding on livestock (Rift Valley fever, Crimean–Congo haemorrhagic fever (CCHF).

Since the biting activity of arthropod vectors, and hence the infection of vertebrates, is seasonal, the fate of arthropod-borne viruses during winters or dry seasons of inactivity has long constituted a central enigma in the epidemiology of arbovirus diseases. Many plausible mechanisms for overwintering or hibernation of arboviruses have been described, including persistent infection of vertebrates, migration of infected birds or mammals, hibernation of infected adult vectors and continuous vector activity in tropical locations (Reeves, 1974). Transovarial transmission of infection in arthropod vectors, however, theoretically constitutes an ideal mechanism for ensuring the perpetuation of the viruses, and comparatively early in the history of the investigation of arbovirus diseases convincing evidence was produced to indicate that the phenomenon occurs in phlebotomids and ixodid ticks (Tesh, 1984). The evidence for mosquito-borne viruses long remained in doubt, but in recent years many investigators have demonstrated transovarial transmission of bunyaviruses, particularly members of the California encephalitis serogroup, as well as Rift Valley fever virus and members of the *Togaviridae* and *Flaviviridae* families in mosquitoes (Linthicum *et al.*, 1985; Peters and LeDuc, 1991; Swanepoel, 2003; Tesh, 1984). Even in the absence of transovarial transmission of infection, the overwintering of viruses transmitted by ixodid ticks can be explained by the long intervals that occur between the feeding of successive instars of the vectors.

In general, viruses transmitted by dipterid flies (mosquitoes, midges and sandflies) may cause sporadic infections, but are capable of causing explosive epidemics at irregular intervals of years when climatic conditions are particularly favourable for the breeding of vectors, or human manipulation of the environment results in large-scale juxtaposition of susceptible people or livestock and vectors. Viruses transmitted by ixodid ticks

Table 29.2 Abridged list of members of the genus *Orthobunyavirus* showing viruses known to cause infection of humans and farm animals. Information derived from sources cited in the text

Virus genotype/subtype	Putative vectors	Human infection		Livestock disease	Distribution
		Natural	Laboratory		
Bunyamwera	Mosquitoes	+	+	—	Africa
Batai	Mosquitoes	+	—	—	Europe, Asia, Africa
Ngari	Mosquitoes	+	+	—	Africa
Ilesha	Mosquitoes	+	—	—	Africa
Shokwe	Mosquitoes	+	—	—	Africa
Germiston	Mosquitoes	—	+	—	Africa
Cache Valley	Mosquitoes	+	—	+	North America
Fort Sherman	Mosquitoes	+	—	—	Central America
Maguari	Mosquitoes	+	—	?	South America
Tensaw	Mosquitoes	+	—	—	North America
Wyeomyia	Mosquitoes	+	—	—	Central and South America
Bwamba	Mosquitoes	+	—	—	Africa
Pongola	Mosquitoes	+	—	—	Africa
Nyando	Mosquitoes	+	—	—	Africa
California encephalitis	Mosquitoes	+	—	—	North America
La Crosse	Mosquitoes	+	+	+	North America
Snowshoe hare	Mosquitoes	+	—	—	North America
Jamestown Canyon	Mosquitoes	+	—	—	North America
Keystone	Mosquitoes	—	+	—	North America
Tahyna	Mosquitoes	+	—	—	Europe, Asia
Inkoo	Mosquitoes	+	—	—	Europe
Oropouche	Ceratopogonids	+	+	—	Central and South America
Caraparu	Mosquitoes	+	+	—	Central and South America
Apeu	Mosquitoes	+	+	—	South America
Ossa	Mosquitoes	+	+	—	Central America
Madrid	Mosquitoes	—	+	—	Central America
Marituba	Mosquitoes	+	+	—	South America
Murutucu	Mosquitoes	+	+	—	South America
Nepuyo	Mosquitoes	+	—	—	Central and South America
Restan	Mosquitoes	+	—	—	South America
Oriboca	Mosquitoes	+	+	—	South America
Itaqui	Mosquitoes	+	—	—	South America
Guaroa	Mosquitoes	+	—	—	Central and South America
Catu	Mosquitoes	+	+	—	South America
Guama	Mosquitoes	+	—	—	Central and South America
Tacaiuma	Mosquitoes	+	—	—	South America
Turlock	Mosquitoes	—	—	+	North, Cental. and South America
Akabane	Mosquitoes, Ceratopogonids	—	—	+	Asia, Africa, Australasia
Tinaroo	Ceratopogonids	—	—	+	Australasia
Shuni	Ceratopogonids, Mosquitoes	+	—	+	Africa
Aino	Ceratopogonids, Mosquitoes	—	—	+	Asia, Australasia

Table 29.3 Abridged list of members of the genera *Phlebovirus* and *Nairovirus*, plus viruses not yet assigned to a genus, showing viruses known to cause infection of humans and domestic animals. Information derived from sources cited in the text

Virus genotype/subtype	Putative vectors	Human infection		Livestock disease	Distribution
		Natural	Laboratory		
Genus: <i>Phlebovirus</i>					
Sandfly fever Naples	Phlebotomids	+	—	—	Europe, Africa, Asia
Toscana	Phlebotomids	+	—	—	Europe
Sandfly fever Sicilian	Phlebotomids	+	—	—	Europe, Africa, Asia
Candiru	Phlebotomids?	+	—	—	South America
Alenquer	Phlebotomids?	+	—	—	South America
Punta Toro	Phlebotomids	+	—	—	Central America
Chagres	Phlebotomids	+	—	—	Central America
Rift Valley fever	Mosquitoes	+	+	+	Africa, Arabian Peninsula
Uukuniemi	Ixodids	+	—	—	Europe
Genus: <i>Nairovirus</i>					
Crimean–Congo haemorrhagic fever	Ixodids	+	+	—	East Europe, Asia, Africa
Dugbe	Ixodids	+	+	—	Africa
Nairobi sheep disease	Ixodids	+	+	+	Africa, India
Bunyaviruses unassigned to genus					
Bhanja	Ixodids	+	+	—	Europe, Africa, Asia
Kasokero	Unknown		+	—	Africa
Bangui	Unknown	+	—	—	Africa
Issyk-Kul	Mosquitoes?	+	+	—	Asia
Tataguine	Mosquitoes	+	—	—	Africa, Asia
Wanowrie	Ixodids	+	—	—	—

tend to cause sporadic disease in locations where there is occupational or recreational exposure of humans to ticks, but human intervention can precipitate the occurrence of larger outbreaks of disease.

Clinical syndromes associated with members of the *Bunyaviridae* range from inapparent infections known from routine monitoring of laboratory workers, through moderate to severe influenza-like illness with or without a maculopapular rash and characterized by fever (often biphasic), headache, myalgia, arthralgia and malaise, to encephalitis or haemorrhagic disease with necrotic hepatitis, while the hantaviruses of Asia and Europe are associated with a group of diseases known collectively as haemorrhagic fever with renal syndrome (HFRS), and the hantaviruses of the Americas are associated with an acutely fatal respiratory disease known as hantavirus pulmonary syndrome (HPS). The information presented below on the epidemiology and disease associations of individual viruses is derived from a few collated sources, except where indicated otherwise (Calisher and Karabatsos, 1989; Gonzalez-Scarano and Nathanson, 1990; Karabatsos, 1985; McKee *et al.*, 1991; Peters and LeDuc, 1991; Porterfield, 1990).

LABORATORY DIAGNOSIS

The appropriate specimens and laboratory methods required for confirming diagnoses of the more important diseases are indicated in the relevant sections dealing with the individual infections below. Procedures developed and applied over decades for the isolation and identification of arthropod-borne members of the family, or for demonstrating immune responses, remain valid (Shope and Sather, 1979). However, there are residual problems concerning the sensitivity, specificity and rapidity with which certain infections can be diagnosed, and these are being solved through increasing utilization of newer serological and molecular biological techniques. Isolation and identification of virus remains the definitive way of making a diagnosis, and this is especially true for what is perceived as a novel or as an undifferentiated febrile illness: it is easier to arrive at a serological diagnosis in diseases which are recognizable from their clinical presentation or from the circumstances under which patients become infected, such as Oropouche fever, sandfly fever, CCHF, Rift Valley fever or HFRS. Sporadic undifferentiated febrile illnesses, in contrast, have usually been identified in the course of surveys, and the diagnosis of

Table 29.4 Abridged list of members of the genus *Hantavirus* shown in relation to the phylogeny of the natural hosts of the viruses. Information derived from sources cited in the text

Virus genotype/subtype	Vertebrate host: Order: Subfamily		Disease	Distribution
	Known/suspected host			
Rodentia: Murinae				
Hantaan	<i>Apodemus agrarius manichurichus</i> ; <i>Apodemus agrarius coreae</i>		HFRS	Asia
Amur	<i>Apodemus peninsulae</i>		HFRS	Asia
Da Bie Shan	<i>Niniventer confucianus</i>			China
Dobrava-Belgrade	<i>Apodemus flavicollis</i>		HFRS	Europe
Saaremaa	<i>Apodemus agrarius agrarius</i>		HFRS	Europe
Seoul	<i>Rattus rattus</i> ; <i>Rattus norvegicus</i>		HFRS	Worldwide
Thailand	<i>Bandicota indica</i>		—	Thailand
Sangassou	<i>Hylomyscus simus</i>		—	Guinea
Rodentia: Arvicolinae				
Puumala	<i>Myodes glareolus</i>		HFRS (NE)	Europe
Muju	<i>Myodes regulus</i>		HFRS (NE)?	Asia
Hokkaido	<i>Myodes rufocanus</i>		—	Japan
Topografov	<i>Lemmus sibiricus</i>		—	Siberia
Khabarovsk	<i>Microtus fortis</i>		—	Siberia
Tula	<i>Microtus arvalis</i> ; <i>Microtus rissiaemeridionalis</i>		HFRS	Europe
Prospect Hill	<i>Microtus pennsylvanicus</i>		—	North America
Bloodland Lake	<i>Microtus ochrogaster</i>		—	North America
Isla Vista	<i>Microtus californicus</i>		—	West USA, Mexico
Rodentia: Sigmodontinae				
Sin Nombre	<i>Peromyscus maniculatus (Grassland form)</i>		HPS	West and Central USA, Canada
Monongahela	<i>Peromyscus maniculatus nubiterrae (Forest form)</i>		HPS	East USA, Canada
Blue River	<i>Peromyscus leucopus (SW/NW haplotypes)</i>		HPS	Central USA
New York	<i>Peromyscus leucopus (Eastern haplotype)</i>		HPS	East USA
Limestone Canyon	<i>Peromyscus boylii</i>			Southwest USA, Central Mexico
Bayou	<i>Oryzomys palustris</i>		HPS	Southwest USA
Black Creek Canal	<i>Sigmodon hispidus (Eastern form)</i>		HPS	Southeast USA
Muleshoe	<i>Sigmodon hispidus (Western form)</i>		—	South USA
Cano Delgadito	<i>Sigmodon alstoni</i>		—	Venezuela
Andes	<i>Oligoryzomys longicaudatus</i>		HPS	Argentina, Chile
Oran	<i>Oligoryzomys longicaudatus</i>		HPS	Northwest Argentina
Lechiguanas	<i>Oligoryzomys flavescens</i>		HPS	Central Argentina
Central Plata	<i>Oligoryzomys flavescens</i>		HPS	Argentina, Uruguay
Bermejo	<i>Oligoryzomys chacoensis</i>		—	Northwest Argentina
Hu 39694 ^a	<i>Oligoryzomys sp.?</i>		HPS	Central Argentina
Pergamino	<i>Akadon azarae</i>		—	Central Argentina
Maciel	<i>Necomys benefactus</i>		—	Central Argentina
Laguna Negra	<i>Calomys laucha</i>		HPS	Paraguay, Bolivia
Juquitiba	<i>Oligoryzomys nigripes</i>		HPS	Brazil
Castelo dos Sonhos	Unknown		HPS	Brazil
Araraquara	<i>Bolomys lasiurus</i>		HPS	Brazil
Rio Mamore	<i>Oligoryzomys microtis</i>		HPS	Bolivia, Peru
El Moro Canyon	<i>Reithrodontomys megalotis</i>		—	West USA, Mexico
Rio Segundo	<i>Reithrodontomys mexicanus</i>		—	Costa Rica
Choclo	<i>Oligoryzomys fulvescens</i>		HPS	Panama
Calabazo	<i>Zygodontomys brevicauda</i>		—	Panama
Insectivora: Crocidurinae				
Thottapalyam	<i>Suncus murinus</i>		—	India
Tanganya	<i>Crocidura theresae</i>		—	Guinea

^aVirus will be named when the rodent host or distribution is identified. HFRS, haemorrhagic fever with renal syndrome; HPS, hantavirus pulmonary syndrome.

individual cases requires clinical acumen and recourse to the services of a specialized laboratory able to screen for a range of viruses known or considered likely to occur in the area where the infection was acquired.

Virus Detection and Identification

Most members of the family were discovered through intracerebral inoculation of suckling mice, and this method is still widely used for isolating the viruses. A few of the viruses are also pathogenic for weaned mice or hamsters, some even by peripheral route, and this constitutes a useful screening method for preliminary identification of isolates, for example Rift Valley fever virus. Some viruses which are nonpathogenic for laboratory rodents, such as the hantaviruses (not known to be arthropod-borne), can nevertheless be isolated in rodents through demonstrating the presence of viral antigens in tissues. Many cytopathic, as well as noncytopathic viruses such as CCHF, can be isolated in mammalian cell cultures and detected by immunofluorescence. The method has the advantage that it is usually rapid, and therefore clinically useful, but it is not invariably more sensitive than the use of suckling mice for isolating viruses which are present in low concentrations in pathological specimens. Certain viruses which are nonpathogenic for laboratory mice, such as some of the neotropical phleboviruses, were only discovered because they proved to be cytopathic in mammalian cell cultures. Although it has not been proven for members of the *Bunyaviridae*, some arboviruses can be isolated most successfully by inoculation of mosquito cell cultures, which do not manifest cytopathic effect, or live mosquitoes, and in these instances the isolation of virus has to be demonstrated by immunological or molecular means.

In some diseases, such as Rift Valley fever or CCHF, rapid diagnoses can sometimes be achieved without culturing virus, by demonstrating the presence of viral antigens directly in infected blood or other tissues by enzyme-linked immunoassay, immunofluorescence, immunohistochemistry or a variety of other immunological methods. This approach was used in the discovery of hantaviruses in rodent tissues. In certain diseases, such as La Crosse encephalitis, it appears that virus is seldom present in blood or other tissues in infective concentrations at the time that the disease is recognized, while other viruses, such as the hantaviruses, appear to be present but may be extremely difficult to isolate and adapt to laboratory host systems. An alternative is to detect viral nucleic acids in tissue extracts or histological sections by hybridization with specific radiolabelled nucleic acid probes. A more sensitive technique, which has become a standard approach to the diagnosis of virus infections,

is the use of reverse transcription polymerase chain reaction (RT-PCR) to detect viral nucleic acids. This was used with notable success in the detection of novel hantaviruses in the blood and other tissues of human patients and rodents. Recent refinements of RT-PCR applied to the diagnosis of bunyavirus infections include the selection of consensus sequence primers which are specific for either individual viruses, groups of viruses or all potential members of a genus, and the use of pyrosequencing or hybridization of the PCR products to DNA microarrays to obtain rapid and accurate identification of the virus concerned (Ahmed *et al.*, 2005; Andreadis, 2006; Armstrong and Kramski *et al.*, 2007; Arthur *et al.*, 1992; Drosten *et al.*, 2002; Monroe *et al.*, 1999; Nichol *et al.*, 1993; Nordström *et al.*, 2004; Puthavathana *et al.*, 1992; Weidmann *et al.*, 2008; Xiao *et al.*, 1994).

Until recently, isolates were generally identified exclusively by serological means, and by definition viruses react most specifically with antisera in neutralization tests, but these are technically difficult to perform with some viruses, or may not yield results sufficiently rapidly to be clinically useful. Antisera tend to be more cross-reactive in other serological tests, such as complement fixation, haemagglutination inhibition, immunofluorescence and enzyme-linked immunoassay which appear to detect mainly the immunodominant nucleocapsid protein antigens (Briese *et al.*, 2004), but the problem can be overcome by use of monospecific monoclonal antibodies. In instances where potentially new viruses, or viruses associated with undifferentiated illnesses, have to be identified, cross-reactivity can be useful, and isolates may be subjected to preliminary screening with pools of antisera, or antisera which have deliberately been rendered cross-reactive by immunizing laboratory animals sequentially with several viruses. Morphological or partial biochemical characterization of isolates, by performance of electron microscopic examination or tests for sensitivity to ether and bile salts, for instance, may also facilitate the process of identifying a virus.

However, as indicated above, virus isolates are now usually identified by molecular biological techniques, including hybridization of viral nucleic acids with labelled DNA probes specific for individual viruses, by demonstrating specific endonuclease restriction enzyme digestion patterns with the nucleic acids, or more commonly by performing nucleotide sequencing of RT-PCR products or even whole viral genomes (Monroe *et al.*, 1999).

Serology

Serological diagnosis of infections is beset with the same problems of cross-reactivity which apply to antigenic identification of isolates, and the difficulties are compounded where patients have previously been infected

with an antigenically related virus: antibody response tends to be broadly cross-reactive within serogroups following sequential infections. Neutralizing antibody, which reacts most specifically for individual viruses, usually becomes demonstrable by day 7–10 of illness (earlier in Rift Valley fever) and after an initial post-convalescent decline in titre, tends to remain demonstrable for life, but the response is usually weak and difficult to demonstrate following nairovirus infections. Complement fixing antibody becomes demonstrable in the second or third week of illness, declines after several months, and tends to be group-specific with bunyaviruses and nairoviruses, but more specific among phleboviruses. Antibody demonstrable by haemagglutination inhibition, indirect immunofluorescence or enzyme-linked immunoassay becomes detectable at about the same time as neutralizing antibody, and after a post-convalescent decline in titre, remains demonstrable for a period of at least several years, and varies in specificity in different groups of viruses. Demonstration of IgM antibody activity in indirect immunofluorescence tests or enzyme-linked immunoassays is most useful for establishing a rapid diagnosis.

Antibody titres tend to be highest against the homologous infecting virus, so that problems of cross-reactivity can sometimes be overcome by screening patients' sera with a range of antigens prepared from all members of the virus serogroup known to occur in the area concerned, as is done with members of the California encephalitis serogroup. An alternative is the preparation of purified antigens which contain virus proteins or peptides that react specifically with serotype antibody, and this includes preparation of antigens by recombinant DNA technology (Feldmann *et al.*, 1993). The same effect may be obtained by selective capture of viral proteins with monoclonal coating antibody in enzyme-linked immunoassays.

GENUS ORTHOBUNYAVIRUS

Bunyamwera, Batai, Ngari, Ilesha, Germiston and Shokwe Viruses

Bunyamwera virus is widely distributed in Africa and has been isolated from aedines and other culicine mosquitoes, and/or human blood in Uganda, South Africa, Kenya, Nigeria, Central African Republic, Cameroon and Senegal. Antibody has been found in humans and/or domestic animals, rodents, bats and sub-human primates in the same countries as well as in Mozambique, Tanzania, Angola, Congo, Egypt and Tunisia. However, some of the antibody reactions recorded in surveys may have been due to infection with related viruses. Despite the widespread

occurrence of antibody, human disease has seldom been recognized and the few cases which have been described include several laboratory infections. Clinical findings included fever, maculopapular rash, arthralgia, neck stiffness, vertigo and temporary loss of visual acuity. Severe encephalitis occurred in experimental infection of a tumour patient. Infection was confirmed in patients by isolation of virus from blood or demonstration of an immune response. It seems likely that disease may be more common than at present realized.

Batai virus was first isolated from culicine mosquitoes in Malaysia in 1955. Antigenically related Calovo virus, isolated from anopheline mosquitoes in the former Czechoslovakia, Olyka virus isolated from mosquitoes in the Ukraine, and Chittoor virus isolated from anophelines in India, have all been confirmed to be strains of Batai virus (Briese *et al.*, 2006). The cluster of viruses has been isolated from anopheline and culicine mosquitoes in Malaysia, Thailand, Cambodia, India, former Yugoslavia, Austria, the former USSR and former Czechoslovakia. Recently, an unidentified virus which had been isolated from mosquitoes in Uganda in 1967 was also confirmed to be Batai virus (Briese *et al.*, 2006). Antibody to Batai virus has been found in the above countries, as well as in Sri Lanka, Romania, Hungary, Germany, Portugal and Finland in the sera of humans and/or birds, rodents, domestic ruminants and deer. The findings in seroprevalence studies suggest that human infection is seldom accompanied by overt disease, but febrile illness with malaise, myalgia, anorexia, and sometimes abdominal pain, tonsillitis, cough and dyspnoea (associated with lung infiltration), has been reported from former Czechoslovakia and Malaysia on the basis of serological diagnoses.

Ngari virus has been isolated from mosquitoes in Senegal, Burkina Faso and the Central Africa Republic, as well as from a sheep in Mauritania and the liver of a person who succumbed to cerebral malaria in Senegal. A mosquito isolate of Ngari virus reported from Madagascar may be Batai virus. A virus isolated from the blood of two human patients during the 1997–1998 Rift Valley fever epidemic in north-east Kenya and adjacent Somalia was thought to be a new bunyavirus and given the name Garissa virus, but was subsequently been found to be Ngari virus (Bowen *et al.*, 2001; Nichol, 2003). Later it was established that the nucleotide sequences of the L and M RNA genome segments of Ngari virus correspond to Bunyamwera virus sequences, and it was concluded that Ngari virus represents a reassortant between Bunyamwera virus and an unidentified M segment RNA donor (Gerrard *et al.*, 2004). Recently determined sequence homologies suggest that Batai virus is the most likely donor of the Ngari virus M RNA segment (Briese *et al.*, 2006). There

were many deaths from haemorrhagic disease which could not be confirmed as cases of Rift Valley fever in Kenya and Somalia in 1997–1998, and antibody to Ngari virus was found in a proportion of the patients (Bowen *et al.*, 2001; Gerrard *et al.*, 2004). Moreover, virus which had been isolated from two patients during an outbreak of febrile disease in Sudan in 1988 was recently shown to be Ngari virus, so it appears that Ngari virus could prove to be an important human pathogen.

Ilesha virus has been isolated from the blood of febrile humans in Nigeria, Uganda, Cameroon and the Central African Republic, and from anopheline mosquitoes in the latter country. In addition there is serological evidence that the virus occurs in Senegal and Ghana. Few cases of disease have been reported, and these consisted of undifferentiated febrile illness with a rash.

Shokwe virus has been isolated from mosquitoes, mainly aedines but also from other culicines in South Africa, Senegal, Ivory Coast and Kenya, and from rodents and the blood of a febrile human in Ivory Coast. Little is known of the pathogenic potential of the virus. Germiston virus has been isolated in South Africa, Zimbabwe, Mozambique, Kenya and Uganda from *Culex rubinotus*, a mosquito which selectively feeds on rodents, and from myomorph rodents (rats and mice) in Uganda. Antibody has been found in the sera of humans and/or cattle and rodents in South Africa, Botswana, Namibia and Angola. Two laboratory infections have been reported; one with undifferentiated febrile illness with rash, and the other with signs of mild encephalitis. Virus was isolated from the blood of the patients.

Cache Valley, Maguari, Fort Sherman, Tensaw and Wyeomyia Viruses

Cache Valley virus has been isolated from culicine and anopheline mosquitoes from widely separated locations in the United States and from Jamaica, and antibody has been found in the sera of humans and/or horses, sheep, cattle, wild rodents, raccoons, deer and monkeys in the United States, Canada, Trinidad and Guyana. A single case of fatal aseptic meningitis was diagnosed in a patient in North Carolina in 1995, and in 2003 a second, nonfatal, case of meningitis was diagnosed in Wisconsin, but the occurrence of human disease may be underestimated due to lack of awareness and failure to perform specific tests (Campbell *et al.*, 2006). Serological studies and pathogenicity trials, and the isolation of virus from a sentinel sheep, incriminated Cache Valley virus as the causative agent of an outbreak of congenital abnormalities (hydranencephaly–arthrogryposis syndrome) among sheep in Texas (Chung *et al.*, 1990a, 1990b). Maguari virus has been isolated from mosquitoes, mainly

aedines, in Brazil, Argentina, French Guiana, Colombia and Trinidad, and from horse blood in Guyana and Colombia. Antibody has been found in the same countries as well as in Peru, Surinam and Venezuela in the sera of humans and/or horses, cattle, sheep, water buffalo and birds. Human disease has not been reported, but the virus is suspected of causing disease in horses. Fort Sherman virus was isolated from the blood of a patient with fever, malaise, myalgia and sore throat in Panama, but no further information on the virus is available. Tensaw virus has been isolated from several species of anophelines in south-eastern United States, where antibody has been found in humans, dogs, cattle and raccoons. A single case of encephalitis was reported in 1973. Wyeomyia virus has been isolated from a range of culicine mosquitoes in Colombia, Panama, French Guiana and Trinidad, and antibody has been found in human sera in Panama and Trinidad. The virus has been isolated once from the blood of a patient with febrile illness in Panama.

Bwamba, Pongola and Nyando Viruses

Bwamba virus was originally isolated from blood samples from nine road workers with febrile illness in Uganda, and subsequently from eight febrile patients in Nigeria, three in the Central African Republic and one in Kenya, and from anopheline mosquitoes in Uganda, Nigeria and Senegal. In 1997, a large outbreak of infection with the mosquito-borne o'nyong-nyong virus (*Alphaviridae*) occurred in southern Uganda and adjacent Tanzania, and Bwamba virus was isolated from two patients and from mosquitoes collected during the outbreak, suggesting that Bwamba virus infection may be more common than realized (Lutwama *et al.*, 2002). Antibody has been found in human sera in Uganda, Tanzania, Mozambique, South Africa, Botswana, Angola, Congo, Nigeria and Guinea; generally with very high prevalence, up to 97% in some populations, and including both children and adults. Antibody was also found in donkeys and a bird in South Africa. Bwamba virus appears to be an important pathogen and the eight isolations in the Nigerian series represented 5% of all arbovirus isolations from febrile patients over a seven-year period, while 18 diagnoses (virological and serological) made in similar patients in the Central African Republic represented 25% of arbovirus infections diagnosed over a 13-year period. The patients suffered prostrating illness with fever, headache, conjunctivitis, rash, epigastric pain and myalgia, and many had meningeal signs.

There have been numerous isolations of Pongola virus from mosquitoes, mainly aedines and other culicines, in South Africa, Mozambique, Kenya, Uganda, Ethiopia, Central African Republic and Ivory Coast. There has been one isolation of the virus from a febrile patient with

headache and myalgia in Uganda (Kalunda *et al.*, 1985). Neutralizing antibody has been found in humans in South Africa, Mozambique, Botswana, Namibia and Angola, and in cattle, sheep, goats and donkeys in South Africa, but interpretation of the findings is complicated by the fact that there is unidirectional cross-neutralization of Pongola virus by antibody to its close relative, Bwamba virus. Moreover, the fact that most human isolates have reacted as Bwamba serotype while most mosquito isolates have reacted as Pongola serotype merits further investigation, particularly in view of a report that passage of a Bwamba isolate in mosquitoes led to selection of virus reacting as Pongola serotype (Johnson *et al.*, 1978).

Nyando virus has been isolated from anopheline and aedine mosquitoes in Kenya, Central African Republic and Senegal. Antibody has been found in human sera in Kenya and Uganda, and the virus was isolated from the blood of a single human patient with biphasic fever, myalgia and vomiting in the Central African Republic.

California Encephalitis, La Crosse, Snowshoe hare, Jamestown Canyon and Keystone Viruses

California encephalitis virus, which is distributed across the western United States and into Canada, was isolated from mosquitoes in the early 1940s and shortly thereafter serological evidence was produced to indicate that it causes encephalitis. However, from the mid 1960s onwards it became clear that most cases of what are loosely termed 'California encephalitis' are in fact due to infection with the La Crosse subtype of virus, and this agent is responsible for the majority of the approximately 100 cases of arbovirus encephalitis diagnosed in the United States annually, except in years when there are epidemics of St Louis encephalitis (a flavivirus). It must also be borne in mind that since its introduction into North America in 1999, the mosquito-borne flavivirus West Nile has been responsible for a major proportion of cases of encephalitis recognized each year, particularly in elderly or immunocompromised patients.

Most cases of disease due to La Crosse virus are recorded in the Mid-West states of Wisconsin, Iowa, Indiana, Minnesota and Ohio, but the virus is widely distributed and the infection is probably underdiagnosed elsewhere in the United States. Recent isolation of a distinct lineage of La Crosse virus from mosquitoes in Connecticut suggests that it has long been present in north-eastern United States (Armstrong and Andreadis, 2006). The principal vector of the virus is *Aedes triseriatus*, a tree-hole breeding mosquito, and accordingly the virus tends to be focally distributed in woodlands, but also occurs in suburban situations where water that collects in discarded containers such as motor vehicle tyres, affords mosquito breeding sites. The virus is passed transovarially in the

vector and overwinters in mosquito eggs; infection is amplified in the succeeding spring and summer in small mammals such as chipmunks and squirrels. The vector is a diurnal feeder and the infection is seen most commonly in forest workers, and children who enter woodlands for recreational purposes, but also occurs focally in rural and suburban residents. Males are more commonly infected than females, among both children and adults. Seroprevalence surveys and prospective studies indicate that most infections are inapparent or benign. Probably less than 1% of infected adults develop encephalitis, but the incidence may be up to four times greater in young children.

After an incubation period of three to seven days there is sudden onset of fever, headache, lethargy, nausea and vomiting, pharyngitis and sometimes respiratory illness. There is seldom a cutaneous rash. In mild cases of overt disease there may be transient meningismus and disorientation, and recovery within one week. In severe disease there may be greater disturbance of consciousness, aphasia, tremors, chorea, positive Babinski signs and other abnormal reflexes, and hemiparesis in about 20% of patients. Seizures may occur from the second day of illness onwards: in about half of severely ill patients there may be generalized, life-threatening convulsions, and in a further 25% there are focal convulsions associated with frontal or parietal brain lesions. Approximately one third of severely ill patients become comatose. There may be marked leukocytosis, and examination of cerebrospinal fluid (CSF) reveals elevated mononuclear and polymorphonuclear cell counts, but protein and glucose levels tend to remain normal. Electroencephalograms show generalized slow-wave activity or localized changes and sometimes epileptiform discharges. Treatment is symptomatic and includes monitoring and control of intracranial pressure, and vigorous anticonvulsant therapy as indicated. Less than 1% of patients with severe disease succumb and most are discharged from hospital after about two weeks of illness, but may remain irritable and emotionally labile for a few weeks. There are seldom residua, but patients who suffer seizures in the acute illness may have recurrent convulsions over a period of one or more years, and lasting hemiparesis occurs in about 1% of patients. Virus has not been isolated from throat swabs, blood, stools or CSF, and only with difficulty from brain specimens. Histopathological lesions are not pathognomonic and include cerebral oedema, perivascular cuffing and focal gliosis in grey matter. A variety of methods are used for making a serological diagnosis, but demonstration of IgM antibody activity by means of enzyme-linked immunoassay holds greatest promise as a rapid diagnostic technique. There is no vaccine.

Snowshoe hare is a mosquito-borne subtype of California encephalitis virus with a distribution extending from

north-western United States, across most of Canada to Alaska. The natural host appears to be the snowshoe hare, *Lepus americanus*, and serological evidence suggests that the virus is occasionally associated with encephalitis in children and adults.

Jamestown Canyon is a mosquito-borne virus which occurs widely in the United States, and high prevalence rates of antibody are found in white-tailed deer, and in humans where there are high concentrations of the deer. Serological evidence of an association with encephalitis in humans has been found from the early 1980s onwards (Grimstad *et al.*, 1986). Since antigens and tests commonly used for the serodiagnosis of 'California encephalitis' fail to allow response to Jamestown Canyon virus to be distinguished from response to other members of the serogroup, it is felt that the infection has probably been missed or underdiagnosed as a cause of encephalitis in the past, both in the Mid-West states and elsewhere where the deer occurs. In contrast to La Crosse virus, Jamestown Canyon appears to cause encephalitis more frequently in adults than in children, and nervous disease is often preceded by respiratory illness. Keystone virus is associated with swamp-breeding aedines and cotton rats and rabbits in south-eastern United States, and has been known to cause inapparent accidental infection in the laboratory.

Tahyna and Inkoo Viruses

Tahyna virus is widely distributed in countries of central Europe, including Yugoslavia, Germany and Italy, with antibody prevalence rates being particularly high in the Rhone Valley of France, the Danube basin in Austria, and in the southern Moravia region of the former Czechoslovakia, where up to 95% of adults may be immune in some communities. Viruses described as being Tahyna-like have been isolated in the former USSR, while Lumbo virus, which was isolated from saltwater-breeding mosquitoes on the coast of Mozambique, is considered to be indistinguishable from Tahyna virus. Antibody to Tahyna virus has also been found in southern China and Sri Lanka. It is not yet clear whether a single virus occurs throughout this range or whether, as seems more likely, a cluster of closely related viruses, subtypes or varieties is involved.

The epidemiology of the disease has been studied most intensively in Moravia where seasonal flooding of level woodlands provides extensive breeding sites for mosquitoes. The virus is transmitted transovarially in *Aedes vexans* which overwinters as eggs, and in *Culiseta annulata* which overwinters as larvae. Amplification of infection in spring occurs in small mammals such as hedgehogs, hares and rabbits, as well as in domestic animals such as horses. Hedgehogs may themselves serve as reservoir hosts for overwintering of virus in instances

where they undergo chronic infection during hibernation, with viraemia which persists for a few days after awakening. Once infection of vertebrates occurs in summer other species of mosquito also become infected and serve as vectors for transmission of the virus.

Seroprevalence surveys indicate that infection is much more common than overt disease in rural residents of Moravia. Nevertheless, the infection accounts for up to 20% of patients hospitalized with febrile illness in the region, including both adults and children. Patients may present with undifferentiated febrile illness with leukocytosis, but pharyngitis, cough and chest pain, and infiltration (demonstrable by X-ray imaging) may predominate, or gastrointestinal symptoms such as nausea, vomiting and abdominal pain may be dominant. Aseptic meningitis occurs in a minority of patients but fatal disease is unknown. Virus can be isolated from blood early in the illness, but the infection is usually diagnosed by demonstration of an antibody response. There is no vaccine.

Inkoo virus was isolated from aedine mosquitoes in Finland. Distribution of the virus extends to the Lapland region in the north of the country, and antibody occurs in the sera of humans, cattle, deer and hares. Antibody prevalence rates of up to 25% have been recorded in humans, and a few cases of febrile illness have been confirmed serologically.

Oropouche Virus

Oropouche virus was originally isolated from the blood of a febrile patient in Trinidad, but large epidemics involving thousands of people have occurred almost exclusively in northern Brazil over the past 20 years, with the most recent outbreaks being recorded in 2003 and 2004 (Azevedo *et al.*, 2007). In addition to humans, the virus has been isolated from a sloth, a few species of culicine mosquitoes plus the midge *Culicoides paraensis*, while antibody has been found in the sera of humans and/or monkeys and birds, in Brazil, Trinidad, Panama and Colombia. Virus is thought to be maintained in nature by circulation in forest primates, sloths or birds and an unidentified vector, and is introduced into urban settings by infected travellers or by extension from the sylvatic cycle. Humans serve as amplifier hosts in the urban cycle, and the vector is the *Culicoides* midge which breeds in decaying waste from tropical agricultural products. Epidemics occur when there are large concentrations of vectors and susceptible humans. Aerosol infection is suspected to have occurred in laboratory workers. The incubation period is four to eight days, and there is sudden onset of fever, chills, headache, myalgia, arthralgia and prostration. There may be a rash, and occasionally signs of meningitis or encephalitis, but there are no deaths or sequelae. Viraemia lasts from two to five days, as does illness, but myalgia persists for a

further three to five days, and strenuous exertion in early convalescence can precipitate a relapse of symptoms.

Caraparu, Apeu, Ossa, Madrid, Marituba, Murutucu, Nepuyo, Restan, Oriboca and Itaqui Viruses

These viruses occur in Central and South America and tend to be associated with tropical forests. They have all been isolated from the blood of febrile humans, and variously from sentinel monkeys, rodents, occasional marsupials and fruit bats, and from a range of culicine mosquitoes in Brazil, Surinam, French Guiana, Guatemala, Honduras, Trinidad, Panama or Mexico. Serological evidence suggests that some of the viruses may also occur in Venezuela, Colombia and Peru. Pairs of the viruses which are closely related antigenically may circulate in the same geographic location yet occupy separate ecologic niches. For instance, one virus circulates in arboreal monkeys and mosquitoes which feed in the canopy layer, while a closely related virus circulates in rodents and mosquitoes which feed at the level of the forest floor. Other pairs of closely related viruses coexist in the same habitat simply by utilizing different vectors. Sporadic infections occur in people who enter forests. No large outbreaks of disease have been reported, but disease is observed when susceptible outsiders such as military personnel enter endemic regions. Laboratory infections are relatively common. Disease, which lasts for up to a week and runs a benign course, is characterized by fever, rigors, headache, photophobia, conjunctivitis, tachycardia, myalgia, arthralgia, prostration, leukopenia and occasionally pain in the right upper quadrant of the abdomen and jaundice.

Guaroa, Guama, Catu, Tacaiuma and Turlock Viruses

Guaroa virus has been isolated from 12 febrile patients in Colombia and the Amazon region of Brazil, and from anopheline mosquitoes in Panama and Colombia. Antibody has been found in human sera in Colombia, Brazil, Argentina and Peru. Disease ascribed to infection with the virus was characterized by fever, headache, myalgia, arthralgia, prostration and leukopenia. Virus was isolated from the blood of the patients, and from a liver biopsy in one instance.

Catu virus has been isolated from sentinel monkeys in Brazil, febrile humans and forest rodents in Brazil and Trinidad, and from culicine and anopheline mosquitoes in the same two countries plus French Guiana. Guama virus has been isolated from febrile humans and sentinel monkeys in Brazil, and from rodents and/or culicine mosquitoes in Brazil, Trinidad, Surinam, French Guiana and Panama. The two viruses cause isolated cases of benign disease characterized by fever, headache, myalgia

and leukopenia, in people who enter tropical forests. Catu has also been associated with laboratory infection.

Tacaiuma virus was isolated from the blood of a sentinel monkey and from forest mosquitoes in the Amazon region of Brazil, as well as from mosquitoes in Argentina. Antibody has been found in humans in Brazil and French Guiana, and in horses, rodents, bats and birds in Brazil. The virus has been isolated once from the blood of a patient with febrile illness in Brazil.

Turlock virus was first isolated from *Culex tarsalis* mosquitoes in California in 1954. In 1995, the virus was isolated from the brain of an ostrich chick with ataxia in California, and antibody was found in a proportion of ostrich sera, but there has been no further reported association of the virus with disease in vertebrates (Shivaprasad *et al.*, 2002). There were slight quantitative differences in cross-neutralization tests between the ostrich isolate and prototype virus.

Akabane, Aino, Tinaroo and Shuni Viruses

Most members of the Simbu serogroup do not occur in the Americas, but are widely distributed in Africa, Asia and Australasia. Of these latter viruses, only Shuni has been marginally implicated in causing human disease, but a few have been incriminated as causative agents in large outbreaks of abortion, stillbirth and congenital defects (hydranencephaly–arthrogryposis syndrome) in domestic ruminants, particularly sheep and cattle, and the suspicion exists that most members of the serogroup have the potential for producing this type of disease. Akabane has been incriminated in outbreaks of the disease in Japan, Australia and Israel, Aino in Japan and Australia, Tinaroo in Australia, and Peaton virus has been shown to be teratogenic in experimental infections in Australia. Most recently, Akabane virus was incriminated on serological grounds of association with an outbreak of teratology in cattle in Israel in 2002, and the virus was isolated from mosquitoes collected in Vietnam in 2002–2004 (Brenner *et al.*, 2004; Bryant *et al.*, 2005). The main vectors of these viruses are believed to be species of *Culicoides* midges which breed in dung, independently of available surface water, but which are favoured by the humid conditions created by heavy rainfall. The natural hosts of the viruses are unknown, but antibodies have been found in wild herbivores. Infection is usually inapparent in domestic ruminants. However, if infection occurs in pregnant animals at critical stages of gestation, there may be embryonal or fetal death, or arrest of brain development (hydranencephaly), and the consequent lack of trophic effect of nervous stimulation on skeletal muscles of the fetus results in postural defects of the limbs, with joints locked in flexion (arthrogryposis). Once the stage of organogenesis in gestation is passed, fetuses are less susceptible to

the harmful effects of infection (the timing varies with length of gestation in different species).

Shuni virus has been isolated from cattle, sheep, midges and once from the blood of a febrile human in Nigeria, and from cattle and mosquitoes in South Africa. It was also isolated from the brain of a horse with histopathological lesions of meningoencephalitis, which was submitted for laboratory examination for suspected rabies in Zimbabwe (Foggin and Swanepoel, unpublished).

GENUS PHLEBOVIRUS

Sandfly Fever Naples, Sandfly Fever Sicilian and Toscana Viruses

Sandfly fever (also known as phlebotomus fever or papataci fever from the vector, *Phlebotomus papatasi*) has been known for at least two centuries as a febrile illness encountered by armies invading the Mediterranean basin. It was demonstrated shortly after the start of the twentieth century that the disease was caused by a virus transmitted by sandflies, but it was only during the Second World War that it was shown that there are in fact two distinct viruses and that there is no cross-immunity. Between them, the two viruses are known to occur in Morocco, Tunisia, Egypt, Sudan, Somalia, Italy, Greece, former Yugoslavia, Turkey, the former USSR, Israel, Saudi Arabia, Iraq, Iran, Pakistan, India and Bangladesh, and are probably present in many intervening countries. Throughout this range *P. papatasi* is the vector, and it breeds in moist soil in dark niches such as in rubble, drains, cracks in soil and in animal burrows, equally successfully in large cities as in remote rural locations. The viruses are transmitted transovarially in the vector, which overwinters in the larval stage, and adult flies emerge to assume biting activity in summer. High infection rates have been recorded in newly emerged sandflies and it is not known whether amplification in vertebrates is essential to ensure perpetuation of the viruses, but humans develop sufficiently intense viraemia to serve as source for the infection of the vector. No wild vertebrate hosts of the viruses are known, but antibody has been found in gerbils (whose burrows are utilized by the vector). Sandflies are nocturnal feeders, but will feed in dark rooms where they rest during daylight hours. Human infection rates recorded in outbreaks range from 3 to 75%, but the attack rate varies focally and is influenced by background immunity in the human population. Large epidemics have often occurred in association with socio-economic upheavals, wars or natural disasters such as earthquakes which create ideal breeding conditions for sandflies and/or lead to widespread exposure of susceptible humans to sandflies.

Experimental evidence suggests that most sandfly fever infections are symptomatic. Typically, there is sudden onset of fever of two to four days duration, severe headache, sore eyes and photophobia, myalgia, arthralgia, anorexia and malaise. Occasionally there may be sore throat, nausea and vomiting, abdominal pain and diarrhoea, epistaxis and dizziness. Patients may have injected conjunctivae and a flushed appearance, but there is seldom a rash, and meningeal signs are rare. The disease may be milder in children. Treatment is symptomatic. Recovery is complete and no deaths have been recorded. There appears to be lifelong immunity to the homologous virus. The diagnosis can be confirmed by detection of viral nucleic acid by RT-PCR or isolation of virus from blood taken in acute illness (Liu *et al.*, 2003; Weidmann *et al.*, 2008), or by demonstration of IgM antibody activity, or rising antibody titres in convalescence. Prevention of infection includes the use of insect repellants, but the treatment of walls (sandfly resting sites) with residual insecticides is highly effective.

Toscana virus was first isolated in 1971 in Tuscany, Italy, from *Phlebotomus perniciosus*, a sandfly which breeds in forest litter. Antibody was found to be common in the sera of rural and suburban residents of the region, and an association was established between the occurrence of aseptic meningitis and serological evidence of infection with Toscana virus. Since then the virus has come to be recognized as a major cause of meningitis and encephalitis each year in summer months in the Mediterranean region, including parts of Italy, France, Spain, Portugal and Greece (Charrel *et al.*, 2005; De Lamballerie *et al.*, 2007; Peyrefitte *et al.*, 2005). The virus is transmitted transovarially in the vector, *P. perniciosus*, which is widely distributed in Europe. No vertebrate maintenance host has been identified, but antibody has been found in rodent sera and the virus has been isolated from an insectivorous bat. The disease can sometimes be diagnosed by isolation of virus from CSF in acute illness, but usually by demonstrating IgM antibody activity or rising antibody titres in sera taken during convalescence.

Alenquer, Candiru, Punta Toro and Chagres Viruses

Alenquer and Candiru viruses were isolated from the blood of febrile patients in the Amazon region of Brazil, but otherwise little is known of their biology, and it is surmised that they are transmitted by phlebotomid flies. Punta Toro and Chagres viruses were isolated from febrile patients and from phlebotomids (*Lutzomyia* spp.) in Panama. These two viruses are known to be transmitted transovarially in phlebotomids, and antibodies to them have been found in primates, sloths, porcupines and other rodents. Disease thus far associated with all four viruses fits the description of classical sandfly fever, with the

difference that epidemics are unknown and only isolated cases have been seen in people who entered tropical forests for occupational or recreational purposes. Primers have been developed for use in RT-PCR for diagnosis of infection with these viruses (Liu *et al.*, 2003).

Rift Valley Fever Virus

The literature on Rift Valley fever has been reviewed extensively (Easterday, 1965; Flick and Bouloy, 2005; Henning, 1956; Meegan and Bailey, 1989; Peters and Meegan, 1981; Shimshony and Barzilai, 1983; Swanepoel and Coetzer, 2003; Weiss, 1957). It is an acute disease of domestic ruminants in mainland Africa and Madagascar, caused by a mosquito-borne virus and characterized by necrotic hepatitis and a haemorrhagic state, but infections are frequently inapparent or mild. Large outbreaks of the disease in sheep, cattle and goats are distinguished by heavy mortality among newborn animals and abortion in pregnant animals. Humans become infected from contact with tissues of infected animals or from mosquito bite, and usually develop mild to moderately severe febrile illness, but severe complications occur in a small proportion of patients. The virus recently escaped from the African region to cause a major outbreak of disease on the Arabian Peninsula in 2000–2001.

The disease was first recognized in the Rift Valley in Kenya early in the twentieth century, but the causative agent was not isolated until 1930. Since then large outbreaks of the disease have been recorded in Kenya, South Africa, Namibia, Mozambique, Zimbabwe, Zambia, Sudan, Egypt, Mauritania and Senegal, while lesser outbreaks, periodic isolations of virus or serological evidence of infection have been recorded in Angola, Botswana, Burkina Faso, Cameroon, Central African Republic, Chad, Gabon, Guinea, Madagascar, Malawi, Mali, Nigeria, Somalia, Tanzania, Uganda and Democratic Republic of the Congo. Epidemics may be extremely severe and, for example, it is estimated that 500 000 ewes aborted and a further 100 000 sheep died in the first outbreak of the disease to be recognized in South Africa in 1950–1951.

Prior to the 1970s, epidemics were seen only in eastern and southern Africa, where they tend to occur at irregular intervals of 5–15 years or longer when above-average rainfall favours the breeding of the mosquito vectors. Meteorological conditions conducive to the occurrence of epidemics usually prevail over large tracts of Africa, so there has been some tendency for outbreaks in adjacent territories such as Zimbabwe and Mozambique, Kenya and Tanzania, or South Africa and Namibia to coincide. The fate of the virus during inter-epidemic periods was unknown for decades, but on the basis of observations made in Uganda, Kenya and South Africa, it was widely accepted that the virus was endemic in indigenous forests

which extend in broken fashion from East Africa to the coastal regions of South Africa. The virus was thought to circulate in *Eretmapodites* spp. mosquitoes and unknown vertebrates in the forests, and to spread in seasons of exceptionally heavy rainfall to livestock-rearing areas where the vectors were believed to be floodwater-breeding aedine mosquitoes of the subgenera *Aedimorphus* and *Neomelaniconion*, which attach their eggs to vegetation at the edge of stagnant surface water. In contrast to other culicine mosquitoes, it is obligatory for the eggs of aedines to be subjected to a period of drying as the water recedes before they will hatch on being wetted again when next the area floods. Thus, the aedine mosquitoes overwinter as eggs which can survive for long periods in dried mud, possibly for several seasons if the area remains dry.

On the inland plateau of South Africa, where sheep rearing predominates, surface water gathers after heavy rains in undrained shallow depressions (pans) and farm dams which afford ideal breeding environments for aedines. On the watershed plateau of Zimbabwe, where cattle farming predominates, aedines breed in vleis, low-lying grassy areas which constitute drainage channels for surrounding high ground, and which are flooded by seepage after heavy rains. Vleis correspond to what are termed dambos in the livestock-rearing areas of central and eastern Africa. Sustained monitoring in Zimbabwe revealed that a low level of virus transmission to livestock occurred each year in the same areas where epidemics occurred. The generation of epidemics, therefore, was associated with the simultaneous intensification of virus activity over vast livestock-rearing areas where it was already present, rather than lateral spread from cryptic endemic foci. Comparison of the distributions of canopy forests and vleis in Zimbabwe, plotted from satellite images and aerial photographs, with the distribution determined for endemic Rift Valley fever, revealed remarkable overlap between the endemic areas and areas where vleis were common.

A major advance in the understanding of the epidemiology of the disease was made when the virus was isolated from unfed *Aedes mcintoshi* mosquitoes (= *Aedes lineatopennis* sensu lato) hatched in dambos on a ranch in Kenya during inter-epidemic periods in 1982 and 1984, confirming that the virus is endemic in livestock-rearing areas and indicating that it appears to be maintained by transovarial transmission in aedines. The available evidence suggests that in Zimbabwe, as in Kenya, *A. mcintoshi* is the most important maintenance vector of the virus while *Aedes dentatus* is probably also a maintenance vector; the same two species and possibly *Aedes unidentatus* and *Aedes juppi* are maintenance vectors on the inland plateau of South Africa. Heavy rainfall

and the humid conditions that prevail during epidemics favour the breeding of other biting insects besides aedine mosquitoes. Following extensive flooding of aedine breeding sites, significant numbers of livestock become infected and circulate high levels of virus in their blood during the acute stage of infection. Other culicines and anopheline mosquitoes then become infected and serve as epidemic vectors, particularly *Culex theileri* in southern Africa, and biting flies such as midges, phlebotomids, stomoxids and simuliids serve as mechanical transmitters of infection. Although contagion has been demonstrated on occasion under artificial conditions, nonvectorial transmission is not considered to be important in livestock, as opposed to humans. Epidemics generally become evident in late summer after there has been an initial increase in vector populations and in circulation of virus, and terminate in late autumn when the onset of cold weather depresses vector activity, or when most animals are immune following natural infection, or after there has been successful intervention with vaccine.

Antibody surveys and laboratory studies have failed to prove that the virus is maintained in transmission cycles in rodents, birds, monkeys, baboons or other wild vertebrates, although it is felt that wild ruminants could play a role similar to their domestic counterparts in areas where they predominate. It is also believed that the possibility of endemicity of the virus in forests cannot be dismissed entirely, and merits further investigation.

It was recognized from the time of the original investigations in Kenya that febrile illness in humans accompanied outbreaks of disease in livestock, and that some patients experienced transient loss of visual acuity, but the occurrence of serious ocular sequelae was first reported in the 1950–1951 epidemic in South Africa. Human deaths following natural infection were first recorded in South Africa during the epidemic of 1974–1976 when seven patients are known to have died of encephalitis and haemorrhagic fever associated with necrotic hepatitis. Subsequently deaths were also observed in Zimbabwe.

Outbreaks of Rift Valley fever were reported in the Sudan in 1973 and 1976. In 1977 and 1978 a major epidemic occurred along the Nile delta and valley in Egypt, causing an unprecedented number of human infections and deaths, as well as numerous deaths and abortions in sheep and cattle and some losses in goats, water buffaloes and camels. Estimates of the number of human cases of disease range from 18 000 to more than 200 000 with at least 598 deaths occurring from encephalitis and/or haemorrhagic fever. A severe epidemic occurred in 1987 in the Senegal River basin of southern Mauritania and northern Senegal. In Mauritania alone an estimated 224 human patients died of the disease, and there was a high rate of abortion in sheep and goats. Further outbreaks of Rift

Valley fever occurred in Mauritania in 1993 and 1998 with smaller numbers of human casualties, while a minor outbreak of disease in livestock occurred in Senegal in 1994–1995.

The outbreaks of Rift Valley fever which occurred in North and West Africa differed in many respects from the pattern of disease which had hitherto been observed in sub-Saharan Africa; in particular they occurred independently of rainfall in arid countries, apparently in association with vectors which breed in large rivers and dams. The presence of the virus in the Sudan and certain West African countries had long been known from antibody studies, and there had been periodic isolations of the virus in West Africa, where it was sometimes reported as Zinga virus, which is now known to be identical to Rift Valley fever virus. Various theories were advanced to account for the first known appearance of the virus in Egypt in 1977, including the carriage of infected mosquitoes from the Sudan at high altitude by prevailing winds associated with the intertropical convergence zone. The introduction of the virus through the transportation of infected sheep and cattle on the Nile or overland from northern Sudan to markets in southern Egypt was considered to have been the strongest possibility, and the movement of slaughter animals by sea could account for the evidence of infection detected in the northern and eastern coastal areas of Egypt. Although transportation on some routes would take a long time in relation to the course of the infection, Rift Valley fever virus has been shown to persist for prolonged periods in various organs of sheep, particularly the spleen, for up to 21 days after infection. The same could be true for goats and cattle, or even the camels brought in by overland caravan routes. It is believed that humans slaughtering or handling the tissues of such animals could have become infected and served as the amplifying hosts for the infection of mosquitoes since the main vector in the Egyptian epidemic, *Culex pipiens*, is known to be peridomestic and anthropophilic. In at least one instance there were indications that human infections centred on a location where introduced camels were slaughtered.

The occurrence of the epidemic in Egypt raised the spectre that Rift Valley fever could be introduced to the mainland of Eurasia, and the possibility was underscored by the fact that the virus is apparently capable of utilizing a wide range of mosquitoes as vectors. Extensive preventive vaccination of livestock was undertaken at the time in the Sinai peninsula and Israel. However, only isolated outbreaks of Rift Valley fever were recorded in Egypt in 1979 and 1980, and thereafter the country remained free of the disease for 12 years until ocular complications of the infection in humans, and abortions in cattle and water buffalo, were noted in the Aswan Governate in

May 1993. On this occasion there was not the same tendency for an explosive outbreak of the disease to occur as in 1977–1978, but by October 1993 infections of humans and livestock, including sheep, had been recognized across the length of the country in Sharqiya, Giza and El Faiyum Governates, and further infections were observed in 1994 (Arthur *et al.*, 1993; Botros *et al.*, 1997; World Health Organization, 1993, 1994).

From late October 1997 to February 1998, a large outbreak of Rift Valley fever occurred in north-eastern Kenya and adjoining southern Somalia, following the occurrence of heavy rains and extensive flooding in what is essentially an arid area where people had been receiving food relief owing to the extreme drought conditions that had prevailed in the preceding two years (Woods *et al.*, 2002; World Health Organization, 1998). There were heavy losses of livestock and an estimated 500 human deaths, but Rift Valley fever could not be confirmed in all instances. An agent isolated from human blood was thought to be a new bunyavirus and given the name Garissa virus, but was later found to be Ngari virus, originally isolated from mosquitoes in West Africa and recently shown to be a reassortant bunyavirus (Bowen *et al.*, 2001; Briese *et al.*, 2006; Gerrard *et al.*, 2004; Nichol, 2003). Antibody to Ngari virus was found in people in both Kenya and Somalia, but the importance of the virus as a human pathogen remains to be determined; many of the deaths could have been due to the appearance of malaria in an area not normally affected by this disease. It was subsequently established that extensive outbreaks of Rift Valley fever had also occurred elsewhere in Kenya and northern Tanzania following heavy rains in the region, and a few human deaths in southern Kenya were also ascribed to the disease. An outbreak of Rift Valley fever was again recognized in the North-Eastern Province of Kenya in November 2006 following the occurrence of heavy rains, and by the end of January 2007 it had also appeared in the Coastal, Central, Rift Valley and Eastern Provinces. A total of 684 human cases of the disease were recorded, with a 20% death rate. The disease also occurred in neighbouring Somalia with 114 cases and a 45% death rate being reported. Outbreaks were recognized in Tanzania in January 2007, but investigations revealed that livestock and human disease had occurred in late 2006, with a total of 191 cases and a 21% death rate being recorded. Following heavy rains an outbreak of Rift Valley fever occurred in October–November 2007 in White Nile, Gezira and Senar Provinces of Sudan, with 451 cases of the disease and an approximately 36% death rate being reported (World Health Organization, 2007a, 2007b). The high death rates reported in these outbreaks were estimated from cases which were diagnosed mainly on clinical grounds, without reference to mild or inapparent infections.

In September, 2000, Rift Valley fever broke out simultaneously in Jizan Province in south-west Saudi Arabia and in adjoining Yemen (Abdo-Salem *et al.*, 2006; Al Hazmi *et al.*, 2003; Madani *et al.*, 2003). The outbreaks lasted until early 2001, and resulted in 245 human deaths and the loss of thousands of sheep and goats. There had been heavy rains in the inland mountain range which runs parallel to the coast, with drainage from the mountains resulting in the creation of ideal mosquito breeding habitats (Jupp *et al.*, 2002). There was speculation that the virus may have been imported from Africa with slaughter animals, or carried from Africa by wind-borne mosquitoes in 2000, but there were no known epidemics in the Horn of Africa at the time. It is much more likely that infected animals were imported during the 1997–1998 epidemic in East Africa, and that infection had smouldered on the Arabian Peninsula until ideal circumstances for an epidemic occurred following heavy rains in 2000. Recent detection of IgM antibody in sentinel sheep suggests that the virus may have become endemic on the Arabian Peninsula (Elfadil *et al.*, 2006).

In contrast to the main vector in the Egyptian epidemic of 1977–1978, the principal mosquito vectors of Rift Valley fever virus in sub-Saharan Africa tend to be zoophilic and sylvatic, with the result that humans become infected mainly from contact with animal tissues, although there are instances where no such history can be obtained and it must be assumed that infection has resulted from mosquito bite. Occasional infections diagnosed in tourists from abroad who visited countries in Africa are also thought to have resulted from mosquito bite. Generally, people who become infected are involved in the livestock industry, such as farmers who assist in dystocia of livestock, farm labourers who salvage carcasses for human consumption, veterinarians and their assistants, and abattoir workers. There are numerous reports of humans becoming infected while investigating the disease in the field or laboratory, and the first known human fatality was recorded in 1934 in a laboratory worker, but since the infection was complicated by thrombophlebitis and the patient died from pulmonary embolism, the potential lethality of the virus for humans was overlooked until fatal infections were recognized during the 1974–1976 epidemic in South Africa. The results of surveys following epidemics in southern Africa indicated that 9–15% of farm residents became infected, with a slight pre-ponderance of adult males, although it appeared that housewives also gained infection from handling fresh meat.

No outbreaks of the disease have been recognized in urban consumer populations and it is surmised that the fall in pH associated with the maturation of meat in abattoirs is deleterious to the virus. Moreover, highest infection rates were found in workers in the by-products

sections of abattoirs in Zimbabwe and the implication is that the carcasses of infected animals which reach abattoirs are generally recognized as being diseased and are condemned as unfit for human consumption, and are then sterilized in the process of preparing carcase meal for fertilizers.

Human infection presumably results from contact of virus with abraded skin, wounds or mucous membranes, but aerosol and intranasal infection have been demonstrated experimentally and circumstantial evidence suggests that aerosols have been involved in some human infections in the laboratory, and in the field during the Egyptian epidemic. Many infections in Egypt are thought to have resulted from the slaughter of infected animals outside of abattoirs, and the fact that the mosquito vector was anthropophilic is thought to explain the high incidence of infection which occurred in people of all ages and diverse occupations. Low concentrations of virus have been found in milk and body fluids such as saliva and nasal discharges of sheep and cattle, and it appears that there may have been a connection between human infection and consumption of raw milk in Mauritania. In view of the intense viraemia which occurs in humans and the fact that virus has been isolated from throat washings, it is curious that there are no records of person-to-person transmission of infection.

Despite the sudden and dramatic change perceived in the nature of the human disease in the mid 1970s, it was deduced from the 598 reported deaths and 200 000 estimated cases of disease that Rift Valley fever had a case fatality rate of less than 1% in Egypt where a high prevalence of schistosomiasis may have predisposed the population to severe liver disease. The fatality rate may even have been lower in relation to total infections, since an antibody prevalence rate of 30% was detected and the human population estimated at 1 to 3 million in the areas affected by the epidemic. On the other hand, remarkably high estimates of 5 and 14% were made for case fatality rates in two separate populations in the 1987 epidemic in Mauritania, on the basis of the proportion of IgM antibody-positive people who actually reported illness considered to be compatible with Rift Valley fever, but it can be deduced that the fatality rates in terms of total IgM antibody-positive patients are much closer to the corresponding fatality rate in Egypt.

The majority of Rift Valley fever infections in humans are inapparent or associated with moderate to severe, non-fatal, febrile illness. After an incubation period of two to six days, the onset of the benign illness is usually very sudden and the disease is characterized by rigor, fever that persists for several days and is often biphasic, headache with retro-orbital pain and photophobia, weakness, and muscle and joint pains. Sometimes there is

nausea and vomiting, abdominal pain, vertigo, epistaxis and a petechial rash. Defervescence and symptomatic improvement occur in four to seven days in benign disease and recovery is often complete in two weeks, but in a minority of patients the disease is complicated by the development of ocular lesions at the time of the initial illness or up to four weeks later. Estimates for the incidence of ocular complications range from less than 1 to 20% of human infections, and possibly the differences stem from failure to record mild cases in populations where illiterate persons are less likely to report minor disturbances of vision. The ocular disease usually presents as a loss of acuity of central vision, sometimes with development of scotomas. The essential lesion appears to be focal retinal ischaemia, generally in the macular or paramacular area, associated with thrombotic occlusion of arterioles and capillaries, and is characterized by retinal oedema and loss of transparency caused by dense white exudate and haemorrhages. Sometimes there is severe haemorrhage and detachment of the retina. The lesions and the loss of visual acuity generally resolve over a period of months with variable residual scarring of the retina, but in instances of severe haemorrhage and detachment of the retina there may be permanent uni- or bilateral blindness.

Probably less than 1% of human patients develop the haemorrhagic and/or encephalitic forms of the disease. Underlying liver disease may predispose to the haemorrhagic form of the illness. The haemorrhagic syndrome starts with sudden onset of febrile illness similar to the benign disease, but within two to four days there may be development of a petechial rash, purpura, ecchymoses and extensive subcutaneous haemorrhages, bleeding from needle puncture sites, epistaxis, haematemesis, diarrhoea and melaena, sore and inflamed throat, gingival bleeding, epigastric pain, hepatomegaly or hepatosplenomegaly, tenderness of the right upper quadrant of the abdomen and deep jaundice. This is followed by pneumonitis, anaemia, shock with racing pulse and low blood pressure, hepatorenal failure, coma and cardiorespiratory arrest. Factors contributing to fatal outcome in the hepatic form of the disease include anaemia, shock and hepatorenal failure, with the kidney lesions possibly being as important as shock in producing anuria. A proportion of the less severely affected patients may make a protracted recovery without sequelae.

Encephalitis may occur in combination with the haemorrhagic syndrome. Otherwise, signs of encephalitis in humans may supervene during the acute illness, or up to four weeks later and include severe headache, vertigo, confusion, disorientation, amnesia, meningismus, hallucinations, hypersalivation, grinding of teeth, choreiform movements, convulsions, hemiparesis, lethargy, decerebrate posturing, locked-in syndrome, coma and death. A

proportion of patients may recover completely, but others may be left with sequelae, such as hemiparesis.

Abortion is the usual, if not invariable, outcome to infection of pregnant ruminants, but an attempt to relate the occurrence of abortion in humans to evidence of Rift Valley fever infection in Egypt produced inconclusive results. Diagnosis of the infection in a neonatal child in Saudi Arabia in 2000 implied that there had been vertical transmission of infection, but the mother was not tested (Arishi *et al.*, 2006).

By analogy with the course of events believed to follow natural infection with other arthropod-borne viruses, it can be surmised that the pathogenesis of the disease may involve some replication of virus at the site of inoculation, conveyance of infection by lymphatic drainage to regional lymph nodes where there is further replication with spillover of virus into the circulation to produce primary viraemia, which in turn leads to systemic infection, and that intense viraemia then results from release of virus following replication in major target organs. Wild Rift Valley fever virus, which has not been subjected to serial passaging in laboratory host systems, is described as being hepato-, viscer- or pantropic, and immunofluorescence studies in laboratory animals indicate that replication occurs in littoral macrophages of lymph nodes, most areas of the spleen except T-dependent peri-arteriolar sheaths, foci of adrenocortical cells, virtually all cells of the liver, most renal glomeruli and some tubules, lung tissue and scattered small vessel walls, as well as in necrotic foci in the brains of individuals which develop the encephalitic form of the disease. These sites correspond to the lymphoid necrosis in lymph nodes and spleen, hepatic necrosis and adrenal, lung and glomerular lesions seen in humans and livestock, and the brain lesions in humans (encephalitis has not been described in natural disease of ruminants). Titration of infectivity in organ homogenates indicates that the liver and spleen are the major sites of virus replication. Cell damage is ascribed directly to the lytic effects of the virus, but the inflammatory response seen in human brain tissue suggests that there may also be an immunopathological element to the pathogenesis of encephalitis. The same may be true for ocular lesions. Recovery is mediated by nonspecific and specific host responses, and the clearance of viraemia correlates with the appearance of neutralizing antibody. No significant antigenic differences have been detected between isolates of the virus, although differences in pathogenicity for laboratory rodents have been demonstrated, and immunity appears to be lifelong.

The haemostatic derangement that occurs in Rift Valley fever has been investigated in rhesus monkeys, but the mechanisms involved remain speculative. Impairment of coagulation occurs even in benign infection of monkeys,

and moderate thrombocytopenia has been observed in benign infection in sheep, but haemostatic derangement is most severe in the fatal hepatic syndrome. It is postulated that the critical lesions are vasculitis and hepatic necrosis. Destruction of the antithrombotic properties of endothelial cells is thought to trigger intravascular coagulation, and the widespread necrosis of hepatocytes and other affected cells to results in the release of procoagulants into the circulation. Severe liver damage presumably limits or abolishes production of coagulation proteins and reduces clearance of activated coagulation factors, thereby further promoting the occurrence of disseminated intravascular coagulopathy, which in turn augments tissue injury by impairing blood flow. Vasculitis and haemostatic failure result in purpura and widespread haemorrhages.

Clinical pathology findings in humans are compatible with observations made in haematological and coagulation studies on monkeys, except that leukocytosis and anaemia may be more marked in severe human disease (Al Hazmi *et al.*, 2003). In most species there is an initial leukopenia followed by leukocytosis, and the same may be true for humans. Monkeys may have prolonged activated partial-thromboplastin times and prothrombin times even in benign infection, and in severe liver disease there may be depletion of coagulation factors II, V, VII, IX, X and XII, thrombocytopenia and platelet dysfunction, increased schistocyte counts and depletion of fibrinogen together with raised fibrin degradation product levels. Raised serum aspartate aminotransferase and alanine aminotransferase levels have been recorded even in benign disease in humans.

Treatment is essentially symptomatic, and supportive therapy in the haemorrhagic disease includes replacement of blood and coagulation factors. Results obtained in animal models suggest that the administration of immune plasma from recovered patients may be beneficial. The antiviral drug ribavirin inhibits virus replication in cell cultures and laboratory animals, and it has been suggested that it could be used even in benign disease in order to obviate the potentially serious complications which may occur in humans.

Specimens to be submitted for laboratory confirmation of the diagnosis include blood from live patients, and tissue samples, particularly liver, but also spleen, kidney, lymph nodes and heart blood of deceased patients. Tissue samples should be submitted in duplicate in a viral transport medium, and in 10% buffered formalin for histopathological examination.

Viral antigen can frequently be detected rapidly in tissues and/or blood by a variety of immunological methods, including immunodiffusion, complement fixation, immunofluorescence and enzyme-linked immunoassay, and viral nucleic acid can readily be detected by RT-PCR

(Bird *et al.*, 2007; Drosten *et al.*, 2002; Jupp *et al.*, 2002; Weidmann *et al.*, 2008). Viraemia lasts for up to a week. The virus is cytopathic and can be isolated readily in almost all cell cultures commonly used in diagnostic laboratories, and identified rapidly by immunofluorescence. Virus can also be isolated in suckling or weaned mice, or hamsters, inoculated intracerebrally or intraperitoneally, and antigen can be identified in harvested brain or liver by the immunological methods mentioned above. Definitive identification of isolates is achieved by performing neutralization tests with reference antiserum, or by nucleotide sequencing of RT-PCR products.

Histopathological lesions, particularly those in the liver, are considered to be pathognomonic, and are essentially similar in humans and domestic ruminants. The severity of the lesions varies from primary foci of coagulative necrosis, consisting of clusters of hepatocytes with acidophilic cytoplasm and pyknotic nuclei, multi-focally scattered throughout the parenchyma, to massive liver destruction in which the primary foci comprising dense aggregates of cytoplasmic and nuclear debris, some fibrin and a few neutrophils and macrophages, can be discerned against a background of parenchyma reduced by nuclear pyknosis, karyorrhexis and cytolysis to scattered fragments of cytoplasm and chromatin, with only narrow rims of degenerated hepatocytes remaining reasonably intact close to portal triads. Intensely acidophilic cytoplasmic bodies which resemble the Councilman bodies of yellow fever are common, and rod-shaped or oval eosinophilic intra-nuclear inclusions may be seen in intact nuclei. Icterus may be evident.

Antibody to Rift Valley fever virus can be demonstrated in complement fixation, enzyme-linked immunoassay, indirect immunofluorescence, haemagglutination inhibition or neutralization tests. Diagnosis of recent infection is confirmed by demonstrating seroconversion or a fourfold or greater rise in titre of antibody in paired serum samples, or by demonstrating IgM antibody activity in an enzyme-linked immunoassay.

Benign Rift Valley fever in humans must be distinguished from other zoonotic diseases such as brucellosis and Q fever, while the fulminant hepatic disease must be distinguished from the so-called formidable viral haemorrhagic fevers of Africa: Lassa fever, CCHF, Marburg disease and Ebola fever. The occurrence of HFRS associated with hantavirus infections, is also a theoretical possibility in Africa.

An inactivated and a live attenuated vaccine are available for immunization of livestock, but it is usually difficult to persuade farmers to vaccinate livestock during long inter-epidemic periods. The attenuated vaccine confers lifelong immunity in sheep, but is abortigenic and

teratogenic in a small proportion of pregnant ewes. The attenuated vaccine is poorly immunogenic in cattle and they are immunized annually with the inactivated vaccine. A formalin-inactivated cell culture vaccine produced in the United States is used on an experimental basis to immunize people such as laboratory and field workers who are regularly exposed to Rift Valley fever infection.

Uukuniemi Virus

Uukuniemi virus was originally isolated in Finland in 1960s from *Ixodes ricinus* ticks, which parasitize livestock but also bite humans. The virus has subsequently been isolated from ticks, birds and field mice in Finland, Norway, Poland, Lithuania and the former USSR and Czechoslovakia. Antibody to the virus has been found in human sera in Finland, Hungary and former Czechoslovakia, but no evidence has been presented to indicate that infection is associated with disease. The remaining members of the serogroup have been isolated from ticks associated with passerine or sea birds, and have no known medical or veterinary significance.

GENUS *NAIROVIRUS*

Crimean–Congo Haemorrhagic Fever Virus

The literature on CCHF is the subject of several comprehensive reviews (Chumakov, 1974; Hoogstraal, 1979, 1981; Watts *et al.*, 1989). A disease given the name Crimean haemorrhagic fever was first observed on the Crimean peninsula in 1944, and it was demonstrated through the inoculation of human subjects that the disease was caused by a tick-transmitted virus, but the virus itself was only isolated in laboratory hosts, namely mice, in 1967. In 1969, it was shown that the agent of Crimean haemorrhagic fever was identical to a virus named Congo which had been isolated in 1956 from the blood of a febrile child in Stanleyville (now Kisangani) in what was then the Belgian Congo (now Democratic Republic of the Congo), and since that time the two names have been used in combination.

The distribution of CCHF virus extends over Eastern Europe, Asia and Africa: the presence of the virus or antibody to it has been demonstrated in the former USSR, Albania, Bulgaria, Greece, Turkey, Hungary, former Yugoslavia, France, Portugal, Serbia, Kosovo, Macedonia, Kuwait, Saudi Arabia, Oman, Dubai, Sharjah, Iraq, Iran, Afghanistan, Pakistan, India, China, Egypt, Ethiopia, Mauritania, Senegal, Burkina Faso, Benin, Nigeria, Central African Republic, Democratic Republic of the Congo, Kenya, Uganda, Tanzania, Zimbabwe, Namibia, South Africa and Madagascar. However, the evidence for France

and Portugal is based on limited observations and needs to be confirmed.

In many instances virus or antibody was discovered in deliberate surveys, but in some countries of Eastern Europe and Asia the presence of CCHF first became evident in nosocomial outbreaks of disease, or in epidemics that arose in circumstances where humans were exposed to ticks and livestock on a large scale, such as in major land reclamation or resettlement schemes in Bulgaria and parts of the former USSR. Notable outbreaks of the disease in Eurasia during recent years have resulted from the exposure of people to blood and ticks from slaughter stock imported from Africa and Asia to Saudi Arabia in 1990, the United Arab Emirates in 1994–1995, and Oman in 1995, plus large scale exposure of war refugees to outdoor conditions in Kosovo in 2000 and Albania in 2001 (El Azazy and Scrimgeour, 1997; Khan *et al.*, 1997; Papa *et al.*, 2002a, 2002b; Scrimgeour *et al.*, 1996; Williams *et al.*, 2000). Prior to 1981, a total of 15 cases of the disease had been reported in Africa, eight of them laboratory infections, and only one patient had developed haemorrhagic manifestations and died. Since then sporadic cases of haemorrhagic disease and deaths have been diagnosed regularly each year in southern Africa, probably as a result of increased awareness among clinicians, and severe disease has also been recorded elsewhere in Africa.

Phylogenetic studies indicate that there is marked diversity between CCHF isolates from geographically distinct regions, and although the differences do not necessarily correlate with pathogenicity, they need to be taken into account in designing diagnostic assays and vaccines (Ahmed *et al.*, 2005).

The virus has been isolated from at least 29 species of ixodid ticks, but for most species there is no definitive evidence that they are capable of serving as vectors, and in some instances the virus recovered from engorged ticks may merely have been present in the bloodmeal imbibed from a viraemic host. Members of three genera of ixodid ticks, *Hyalomma*, *Dermacentor* and *Rhipicephalus*, have been shown to be capable of transmitting infection trans-stadially and transovarially, but *Hyalomma* is considered to be the principal vector, and with the exception of Madagascar the known distribution of CCHF virus coincides with the world distribution of members of this genus of ticks. Moreover, the prevalence of antibody to CCHF virus detected in the sera of wild vertebrates in southern Africa was highest in large herbivores known to be the preferred hosts of adult *Hyalomma* ticks, and in small mammals such as hares which are the preferred hosts of immature *Hyalomma* ticks. Mammals of intermediate size, and passerine and water birds, generally lacked evidence of infection, but antibody was found in ostriches which are known to be parasitized by adult *Hyalomma*

ticks. Virus or antibody has also been demonstrated elsewhere in the sera of small mammals of Eurasia and Africa, such as little susliks, hedgehogs, hares and certain myomorph rodents, and in some instances it has been shown that these hosts develop viraemia of sufficient intensity to infect ticks.

High prevalences of antibody occur in domestic ruminants in areas infested by *Hyalomma* ticks and the virus causes inapparent infection or mild fever in cattle, sheep and goats, with viraemia of sufficient intensity to infect ticks. It is doubtful whether transovarial transmission occurs with sufficient frequency in ticks to ensure indefinite perpetuation of the virus in the absence of amplification of infection in vertebrate hosts, and in particular, it is believed that the infection of small vertebrates constitutes an important amplifying mechanism which facilitates trans-stadial transmission of virus by adult ticks to large vertebrates.

Young ruminants generally acquire natural infection early in life and are viraemic for about a week. Humans become infected when they come into contact with the viraemic blood of young animals in the course of performing procedures such as castrations, vaccinations, inserting ear tags or slaughtering the animals. Animals that are raised under tick-free conditions and moved to infested locations later in life may acquire tick-borne diseases of livestock at the same time that they become infected with CCHF virus, and consequently humans become infected from contact with viraemic blood in the course of treating sick animals or butchering those that die. The available evidence suggests that the infection in humans is acquired through contact of viraemic blood with broken skin, and this accords with the fact that nosocomial infection in medical personnel usually results from accidental pricks with needles contaminated with the blood of patients, or similar mishaps. Common source outbreaks involving more than one case of the disease can occur when several people are exposed to infected tissues. Infection appears to be limited to those who have contact with fresh blood or other tissues, probably because infectivity is destroyed by the fall in pH which occurs in tissues after death, and there has been no indication that CCHF virus constitutes a public health hazard in meat processed and matured according to normal health regulations. Many human infections result directly from tick bite, and it has been observed that people can also become infected from merely squashing ticks between the fingers. Some patients are unable to recall contact with blood or other tissues of livestock, or having been bitten by ticks, but live in or have visited a rural environment where such exposure to infection is possible. Town dwellers sometimes acquire infection from contact with animal tissues or tick bite while on hunting or hiking trips.

The majority of patients tend to be adult males engaged in the livestock industry, such as farmers, herdsmen, slaughtermen and veterinarians. Seroprevalence studies indicate that infection of humans is uncommon despite the widespread evidence of infection in livestock, and this may be explained by the facts that viraemia in livestock is short-lived, and of low intensity compared to that in other zoonotic diseases such as Rift Valley fever, and that humans are not the preferred hosts of *Hyalomma* ticks. The low prevalences of antibody generally found in populations at risk, and the paucity of evidence of inapparent infection encountered among the cohorts of cases of the disease, suggests that infection is frequently symptomatic.

Incubation periods are generally short, ranging from one to three days (maximum nine) following infection by tick bite, and are usually five or six days (maximum 13) in people exposed to infected blood or other tissues of livestock or human patients. Onset of the disease is usually very sudden. Patients develop fever, rigors, chills, severe headache, dizziness, neck pain and stiffness, sore eyes, photophobia, myalgia and malaise, with intense backache or leg pains. Nausea, sore throat and vomiting commonly occur early in the illness and patients may experience non-localized abdominal pain and diarrhoea at this stage. Fever is often intermittent and patients may undergo sharp changes of mood over the next two days, with feelings of confusion and aggression. By the second to fourth day of illness patients may exhibit lassitude, depression and somnolence, and have a flushed appearance with injected conjunctivae or chemosis. By this time, tenderness may be localized in the right upper quadrant of the abdomen, and hepatomegaly may be discernible. Tachycardia is common and patients may be slightly hypotensive. There may be lymphadenopathy, and enanthem and petechiae of the throat, tonsils and buccal mucosa.

A petechial rash appears on the trunk and limbs on days 3–6 of illness, and this may be followed rapidly by the appearance of large bruises and ecchymoses, especially in the antecubital fossae, upper arms, axillae and groin. Epistaxis, haematemesis, haematuria, melaena, gingival bleeding and bleeding from the vagina or other orifices may commence on day 4–5 of illness, or even earlier. Sometimes a haemorrhagic tendency is evident only from the oozing of blood from injection or venipuncture sites. There may be internal bleeding, including retroperitoneal and intracranial haemorrhage. Severely ill patients may enter a state of hepatorenal and pulmonary failure from about day 5 onwards and progressively become drowsy, stuporous and comatose. Jaundice may become apparent during the second week of illness. The mortality rate is approximately 30% and deaths generally occur on

days 5–14 of illness. Patients who recover usually begin to improve on day 9 or 10 of illness, but asthenia, conjunctivitis, slight confusion and amnesia may continue for a month or longer.

Changes in clinical pathology values recorded during the first few days of illness include leukocytosis or leukopenia, and elevated aspartate transaminase, alanine transaminase, gamma-glutamyl transferase, lactic dehydrogenase, alkaline phosphatase and creatine kinase levels, while bilirubin, creatinine and urea levels increase and serum protein levels decline during the second week. Thrombocytopenia, elevation of prothrombin ratio, activated partial thromboplastin time, thrombin time and fibrin degradation products, and depression of fibrinogen and haemoglobin values are also evident during the first few days of illness, indicating that the occurrence of disseminated intravascular coagulopathy is probably an early and central event in the pathogenesis of the disease. Changes are more severe in fatal than in nonfatal infections, and the occurrence of certain markedly abnormal clinical pathology values during early illness are predictive of fatal outcome (Ergonul *et al.*, 2006a; Swanepoel *et al.*, 1987, 1989).

It is surmised that peripherally introduced CCHF virus undergoes some replication at the site of inoculation, and that haematogenous and lymph-borne spread of infection occurs to organs such as the liver which are major sites of replication. Although it has not been shown conclusively that there is infection of endothelium, capillary fragility is a feature of the disease and there is evidence of formation of circulating immune complexes with complement activation, and this would contribute to damage of the capillary bed and the genesis of renal and pulmonary failure. Endothelial damage would account for the occurrence of a rash and would contribute to haemostatic failure through stimulating platelet aggregation and degranulation, with consequent activation of the intrinsic coagulation cascade. It is clear from the results of therapeutic administration of platelets to patients that they are consumed, and evidence of depression of thrombopoiesis in bone marrow has been reported. Widespread tissue damage in organs such as the liver would result in further release of procoagulants such as tumour necrosis factor into the bloodstream, and impairment of the circulation through the occurrence of a disseminated intravascular coagulopathy would contribute to further tissue damage. Damage to the liver would also impair synthesis of coagulation factors to replace those which are consumed.

Limited studies indicate that elevated serum levels of the pro-inflammatory cytokines tumour necrosis factor- α and interleukin-6 are significantly higher in patients with fatal outcome (Ergonul *et al.*, 2006b; Papa *et al.*, 2006). It is postulated that release of pro-inflammatory cytokines

by T-helper lymphocytes and macrophages triggers the occurrence of haemophagocytosis in bone marrow, and that endothelial damage associated with the occurrence of disseminated intravascular coagulopathy is caused by cytokine storms rather than directly by viral infection (Doganci, 2007; Fisgin *et al.*, 2008; Karti *et al.*, 2004).

Lesions in the liver vary from disseminated foci of coagulative necrosis, mainly mid-zonal in distribution, to massive necrosis involving over 75% of hepatocytes, and a variable degree of haemorrhage, with little or no inflammatory cell response. Lesions in other organs include congestion, haemorrhage and focal necrosis in the central nervous system, kidneys and adrenals and general depletion of lymphoid tissues. Fibrin deposits may be seen in small blood vessels in parenchymatous organs including the liver, and thrombus formation and infarction may contribute to the pathogenesis of the necrotic lesions in these organs.

Where possible, patients are treated by specially trained staff in institutions equipped for handling formidable viral haemorrhagic fevers, and barrier-nursing techniques are used for the protection of medical personnel. Therapy appropriate for disseminated intravascular coagulopathy, such as the use of heparin, may be contemplated early in the course of the disease by clinicians well versed in the treatment of haemostatic failure, but the procedure is considered to be risky, and generally only patients who acquire nosocomial infection come to medical attention at a sufficiently early stage. Standard treatment consists of replacement of red blood cells, platelets, other coagulation factors, protein (albumin) and intravenous feeding as indicated by clinical pathology findings. Immune plasma from recovered patients has been used in therapy, but there is no firm evidence from controlled trials of the value of the treatment, and there has been a lack of a uniform product with proven virus-neutralizing activity. Ribavirin has been found to inhibit virus replication in cell cultures, and in suckling mice, and preliminary results of a trial in human patients are promising.

On account of the propensity of the virus to cause laboratory infections, and the severity of the human disease, investigation of CCHF is generally undertaken in maximum security laboratories in countries which have biosafety regulations. Specimens to be submitted for laboratory confirmation of the diagnosis include blood from live patients and, in order to avoid performing full autopsies, heart blood and liver samples taken with a biopsy needle from deceased patients. During the acute phase of illness viral nucleic acid can be detected in blood by RT-PCR, viral antigen can be detected by enzyme-linked immunoassay, and virus can be isolated in cell cultures or mice (Burt *et al.*, 1998; Drosten *et al.*, 2002). Virus can be isolated from blood and organ suspensions in a wide

variety of primary and line cell cultures, including Vero, CER and BHK-21 cells, and identified by immunofluorescence. Isolation and identification can be achieved in one to five days, but cell cultures lack sensitivity and usually only detect high concentrations of virus present in the blood of severely ill patients during the first five days or so of illness. Intracerebral inoculation of suckling mice is more sensitive and can be used to demonstrate low concentrations of virus present in blood up to 13 days after the onset of illness.

Antibodies, both IgG and IgM, become demonstrable by indirect immunofluorescence from about day 7 of illness (slightly earlier by enzyme-linked immunoassay), and are present in the sera of all survivors of the disease by day 9 at the latest. The IgM antibody activity declines to undetectable levels by the fourth month after infection, and IgG titres may begin to decline gradually at this stage, but remain demonstrable for at least five years. Recent or current infection is confirmed by demonstrating seroconversion, or a fourfold or greater increase in antibody titre in paired serum samples, or IgM antibody activity in a single sample. Patients who succumb rarely develop a demonstrable antibody response and the diagnosis is confirmed by isolation of virus from serum, or from liver specimens, or by demonstration of antigen in liver sections by immunohistochemistry. Observation of necrotic lesions compatible with CCHF infection in sections of liver, provides presumptive evidence in support of the diagnosis.

The disease must be distinguished from the other so-called formidable viral haemorrhagic fevers: Lassa fever, Marburg disease, Ebola fever and HFRS (hantavirus infections), other febrile illnesses such as Rift Valley fever, Q fever and brucellosis which can be acquired from contact with animal tissues, as well as tick-borne typhus (*Rickettsia conorii* infection commonly known as tickbite fever), but many other conditions including bacterial septicaemias may resemble CCHF.

The control of CCHF through the application of acaricides to livestock is impractical, particularly under the extensive farming conditions which prevail in the arid areas where *Hyalomma* ticks are most prevalent. Pyrethroid preparations are available which can be used to kill ticks that come into contact with human clothing. Veterinarians, slaughtermen and others involved with livestock should be aware of the disease and take practical steps, such as the wearing of gloves, to limit or avoid exposure of naked skin to fresh blood and other tissues of animals. Inactivated mouse brain vaccine for the prevention of human infection has been used on a limited scale in Eastern Europe and the former USSR. Development of a safe and effective modern vaccine is inhibited by the limited potential demand for such a vaccine.

Nairobi Sheep Disease Virus

Nairobi sheep disease virus was first isolated from sheep blood in Kenya in 1910 and is known to be associated with disease of small ruminants, specifically sheep and goats, in a narrow band straddling the equator from Kenya in the east to Congo in the west. Antibody, but not disease, has also been found to the north of Kenya in Ethiopia and Somalia, and southwards along the east of the continent to Mozambique and Botswana. The virus can be transmitted trans-stadially by a range of ixodid ticks, including *Amblyomma variegatum*, but the endemic vector appears to be *Rhipicephalus appendiculatus*, in which transovarial transmission occurs. The disease in sheep and goats is characterized by fever, haemorrhagic gastroenteritis and abortion in pregnant animals. High mortality occurs when susceptible sheep or goats are introduced into an endemic area, but within such areas young animals appear to undergo benign infection as maternal immunity wanes, and there is a high prevalence of immunity in adult animals. Attenuated live and killed vaccines for sheep and goats are available in East Africa. Small antelope are susceptible to the disease, and rodents develop viraemic infection, but seroprevalence studies have failed to identify wild maintenance hosts of the virus. Larger ruminants such as cattle and buffalo are not susceptible to the disease. The virus has been isolated from human blood in association with febrile illness with arthralgia and malaise in Uganda, and laboratory infection has been recorded. Antibody prevalence rates of up to 20% have been found in humans in endemic areas.

Ganjam virus, first isolated from ixodid ticks in India in 1954, is considered to be identical to Nairobi sheep disease virus. It has been isolated from the blood of sheep and humans with febrile illness in India, where it is associated with ticks of the genus *Haemaphysalis*. There is speculation that the virus may have been translocated from India to Africa with ectoparasites on sheep and goats, which have been traded along sea routes for centuries.

Dugbe Virus

There have been approximately 600 isolations of Dugbe virus from ixodid ticks, mainly *A. variegatum*, in Nigeria, Central African Republic, Kenya and Ethiopia. The virus has also been isolated frequently from cattle blood in surveys, and from a giant rat (*Cricetomys gambianus*), aedine mosquitoes and *Culicoides* midges in Nigeria, and there is serological evidence of the occurrence of the virus in Senegal and Uganda. There have been seven isolations of the virus from the blood of patients with benign febrile illness in Nigeria and Central African Republic (including a laboratory infection). One patient

had mild meningitis and virus was isolated from CSF. Surprisingly, serosurveys have not revealed widespread human infection.

GENUS HANTAVIRUS

Hantaan, Dobrava, Seoul, Puumala, Sin Nombre and Related Viruses

Hantaviruses are associated with a range of nephrotic diseases in Asia and Europe known variously as haemorrhagic nephrosonephritis, Korean haemorrhagic fever, Songo fever, epidemic haemorrhagic fever and nephropathia epidemica (NE), but use of the generic term haemorrhagic fever with renal syndrome (HFRS) is advocated, while the term hantavirus pulmonary syndrome (HPS) is preferred for respiratory disease associated with hantaviruses in the Americas although some authors consider hantavirus cardiopulmonary syndrome (HCPS) to be more appropriate.

The existence of a febrile disease with haemorrhagic and renal manifestations has been recognized in Eurasia at least since the early years of the twentieth century and, in fact, descriptions of similar disease can be traced back to antiquity. A disease known by various names, including haemorrhagic nephrosonephritis, and which caused outbreaks among civilians and soldiers, was investigated independently in the far eastern region of the former USSR and in Manchuria prior to the Second World War, and by the early 1940s it was established that the condition could be transmitted to human volunteers by inoculation of filtrates of patients' blood or urine, or tissue extracts from *Apodemus* field mice (it had been observed that the incidence of disease was greatest at the end of summer when the mice were most numerous). At the same time, a febrile syndrome with abdominal pain, backache and renal manifestations was recognized in Scandinavia and numerous cases of this disease, later named nephropathia epidemica, were observed in soldiers during the Second World War.

Thousands of cases of a disease named Korean haemorrhagic fever were observed in soldiers and civilians during the Korean War of the early 1950s, and the disease continued to be seen after the war. In 1976 it was found that convalescent sera from patients in Korea could be used to demonstrate the presence of an antigen by immunofluorescence in the tissues of striped field mice (*Apodemus agrarius*) caught near the Hantaan river. The antigen was shown to be associated with a virus which could be subcultured in field mice. The virus, named Hantaan, was successfully grown in cell cultures in 1981, and shortly thereafter characterized as a member of the family *Bunyaviridae* and placed in a new genus *Hantavirus*.

The virus is widely distributed as the causative agent of HFRS in Asia, particularly in the eastern portion of the former USSR, China and Korea in association with *A. agrarius manichuricus* and *A. agrarius coreae*. A subtype of Hantaan virus, designated Amur virus, was isolated from *Apodemus peninsulae* (Korean field mice) in eastern Russia and subsequently shown to be associated with HFRS in China. Virus isolated from *A. peninsulae* in Korea and reported as Soochong virus, is probably synonymous with Amur virus (Baek *et al.*, 2006; Jiang *et al.*, 2007). Da Bie Shan, a further subtype of Hantaan virus isolated from Chinese white-bellied rats (*Niniventer confucianus*), is not known to be pathogenic for humans (Wang *et al.*, 2000). Virus associated with a severe form of HFRS in the Balkans (Albania, Greece, former Yugoslavia and Bulgaria) and found to be distinct from Hantaan virus, was independently isolated and described as Dobrava virus and Belgrade virus, and is currently designated Dobrava–Belgrade virus. It is associated with the yellow-necked field mouse, *A. flavicollis*. Subsequently, Saarema virus isolated from *A. agrarius agrarius* field mice in Europe, was found to be a subtype of Dobrava–Belgrade virus, but it causes milder disease.

Seoul virus was isolated in 1980s at Korea from the tissues of peridomestic rats, *Rattus rattus* and *Rattus norvegicus*, in association with human disease which occurred in urban as opposed to rural environments. It has been implicated in human disease in Japan, China and Korea, but has been isolated from rats in Egypt, the United States and elsewhere, and probably has a worldwide distribution. Isolation of the virus from rats led to speculation that hantaviruses in general may have been disseminated worldwide with ship-borne rodents. However, the distribution patterns of most hantaviruses within the interiors of continents, and the evolution of particular host relationships constitute evidence against recent spread of the viruses.

The last of the currently recognized hantaviruses associated with rodent hosts of the subfamily Murinae (Old World rats and mice), comprise Thailand virus isolated from bandicoots (*Bandicota indica*) in Thailand and Sangassou virus recently identified from partial genome nucleotide sequences obtained from tissues of African wood mice (*Hylomyscus simus*) in Guinea (Table 29.4) (Klempa *et al.*, 2006). Neither of these two viruses are known to be pathogenic for humans.

In the 1980s, the presence of the causative agent of nephropathia epidemica was demonstrated by immunofluorescence in the tissues of bank voles (*Myodes glareolus*) in Finland, and subsequently the agent, named *Puumala virus*, was grown in cell cultures and shown to be related to but distinct from Hantaan virus. Although evidence obtained in former Yugoslavia, Germany, Belgium,

France and Britain suggests that Puumala virus occurs widely in Europe, it is most prevalent at northerly latitudes, extending into the Arctic Circle in Scandinavia and the adjoining western portion of the former USSR, where highest concentrations of the bank vole occur. Muju virus, a subtype of Puumala virus recently identified from nucleotide sequences detected in *M. regulus* voles in Korea, is considered to be a possible cause of nephropathia epidemica-like infections in that country (Song *et al.*, 2007), while Hokkaido virus, associated with the red-backed vole (*Myodes rufocanus*) in Japan (Daud *et al.*, 2007; Kariwa *et al.*, 1999), is not known to be pathogenic for humans. Other hantaviruses associated with rodents of the subfamily Arvicolinae (voles, lemmings and muskrats) in Europe and Asia, and not known to cause human disease, include Topografov virus, Khabarovsk virus and Tula virus (Table 29.4). The findings in Asia and Europe prompted interest elsewhere, and as a result Prospect Hill virus, and later two other viruses, were isolated from voles in the United States, but no disease associations have been described for these viruses (Table 29.4).

In May 1993, an outbreak of an acute respiratory disease in adults, with a high fatality rate, was recognized in the Four Corners region of south-western USA where the borders of the states of Utah, Colorado, Arizona and New Mexico meet, but the initial occurrence of cases could be traced back to late 1992. Antibody cross-reactive with the antigens of known hantaviruses was found in the sera of patients, and by means of RT-PCR with consensus sequence hantavirus primers, it was possible to demonstrate the presence of nucleic acid of a novel hantavirus in the tissues of patients. The nucleotide sequence of the entire genome of the virus was determined even before it could be isolated and grown in cell cultures. The outbreak was apparently associated with a population explosion of the deer mouse *Peromyscus maniculatus* (a member of the Sigmodontinae subfamily of rodents), which is thought to be the natural host of the virus. Sporadic cases of similar disease were recognized elsewhere in the United States, some retrospectively, and by the end of 1993 a total of 53 cases had been confirmed (Bremner, 1994; Duchin *et al.*, 1994; Nichol *et al.*, 1993). After objections were raised to various names proposed for the new virus, Sin Nombre (Spanish for 'without name') was adopted, and the disease was referred to as hantavirus pulmonary syndrome (HPS).

Isolated cases and outbreaks of HPS were subsequently recognized beyond the distribution range of *P. maniculatus* in the United States, in Canada, and in several countries of South America. A succession of new hantaviruses was discovered in association with HPS, or in rodents tested speculatively in surveys (Table 29.4).

Thottapalayam virus, which had been isolated from suncid shrews in India in 1964, was found to be a distinct hantavirus, and recently nucleotide sequences of a putative new Tanganya virus were detected in a crocidurid shrew in Guinea (Table 29.4) (Klempa *et al.*, 2007).

Many of the new viruses were discovered through the detection of cross-reactive antibody activity to the antigens of existing hantaviruses, followed by the application of the PCR to detect viral nucleic acid, and genetic characterization. Adaptation to cell cultures followed the initial identification of the viruses, but *in vitro* culture has not yet been achieved in all instances. It is suspected that in addition to the hantaviruses currently known to be human pathogens, some of the remaining viruses may also prove to be associated with disease (Table 29.4) (Kanerva *et al.*, 1998; Monroe *et al.*, 1999; Schmaljohn and Hjelle, 1997; Schmaljohn *et al.*, 2002).

Serological classification of hantaviruses has lagged behind genetic characterization, primarily because the lack of *in vitro* culture systems for some of the viruses has prevented the performance of definitive cross-neutralization tests, but the extant information on antigenic affinities is in agreement with the genetic clustering of the viruses, which in itself conforms with the phylogeny of the rodent hosts (Table 29.4) (Puthavathana *et al.*, 1992; Arthur *et al.*, 1992; Chu *et al.*, 1994, 1995; Klein and Calisher, 2007; Kanerva *et al.*, 1998; Monroe *et al.*, 1999; Schmaljohn and Hjelle, 1997; Zeier *et al.*, 2005). In brief, all hantaviruses are antigenically related, with greatest affinities existing within clusters designated as Hantaan-like, Puumala–Prospect Hill-like and Sin Nombre-like, while Thottapalayam virus from shrews in India is more distantly related to the others. Viruses of the Hantaan-like group are associated with rodent hosts of the subfamily Murinae and with HFRS; Puumala–Prospect Hill-like viruses are associated with the subfamily Arvicolinae and with NE, and Sin Nombre-like viruses with the subfamily Sigmodontinae and with HPS (Table 29.4). Despite the fact that Seoul virus appears to be very widely distributed, incontrovertible evidence of disease associated with hantaviruses (detection of virus) has been obtained only for Asia, Europe and the Americas, while elsewhere there has only been inconclusive serological evidence of infection. However, Seoul, Hantaan and Puumala viruses have been encountered as contaminants of laboratory rodent colonies and are known to have caused infections in laboratory workers in the former USSR, Korea, Japan, Belgium, France and England. In one instance virus was inadvertently preserved for years in rat tissues kept in frozen storage. Hence, the potential exists for inadvertent dissemination of the viruses.

The distributions of the hantaviruses, insofar as they are known, tend to overlap, but conform to the distributions of the rodent hosts. Individual viruses have been isolated from more than one type of rodent, but each tends to have a particular association with a single species of rodent. The viruses appear to be apathogenic for their reservoir hosts. After the rodents become infected there is an initial viraemia followed by the persistence of infection, probably for life, in lungs, kidneys and possibly other organs, with chronic excretion of virus in urine, faeces and saliva, despite the occurrence of a demonstrable immune response. There does not appear to be intrauterine transfer of infection, and transmission between rodents is thought to occur by bite, aerosol or contamination of dust, food and other fomites with excreted virus. Gamasid mite parasites of rodents are suspected to be capable of transmitting infection, but transmission occurs in the laboratory in the absence of mites. Foci with very high infection rates are observed among rodents in nature.

Humans become infected by the same means as rodents, but airborne infection from dust contaminated with rodent urine and faeces appears to be the principal mechanism, and has been observed to occur even in people who briefly visited infected colonies of laboratory rodents. Infection occurs in three main situations: rural or sylvatic infection with Hantaan-like, Puumala-like or Sin Nombre-like viruses occurs in people who have occupational, residential or recreational exposure to rodent-infested buildings or to the outdoors, urban infection with Seoul virus occurs indoors in association with rat infestations, while all of the viruses may cause infections associated with laboratory rodents. Rodents are subject to periodic population explosions and crashes, and the incidence of human infection with Hantaan-like, Puumala-like and Sin Nombre-like viruses increases in years when the rodents are most numerous. Person-to-person spread of hantavirus infection has been observed in outbreaks of HPS caused by Andes virus in Argentina, but it has not been established whether transmission is associated with direct contact, droplets, aerosols or contaminated fomites (Wells *et al.*, 1997). Numerous cases of HPS have been reported in the Americas, and up to 200 000 hospitalized cases of HFRS are recorded in Eurasia each year, with more than half occurring in China (Monroe *et al.*, 1999; Schmaljohn and Hjelle, 1997).

Four clinical forms of HFRS are recognized and these vary in order of increasing severity from NE associated with Puumala virus infection, through mild or rat-borne HFRS associated with Seoul virus infection, to Far Eastern HFRS associated with Hantaan virus carried by

A. agrarius field mice, and so-called Balkan HFRS associated with Dobrava–Belgrade virus carried by *A. flavicollis* mice.

Far Eastern HFRS occurs in China, the eastern part of the former USSR and Korea, mainly in adult males with occupational exposure to the outdoors, such as farmers, forest workers and soldiers stationed in the field, and seldom occurs in children under 10 years of age. Most cases are seen in autumn and early winter when crops are harvested and the rodents are most numerous, and subsequently when the agricultural products are stored in proximity to homesteads. The incidence of asymptomatic infection is unknown, but it was noted that American soldiers participating in an exercise in Korea who seroconverted had all been ill, while in parts of Korea high-antibody prevalence rates without corresponding levels of disease have been observed.

The classical form of Far Eastern HFRS described in Korea has well-marked phases, but these may overlap and be obscured in mild cases (Lee, 1982). An incubation period of two to three weeks is followed by the abrupt onset of a febrile phase which lasts three to seven days and is marked by high fever, chills, malaise, myalgia, anorexia, headache, dizziness, ocular pain and abdominal and back pain, which is felt particularly in the renal area as a result of peritoneal and retroperitoneal oedema. Proteinuria is marked during this phase. Towards the end of the phase there is characteristic flushing of the face, neck and anterior chest, with the conjunctivae, palate and pharynx assuming an injected appearance, followed by the emergence of fine petechiae on the face, neck, soft palate and chest, together with conjunctival haemorrhages. Patients next enter a hypotensive phase which lasts hours to two days, and is marked by classical shock: tachycardia, narrowed blood pressure, cold and clammy skin, dulled senses and confusion. One third of fatal cases enter irreversible shock at this stage. There is marked proteinuria, microscopic haematuria, raised haematocrit levels (haemoconcentration), leukaemoid reaction and thrombocytopenia. Capillary haemorrhages are prominent. The patients then enter an oliguric phase which lasts three to seven days. Blood urea and creatinine levels increase, blood pressure begins to normalize, but hypertension may result from a hypervolaemic state. Bleeding tendencies increase markedly, and there may be epistaxis, conjunctival, cerebral and gastrointestinal haemorrhages and extensive purpura. There may be severe nausea and vomiting, lung oedema and symptoms referable to the central nervous system. Most deaths occur at this stage. A diuretic phase which follows may last days or weeks, and marks the start of clinical recovery. Diuresis of 3–6 l per day is common, but is influenced by dehydration, electrolyte imbalance or secondary infections. Severely ill patients are at risk in

this phase and may lapse into shock. A convalescent phase with progressive recovery of glomerular filtration rate, renal blood flow and urine-concentrating ability, may last two to three months. Mortality has been reduced from the 10–15% observed during the Korean War to 5%, with intensive supportive therapy and renal dialysis.

Balkan HFRS, associated with Dobrava–Belgrade virus, is also seen mainly in adult males, including woodcutters, shepherds and military personnel, but cases generally occur in spring and summer, possibly because there is not the same type of cereal crop farming as in the Far East, and the reservoir host is encountered in outdoor activities and at campsites during the warmer months of the year. The disease is essentially similar to Far Eastern HFRS, but is more severe, with a higher proportion of patients requiring renal dialysis, and with a greater tendency for the development of disseminated intravascular coagulopathy and haemorrhages. A case of the disease involving focal encephalitis with epileptic seizures and hemiparesis has been described (Cerar *et al.*, 2007). Reported death rates for Balkan HFRS range from 5 to 35%.

Natural outbreaks of mild or rat-borne HFRS, as opposed to outbreaks associated with laboratory rodent colonies, have been recorded in cities in Japan, China and Korea. The disease occurs in urban residents who have no contact with field rodents, and most cases are seen in spring and early summer. The disease is less severe and runs a shorter course than disease associated with Hantaan virus infections, and has less distinct clinical phases. There is also less tendency for haemorrhages and renal failure to occur, and frequently signs of liver involvement are dominant: abdominal pain, hepatomegaly and hepatic dysfunction. There are few deaths and mortality has been estimated at 1% or less.

Infection with Puumala-type virus occurs widely in Europe, but the disease, nephropathia epidemica, is recognized most frequently in Scandinavia and the neighbouring western region of the former USSR. The disease affects mainly adult males and infection appears to be associated principally with outdoor activities. It is seen most commonly in late autumn and early winter, but many cases occur in late summer following the traditional holiday season. Cases seen during the colder months are ascribed to the invasion of homes and barns by voles at the onset of winter. The incubation period is thought to be about one month, but a range of three days to six weeks has been reported. There is abrupt onset of fever, headache and malaise. By the third or fourth day of illness there is nausea, vomiting, and abdominal and lumbar pain. At this stage there may be azotemia, oliguria and proteinuria which peaks one week after the onset of illness and declines over the next three to six days. Patients

are extremely ill during the oliguric phase, and may manifest somnolence, restlessness, confusion and meningismus. Transient myopia or blurred vision is regarded as pathognomonic. Facial flushing and maculopapular rash of the neck and trunk are seen occasionally, as are hepatomegaly, cervical lymphadenopathy and haemorrhages such as epistaxis and gastrointestinal bleeding. Patients seldom require renal dialysis. The oliguria is followed by polyuria of 3–4 l daily for 7–10 days. At one stage it was thought that HFRS/NE and HPS were entirely distinct syndromes, but it is now recognized that there is some overlap, and in particular a proportion of NE patients may develop pulmonary infiltration similar to HPS, and some may even exhibit respiratory distress. Clinical improvement begins with the onset of polyuria, and two weeks after the onset of fever patients are subjectively well, but backache and lassitude may recur over weeks, and hyposthenuria may persist for months. Recovery is usually complete, and mortality is consistently less than 1%. The relatively high prevalence of antibody found in surveys suggests that inapparent infections may outnumber cases of overt disease by up to 20-fold.

It should be stressed that the hantaviruses overlap in distribution and in the severity of HFRS which they induce, and for instance, neither rural or urban domicile of patients, nor season of occurrence of disease, allow Hantaan and Seoul virus infections to be distinguished with certainty in Asia. Infections with Dobrava and Puumala-type viruses in the Balkans may be equally difficult to distinguish on occasion.

People who develop HPS are often healthy young adults, but may be of any age and either sex, although the disease occurs infrequently in children. Infection is acquired in similar manner to HFRS from occupational, residential or recreational exposure to the outdoors or rodent-infested buildings, and in many instances infected rodents have been found in the homes of victims. Incubation periods are similar to HFRS, generally falling into the range two to three weeks, but the disease is characterized by severe cardiopulmonary dysfunction rather than renal failure and haemorrhage, despite the facts that there is similar underlying capillary permeability and marked thrombocytopenia. Onset of the prodromal phase of the disease is marked by sudden development of fever, headache, severe myalgia and a cough which may be productive in some instances. Gastrointestinal manifestations in some patients include abdominal pain, nausea, vomiting and diarrhoea. After three to six days of illness there is progressive tachypnoea, tachycardia and hypotension preceding the onset of acute respiratory distress with pulmonary oedema. Patients are generally hospitalized at this stage, but some may die before they can be admitted. In addition to tachypnoea, tachycardia and hypotension,

on admission patients may be found to have proteinuria, leukocytosis with neutrophilia plus increased myeloid precursors and atypical lymphocytes, haemoconcentration, and thrombocytopenia, plus increased prothrombin and partial thromboplastin times, although there is no rash and seldom a tendency towards overt or internal bleeding. Within two days of being admitted to hospital most patients develop diffuse bilateral interstitial and alveolar pulmonary infiltration and pleural effusions demonstrable on radiographs, with hypoxaemia which has necessitated intubation, mechanical ventilation and oxygen supplementation in up to 88% of patients in some outbreaks. Renal insufficiency can occasionally follow prolonged hypoperfusion, but early renal insufficiency and increased serum creatine kinase levels (evidence of skeletal muscle inflammation) are not uncommon in infection with Andes, Bayou and Black Creek Canal viruses. Death generally occurs six to eight days after the onset of illness, often within 48 hours of admission to hospital, but can range from two days after the observed onset of illness to more than two weeks. Fatality rates often exceed 40%, and incurable shock and myocardial dysfunction may contribute to the high mortality. Autopsies reveal noncardiogenic pulmonary oedema and serous pleural effusions, with scant lymphoid infiltration of the lung tissue. Some survivors manifested transient diuresis, but otherwise they make an uneventful recovery without sequelae (Bremner, 1994; Duchin *et al.*, 1994; Kanerva *et al.*, 1998; Nichol *et al.*, 1993; Schmaljohn and Hjelle, 1997).

The underlying lesion in the pathogenesis of hantavirus syndromes appears to be vascular damage, and this is thought to be mediated by both viral invasion of endothelial cells and immunopathological mechanisms. Capillaries and small blood vessels dilate and there is extravasation of plasma and cellular elements into tissues, and the pathological changes observed in multiple systems all appear to be referable to the vascular damage (Borges *et al.*, 2006; Kanerva *et al.*, 1998).

Treatment of HFRS involves complex, phase-specific monitoring and support of homeostasis, including fluid and electrolyte balances. Trials of ribavirin for the treatment of the disease in China have been complicated by difficulties such as lack of uniformity of clinical status of patients at the time of protocol entry, but there are indications that use of the drug reduces mortality and leads to improvement of objective markers of patient well-being, and clinical pathology values. Epidemiological evidence suggests that there is lifelong immunity to hantaviruses, at least to the homologous serotype. There has been research on recombinant vaccines, but these are likely to find application only in Asia where suitable populations at risk can be identified. Inactivated vaccine has been used with some success in Korea (Cho *et al.*, 2002).

Investigation of hantavirus infections is usually undertaken in high security laboratories to minimize the exposure of staff to infection. Isolation and identification of hantaviruses is a notoriously difficult and time-consuming procedure, and is rarely successful on serum and urine specimens from patients. Demonstration of viral antigens in sera and urine is equally unsuccessful. Viral nucleic acids of hantaviruses can be detected in the tissues of human patients and experimentally and naturally infected rodents by means of real-time and conventional RT-PCR with appropriate primers (Arthur *et al.*, 1992; Grankvist *et al.*, 1992; Kramski *et al.*, 2007; Mertz *et al.*, 2006; Monroe *et al.*, 1999; Nichol *et al.*, 1993; Nordström *et al.*, 2004; Xiao *et al.*, 1992). Detection of IgM antibody by enzyme-linked immunoassay is also useful as a rapid diagnostic technique. Antibody activity appears to be present in the sera of HFRS patients from the time of hospitalization, and titres increase rapidly over the next two weeks. Owing to the antigenic cross-reactivity between hantaviruses, it may be difficult to determine the serotype of the virus responsible for the infection from antibody tests, but this can sometimes be inferred by using a range of antigens in enzyme-linked immunoassays (Feldmann *et al.*, 1993), or by performing neutralization tests with the full range of serotypes: antibody titres tend to be highest against the homologous infecting serotype. In attempts to diagnose infection by an unknown hantavirus, particularly in an locations where local viruses are unknown, it is advisable that antigens representative of all four antigenic types be included in the tests: Hantaan-like, Puumala-Prospect Hill-like, Sin Nombre-like and Thottapalyam viruses.

BUNYAVIRUSES UNASSIGNED TO GENUS

Bhanja, Kasokero, Bangui, Issyk-Kul, Tataguine and Wanowrie Viruses

Bhanja virus has been isolated from ixodid ticks of five genera –*Haemaphysalis*, *Amblyomma*, *Dermacentor*, *Boophilus* and *Hyalomma* –variously in India, the former USSR, former Yugoslavia, Bulgaria, Slovakia, Somalia, Central African Republic, Nigeria and Senegal. It has been suggested that the wide distribution of the virus could have resulted from the carriage of immature ticks on migrating birds, although birds do not themselves appear to be susceptible to the virus. Isolations of the virus have also been made from a hedgehog, ground squirrel and blood samples from cattle and sheep in Nigeria. Antibody has been found in cattle, sheep and goats parasitized by the ticks, and in human sera in Slovakia, former Yugoslavia, Italy and the Central African Republic. Mild febrile illness

was observed in two human patients who acquired laboratory infection, and serological evidence of infection was obtained in a patient who suffered meningoencephalitis in former Yugoslavia.

Kasokero virus was isolated from fruit bats in Uganda, and caused four laboratory infections marked by febrile illness, headache, myalgia, arthralgia, abdominal pain, diarrhoea, chest pain, cough, as well as hyperactive reflexes in one patient. Bangui virus was isolated from the blood of a patient with febrile illness, headache and rash in the Central African Republic, and antibody was found in the sera of local residents.

Issyk-Kul virus was isolated from several species of insectivorous bat and from argasid tick parasites of bats, birds and anopheline and aedine mosquitoes in the Central Asian Republics of the former USSR. It was demonstrated that aedine mosquitoes and argasid ticks are able to transmit the virus. Antibody was found in human sera and virus was isolated on at least 19 occasions from the blood of patients with febrile illness, headache, dizziness, cough, nausea and vomiting. The cases included laboratory infections. Keterah virus, isolated from argasid tick parasites of bats and from bat blood in Malaysia, has been shown to be closely related or identical to Issyk-Kul virus. It is difficult to be certain whether the natural vectors of Issyk-Kul/Keterah virus are argasid ticks or mosquitoes.

Tataguine virus has been isolated from anopheline mosquitoes in Senegal, Nigeria, Cameroon and Central African Republic. It appears to be a potentially important pathogen: antibody has been found in human sera in Senegal and Nigeria, and there have been at least 31 isolations of the virus from the blood of febrile humans in Senegal, Nigeria, Central African Republic and Cameroon. The infections were characterized by febrile illness with headache, rash and arthralgia.

Wanowrie virus has been isolated from *Hyalomma* ticks in India, Iran and Egypt. Little else is known about the virus except that it was isolated in Sri Lanka from the brain of a human patient who succumbed to febrile illness with abdominal pain, vomiting, haematemesis and passing of blood per rectum.

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Arenaviruses

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INTRODUCTION

The arenaviruses are a family of enveloped, single-stranded RNA viruses, the study of which has been pursued for two quite separate reasons. First, lymphocytic choriomeningitis virus (LCMV) has been used as a model of persistent virus infections for over half a century; its study has contributed a number of cardinal concepts to our present understanding of interactions between viruses and the host immune system. Although LCMV infections of humans are rare, there is significant evidence of LCMV associated with the keeping of pet rodents. LCMV remains the prototype of the *Arenaviridae* and was once a common infection of laboratory mice, rats and hamsters. Second, at least six certain arenaviruses cause severe haemorrhagic diseases in humans, notably Lassa fever in Africa, and Argentine haemorrhagic fever (AHF) in South America. Several other arenaviruses have been described from South America, two of which are associated with human infections (Guanarito and Sabia). In common with LCMV, the natural reservoir of these infections is a limited number of rodent species (Table 30.1). Although the initial isolates from South America were at first erroneously designated as newly defined arboviruses, there is no evidence to implicate arthropod transmission for any arenavirus. However, similar methods of isolation and the necessity of trapping small animals meant historically that the majority of arenaviruses have been isolated by workers in the arbovirus field. The discovery of hantaviruses as a cause of hantavirus pulmonary syndrome has led to a resurgence of interest in the link between zoonoses and persistent virus infections of rodents (see Chapter 29 for a description of the hantaviruses). This was particularly well illustrated in Argentina where virologists and clinicians specializing in AHF were in the

vanguard of national efforts linking respiratory disease with the discovery of new hantaviruses coexisting with arenaviruses in the same rodent populations.

With the current interest in emerging infections, the arenaviruses are an excellent illustration as to how environmental changes may result in an altered balance between humans and natural animal hosts, leading to unexpected diseases which can severely challenge local and national public health resources resulting from sporadic introduction into human populations outside of endemic areas. In addition, arenaviruses are classified as category A bioterrorism agents by the United States Centers for Disease Control and Prevention, thus heightening the need for public health vigilance.

There is a wide spectrum of pathological processes associated with these viruses that give useful insights into other zoonotic infections, particularly those with rodent reservoirs. The public health challenge is to recognize the likelihood of arenavirus infection as early as possible given the insidious onset of clinical disease. With new zoonotic infections emerging at least annually, the risk of new arenaviruses causing previously unknown human infections is high.

Nomenclature and Natural History

The members of the family currently identified are listed in Table 30.1. The various strains and isolates of LCMV are now considered to be a genus within the family *Arenaviridae*. A close serological relationship exists between LCMV, Lassa virus and other arenaviruses from Africa (LCMV-Lassa serocomplex). For this reason, they are loosely referred to as the 'Old World' arenaviruses, in contrast to those from the Americas, although LCMV can be found worldwide except in Australia. The 'New World'

Table 30.1 The arenaviruses: hosts, disease risk and geographical distribution

Virus	Natural host	Human disease	Distribution
LCMV-Lassa serocomplex (Old World)			
Ippy	<i>Arvicanthus</i> spp.	Not recorded	Central Republic African
Lassa	<i>Mastomys natalensis</i>	Lassa fever	West Africa
Lymphocytic choriomeningitis	<i>Mus musculus</i> , <i>Mus domesticus</i>	Aseptic meningitis	Worldwide except Australasia
Mobala	<i>Praomys jacksoni</i>	Infection possible	Central African Republic
Mopeia	<i>Mastomys natalensis</i>	Infection possible	Mozambique, Zimbabwe
Tacaribe serocomplex (New World)			
Clade A			
Allpahuayo	<i>Oecomys bicolor</i>	Not recorded	Peru
Bear Canyon	<i>Peromyscus californicus</i>	Infection possible?	California, USA
Catarina	<i>Neotoma micropus</i>	Not recorded	Texas, USA
Flexal	<i>Neocomys</i> spp.	Not recorded	Brazil
Paraná	<i>Oryzomys buccinatus</i>	Not recorded	Paraguay
Pichinde	<i>Oryzomys albigularis</i>	Not recorded	Columbia
Pirital	<i>Sigmodon alstoni</i>	Not recorded	Venezuela
Tamiami	<i>Sigmodon hispidus</i>	Not recorded	Florida, USA
Whitewater Arroyo	<i>Neotoma albigula</i>	Infection possible?	New Mexico, USA
Clade B			
Amapari	<i>Oryzomys gaedi</i> , <i>Neocomys guianae</i>	Not recorded	Brazil
Cupixi	<i>Oryzomys capito</i>	Not recorded	Brazil
Guanarito	<i>Zygodontomys brevicuda</i>	Haemorrhagic fever	Venezuela
Junín	<i>Calomys musculinus</i> , <i>Calomys laucha</i> , <i>Akadon azarae</i>	Haemorrhagic fever	Argentina
Machupo	<i>Calomys callosus</i>	Haemorrhagic fever	Bolivia
Sabiá	Unknown	Haemorrhagic fever	Brazil
Tacaribe	<i>Artibeus literatus</i> (bat)	Infection possible	Trinidad
Clade C			
Latino	<i>Calomys callosus</i>	Not recorded	Bolivia
Oliveros	<i>Bolomys obscurus</i>	Not recorded	Argentina

arenaviruses show varying degrees of serological relationships with Tacaribe virus, first isolated in Trinidad. For this reason, viruses from the Americas are frequently regarded as members of the Tacaribe serocomplex.

The arenaviruses take their name from the sand-sprinkled appearance when viewed in the electron microscope (Latin *harena* = 'sand'). With the exception of LCMV, all are referred to by names that reflect the geographical area in which they were isolated (Figure 30.1). Various strain designations are also commonly used, in particular for LCMV and human arenavirus isolates. There are increasing numbers of nonpathogenic arenaviruses being isolated from rodents in the Americas. Determining a causal link with human disease is often difficult, however, as is the discrimination of these new agents as new species distinct from other arenaviruses that may be variants of existing family members.

All but one of the 23 members of the *Arenaviridae* so far described have rodents as their natural reservoir hosts. The pre-eminent property of the arenaviruses is a capacity to establish long-term persistent infections in their principal host species. Although rodents are divided into over 30 families distributed worldwide, arenaviruses are predominantly found within two major families: Muridae (e.g. mice and rats) and Cricetidae (e.g. voles, lemmings, gerbils). Each arenavirus, except Tacaribe virus, is associated with a primary rodent host species, but seroprevalence studies indicate that virus is not uniformly distributed over the host's geographical range. Nearly all arenaviruses isolated from the South American continent are associated with cricetid rodents whose members frequent open grasslands and forest. Tacaribe virus was originally isolated from the fruit bat, *Artibeus literatus*, and, inexplicably, has never been recovered from wild rodents.

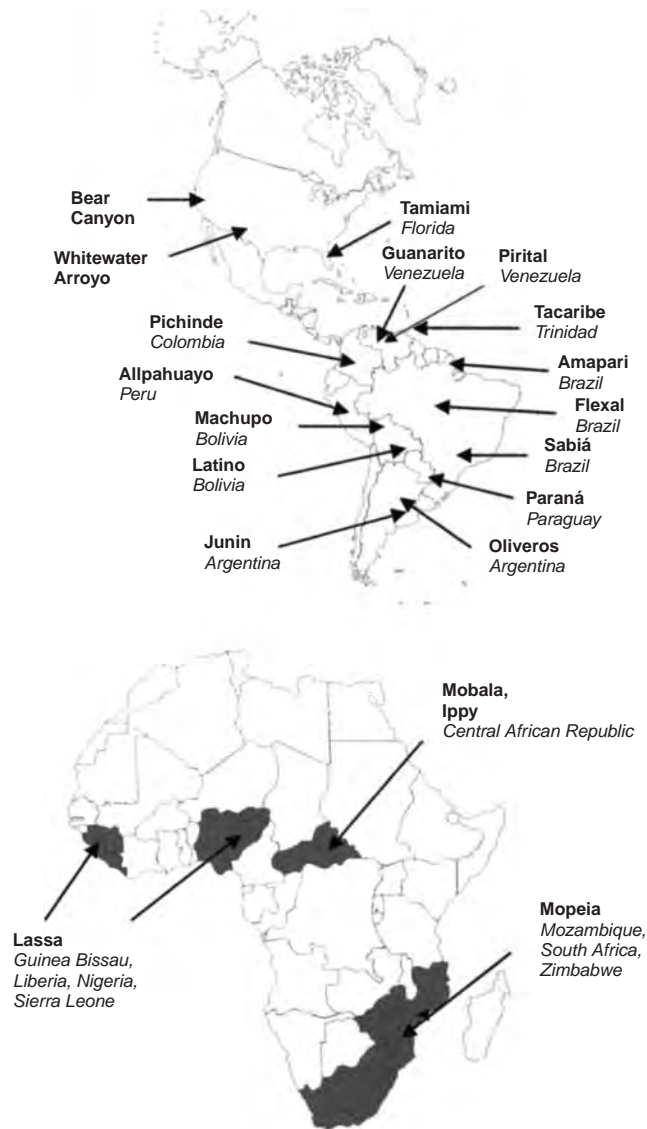


Figure 30.1 Geographical distribution of (a) New World and (b) Old World arenaviruses. Lymphocytic choriomeningitis virus (LCMV) can be found on all continents except Australasia.

The natural reservoirs of Lassa virus and the remaining Old World arenaviruses are members of the genus *Mastomys*. This is also a member of the Muridae and, in common with the host of LCMV, frequents human dwellings and food stores. The nature of the original reservoir for LCMV remains obscure, but it appears to be mainly in species of the Muridae which evolved in the Old World and subsequently spread to most parts of the globe. Interestingly, there is a wide range of tropism and virulence among those strains

of LCMV originally isolated from laboratory mouse colonies.

ULTRASTRUCTURE OF ARENAVIRUSES AND INFECTED CELLS

Negative-staining electron microscopy of extracellular virus shows pleomorphic particles ranging in diameter from 80 to 150 nm (Figure 30.2). The virus envelope

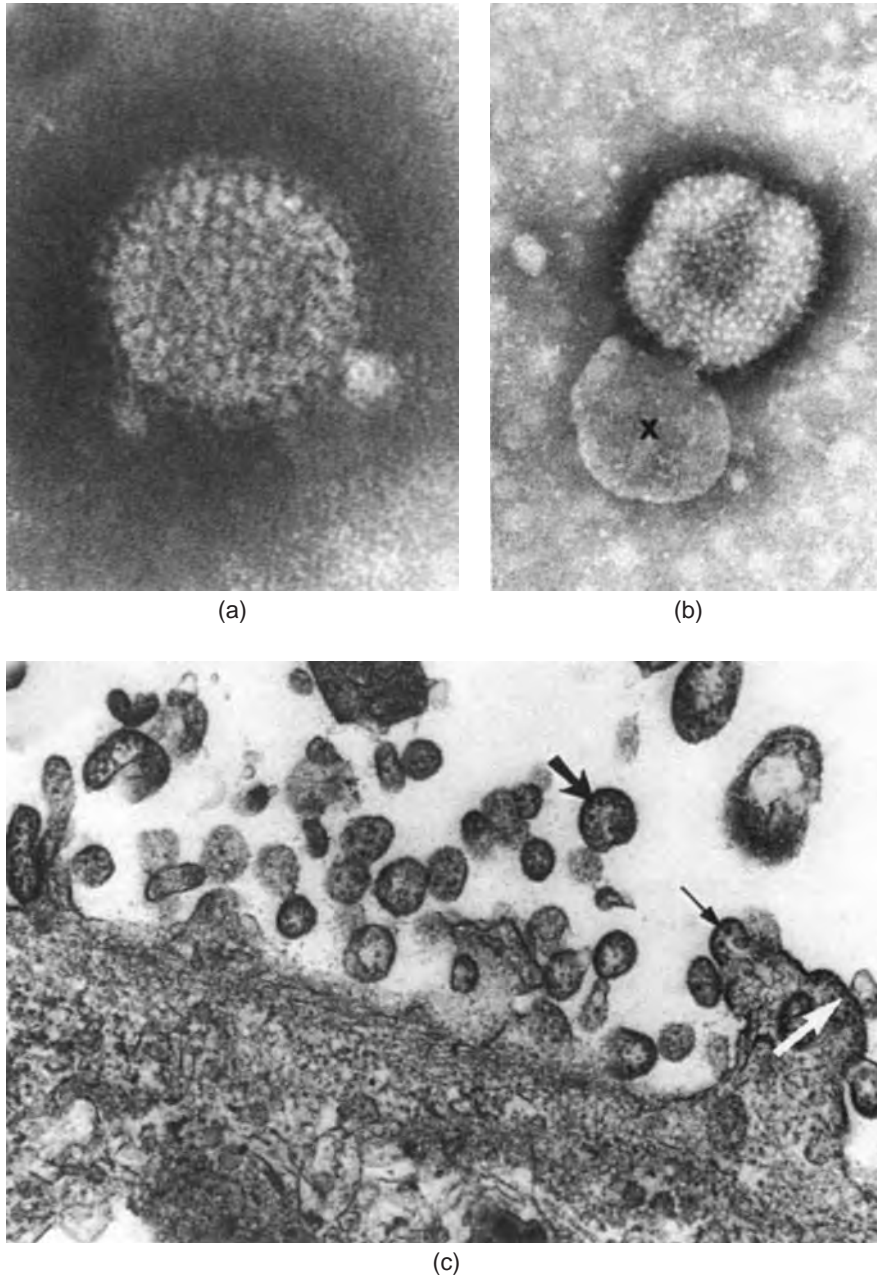


Figure 30.2 Electron microscopy of arenaviruses. (a) Negatively stained Lassa fever particle showing the whole surface covered in projections. Few particles are less than 100 nm, and many are twice this size, $\times 300\ 000$. (b) Mopeia virus from southern Africa. Here the negative stain has disrupted the particle, the contents of which (x) have been extruded, $\times 150\ 000$. (c) Lassa fever particles budding from an infected Vero cell. The thick arrow shows a mature particle, the thin arrow a maturing particle at the plasmalemma. Nucleocapsids and ribosomes line up immediately below the thickened membrane (white arrow), $\times 39\ 000$. (Source: Micrographs courtesy of Dr D.S. Ellis.)

is formed from the plasma membrane of infected cells. A significant thickening of both bilayers of the membrane together with an increase in the width of the electron-translucent intermediate layer is characteristic of arenavirus maturation. Little is known about the internal structure of the arenavirus particle, although thin sections of mature and budding viruses clearly show the ordered, and often circular, arrangements of host ribosomes that are typical of this virus group and confer the 'sandy' appearance from which its name is derived. Distinct, well-dispersed filaments 5–10 nm in diameter are released from detergent-treated virus. Two predominant size classes are present, with average lengths of 649 and 1300 nm; these lengths do not show a close relationship with the two virus-specific L and S RNA species. Each is circular and beaded in appearance. Convolutated filamentous strands up to 15 nm in diameter can be seen in preparations of spontaneously disrupted Pichinde virus. These appear to represent globular condensations which arise from an association between neighbouring turns of the underlying helix. The basic configuration of the filaments shows a linear array of globular units up to 5 nm in diameter, probably representing single molecules of the viral polypeptide. These filaments progressively fold through a number of intermediate helical structures to produce the stable 15-nm-diameter forms (Young, 1987).

Arenaviruses replicate in experimental animals in the absence of any gross pathological effect. However, cellular necrosis may accompany virus production, not unlike that seen in virus-infected cell cultures. The variable pathological changes associated with arenavirus infections are further complicated by the occasional appearance of particles in tissue sections that react strongly with fluorescein-conjugated antisera. Granular fluorescence with convalescent serum in the perinuclear region of acutely infected Vero cells is often seen. In addition, intracytoplasmic inclusion bodies are a prominent feature in virus-infected cells both *in vitro* and *in vivo*. These usually appear early in the replication cycle and consist largely of single ribosomes which later become condensed in an electron-dense matrix, sometimes together with fine filaments (Murphy and Whitfield, 1975).

CHEMICAL COMPOSITION

Proteins

All arenaviruses contain a major nucleocapsid-associated (N) protein of molecular weight 60–68 kDa with two glycoproteins in the outer viral envelope. These envelope

glycoproteins are not primary gene products but arise by proteolytic cleavage of a larger, 75 kDa glycoprotein precursor polypeptide (GPC). Maturation and release of virus are not inhibited in the presence of tunicamycin, an inhibitor of glycosylation, but glycoprotein processing is essential for infectivity.

The major glycoprotein species (GP2) in the molecular weight range of 34–42 kDa represents the C-terminal cleavage product of the GPC envelope glycoprotein precursor. The first 59 amino acids at the N-terminus of GPC act as a signal sequence, containing two distinct hydrophobic domains that could function during glycoprotein transport and virus assembly. GP1 is cleaved from the N-terminus at a unique cleavage site conserved among all arenaviruses except Tacaribe. GP1 assembles into tetramers linked by disulfide bonds. GP2 forms tetrameric structures proximal to GP1 in the glycoprotein peplomer, penetrating the viral membrane to form electrovalent bonds with the underlying N-RNA nucleocapsids, possibly mediated by the Z protein.

A major antigenic site recognized by antibodies has been located between amino acids 390 and 405, and cross-reactive monoclonal antibodies bind epitopes in this region. The corresponding N-terminal product of GPC cleavage (GP1) is highly glycosylated with at least four antigenic domains. Neutralizing monoclonal antibodies to LCMV map to two of these regions where there is less sequence homology between the GP1 than between the GP2 molecules of different arenaviruses. Polyclonal neutralizing antibody appears to react predominantly with conformation-dependent structures within at least one of these domains.

The internal nucleocapsid-associated (N) protein accounts for over 70% of the protein present in purified virus and infected cells, and remains bound to the virus genome after solubilization of the virus to form the structures resembling a string of beads seen by electron microscopy. However, cleavage products of the N protein are a consistent feature of both virus and virus-infected cells. Cleavage is not noticeable in Vero cells; yields of arenaviruses are lower in these cells, perhaps due to reduced availability of N for packaging. N protein accumulates in the cytoplasm of infected cells and a fragment of the N protein often seen in the nuclei, the exact function of which is not clear. Molecular cloning studies have shown a surprisingly high degree of homology between the 558 and 570 amino acid N proteins of Old and New World arenaviruses with structural motifs and RNA-binding domains particularly conserved. This would account for the serological cross-reactions seen using certain monoclonal antibodies raised against such epidemiologically distinct viruses and may indicate precise functional roles in virus replication for specific domains of the N polypeptide.

A minor component with a molecular weight in excess of 150 kDa is observed in infected cells and is found with purified nucleocapsids. This L protein is coded by the larger RNA genome segment as shown by the study of reassortment viruses and represents the virus-specific RNA polymerase (Fuller-Pace and Southern, 1989; Lukashovich *et al.*, 1997). Amino acid sequences common to all RNA-dependent RNA polymerases are present along the open reading frame coding for the L protein owing to the conservation of certain functional domains. An additional two sequences are shared with the RNA polymerases of bunyaviruses. A small, 11 kDa viral polypeptide, the Z protein, is considered to play a role in controlling the replication and expression of the genome owing to its zinc-binding properties and interactions with cellular proteins. The Z protein may also modulate the interferon response to infection *in vivo* by binding to the nuclear promyelocytic oncoprotein PML (Djavani *et al.*, 2001).

Nucleic Acids

The genome of arenaviruses consists of two single-stranded RNA segments of different sizes, designated L and S, with S RNA being more abundant. Analysis of RNA is complicated by the presence of ribosomal 18S and 28S RNA, although these cellular RNA species are not essential for virus replication. The total ribosomal RNA content may in turn be influenced by the varying proportions of infectious to non-infectious particles present in virus stocks, particularly if cells are infected at a multiplicity above 0.1. In addition there are small quantities of both cell and viral low molecular weight RNA. One of these species, mRNA coding for the viral Z protein, may have a role in the initial stages of infection. There is no obvious role for host RNA molecules in either replication or the establishment of persistent infections (see section on Replication below).

Extracted virion RNA is not infectious and the detection of a viral RNA polymerase led to the belief initially that arenaviruses adopt a negative strand coding strategy with respect to viral protein synthesis. However, the actual coding strategy from the L and S RNA strands is not entirely in accord with all negative-strand RNA viruses as some genetic information can only be expressed by a genomic sense mRNA. This 'ambisense' strategy is also a characteristic of some bunyavirus genomes (see Chapter 29). Such a coding strategy allows for the independent regulation of arenavirus envelope and nucleocapsid proteins.

The S strand codes for the nucleoprotein (N) and the envelope glycoprotein precursor (GPC) in two main open reading frames located on RNA molecules of opposite polarity. The 3' half of the S RNA codes for the N protein by production of an mRNA with a viral-sense

sequence specific for the GPC protein. Thus expression of the genome is by synthesis of subgenomic RNA from full-length templates of opposite polarities. The reading frames for the two major gene products are separated by a hairpin structure of approximately 20 paired nucleotides. This intergenic region may act as a control mechanism for genome expression by forming stable stem-loop structures that in turn regulate transcription. The S RNA of Tacaribe and Junín viruses are predicted to form a second stem-loop structure.

The L RNA strand represents about 70% of the viral genome; reassortment studies with virulent and avirulent strains of LCMV have shown that the lethality of the disease in guinea-pigs is associated with the properties of the L RNA. The L protein is encoded by a large open reading frame covering 70% of the L RNA strand: it is expressed via mRNA complementary in sense to the viral genome. The mRNA for the Z protein is also expressed from the L RNA strand.

All arenavirus genomes have a conserved 3' terminus at the ends of the L and S RNA; this sequence is inversely complementary to the 5' terminus of the same RNA strand. There is evidence obtained by electron microscopy of intramolecular and intramolecular complexes promoted by this arrangement of 3' and 5' termini nucleotide complementarity.

Phylogenetic Analysis

Sequencing of polymerase chain reaction (PCR) products can give useful quantitative comparisons between newly discovered isolates and those already characterized, providing caution is exercised both in the choice of primer sets and the method of analysis. Bowen and colleagues (Bowen *et al.*, 1997) analysed at least one strain of all arenaviruses known at that time using maximum parsimony to generate an unrooted phylogenetic tree. The results confirmed that the distant relationship between Old World and New World arenaviruses are broadly consistent with the previously determined serological relationships using polyclonal and monoclonal antibodies. The New World arenaviruses are divisible into three lineages (Figure 30.3): clade A includes the viruses Pichinde, Tamiami, Paraná, Flexal and the newly described Whitewater Arroyo Virus; clade B the viruses Sabiá, Tacaribe, Amaparí, Guanarito and the human pathogens Machupo and Junín; and clade C consists of Oliveros and Latino viruses. Interestingly, Whitewater Arroyo Virus isolated in the United States appears closely related to Tamiami, for many years the only arenavirus reported from North America. However, full-length analysis of Whitewater Arroyo Virus S RNA strand has shown a quite separate ancestry for the nucleocapsid (N) and envelope (GP1, GP2) proteins, almost certainly the result of

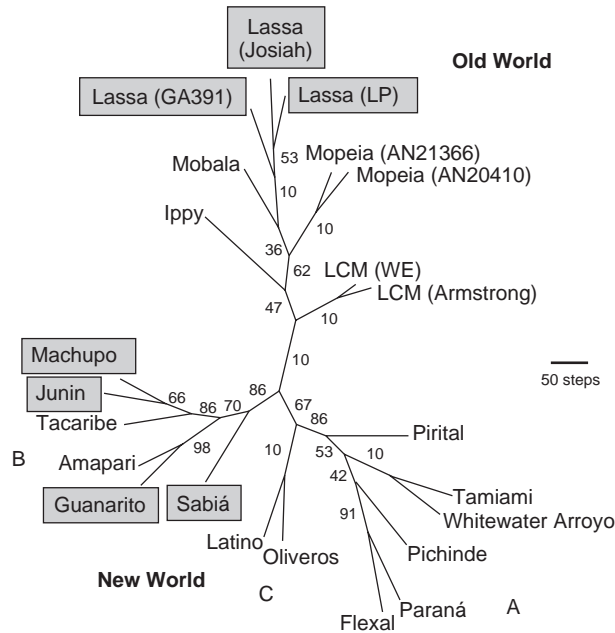


Figure 30.3 Phylogenetic analysis of Old and New World arenaviruses using nucleocapsid (N) gene sequences. The New World viruses are divisible into at least three clades. (Modified from Clegg, 2002.)

recombination between two ancestral arenaviruses. There is less variability among the Old World members. As may be expected from their natural history, Mopeia and Mobala viruses are closely related to Lassa fever virus. LCMV occupies the distinctive position of being closely related to the probable ancestral virus.

Nucleotide sequencing of isolates reveals a remarkable genetic diversity, not always correlated with geographical area and host. For example, Lassa virus may show over 25% nucleotide variability (Bowen *et al.*, 2000). Similarly, Guanarito virus isolates from clinical cases in Venezuela show a high degree of variability, the heterogeneity being greater among rodent isolates (Weaver *et al.*, 2000). Similar diversity has been reported among Whitewater Arroyo virus isolates collected from rodents captured in the same locality (Fulhorst *et al.*, 2002). It has been speculated that such diversity arises as a mechanism whereby the virus avoids the host immune response and thus persists within the rodent host.

The propensity to cause serious human illness appears to have evolved on two distinct occasions. The South American pathogens are all confined to clade B, suggesting these viruses have acquired the capacity to infect humans as a result of a common mutational event. Lassa fever virus, by contrast, has likely acquired its ability to cause serious haemorrhagic disease in humans by a quite separate series of evolutionary events.

REPLICATION

Arenaviruses replicate in a wide variety of mammalian cells, although either BHK-21 cells or monkey kidney cell lines are preferred for molecular studies (Howard, 1986). Arenaviruses can also infect a number of primary human cell lines and macrophages, including some members of the family that do not otherwise cause human infections. Most arenaviruses also grow well in mouse L cells but the simultaneous production of C-type retroviruses restricts the usefulness of such cells. The widely conserved cell protein α -dystroglycan has been identified as the cellular receptor for Old World arenaviruses, such as Lassa fever and Mobala, and the New World arenaviruses Latino and Oliveros in clade C (Cao *et al.*, 1998). Other cell surface proteins and cofactors may also be involved.

At low multiplicities of infection (i.e. below 0.1) the latent period is approximately 6–8 hours, after which cell-associated virus increases exponentially. The titre of extracellular virus reaches a maximum 36–48 hours after infection. The passage history of any particular virus stock is probably one of the most critical factors in determining the kinetics of arenavirus replication.

Infected cells undergo only limited cytopathic changes in the cell lines commonly employed, with little or no change in the total level of host cell protein synthesis; virus yields vary in different susceptible cell types.

Plaque assays are possible, but only under well-defined conditions. Cultures of persistently infected cells are readily established, with morphology and growth kinetics similar to those of uninfected cells.

The major feature of an ambisense coding strategy is that it allows for independent expression and regulation of the N and GPC genes from the S RNA segment. The N protein is expressed late in acute infection and continues to be expressed in persistently infected cells in the absence of glycoprotein production. This is explained by the production of a subgenomic mRNA from a negative polarity, virus-sense template. A control mechanism must therefore exist which determines the fate of nascent RNA of negative polarity, destined either for encapsidation or as a template for N protein-specific mRNA. In contrast, the template for glycoprotein-specific mRNA is of complementary sense to viral RNA and as such would not be required for nascent virus production. The lack of glycoprotein late in the replicative cycle or in persistently infected cells would therefore imply selective transcriptional or translational control of this gene product.

Both viral RNA and its complementary strand contain at least one hairpin sequence which may provide recognition points for termination of transcription by viral RNA polymerase. The nucleotide sequence in the hairpin region is of coding sense and may be transcribed, either as a discrete mRNA species or as a result of extended transcription of N or GPC messengers through this region. The reading frames for viral gene products transcribed from LCMV and Pichinde viral genomes would fit this hypothesis. In addition, a sequence for ribosomal 18S subunit binding is present on both mRNA molecules, although its significance remains uncertain.

DIAGNOSIS OF HUMAN ARENAVIRUS INFECTIONS

Early clinical signs are relatively nonspecific and therefore laboratory methods need to be used in order to reach a definitive diagnosis. A history of travel to a region where an arenavirus is endemic is also indicative. The importance of informing local public health officials as soon as an arenaviral case is suspected cannot be stressed too highly. Owing to the insidious onset and early influenza-like nature of the disease, there are a wide number of other causes which need to be excluded (Table 30.2). In the event that the patient presents with neurological signs, other causes of encephalitis or meningitis must be excluded.

Serology is often the only practical approach for making a differential diagnosis. Antibodies can be detected in the late acute phase and into early convalescence, but Old

Table 30.2 Differential diagnosis of arenavirus fevers.

Disease
Yellow fever
Malaria
Bacterial septicaemia
Enteric fevers (typhoid, paratyphoid)
Streptococcal pharyngitis
Typhus
Trypanosomiasis
Leptospirosis
Other viral haemorrhagic fevers

World arenaviruses are known to be immunosuppressive and thus caution needs to be exercised in interpreting negative results. The diagnosis of arenavirus infections ideally should be by virus isolation. Although arenaviruses can easily be grown in a variety of mammalian cell cultures, it must be remembered that clinical specimens from patients suspected as having a viral haemorrhagic fever should always be handled in biologically secure containment facilities. For this reason tests for antibody are more useful since inactivated viral antigens for serology can be prepared easily. For routine isolation, the E6 clone of Vero cells is the cell line of choice, although all arenaviruses grow well in primate and rodent-derived fibroblast cell lines. However, a cytopathic effect (CPE) is often difficult to see, and inoculated cultures often require examination by immunofluorescence (IF) or immunoperoxidase assay in order to detect viral antigens.

As a first step towards diagnosis, the use of PCR can be considered, provided primer sets have been rigorously tested beforehand and the temperature cycling conditions optimized. The need is often to give a first indication as to which of the various causes of viral haemorrhagic fever may be present, and thus it is often the case that PCR reactions need to be conducted in parallel using a range of primer sets specific for as many as six different agents, whether these be arenaviruses or other suspected causes, for example filoviruses. Drosten *et al.* (2002) have shown that this is possible; overcoming the common problems of low sensitivity and nonspecific amplification often associated with such multiplex PCR tests. The advantages of PCR include the opportunity to obtain sequence information of increasing relevance to the identification of new family members. Also PCR is useful for diagnosis in the early stages of disease when antibodies have yet to develop. The drawback, however, is that PCR does not discriminate between the presence of RNA fragments and infectious virus. Thus isolation of virus using cell cultures in a high-security facility should be attempted whenever possible.

Immunofluorescence-based specific viral antibody tests have been used successfully for the diagnosis of human arenavirus infections. In the case of Lassa fever, infected cell substrates are used that have been treated by ultraviolet (UV) light, acetone and cobalt irradiation to ensure safety. Drops of cell cultures dried onto glass slides can be prepared in a central laboratory and these preparations remain stable for many months. Most of the antigen detected within acetone-fixed infected cells represents cytoplasmic nucleocapsid protein. In the case of the New World arenaviruses, serological cross-reactions in the immunofluorescence test (e.g. with sera from patients with Bolivian (Machupo) and Argentine (Junín) haemorrhagic fevers) are found with fixed cultures. Substrates prepared from other members of the Tacaribe complex, which includes Junín and Machupo viruses, also react with sera taken from these patients during the acute phase and into early convalescence. Greatest cross-reactivity is seen between the closely related Junín and Machupo antigens, closely followed by Tacaribe virus-infected cells. Initially the use of enzyme-linked immunosorbent assay (ELISA) was restricted by the small amounts of antigen available for coating the solid phase but this has changed with the availability of recombinant antigens.

The difficulty in correlating the presence of hitherto unknown arenaviruses and human disease is illustrated by the emergence of Whitewater Arroyo virus. Initially fatal infections were considered as due to this virus on the basis that all three cases were found positive for Whitewater Arroyo virus RNA; however, virus was only isolated in one case and a specific diagnosis could not be confirmed in a second (Centers for Disease Control and Prevention, 2000). Until further cases come to light, assessing the zoonotic potential of this and similar newly described arenaviruses will remain challenging.

ANTIGENIC RELATIONSHIPS

Monoclonal antibodies are used to distinguish between virus strains because they can be prepared against epitopes that go unrecognized when polyclonal antisera are used. Buchmeier *et al.* (1981) summarized the patterns of reactivity with a panel of monoclonal antibodies directed against laboratory strains of the homologous LCMV, Lassa and Mopeia viruses. Antibodies directed against the smaller, GPC envelope glycoprotein cross-reacted by immunofluorescence with all substrates examined, whereas antibodies directed against the larger GP1 glycoprotein were either strain-specific or reacted with only a subset of the strains examined, presumably by binding to previously unrecognized epitopes. Certain of these broadly cross-reactive antibodies also reacted with Pichinde virus,

suggesting that epitopes on surface envelope structures among Old World and New World arenaviruses are conserved. A similar comparison has also been undertaken with monoclonal antibodies to Lassa tested against the Mopeia and Mobala viruses from Africa. Again, various degrees of cross-reactivity were observed, with reagents specific for the GP2 external glycoprotein. Mobala virus from the Central African Republic, however, appears to be distinct, as several cross-reactive monoclonal antibodies originally prepared against LCMV failed to recognize Mobala-infected cells.

The plaque-reduction neutralization test is highly specific for all members of the *Arenaviridae*; it is notable that the few examples of cross-reactivity were obtained with high-titre animal antisera raised against Junín, Tacaribe and Machupo viruses. However, the ease with which neutralizing antibodies can be quantified varies greatly. No cross-reactions have been observed between Junín and Machupo viruses in plaque reduction tests with human convalescent sera despite sharing a close antigenic relationship. A similar marked specificity of neutralization has been demonstrated with LCMV and Lassa sera, although neutralizing antibodies to Lassa virus are difficult to detect. The sensitivity of the neutralization test for LCMV can be increased by incorporating either complement or anti-gammaglobulin into the test system.

CLINICAL AND PATHOLOGICAL ASPECTS

Immune Response

Lymphocytic choriomeningitis virus infection of adult mice is the classic example of virus-induced immunopathological disease in which intracerebral inoculation causes severe disease and death. In contrast, if mice are infected before or shortly after birth they develop a nonpathogenic lifelong carrier state. The newborn mouse is immunologically immature and the virus does stimulate an immune response; in these circumstances the virus causes no illness. The immunologically mature mouse mounts an immune response following LCMV infection and a fatal choriomeningitis results, but without evidence of neuronal damage (Lehmann-Grube and Lohler, 1981). Immunosuppression, either by neonatal thymectomy or by use of anti-lymphocytic serum, protects adult mice against fatal LCMV infection; the pathological damage thus appears to be immune-mediated.

The immune responses are best understood in acute infection of mice. Intraperitoneal injection of adults gives rise to an asymptomatic acute infection of two to three weeks' duration. Studies of such infections have resulted in a number of findings with implications beyond

the field of arenavirus research. First, the description by Rowe (1954) of the immune-mediated pathology of acute LCMV infection was the seminal demonstration that the pathogenicity of the viruses may not be solely related to their cytolytic effects. The observation that LCMV-infected cells were lysed by cytotoxic T cells led to the understanding that recognition of a target cell requires the presence of both viral antigen and class I antigen of the host's major histocompatibility complex (Zinkernagel and Doherty, 1997). Second, the persistence of virus in mice infected shortly after birth has provided a model for both host and viral factors involved in the establishment and maintenance of chronic infection. The finding of virus antigen-antibody complexes in persistently infected animals shows that B-cell tolerance is not involved.

Activation of natural killer cell activity early in acute infection, which coincides with the production of interferon, has helped increased our knowledge of innate immunity against virus infection.

The direct demonstration of virus replication in lymphocytes is of substantial importance for understanding arenavirus pathogenesis, as these cells provide a continued source of virus entering the circulation. These cells play a key role in the temporal and quantitative control of the immune response (Murphy and Whitfield, 1975). Viral antigen is present in the cells of the lymphatic system in mice persistently infected with LCMV. Most of the virus in the blood of carrier mice is associated with approximately 2% of the total circulating lymphocyte population. Precursor or immature lymphocytes may support the replication *in vitro* of LCMV when stimulated to proliferate by phytohaemagglutinin, in agreement with the general finding that arenaviruses grow best in actively dividing cells. Such clonal expansion may be triggered *in vivo* by viral antigen binding to appropriate lymphocyte receptors.

Key to understanding LCMV outcome is T-cell immunosuppression or 'tolerance'. Immunocompetent mice induce a marked adaptive immune response that rapidly controls the acute infection and results in virus elimination. The effector CD8⁺ T cells directly lyse infected cells and this function is augmented by the production of interferon- γ and tumour necrosis factor, which further limit the extent of virus replication. Virus heterogeneity also plays a role: a normally cytolytic isolate of LCMV will produce persistence after intracerebral inoculation of newborn mice, but during the course of chronic infection clones can be obtained from lymphoid organs with a different phenotype characterized as being able to induce persistent infection. Passage of this virus results in persistence owing to the absence of a functioning cytolytic CD8⁺-mediated T-cell response. Comparison of

these isolates has shown that acquisition of the persistence phenotype correlated with a change in sequence along the GPI surface glycoprotein (Sevilla *et al.*, 2000), with the consequence that virus binds with higher affinity to α -dystroglycan, a receptor molecule found predominantly on the surface of cells in lymphoid tissue. Here there is a similarity with Lassa virus which also binds α -dystroglycan with high affinity, leading to dendritic cell activation and downstream immunosuppression (Mahanty *et al.*, 2003). Interleukin (IL)-10 plays a critical role in this process of T-cell suppression as mice deficient in IL-10 production fail to become chronically infected with LCMV.

Interferon

Interferon is induced early in acute LCMV infection of mice, and its appearance correlates with the appearance of infectious virus in the blood. There have been few studies of the levels of α -interferon in acute human arenavirus infection. Elevated levels can be detected in the early stages of AHF, and these coincide with the onset of fever and backache. Although there is no correlation between the titres of interferon and circulating virus, Levis *et al.* (1984) suggested that at least some of the clinical signs may be directly attributable to interferon, particularly the depression of platelet and lymphocyte numbers resulting from Junín virus infection of leukocytes and macrophages.

The role of natural killer cells in controlling arenavirus infection is not clear, although many are found in the blood and spleen of LCMV-infected mice as early as one day after infection. This response declines rapidly, however, until by the fourth day almost all the cytolytic immune activity is H-2 restricted.

Antibodies

Antibodies against the nucleocapsid can be detected early in the acute phase of most arenavirus infections. Infectious virus-antibody complexes can be detected four days after LCMV infection of mice but there is no evidence that B-cell responses play a role in the pathology of the acute infection. Immunity to arenaviruses appears in general to be type-specific; an infection with one member of the family does not necessarily confer protective humoral or cellular immunity against arenaviruses that can be distinguished by neutralization tests *in vitro*. However, cross-reactive antibodies may confer some degree of protection in some instances. For example, immunization of guinea-pigs with Tacaribe virus protects against subsequent challenge with the normally virulent Junín virus. These responses are clearly different from the secondary immune responses that may be induced as a result of antigenic similarities between the nucleocapsid proteins of the two viruses concerned.

Cellular Immunity

The role of cellular immunity during acute LCMV infection is manifested by a cytotoxic T-cell response associated with the clearing of virus; for example, CD8⁺ T cells cultured and cloned *in vitro* and injected intravenously reduce the amount of virus 100-fold in the spleens of acutely infected mice. Cytotoxic T-cell responses are restricted by the need for activated T cells to recognize both viral antigen and host cell proteins encoded by the H-2 region, a concept developed in LCMV-infected mice which, as referred to above, has radically altered our concept of how the infected host clears virus from infected tissues. The generation of specific cellular toxicity is related to the replication of the virus in target organs; inoculation with live virus appears necessary as a primary cytotoxic T-cell response is not seen if the virus is inactivated. This has implications for the development of inactivated arenavirus vaccines should the stimulation of cellular immunity prove essential for protection, as many workers believe. T-cell clones from mice infected with the Armstrong strain of LCMV lyse a wide range of LCMV strains. This finding demonstrates that cytotoxic responses to arenaviruses are haplotype-restricted but show a broad cross-reactivity for conserved viral determinants. Some of these determinants have now been mapped to an immunodominant domain of GP2 between amino acids 278 and 286 (Whitton *et al.*, 1988). Such T-cell clones can discriminate between cells infected with a given strain of virus containing only a single amino acid substitution in this region; this implies that mutations in this region of the genome may lead to selection of a virus variant with altered pathogenicity.

In contrast to LCMV, the role of cellular immunity in Lassa virus infection seems to play only a minor role. The human host is clearly restricted in its ability to clear the virus and preventing virus replication in tissues. The poor neutralizing antibody response and the high degree of viraemia contrast sharply with those in patients with South American haemorrhagic fevers, with neutralizing antibodies developing rapidly during acute infection and in whom there is little viraemia.

The release of pro-inflammatory mediators and cytokines is a cardinal feature of viral haemorrhagic fever in humans, yet there is suppression in patients with Lassa fever of both antibody and cytotoxic T-cell responses. Mahanty *et al.* (2003) have provided insight into this paradox by showing that Lassa virus productively infects macrophage-derived dendritic cells. The result is that dendritic cells do not become activated and thus the two arms of the immune response are blocked. The apparent contradiction that macrophages and endothelial cells are strongly activated would suggest the differentiation of inflammatory responses between cell

populations and their microenvironments. Whatever the mechanism, the prospects for immunotherapy at first sight seem poor but there is some encouragement in that antibodies directed against immunoregulatory components, for example IL-10, can 'restore' effector CD8⁺ cells in mice chronically infected with LCMV, leading to virus clearance (Barber *et al.*, 2006).

LCMV infection in mice shows the complexity of this process: interferon- γ , a key effector in inhibiting virus replication, has been shown by Whitmire *et al.* (2005) to enhance T-cell help but is not required for functional development. Thus immunosuppression in Lassa virus infection may be due to factors other than the direct activation of effector T cells.

PERSISTENT INFECTION

Antibodies

Mice persistently infected with LCMV produce antibodies to all the major structural proteins. This finding was contrary to the view previously held that viral persistence is established or maintained *in vivo* as a result of an absence of specific B-cell responses to some or all viral antigens. As viral proteins continue to be produced in the tissues of such animals, circulating antigen-antibody complexes are formed which can be detected by binding Clq. Antibodies in the sera of such animals bind to the surface of virus-infected cells, but are unable to mediate complement-dependent cytolysis, suggesting that viral antigens at the plasma membrane may be either masked, thereby preventing further immune reactions, or removed by antigenic modulation. This notion would imply that persistently infected mice are deficient in viral antibody of the complement fixing subclass of IgG, but this has not been proven.

Cell-mediated Immunity

Mice persistently infected with LCMV are expected to mount a normal T-cell response to unrelated immunogens, indicating a state of tolerance only to specific antigens. However, it has been difficult to distinguish T-cell suppression from an absence of virus-specific T-cell clones. Here it is pertinent to mention that persistence of LCMV in mice infected at birth or *in utero* was one of the important observations made by Burnet and Fenner to support the concept of tolerance to 'self' antigens. The time of infection is critical, as LCMV infection induced 24 hours after birth results in a cytotoxic T-cell response typical of acute disease. The failure of mice infected before this time to mount an adequate cytotoxic response is presumably related to maturation of T-cell function; it appears

to be virus-specific because adult carrier mice challenged with other unrelated arenaviruses mount normal cytotoxic T-cell responses. Thus the block appears to be either in recognition of infected cells, or in their expression of type-specific antigenic determinants. The relationship between the virus and the host immune response may be more complex than hitherto believed, however, as there is evidence for arenaviral RNA being transcribed into complementary DNA, presumably mediated by endogenous retroviral reverse transcriptase (Klenerman *et al.*, 1997). This would imply that long-term persistence of viral gene sequences as retroviral elements results in continual low-level expression of viral proteins. Thus immune responsiveness is maintained by the continued presentation of viral sequences as major histocompatibility complex (MHC)–peptide complexes.

PATHOLOGY OF ARENAVIRUS INFECTIONS: GENERAL FEATURES

Haemorrhagic fevers due to arenaviruses are characterized by a rapidly progressing febrile phase accompanied by capillary leakage, the latter giving rise to subcutaneous haemorrhages sometimes accompanied by haemorrhages at mucosal surfaces. It appears to be more likely that disease is caused by direct damage of cells by the virus although disease severity often appears out of proportion to the damage evident by histology. Post-mortem studies on patients who died from Junín virus infection have shown generalized lymphadenopathy, endothelial swelling both in the capillaries and arterioles of almost every organ, abnormalities accompanied by a depletion of lymphocytes in the spleen. Junín virus first replicates in lymphoid tissue, from whence it invades the reticuloendothelial system and those cells concerned in generating the humoral and cellular immune response. Fatal illness is invariably associated with capillary damage, leading to capillary fragility, haemorrhages and irreversible shock (Johnson *et al.*, 1973). Disseminated intravascular coagulation is not a typical feature. Although Lassa fever is often regarded as being hepatotropic, the extent of hepatic damage is insufficient to account for the severity of the infection, serum transaminase values often remaining within normal limits except in more severe cases. Studies of Lassa virus-infected rhesus monkeys have shown that changes in vascular function may play a much greater role in pathogenesis, as a result either of viral replication in the vascular epithelium or secondary effects of virus activity in different organs. Platelet and epithelial cell functions fail immediately before death and are accompanied by a drop in the level of prostacyclin; these functions rapidly return to normal in surviving animals

(Fisher-Hoch *et al.*, 1987). Impairment of vascular epithelial functions in the absence of histological changes appears to be a common feature of the final stages of viral haemorrhagic diseases. Parallel infection of dendritic cells may in turn lead to impairment of immune responses and the release of pro-inflammatory cytokines. There is no general agreement on this point, however, since some work has shown that the virus may actually stimulate dendritic cells when infected *in vitro*.

The pathogenesis of AHF has been studied in guinea-pigs infected with Junín virus, regarded as a suitable model of human disease. There is the pronounced thrombocytopenia and leukopenia characteristic of human infections, and animals die of severe haemorrhagic lesions. Bone marrow cells are destroyed with release of proteases and both acid and alkaline phosphatases into the blood; this leads to consumption of the C4 complement component. These effects may lead in turn to progressive alterations in vascular permeability and platelet function (Rimoldi and de Bracco, 1980). The most extensive histopathological studies have been made on tissues from patients with Lassa fever (Walker and Murphy, 1987). However, there are many similarities in the pathological lesions found in human Junín and Machupo virus infections. Focal non-zonal necrosis in the liver has been described in all three conditions, with hyperplasia of Kupffer cells, erythrophagocytosis and acidophilic necrosis of hepatocytes. Councilman-like bodies can be observed together with cytoplasmic vacuolations and nuclear pyknosis or lysis. As with many organs, there is little evidence of cellular inflammation. Lesions in other organs have been described, including interstitial pneumonitis, tubular necrosis in the kidney, lymphocytic infiltration of the spleen and minimal inflammation of the central nervous system (CNS) and myocardium (Walker and Murphy, 1987). The hepatic changes may range from mild, focal necrosis to extensive zonal necrosis involving up to 50% of hepatocytes. These changes are consistent with a direct cytolytic action of the virus; nevertheless, the simultaneous presence of Lassa virus and specific antibodies during the later stages of the acute disease suggests that antibody-dependent cellular immune reactions may also occur. Microscopic changes in the kidneys are minimal, although it is not clear whether the functional impairment is due to the deposition of antigen–antibody complexes.

Lymphocytic Choriomeningitis

Clinical and Pathological Features

The incubation period is 6–13 days. Infection is often inapparent but may present as an influenza-like febrile illness, as aseptic meningitis or as severe

meningoencephalomyelitis. Up to 20% of patients develop neurological signs, and in such cases the incubation period may extend to 21 days. The great majority of LCMV infections are, however, benign. In the influenza-like illness there is fever, malaise, muscular pains and bronchitis. LCMV infection is biphasic, the first phase being characterized by leukopenia followed by lymphocytosis. In general, the mean value of mononuclear cells is approximately $600 \text{ cells mm}^{-3}$, although counts of up to 3000 mm^{-3} have been recorded. A coryza together with retro-orbital pain, anorexia and nausea are common. During the acute phase a large number of mononuclear cells are present in the cerebrospinal fluid (CSF) as part of a pleocytosis, although the absolute number varies with time after onset.

CNS involvement becomes apparent in the second phase after a short period of remission. As with all CNS diseases, the CSF is at increased pressure, with a slight rise in protein concentration, normal or slightly reduced sugar concentration, and a moderate number of cells, mainly lymphocytes ($150\text{--}400 \text{ mm}^{-3}$). It has been noted that the majority of such patients have a history of influenza-like illness immediately prior to the onset of meningitis. The meningeal form is more common; the same symptoms may remain mild and be of short duration and patients recover within a few days, but there can be a more pronounced illness with severe prostration lasting two weeks or more. Chronic sequelae have been reported on occasion, including parotitis and orchitis. Other symptoms include continuing headache, paralysis and personality changes. The few deaths reported have followed severe meningoencephalomyelitis. In this most severe form, patients may rapidly develop a bilateral papilloedema, confusion and paralysis of the extremities over a one-week period. An erythematous rash followed by haemorrhage and death has also been reported. Virus can be isolated from blood, CSF and, in fatal cases, from brain tissue.

The mechanism of transmission of the virus to humans is not fully understood but is likely to involve dust contaminated by urine, the contamination of food and drink, or via skin abrasions. Intrauterine infection is possible, the fetus being at risk of developing hydrocephaly or microcephaly.

Epidemiology

Humans are usually infected through contact with rodents. In the past, these have been acquired in laboratories, where LCMV may be a contaminant in laboratory colonies of mice and hamsters. In particular, virus is shed from the urine of persistently infected animals, resulting in contamination of skin and working surfaces. Hamsters kept as pet animals have also played a role in human

infection. Human cases of LCMV infection are rare, despite there being widespread evidence of infection among peridomestic rodents, although it must be stated the prevalence of chronic infection in wild mice varies between geographical regions. When human infections do occur, these tend to be benign or asymptomatic. This is consistent with the few reported seroprevalence studies that have shown low numbers (<5%) of adults with have demonstrable anti-LCMV antibodies. Of concern are reports of LCMV transmission by organ transplantation where the donor has a history of keeping rodents as pets. In a study of two clusters of infection, one of these was traced back to a donor who had been exposed to virus from an infected pet hamster (Fischer *et al.*, 2006).

A variant of LCMV has been isolated from captive New World primates. The histopathology in infected marmosets and tamarins is remarkably similar to that seen in Lassa virus infection in humans. It is suspected that these animals acquired the virus from infected *Mus musculus* rodents (Montali *et al.*, 1995; Stephensen *et al.*, 1991).

Diagnosis of Bolivian and Argentine Haemorrhagic Fevers

Although the clinical features of Bolivian and Argentine haemorrhagic fevers are similar, the laboratory diagnosis of these diseases is approached in a somewhat different manner. In the case of Junín, virus can be recovered consistently from the blood from the third to the eighth day of illness; in contrast, direct recovery of Machupo virus from acutely ill patients is much more difficult. In both instances, however, serological methods are more useful.

Antibodies may be detected sufficiently early in both cases, providing suitable paired sera are available or the capability exists for the detection of virus-specific IgM antibodies. Early use of immunofluorescence techniques for the diagnosis of AHF showed that specific antibodies could be detected by the indirect method approximately 30 days after onset of symptoms. Specific staining is generally seen as a bright, granular fluorescence, evenly distributed over the cytoplasm of the fixed infected cell substrate. The titre of immunofluorescent antibodies increases from the 12th to the 20th day of illness and is a mixture of IgG and IgM antibodies.

Neutralizing antibodies to both Machupo and Junín viruses persist for many years at high titre, appearing simultaneously with IgG antibodies. The sensitivity and specificity of neutralization tests for detecting immunity to Junín virus has proven to be of value retrospectively in the detection of subclinically infected individuals. The test may be carried out in Vero cell monolayers by varying virus dilution in the presence of a fixed concentration of

serum. Antibody titres are then expressed as an index calculated by subtracting the logarithmic differences between the virus titre in control and experimental reactions. Inapparent infections have been shown in approximately 20% of laboratory workers handling known or presumptively positive specimens by this method.

Argentine Haemorrhagic Fever (Junín Virus)

Clinical and Pathological Features

Argentine haemorrhagic fever has been known since 1943 and Junín virus, the causative agent, was first described in 1958. The virus causes annual outbreaks of severe illness, with between 100 and 3500 cases, in an area of intensive agriculture known as the wet pampas in Argentina. Mortality in some outbreaks has ranged from 10 to 20%, although the overall mortality is generally 3–15%. After an incubation period of 7–16 days, the onset of illness is insidious, with chills, headache, malaise, myalgia, retro-orbital pain and nausea; these are followed by fever, conjunctival injection and suffusion, a pharyngeal enanthema and erythema and oedema of the face, neck and upper thorax. A few petechiae may be seen, mostly in the axilla. There is hypervascularity and occasional ulceration of the soft palate. Generalized lymphadenopathy is common. Tongue tremor is an early sign, and some patients present with pneumonitis. In the more severe cases the patient's condition becomes appreciably worse after a few days, with the development of hypotension, oliguria, haemorrhages from the nose and gums (Figure 30.4), haematemesis, haematuria and melaena. Oliguria may progress to anuria and pronounced neurological manifestations may develop. There is a suggestion that disease severity correlates with the inhibition of platelet aggregation (Cummins *et al.*, 1990). Laboratory findings have included leukopenia with a decrease in the number of CD4⁺ cells, thrombocytopenia and urinary casts containing viral antigen. Patients recover when the fever falls, followed by diuresis and rapid improvement. Death may result from hypovolaemic shock. Subclinical infections also occur. Person-to-person transmission has not been observed.

Epidemiology

Argentine haemorrhagic fever has a marked seasonal incidence, coinciding with the maize harvest between April and July, when rodent populations reach their peak. Agricultural workers, particularly those harvesting maize, are, not surprisingly, the most commonly affected. The main reservoir hosts of Junín virus are *Calomys* field voles that live and breed in burrows under the maize fields and in the surrounding grass banks (Figure 30.5). Other rodent species may also be infected. *Calomys* spp.



Figure 30.4 Bleeding from the gum margin in a case of Argentine haemorrhagic fever. (Source: From Howard, 1986.)



Figure 30.5 The habitat for the rodent *Calomys* sp. in the wet pampas of Argentina. (Source: From Howard (1986).)

have a persistent viraemia and viraemia, and virus is also present in considerable quantities in the saliva. The mode of transmission of Junín virus to humans has not been conclusively established. The virus may be carried in the air from dust contaminated by rodent excreta or may enter by ingestion of contaminated foodstuffs.

Therapy

In contrast to Lassa fever, antibodies play a major role in recovery from Junín infection. Controlled trials of immune plasma collected from patients at least six months into convalescence have shown a dramatic reduction in mortality if plasma is given within the first eight days of illness. The efficacy of this therapy is directly related to the titre of neutralizing antibody in the plasma; as a result a dose of no less than 3000 'therapeutic units' per kg body weight has been recommended (Enria *et al.*, 1984). The late development of a neurological syndrome is seen in up to 10% of patients treated with immune plasma; it

is often benign and self-limiting but points to the possible persistence of viral antigens on cells of the CNS well into convalescence. Treatment with immune plasma also restores the response of peripheral blood lymphocytes to antigenic stimuli, suggesting that administration of plasma somehow modulates cellular immunity. Interestingly Tacaribe virus infection of mice can be abrogated by injection of CpG oligodeoxynucleotides which stimulate an increase in virus-specific antibodies, again indicating antibody production may play a pivotal role in virus recovery from infection (Pedras-Vasconcelos *et al.*, 2006).

Prophylaxis

There have been attempts to produce a vaccine against AHF. The XJC13 strain of virus grown in the brains of suckling mice is relatively nonpathogenic and was administered to 636 volunteers between 1968 and 1970. Over a period of three years, 70 cases of Junín virus infection occurred among the population but there were no cases amongst those immunized. However, the vaccine often induced a mild febrile reaction or a subclinical infection, and its use was discontinued despite the fact that over 90% of vaccinees maintained neutralizing antibody for up to nine years. There have been renewed attempts to develop a new vaccine strain sufficiently attenuated for human use and meeting modern day requirements as to derivation, manufacture and potency. Several clones have been prepared from the original XJ isolate, one of which exhibits less neurovirulence than the XJC13 strain yet protected rhesus monkeys against challenge with wild-type Junín virus (McKee *et al.*, 1993). This 'Candidate 1' vaccine has been tested in a double blind study in volunteers.

Bolivian Haemorrhagic Fever (Machupo Virus)

Clinical Features

Bolivian haemorrhagic fever was first recognized in 1959 in the Beni region in north-eastern Bolivia with 470 reported cases in the years up to 1962. The disease continued in that region more or less annually for a number of years in the form of sharply localized epidemics. Its incidence has decreased considerably since the late 1960s and human infections are now rarely reported. It is worth noting that the discovery of a common morphology and serological cross-reaction between Machupo and LCMV led to the concept of the arenavirus family. The mortality in individual outbreaks varied from 5 to 30%. The most notable outbreak affected 700 people in the San Joaquin township between late 1962 and the middle of 1964. The mortality was 18%. In July 1994, a fresh outbreak occurred in north-eastern Bolivia, with at least seven deaths. These were the first recorded since 1971; for reasons that

are obscure, this outbreak did not appear linked to any major changes in rodent numbers or behaviour.

The clinical disease is similar to AHF. The incubation period ranges from 7 to 14 days and the onset is insidious, beginning with an influenza-like illness accompanied by malaise and fatigue. This is followed by abdominal pain, anorexia, tremors, prostration and severe limb pain. About one-third of patients show a tendency to bleed, with petechiae on the trunk and palate, and bleeding from the gastrointestinal tract, nose, gums and uterus. Almost half the patients develop a fine tremor of the tongue and hands, and some may have more pronounced neurological systems. The acute disease may last two to three weeks and convalescence may be protracted, generalized weakness being the most common complaint. Clinically inapparent infections are rare. Machupo virus, the responsible agent, is readily isolated from lymph nodes and spleen taken at necropsy. Isolation of the virus from acutely ill patients has proved difficult, with the best results being obtained from specimens taken 7–12 days after onset.

Epidemiology

The rodent reservoir of Machupo virus is the field vole *Calomys callosus*; over 60% of animals caught during the San Joaquin epidemic were found to be infected. The distribution of cases in the township was associated with certain houses and *C. callosus* was trapped in all households where cases occurred. Transmission to humans is probably by contamination of food and water or by infection through skin abrasions. Human transmission is unusual but a small episode took place in 1971, well outside the endemic zone. The index case, infected in Beni, carried the infection to Cochabamba and, by direct transmission, caused five secondary cases, of which four were fatal.

Abnormally low rainfall, combined with an increase in the use of insecticide, led to a rapid decline in the numbers of cats, with the result that the population of Machupo-infected rodents increased dramatically, thus increasing the opportunity for human contact with contaminated soil and foodstuffs. Once this balance was restored, the number of reported cases declined rapidly.

Treatment

As with AHF, treatment is largely supportive. Although attempts have been made to use convalescent immune plasma from survivors of Machupo infection, a combination of a lack of facilities in Bolivia suitable for treating collected plasma and the absence of a controlled trial as to the efficacy of its use means that the treatment of patients with immunoglobulin remains speculative. Ribavirin has been administered during the 1994 outbreak, but again

there is no certain indication that ribavirin is effective against Machupo infection.

Lassa Fever

History

In 1969 Lassa virus made a dramatic appearance in Nigeria as a lethal, highly transmissible disease. The first victim was an American nurse who was infected in a small mission station in the Lassa township in north-eastern Nigeria, whence the virus and the disease derive their names. The origin of the infection was never determined, although it is thought to have been acquired through direct contact with an infected patient in Lassa. When the nurse's condition steadily deteriorated she was flown to the Evangel Hospital in Jos, where she died the following day. While she was in hospital she was cared for by two other American nurses, one of whom also became infected by direct contact, probably through skin abrasion. This nurse became unwell after an eight-day incubation period and died following an illness lasting 11 days. The head nurse of the hospital, who assisted at the post-mortem of the first patient, fell ill seven days after the death of the second patient for whom she had cared, and from whom she probably acquired the infection. This third case was evacuated to the United States by air. After a severe illness under intensive care she slowly recovered. A virus, subsequently named Lassa, was isolated from her blood by workers at the Yale Arbovirus Unit. One of these virologists became ill but improved after an immune plasma transfusion donated by the third case. Five months after this infection, a laboratory technician in the Yale laboratories, who had not been working with Lassa virus, fell ill and died. The manner in which this infection was acquired has never been determined. This trail of events not unnaturally earned for Lassa virus a formidable notoriety, which was sharply enhanced by two more devastating hospital outbreaks—one in Nigeria, the other in Liberia.

The fourth outbreak was seen in Sierra Leone in October 1972. In sharp contrast to the previous outbreaks, this one was not confined to hospitals, although hospital staff were at considerable risk and several became infected. Most of the patients acquired their illness in the community and there were several intrafamilial transmissions. This led to a revision of the initial view—formed from experience of nosocomial infections—of Lassa fever having a high mortality.

Lassa fever has since continued to occur in West Africa, usually as sporadic cases (Monath, 1987). For example, between 1969 and 1978 there were 17 reported outbreaks affecting 386 patients, among whom the mortality was 27%. Eleven of the episodes were in hospitals, where

the case fatality rate reached 44%; two were laboratory infections, two were community-acquired outbreaks, and two were prolonged community outbreaks. Eight patients were flown to Europe or North America. One of them was evacuated with full isolation precautions and the remainder, of whom five were infectious, travelled on scheduled commercial flights as fare-paying passengers. Fortunately, no contact cases resulted.

Clinical Features

Lassa virus causes a spectrum of disease ranging from subclinical to fulminating fatal infection. Although sharing many features with those seen in patients with the South American arenaviruses, neurological involvement in Lassa fever is minimal or absent. Studies in Sierra Leone show that most patients present with only a mild form of the disease and this is resolved by good primary healthcare. The incubation period ranges from 3 to 16 days and the illness usually begins insidiously. Importantly, given that Lassa virus is endemic in West Africa, the disease is difficult to distinguish in the early stages from other systematic febrile illnesses, most notably malaria, septicaemia and yellow fever. The most reliable clinical signs on presentation are a sore throat, myalgia, abdominal and lower back pains, accompanied by vomiting. Occasionally a faint maculopapular rash may be seen during the second week of illness on the face, neck, trunk and arms. Cough is a common symptom, and light-headedness, vertigo and tinnitus appear in a few patients. The fever generally lasts 7–17 days and is variable. Convalescence begins in the second to fourth weeks, when the temperature returns to normal and the symptoms improve. Most patients complain of extreme fatigue for several weeks. Loss of hair is common and deafness afflicts one in four patients, and there may be brief bouts of fever.

In a significant number of cases the symptoms suddenly worsen after the first week, with continuing high fever, severe prostration, chest and abdominal pains, conjunctival injection, diarrhoea, dysphagia and vomiting. One important physical finding is a distinct pharyngitis; yellow-white exudative spots may be seen on the tonsillar pillars together with small vesicles and ulcers. The patient appears toxic, lethargic and dehydrated; the blood pressure is low and there is sometimes a bradycardia relative to the body temperature. Patients in whom the disease is eventually fatal not uncommonly have a high sustained fever. There may be cervical lymphadenopathy, an encephalopathy, and coated tongue, puffiness of the face and neck, and blurred vision. In approximately 25% of cases there is marked involvement of the CNS, manifested by disorientation, ataxia and seizures. Progression to severe haemorrhaging occurs in around a fifth of patients and it

is among such patients that mortality exceeds 50%. Death is due to shock, anoxia, respiratory insufficiency and cardiac arrest. Lassa fever is particularly severe in pregnant women. A study of 75 women in Sierra Leone showed that 11 of 14 deaths were the result of infection during the third trimester; a further 23 patients suffered abortion in the first and second trimesters.

Epidemiology

Lassa virus has been repeatedly isolated from the multimammate rat *Mastomys natalensis* in Sierra Leone and Nigeria. This rodent is a common domestic and peridomestic species, and large populations are widely distributed in Africa south of the Sahara. During the rainy season it may desert the open fields and seek shelter indoors, thus infection rates peak during the summer months. Some genetic variation has been shown in *Mastomys* populations inhabiting different ecological niches; however, there appears to be no difference in the prevalence of antibody and virus in at least two of the karyotypes found in West Africa. The animals are infected at birth or during the perinatal period. Like other arenaviruses, Lassa virus produces a persistent, tolerant infection in its rodent reservoir host with no ill-effects and without any detectable immune response. The animals remain infectious during their lifetime, freely excreting Lassa virus in urine and other body fluids. The correlation between the prevalence of antibody in a community and the degree of infestation by infected rodents, however, is poor.

Studies of the ratio of clinical illness to infections confirm that Lassa fever is endemic in several regions of West Africa. In contrast to the early studies, it has been estimated that only 1–2% of adult infections are fatal—substantially lower than the figures of 30–50% originally associated with the early nosocomial outbreaks. However, there may still be up to 300 000 infections per year with as many as 5000 deaths (McCormick *et al.*, 1987). The seroconversion rates among villagers in Sierra Leone vary from 4 to 22 per 100 susceptible individuals per year; up to 14% of febrile illness in such population groups is due to Lassa virus infection. There is a marked variation as to the severity of the disease according to different geographical regions. However, this may in part be due to genotypic variation of Lassa virus, or dose and route of infection, or a combination of these factors (Fisher-Hoch, 1993). There is a relatively high rate of asymptomatic and mild infections in endemic areas. One reason for this may be the frequency of reinfections; although about 6% of the population lose antibody annually, rises in antibody titre are also often observed. It is not clear if reinfection results in clinical disease. A frequent finding of incomplete immunity after

infection would have profound implications for the use of a vaccine.

There may be secondary spread from person to person in conditions of overcrowded housing and particularly in rural hospitals. There is a particularly high risk to staff and patients on maternity wards as Lassa fever is a major cause of spontaneous abortion. Medical attendants or relatives who provide direct personal care are most likely to contract the infection; as noted above, accidental inoculation with a sharp instrument and contact with blood have resulted in transmission in a few cases. Air-borne spread may take place, as well as mechanical transmission. Although in Sierra Leone there has been no evidence of air-borne spread in hospital outbreaks, one of the 1970 outbreaks in Nigeria is believed to have been caused by air-borne transmission from a woman with severe pulmonary infection.

Lassa fever is a major cause of spontaneous abortion in West Africa. The virus is readily recovered from the blood and placenta of aborted fetuses. Women generally recover quickly, showing a dramatic decline in viraemia, partially due to massive bleeding at the time of abortion (A. Demby, personal communication). Paediatric Lassa fever is known to occur more commonly in male children for unknown reasons. Presenting as an acute febrile illness, the case fatality rate may approach 30% in children, with widespread oedema, abdominal distension and bleeding.

Diagnosis

The diagnosis of Lassa fever is confirmed by isolation of the virus or demonstration of a specific serological response. Infection in the early stages can be confused clinically with a number of other infectious diseases, particularly malignant malaria (Table 30.2) (Woodruff, 1975). The two most reliable prognostic markers of fatal infections are the titres of circulating virus and of aspartate aminotransferase (AST). Patients in whom the titre of virus exceeds 10^4 TCID₅₀ per ml accompanied by AST levels above 150 IU have a poor prognosis, and fatality rates approach 80%. In contrast, patients with virus and enzyme levels below these values have a greater than 85% chance of survival (Johnson *et al.*, 1987). This demonstration of an association between the degree of viraemia and mortality is unique for virus infections and contrasts with the difficulty in predicting the outcome in patients with other arenaviral haemorrhagic fevers. Although Lassa fever can be diagnosed accurately from the presence of IgM antibodies on admission, there is no correlation between the time of appearance, the titre of specific antibodies and clinical outcome.

Lassa virus grows readily in Vero cell culture and virus can usually be isolated within four days. Virus can be cultured from serum, throat washings, pleural

fluid and urine; it is excreted from the pharynx for up to 14 days after the onset of illness and in urine for up to 67 days after onset. Lassa infection can be diagnosed early by detection of virus-specific antigens in conjunctival cells using indirect immunofluorescence. The use of reverse transcription polymerase chain reaction (RT-PCR) is possible, although these techniques require careful use and standardization in endemic areas.

The most sensitive serological test for the detection of Lassa antibodies is indirect immunofluorescence and ELISA; antibodies can be detected by these methods in the second week of illness. Occasionally antibodies are not detected despite the presence of virus. Neutralizing antibodies are difficult to measure *in vitro*, in sharp contrast to infections by the South American arenaviruses, for reasons that are unclear.

Therapy

Although the passive administration of Lassa immune plasma may suppress viraemia and favourably alter the clinical outcome, it does not always do so, particularly if the patient has a high virus burden (McCormick and Fisher-Hoch, 2002). Failure may be due to either the difficulty in assessing accurately the titre of viral neutralizing antibodies in the plasma, the late and non-uniform nature of this response in convalescence, or antigenic variation. The widespread occurrence of human immunodeficiency virus (HIV) infections in West Africa precludes at present the use of immune plasma from convalescent individuals in this region. This is in marked contrast to the benefit of immune plasma in the treatment of Junín infections. This may be due either to the high titre of neutralizing antibodies that develops soon after the acute phase or to the lesser importance of antibody in resolution.

Greater success has been achieved with antivirals. In one study of patients with a poor prognosis, treatment for 10 days with intravenous ribavirin (60–70 mg/kg per day) within six days after the onset of fever showed a reduced case fatality rate of 5% (McCormick *et al.*, 1986). In contrast, patients treated seven or more days after the onset of fever had a case fatality rate of 26%. In the Sierra Leone study, viraemia of greater than $10^{3.6}$ TCID₅₀ per ml on admission was associated with a case fatality rate of 76%. Patients with this risk factor who were treated with intravenous ribavirin within six days of the onset of fever had a case fatality rate of 9% compared with 47% in those treated seven days or more after the onset of illness. Oral ribavirin is less effective. A difficulty with its use, however, is that ribavirin can induce haemolytic anaemia in over 40% of patients.

The introduction of vaccines against Junín virus has stimulated the expectation that a vaccine could also

be developed for the prevention of Lassa virus infections. However, the perceived necessity for a strong cell-mediated response would dictate the development of an attenuated vaccine; this raises concerns as to a possible reversion to virulence of any attenuated Lassa virus vaccine. Given these technological difficulties and the limited numbers globally at risk of infection, it is unlikely that such a vaccine will be developed in the near future.

Control

Containment of Lassa fever depends upon the strict isolation of cases, rigorous disinfection, rodent control and effective surveillance. Nosocomial transmission presents a considerable risk and patient isolation—in isolators if available—is an absolute must. Strict procedures for dealing with body fluids and excreta need to be enforced. Disinfection with 0.5% sodium hyperchlorite or 0.5% phenol in detergent is recommended for instruments and surfaces. Given the higher virus burden in cases of Lassa fever compared with patients with Junín or Machupo infections, surveillance of those having been in contact with Lassa fever patients is also a high public health priority. WHO recommends that those who have been in noncasual contact with cases should be observed for as long as three months after their last contact with the patient. This follow-up should consist of taking body temperature measurements twice daily. Infection should be suspected if the body temperature exceeds 38.3 °C and the contact immediately hospitalized.

Rodent control is frequently difficult, although much can be done to minimize the risk of virus transmission by isolating foodstuffs, preventing rodent entry into dwellings and reducing the chance of inhabitants coming into contact with rodent excreta. The greatest risk factors are housing quality, the presence of rat burrows and external sanitation (Bonner *et al.*, 2007). Minimizing rodent densities in the immediate domestic environment together with improved housing standards could do much to reduce the incidence of infection.

OTHER ARENAVIRUS INFECTIONS

Venezuela Haemorrhagic Fever (Guanarito Virus)

Between May 1990 and March 1991 an outbreak occurred among residents of Guanarito municipality on the central plains of Venezuela. Originally mistaken as dengue fever, a total of 104 cases were recorded with a mortality rate of around 25%. The Guanarito virus was subsequently isolated from the spleens of such cases at autopsy. The principal rodent hosts of this virus have been identified (Table 30.1) (Tesh *et al.*, 1994).

The disease has a clinical profile similar to that of AHF, with patients manifesting a thrombocytopenia, haemorrhaging and neurological signs. Pharyngitis has been observed and deafness reported in convalescent patients. Although initial reports suggest a high mortality for this infection, antibody prevalence rates of up to 3% have been found among healthy individuals and up to 10% of household contacts have anti-Guaranito virus antibodies.

During the course of studying the extent of the natural host for Guaranito virus, a second arenavirus was discovered within the same geographical region; Pirital virus causes chronic infection in the cotton rat (*Sigmodon alstoni*), a species which occupies a separate ecological niche to the main host for Guaranito virus (Fulhorst *et al.*, 1999). Although not a cause of haemorrhagic fever in humans, Pirital virus infection of Syrian golden hamsters has been exploited as a model of human arenaviral haemorrhagic fevers (Xiao *et al.*, 2001).

Brazilian Haemorrhagic Fever (Sabiá Virus)

This arenavirus was isolated in 1990 from human cases at autopsy (Lisieux *et al.*, 1994). The source of this infection was uncertain but is likely to have been acquired by exposure to infected rodents in an agricultural setting in an area immediately outside São Paulo. As a continuing reminder of the potential severity of these infections, a laboratory worker in the United States became critically ill after having been accidentally exposed to an aerosol containing Sabiá virus. This laboratory-acquired infection was characterized by a febrile illness accompanied by leukopenia and thrombocytopenia. There is little information regarding the epidemiology of this virus, although the extensive liver necrosis seen in the first case is a warning that this and other haemorrhagic fevers may on first examination be mistaken for yellow fever.

Oliveros Virus

This new agent has been isolated from a small rodent, *Bolomys obscurus*, within the endemic region of AHF (Bowen *et al.*, 1996). With a rodent host distinct from that of Junín virus, approximately 25% of captured *B. obscurus* have been found to contain antibodies to this virus. At present, there is no indication that this virus causes human infections (Mills *et al.*, 1996).

Whitewater Arroyo Virus and Other Isolates from the United States

As a consequence of the 1993 hantavirus outbreak on the Colorado Plateau in the United States, there followed intensive study of rodent populations in order to gauge the extent of Sin Nombre Virus distribution and

the risk posed by infected rodents to rural populations. During one such study, an unexpectedly high level of arenavirus antibodies was found in pack rats (*Neotoma* spp.) caught in the Whitewater Arroyo of New Mexico (Kosoy *et al.*, 1996). Members of the *Neotoma* genus are ubiquitous throughout the south-western part of the United States. Independently Fulhorst and colleagues (Fulhorst *et al.*, 1996) described the isolation of a hitherto unknown arenavirus from trapped examples of the white-throated woodrat, *Neotoma albigula*. The virus causes chronic infection when passed from dam to progeny, thus infected adults are likely to shed virus into the environment, particularly around isolated human dwellings and in recreational areas. The importance of these findings became evident when in 1999 and 2000 three female patients residing in California presented with symptoms subsequently suggested to be due to infection with the same arenavirus. Although there was no obvious link between the three cases, each presented with nonspecific febrile symptoms and acute respiratory distress. Two developed a lymphopenia and thrombocytopenia, and two showed also signs of liver failure and haemorrhage. All three died within one to eight weeks of onset. Virus was recovered in one and all three gave PCR products that were 87% identical with Whitewater Arroyo virus. Doubt remains, however, as to the zoonotic potential of Whitewater Arroyo virus.

Yet further new isolations have been made recently. A virus closely related to but distinct from Whitewater Arroyo virus has been isolated from the California mouse *Peromyscus californicus*. Infectious virus was recovered from 5 to 27 animals caught in the Santa Ana Mountains of southern California, close to the Bear Canyon trailhead. It cannot be ruled out that the tentatively dubbed Bear Canyon virus represents an additional arenavirus that has yet to be associated with human disease. Catarina virus is a distinct arenavirus found associated with the southern plains woodrat (*Neotoma micropus*) found in southern Texas (Cajimat *et al.*, 2007) but there is no evidence as yet that Catarina virus infects humans.

SUMMARY

The increasing numbers of human infections due to arenaviruses is beginning to require a greater vigilance on the part of public health workers. Arenavirus aetiology for febrile illnesses in individuals residing in endemic areas should be considered, particularly those who are likely to have come into regular contact with rodents by virtue of their lifestyle or occupation.

There is increasing evidence for human arenavirus infection in North America, in part due to a greater awareness of the potential for emerging infections among clinicians and microbiologists, particularly in geographical

areas where the last decades have seen clearance of woodland, forest and scrub in advance of extensive changes in agricultural practices. This potential has been augmented by changing or abnormal weather patterns, these serving to promote behavioural, if not also numerical, changes in rodent populations. Particularly in the Americas, arenavirus investigations have progressively become interleaved with studies on hantavirus distribution, especially in endemic zones where a particular species of rodent may be infected with either a hantavirus or an arenavirus. The only certainty is that the number of arenaviruses identified hitherto will increase as more becomes known regarding the natural history of these agents.

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Filoviruses

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INTRODUCTION

Human infections with filoviruses are rare, and outbreaks both dramatic and mysterious. The first filovirus hit the headlines as the 'Andromeda strain' or 'green monkey disease' following the outbreak in 1967 in Marburg, Germany. It was not until 1990, however, after an outbreak in imported monkeys, that filoviruses, particularly Ebola, hit news headlines, bestseller shelves and the big screen. Some media accounts took over-dramatization to new heights, heavily marketing the fear factor. Media excesses did not serve science or public health well, and included much misinformation. To those closely involved the true stories had drama enough, with many of the best qualities of adventure movies and science fiction. A case of truth being stranger than fiction.

The existence of filoviruses was first recognized in Marburg in 1967 (Martini, 1971). Thirty-one people in this provincial German city were infected: laboratory technicians, medical personnel, animal care personnel and their relatives. Seven died. Those working in Marburg at the time described the fear as palpable whenever the ambulance sirens were heard in the streets. Primary cases had been exposed to tissues and blood from African green monkeys imported into Germany and former Yugoslavia from Uganda; hence the name 'green monkey disease'. Many monkeys also died, but despite efforts the real source of the infection remained obscure (Henderson *et al.*, 1971). A novel virus isolated from patients and animals was found by electron microscopy to be unique among mammalian pathogens, having a strange looped and branched filamentous form, hence the name filovirus (*filo* = 'thread'; Figure 31.1) (Kissling *et al.*, 1968).

The disease then vanished until 1976 when epidemics of a haemorrhagic disease with very high mortality (up to

90%) broke out simultaneously in a remote area of northern Zaire (now the Democratic Republic of the Congo (DRC)) and southern Sudan (World Health Organization, 1978a, 1978b). Again the fear was overwhelming, with abandoned hospitals and fleeing patients, but this time the outbreaks were found to be due to two separate but antigenically related filovirus strains, distinct from the Marburg virus. These new viruses were grouped under the name Ebola virus, after a river in Zaire (Kiley *et al.*, 1982; McCormick *et al.*, 1983).

Over the next 10 years rare, sporadic cases of Marburg infections in Africa were the only continuing evidence of the existence of filoviruses (Gear *et al.*, 1975; Smith *et al.*, 1982). Though the natural host and its ecology remained elusive, evidence pointed to filoviruses being exclusively African, occurring principally in Zaire, Sudan, Uganda and Kenya (Figure 31.2). The mystery of their origin deepened further in 1989 when a filovirus was isolated near Washington, DC, from sick and dying cynomolgus monkeys. These monkeys and other shipments had been imported into the United States from the Philippines (Brown, 1997; Fisher-Hoch *et al.*, 1992a; Jahrling *et al.*, 1990). No epidemiological link with Africa could be found so this virus has to be considered Asian. A few human infections were serologically identified, but in sharp contrast to the previous outbreaks in Africa and Germany, no-one became sick and no-one died.

Later in the 1990s things changed. In 1994 news broke from DRC of a fresh, large outbreak of disease caused by the original Zaire strain of filovirus (Khan *et al.*, 1999). Thereafter came a considerable increase in the filovirus outbreaks and the numbers of patients involved. A series of outbreaks in Gabon were mostly associated with handling of dead chimpanzees (Georges *et al.*, 1999). Later a massive die-off of great apes (chimpanzees and

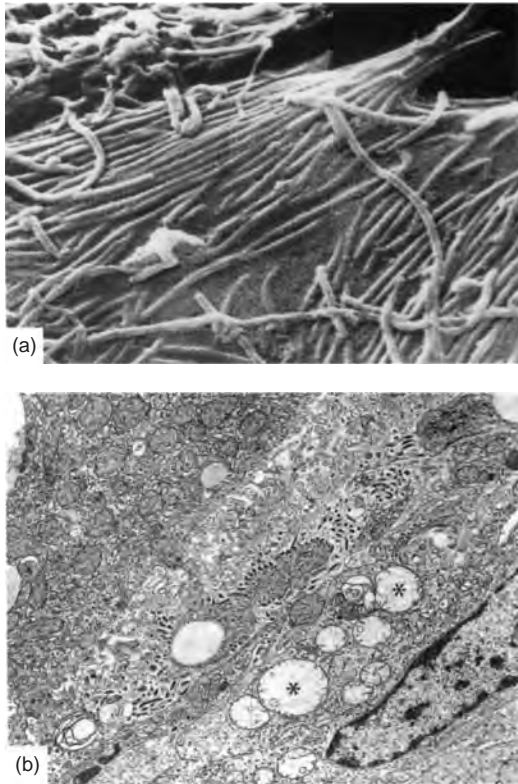


Figure 31.1 Electron micrographs of filoviruses. (a) Scanning electron micrograph of Ebola virus (ZEBOV $\times 500$). (b) Liver section from a cynomolgus monkey that died of Ebola infection (REBOV), showing extruding particles from cells in hepatic sinuses. Stars are in vacuoles also seen in Ebola-infected liver ($\times 8400$). (Source: Both courtesy of C. Goldsmith.)

gorillas) associated with Ebola infection moved through the Gabon and the Republic of Congo (Leroy *et al.*, 2004). Later in the decade (1998–2000) Marburg infections were found to be associated with mining in eastern DRC (Bausch *et al.*, 2006), and in 2004/2005 a very large outbreak of Marburg disease affected northern Angola (World Health Organization, 2005b). Filovirus outbreaks were here to stay.

New classifications within the filoviridae family allow common use of acronyms, which for consistency will be used throughout the remainder of this chapter: The ‘Ebola-like virus’ genus includes Ebola Zaire (Zaire ebolavirus (ZEBOV)), Sudan virus (Sudan ebolavirus (SEBOV)), Ebola Ivory Coast (Cote d’Ivoire ebolavirus (CIEBOV)) and Ebola Reston (Reston ebolavirus (REBOV)). The ‘Marburg-like virus’ genus includes at present only one strain, Lake Victoria marburgvirus (MARV).

EPIDEMIOLOGY

Epidemics: Marburg Haemorrhagic Fever

In 1967, a fulminating haemorrhagic fever struck a number of laboratory workers in Marburg and Frankfurt, Germany, and Belgrade, Yugoslavia (Martini, 1971). The virus name was that of the German city where most of the cases occurred. There were 31 human cases, 25 of which were primary infections with seven deaths (Table 31.1). None of six secondary cases died. Twenty primary cases were in Marburg, four in Frankfurt and one in Belgrade. All primary cases had handled blood or tissues from shipments of African green monkeys (*Cercopithecus aethiops*) soon after they had been imported from Uganda. Twenty out of 29 people with monkey blood contact became infected, and four of thirteen who were exposed to tissue culture. Among the primary human cases in Marburg, ten had assisted in autopsies and three had trephined monkey skulls. One of the laboratory staff had dissected kidneys, one had handled tissue culture from the monkeys, one broke a test tube which had contained contaminated material, and five had cleaned contaminated glassware. One physician accidentally inoculated herself with a needle through a rubber glove. None of the animal attendants who had had no contact with blood were infected, nor were any of the laboratory personnel who had used precautions, such as protective gloves and clothing. There were no further primary cases following the institution of compulsory wearing of protective clothing with gloves and masks for work with monkeys with stringent cleaning procedures and use of disinfectants. Six secondary cases resulted from person-to-person contact at home or in hospital. One was the wife of a veterinarian who became ill several weeks after her husband recovered. Marburg virus was later isolated from his semen. In Belgrade, the single primary case, a veterinarian, performed autopsies towards the end of the monkeys’ six-week quarantine. His wife fell ill 10 days after nursing him at home. Despite the death of a total of 99 animals during quarantine in Belgrade, these were the only two human infections.

About 400–600 animals originating from four shipments reached Europe from Uganda over a three-week period. Frankfurt received only 40–60 animals from two shipments, and Belgrade about 300 animals from three shipments. The remainder went to Marburg. All spent 60–87 days in a holding facility in Uganda before being shipped to London, Heathrow, where they spent between 6 and 36 hours in an animal hostel prior to being forwarded to Germany. In Marburg, monkeys were housed in separate rooms with no recirculation of ‘air-conditioned’ ventilated air. Published data are unclear as to whether ongoing enzootics were observed inside Germany or the

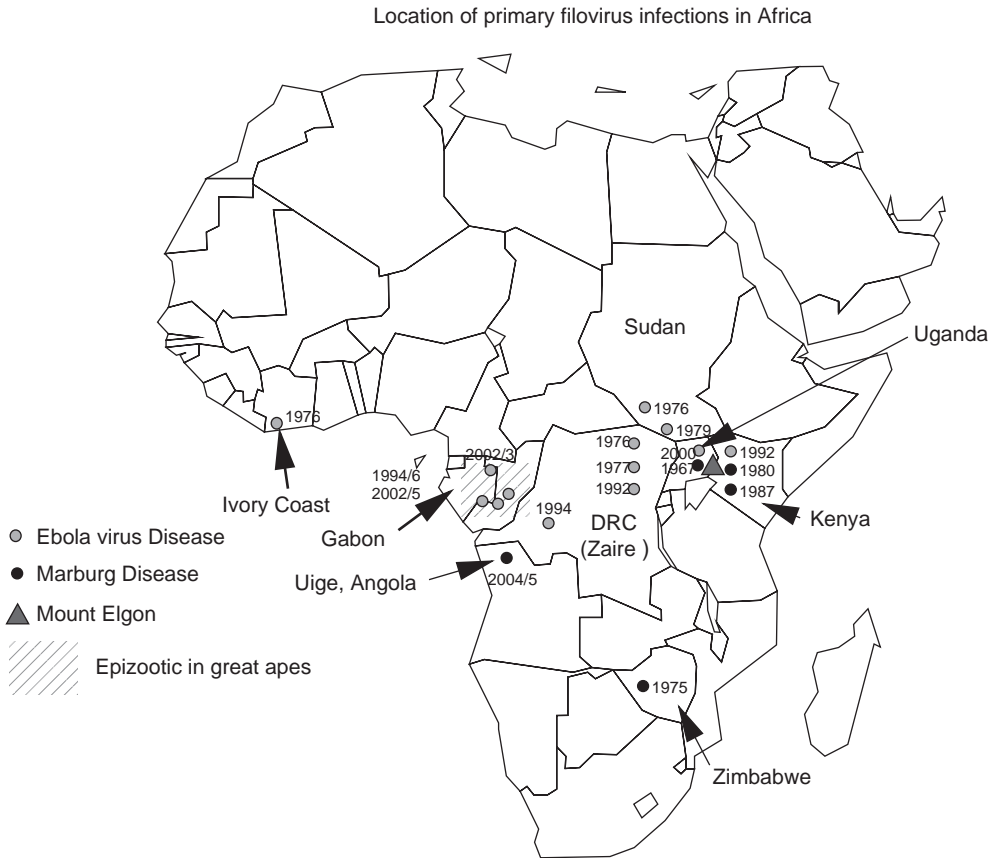


Figure 31.2 Map of Africa showing approximate locations and dates of Ebola and Marburg epidemics and origins of known index cases.

details of animal movements. Data on the Belgrade enzootics are, however, well recorded. Three shipments of monkeys from the same source in Uganda were received, two of which were in transit at London, Heathrow airport. The third arrived directly via Munich. Unusually high mortality during six weeks' quarantine was noted in all three shipments: 46/99 animals died from the first shipment, and 20 and 30 from the second two. The Belgrade epizootic was clearly characterized by ongoing transmission with daily death of one or more animals (Figure 31.3) (Martini, 1971).

Epidemiological studies after the 1967 epidemics concluded that two or three infected monkeys would have been sufficient to initiate the epizootic and all three outbreaks of human disease. It was stated at the time that evidence clearly pointed to transmission between monkeys in quarantine facilities by direct contact with equipment. Direct contact with blood and tissues was documented for all human cases and there was significant evidence ruling out transmission to humans by air. No evidence was

ever produced that supported the hypothesis that the monkeys were infected in transit in London from any number of a wide range of mammals and birds also temporary lodgers at the airport hostel. Furthermore, no evidence could be found of epizootics in Uganda, but later some indirect, controversial information emerged that there had, at the time of the outbreak, been excess deaths in monkey colonies in islands near Lake Kyoga, north of Lake Victoria, to the east of Mount Elgon in Kenya. In Uganda, monkeys were captured in this area and placed in holding facilities, reportedly in single cages (Henderson *et al.*, 1971). They were then transported to Entebbe. Here they were held for at least three days before shipment. At the time of the outbreak the trade had expanded considerably and holding times had been reduced, and it may be imagined that some crowding may have ensued. During July and August 1967, 1772 *C. aethiops* were housed in Entebbe, and 1290 exported, the majority to Germany and Yugoslavia (Henderson *et al.*, 1971).

Table 31.1 Cumulative reported human cases and deaths from filovirus infections between 1967 and August 2007.

Virus	Year	Place of infection	Total cases	Deaths	Mortality
Marburg	1967	Marburg, Germany	31	7	31%
Marburg	1967	Belgrade, Yugoslavia	2	0	—
Marburg	1975	Zimbabwe/South Africa	2	1	—
Marburg	1980	Mount Elgon, Kenya	2	1	—
Marburg	1998–2000	Durba, DRC (Zaire)	154	128	83%
Marburg	2004/2005	Angola (Uige Province)	252	227	90%
Marburg	2004/5	Angola (Uige province)	374	329	88%
<i>Total Marburg</i>			443	364	54%
Ebola Zaire	1976	Yambuku, Zaire	318	280	88%
Ebola Zaire	1976	England (laboratory infection)	1	0	—
Ebola Zaire	1977	Tandala, Zaire	1	1	—
Ebola Zaire	1980	Nzoia, Kenya	1	0	—
Ebola Zaire	1994	Kikwit, Zaire	315	242	77%
Ebola Zaire	1994/1995	Gabon	49	30	61%
Ebola, Zaire	1996	Gabon	60	45	75%
Ebola, Zaire	2000/2002	Gabon	30	22	73%
Ebola Zaire	2000/2002	Republic of Congo	92	80	87%
<i>Total Ebola Zaire</i>			745	598	80%
Ebola Sudan	1976	Maridi, Sudan	284	151	53%
Ebola Sudan	1979	Maridi, Sudan	34	22	65%
Ebola Sudan	1999	Uganda (Gulu district)	425	224	53%
<i>Total Ebola Sudan</i>			743	995	54%
Ebola Reston	1990	Richmond, Virginia	4	0	—
Ebola Reston	1990	Manila, Philippines	12	0	—
<i>Total Ebola Reston</i>			16	0	—
Total cases			3431	2952	

Data are from multiple sources and are approximations. Many cases are not laboratory confirmed, and many other cases may not have been reported.

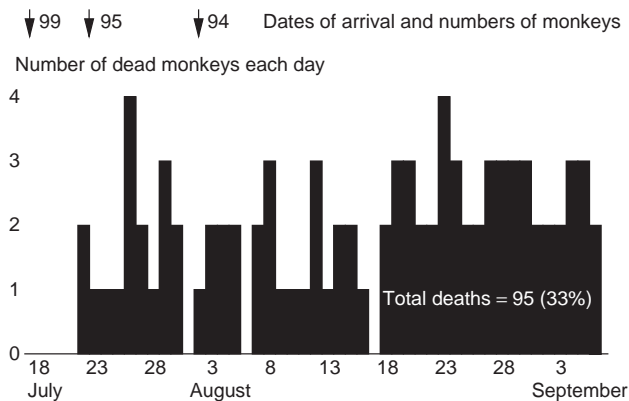


Figure 31.3 Histogram depicting the epizootic of Marburg virus infections in monkeys imported into Belgrade in 1967. (Source: Constructed from published data (Stojkovic *et al.*, 1971).)

For two decades only three further, isolated primary human Marburg infections and only two secondary cases were observed. These were adventurous tourists or expatriate residents. One was a hitchhiker in Zimbabwe who reported an insect bite while waiting for a ride

on the roadside. (It now (2007) appears he had visited bat-infested caves in Zimbabwe.) The two others had both visited the Kitum Cave in the Mount Elgon region of western Kenya, not far from the shores of Lake Victoria (Gear *et al.*, 1975; Smith *et al.*, 1982; Teepe *et al.*, 1983).

The Kitum Cave, a popular tourist destination, is heavily populated with a wide range of exotic species, particularly bats. One of the Marburg victims was a 12-year-old boy, an amateur geologist, who collected specimens in the cave. Extensive epidemiological investigations in Zimbabwe and in the Kitum Cave revealed no clues to the origin of the infections. Medical facilities are limited in these areas, and isolated cases in local residents probably went unnoticed unless dramatic epidemics supervened.

Between 1998 and 2000, Marburg disease re-emerged in the east of the DRC, in a mining community in and around Durba (Feldmann, 2006). This was the first community outbreak of Marburg haemorrhagic fever. A total of 154 cases were identified, 48 of whom were laboratory confirmed. In this outbreak, mortality of 83% contrasted with the 23% in the original outbreak in the city of Marburg. This sharp difference in mortality has not yet been explained by differences in viral strains or other biological factors, but the DRC patients certainly did not have access to quality supportive care. A very difficult investigation was conducted in what was essentially a war zone in a state of anarchy. Cases were found to be associated with illegal working of a partially flooded gold mine infested with all manner of wildlife (Bausch *et al.*, 2003). There appeared to have been a series of primary cases going back many years among these illegal miners rather than as had been seen before, a point source epidemic, with secondary cases in hospitals and households. Genetic analysis of isolates confirmed that, indeed, this outbreak was quite different, with multiple genetically distinct isolates from a series of index cases, most if not all of whom had worked illegally in the same abandoned mine (Bausch *et al.*, 2006). The outbreak stopped abruptly in 2000 when that mine became entirely flooded and no longer accessible.

In 2004/2005 a further, large outbreak of Marburg haemorrhagic fever occurred in northern Angola, in Uige province, which borders southern DRC (Centers for Disease Control and Prevention, 2005). This outbreak was large with 252 cases and 227 deaths (mortality 90%) (Towner *et al.*, 2006). The high reported mortality is consistent with that of the Durba 1998–2000 outbreak. This outbreak was also distinguished by there being many paediatric cases, mostly hospitalized infants (Feldmann, 2006). Molecular analysis of strains from this outbreak indicated it may have been a point source introduction by one index case, with spread in hospitals and the community (Towner *et al.*, 2006).

Epidemics: Ebola Haemorrhagic Fever

It was not until nearly a decade after the Marburg outbreak, in 1976, that simultaneous outbreaks of another lethal haemorrhagic fever struck suddenly in northern

Zaire and southern Sudan (Figure 31.2) (World Health Organization, 1978a, 1978b). Among 318 probable or confirmed cases in Zaire, there were 280 deaths (mortality 88%) (World Health Organization, 1978b). Though initial suspicions were that the outbreaks were linked, it was soon shown that there were no observable or even potential epidemiological connections between the two outbreaks. Mortality in the Sudan outbreak was also lower (53%) than in Zaire (World Health Organization, 1978a). Both outbreaks centred in and around local hospitals, where conditions existed for person-to-person spread. New filoviruses were isolated from specimens from patients from both epidemics.

The excellent and detailed reports of these two outbreaks define the classic filovirus outbreak, with little new knowledge having been added in more recent reports (World Health Organization, 1978a, 1978b). In Yambuku, Zaire, the index case may have been a traveller recently in the northern Equateur region of Zaire. This individual apparently attended the outpatient clinic of the mission hospital in Yambuku for treatment of acute malaria, where he received an injection of chloroquine. It remains unclear whether this man was the source of the epidemic or whether he was infected by his injection, since another patient with a similar illness was admitted to the hospital at about the same time. The subsequent nine cases, however, had all received treatment for other diseases at the hospital. Though all ages and sexes were affected, the highest incidence was in women aged 15–29 years, mostly those who had attended antenatal and outpatient clinics at the hospital. The major risk factor was receiving an injection at this hospital. The hospital was simply run, mostly by Belgian nuns, providing the only medical care in the region. Needles were in short supply, and not sterilized between use. Clearly the medical staff had no concept of what was going on, since 11 of the 17 staff members of the hospital died. The outbreak only terminated four weeks after it began when the hospital was closed. Though transmission was focused in the outpatient clinics of the hospital, there was subsequent dissemination in surrounding villages to people caring for sick relatives, or attending childbirth. The overall secondary attack rate was about 5%, but nearer 20% in close relatives of a case. Illness-to-infection ratios in one village exceeded 10 to one. Ebola virus antibody in people not ill and without contact with cases during the epidemic was only conclusively identified in four individuals. Interestingly, though not reported in the scientific literature, final control of the epidemic was probably achieved by the local village chiefs who, with the recent experience of smallpox eradication, placed their own 'cordon sanitaire' around each village, preventing anyone entering or

leaving (Close, 1995). The outbreak only came to international attention when a nurse infected with Ebola virus fled to the capital Kinshasa, and died in a hospital there.

The following year, 1977, a single fatal case was identified in Tandala, also in northern Zaire (Table 31.1). There was no ensuing epidemic, but serological and epidemiological investigations uncovered two possible cases dating back to 1972, and 7% prevalence of immunofluorescent assay (IFA) antibody in the local population (Heymann *et al.*, 1980). Unfortunately, nonspecific reactions to the Ebola virus antigens make interpretation of serological data uncertain.

At the same time as the 1976 Yambuku epidemic in Zaire, an outbreak of a similar disease occurred in southern Sudan (Figure 31.2) (World Health Organization, 1978a). This outbreak was strongly associated with an index case who worked in a cotton weaving factory. There were 151 deaths among 284 cases identified (case definition not stated). The focus of the infection was in the town of Nzara where the factory was located, and spread was to close relatives (67 cases). The epidemic was augmented by high levels of transmission at nearby Maridi hospital following transfer of one of the Nzara patients, and further cases were transferred to Juba and Khartoum. There were 203 cases in Maridi, 93 of whom were probably infected in the hospital, and 105 in the community. Forty-one staff members died, and at the height of the epidemic all wards contained patients with overt haemorrhage. The highest attack rates were associated with nursing a patient, but not with sleeping in the same room. In 1979 there was a second outbreak originating from an index case working in the same Nzara cotton factory. This time 22 of 34 infections (65%) were fatal (Baron *et al.*, 1983). From the 1976 and 1979 outbreaks two new filovirus strains were isolated, (ZEBOV and SEBOV) (Johnson *et al.*, 1977). It was quickly realized that these two viruses are quite different (Kiley *et al.*, 1982), but the two Sudan virus strains from 1976 and 1979 were identical (Cox *et al.*, 1983).

Between 1979 and 1994 only one case of human Ebola infection was recorded (Teepe *et al.*, 1983). In 1994 all this changed when there was a large, hospital-based outbreak in Kikwit, DRC, resulting in the deaths of 242 of 315 cases. Again a hospital was the amplifying focus of the outbreak, with many needle-borne transmissions. The virus then spilled over into the community with cases among close contacts of the sick (Khan *et al.*, 1999; Reiter *et al.*, 1999). The probable source was traced to a single forest worker (Garrett, 1996), but intensive studies failed to reveal the primary host.

Soon afterwards the first of three epidemics caused by Ebola virus emerged in Gabon (Georges *et al.*, 1999). The index cases may have been gold panners or hunters in the forest. One outbreak resulted from finding and handling

the carcass of a dead chimpanzee in the forest. In this outbreak all the primary cases handled the uncooked meat at some stage. People who ate the cooked meat did not get sick.

The Sudan virus did not reappear until October 2000, and when it did it was by way of a massive epidemic in Uganda (Okware *et al.*, 2002). The origins of this outbreak are obscure, but it appeared to originate in the inaccessible northern part of the country where rebel fighting was ongoing. The epidemic was centred in northern Uganda, in the Gulu district, and was not brought under control until mid January 2001. There were a total of 425 cases and 224 deaths countrywide (case fatality 53%). Attack rates were calculated to be between 4.5 and 12.6/10 000 depending on the case definition (Okware *et al.*, 2002).

Infections of Ebola virus appeared again in Gabon and the neighbouring Republic of Congo in 2000 and continue to date. As of 30 December 2002, WHO had reported 30 confirmed cases (14 laboratory confirmed and 16 epidemiologically linked), including 22 deaths, in Gabon and neighbouring villages across the Congolese border. Epidemics in the Republic of Congo appear to be closely associated with epizootics in great apes (details in the section on Ecology, below). As of 27 May 2005, there were 11 confirmed and many probable cases of Ebola haemorrhagic fever in the Cuvette Ouest Region of the Congo (World Health Organization, 2005a). These outbreaks are proving difficult to detect and control because of the remoteness of the area, the association with the ongoing great ape epizootic and the fears and resistance of the communities affected. These fears were sufficient in one community for a schoolteacher to be attacked and killed following accusations of being the cause of the epidemic.

Epizootics: Reston Virus

In 1989 and early 1990 a filovirus (REBOV) closely related to ZEBOV was isolated from cynomolgus monkeys in quarantine facilities in Reston, Virginia, in Texas and in Pennsylvania (Centers for Disease Control and Prevention, 1989; Jahrling *et al.*, 1990). The monkeys had recently been imported into the United States from the Philippines. The first shipment arrived via Amsterdam, and exhaustive inquiries ruled out a link with African animals during transit. Early the following year more shipments of monkeys from the same source arrived, this time directly from the Philippines, over the Pacific Ocean, and several more REBOV isolations were made from sick and dying monkeys (Centers for Disease Control and Prevention, 1990a). No link with Africa or African animals could be identified in the Philippines, and in the absence of such evidence, REBOV must be considered

an Asian filovirus. Pathogenicity in the outbreak animals was uncertain because of a high rate of co-infection with simian haemorrhagic fever virus (SHFV), a DNA virus which is a known severe simian pathogen unrelated to the *Filoviridae* (Palmer *et al.*, 1968). This co-infection added enormously to the complexity of this episode (SHFV also produces haemorrhagic disease though it does not apparently infect humans). In the first reported SHFV epizootic, 223 of 1050 exposed animals died, with increased handling a risk factor for disease and death. The natural host and geographical distribution are also unknown, though the infected monkeys (*Macaca mulatta*) apparently originated in India (Palmer *et al.*, 1968).

In 1990 concern about the risk to humans led to a temporary ban on importation into the United States of cynomolgus, rhesus and African green monkeys. Evidence for ongoing epizootics and transmission was sought in the Philippine export facilities which had provided the monkeys (Hayes *et al.*, 1992). Antigen-detection enzyme-linked immunosorbent assay (ELISA) assays on liver homogenates revealed that 85/161 (53%) of monkeys that died there over a period of less than three months were positive for filovirus antigen, as were 7% of monkeys tested in an initial serological survey. Incidence was calculated to be 24.4/100 animals, or 0.6/100 monkeys per day of follow-up, similar to the reports from Belgrade in 1967 (Figure 31.3). Documented case fatality at this institution was 82.4%, and survivors developed high-titre IFA antibody. Average duration of viraemia was 5.6–2.4 days. Diarrhoea and respiratory problems were the most frequently recorded manifestations. In the 73% of monkeys positive for filovirus by IFA at this facility the geometric mean antibody titre was 145. A protective factor was the presence of antibody to filovirus at the time of entry to the facility (Hayes *et al.*, 1992). As in the United States, many of the filovirus-infected monkeys were co-infected with SHFV, which means data from these epizootics are difficult to interpret and conclusions are open to question.

Epidemiology: Reston Virus

In the Philippines 186 people were studied who lived in wildlife collection areas or worked in four primate export facilities in Manila (Miranda *et al.*, 1991). Twelve (6%) were filovirus antibody positive by IFA, and in the facility experiencing the epizootic 22% were positive, significantly higher than the other export facilities (relative risk 5.6, 95% confidence interval 1.09–24.14) (Hayes *et al.*, 1992). In that facility the workers in the animal hospital had the highest titres: three of five had titres of >256. However, there was no illness in any of the positive individuals, and no association between seropositivity

and other risk factors, such as bites, scratches or eating monkey meat (a local delicacy). In the facility at Reston, Virginia, five animal handlers had a high level of daily exposure to infected and dying animals (Centers for Disease Control and Prevention, 1990b, 1990c, 1990d). Though four of these had serological evidence of recent infection by IFA, and three were observed to seroconvert during the period of the epizootic none reported any illness. One cut his finger while performing a necropsy on an infected animal.

ECOLOGY

Until the early 1990s outbreaks of human disease had been unusual because transmission from the natural reservoir to humans is rare. Searches for evidence of virus infection in many species of animals captured in Central African countries failed to provide any clues as to the possible reservoir. The original ecological setting of Marburg and Ebola appeared to centre around Central and south Central Africa (Figure 31.2). Surveys of wild monkeys from the Lake Kyoga area in northern Uganda in the 1970s where the original Marburg-infected group originated failed to yield virus, but about 10% of sera reacted with crude Marburg antigen in a complement fixation test, and in at least three animals neutralizing antibody could be demonstrated. The 1987 Marburg disease case was a boy who had spent considerable time in Kitum Cave, collecting minerals and other items, near to the sugarcane factory where the 1980 Marburg disease case had worked. Extensive searches for the reservoir in the area around the sugarcane-processing factory and around Kitum Cave were unsuccessful. The cave contained enormous numbers of bats, and was visited by a wide range of mammals, birds, reptiles and insects. Sentinel animals and other means of searching for the virus failed to identify bats (or any of a number of wild mammals) as the ultimate source. Similarly, because of the history of an insect bite, a thorough study of insects and mammals along the trail of the 1975 traveller in Zimbabwe did not produce evidence of the source of infection.

Bats as the Reservoir

Bats have always been highly suspect, since they were directly implicated in the Kitum Cave cases. In Sudan in 1976 and 1979 both index cases had worked in the same storeroom in a cotton factory where the roof was heavily infested with bats. Antibody could not be detected in sera from six bats captured in 1976, and no virus was isolated from bat tissues in either investigation, but limited sampling could have missed infected animals. (Serological studies in bats and other exotic species presented a

serious challenge in terms of species specific reagents. Similarly all isolation attempts involved tissue culture, since the more sensitive polymerase chain reaction (PCR) methods were not yet available.)

In the 1990s, a heroic laboratory study involving a menagerie of exotic species experimentally infected with ZEBOV was conducted by Swanepoel and co-workers in South Africa in the Pretoria BSL4 suit laboratory. Though technical difficulties led to premature termination of the ZEBOV-infected snake arm of the study, the remainder of the data showed that ZEBOV can replicate in fruit bats without causing disease (Swanepoel *et al.*, 1996). The fruit bats used were *Epomophorus wahlbergi*, a widely distributed species native to the tropical rainforest, home to ZEBOV outbreaks. This was further evidence that bats could carry a silent infection which is a prerequisite for maintaining the virus in a wild population over time. Other suggestions, such as rodents and even plants as primary hosts have never been substantiated. If this were a rodent virus, the ubiquity of most rodents would mean that primary infections in humans would be much more frequent, as is the case with Lassa fever, so rodents are unlikely as hosts. There is no precedent for a plant virus infecting humans.

Fruit bats remained prime suspects, not only because of epidemiological evidence, but because the rarity of human exposure to a canopy-feeding animal would explain the rarity and sporadic nature of primary infections, as well as the association with bat-infested caves and buildings. The evidence, however, remained circumstantial until Leroy *et al.* reported identification of ZEBOV in bats by PCR in Gabon around sites where outbreaks of disease had occurred in great apes and in humans (Leroy *et al.*, 2005). These studies implicated silent infection in three species of fruit bat native to the distribution of known Ebola index cases. The ranges of the bat species identified, *Hypsignathus monstrosus*, *Epomops franqueti* and *Myonycteris torquata*, overlies with precision the known range of ZEBOV primary infections. Captured animals were PCR-positive for seven different RNA fragments from the ZEBOV genome. Studies a few months later at the same sites showed serological evidence of infection in some bats but no PCR-positive animals. Since no animals were simultaneously PCR-positive and serologically reactive, the idea was raised that the infection is not only silent, but transient in bats. This limited window of infectivity in the natural host is also consistent with rarity of human index case infections. Sequencing of the fragments showed them all to be closely related to one another and to locally isolated strains from humans and great apes, and also to the original ZEBOV strains, but more distantly to SEBOV and MARV (Leroy *et al.*, 2005). More recently common ZEBOV ancestry has been demonstrated in bat

reservoirs (Biek *et al.*, 2006). These data are compelling in themselves, but more so since the report in August 2007 that Marburg virus has been identified in four of 1100 fruit bats (*Rousettus aegyptiacus*) captured in two sites in western Gabon (Towner *et al.*, 2007). The distribution of this cave-dwelling species encompasses most of equatorial and southern Africa, including all the known Marburg primary sites. Three of the four infected bats had evidence of both antibody and virus, indicating this time a persistent infection. However, prevalence in this sample was extremely low (0.36%), suggesting that infection is either a rare or a transient event. The infected bats were apparently healthy and active, and no virus could be isolated, which argues for very low titre infection. Isolation of viruses from wild-caught fruit bats would provide an important key to understanding viral dynamics in this reservoir.

Involvement of the Great Apes

Ebola epizootics among great apes in Central Africa have become of great concern to conservationists, to the extent that the virus, combined with intense bush meat hunting, is now threatening chimpanzees and lowland gorillas with extinction. Since the 1990s, large apes, specifically chimpanzees and gorillas have also become increasingly implicated in transmission to humans (Georges *et al.*, 1999). The catastrophic decline in great ape populations is illustrated by a 99% drop in nest groups between 1998 and 2000 in Gabon near Minkébé, an area where there had been a human epidemic (Walsh *et al.*, 2003). In Mekambo, in neighbouring Congo, many ape carcasses have been found, and in Lossi, a population of 143 individually identified gorillas has been reduced to seven. The epizootic has been gradually making its way south and west, and in 2006, the deaths of 5000 gorillas has now been attributed to ZEBOV infection (Bermejo *et al.*, 2006). With such high mortality these apes must be, like humans, an accidental end-host. Ecological disturbances have been hypothesized as an underlying reason for transmission, but it has now been shown that social behaviour among apes facilitates transmission (Walsh *et al.*, 2007).

The question of how the virus is introduced into human populations is now partially resolved. It is known that non-human primates, particularly chimpanzees, will eat any smaller animal they can catch, which could be a bat or an intermediate, canopy-dwelling species, such as a small monkey. Molecular sequencing of strains from dead apes shows infections with differing strains so that, however it happens, there are multiple virus introductions. This also argues against the primates as a natural host (Leroy *et al.*, 2004).

Use of Modelling Techniques

Further light has been cast on these questions by the use of ecological niche modelling (Peterson *et al.*, 2004a). This technique includes large numbers of variables in models, encompassing terrain, climate, flora and fauna. Data from known or suspected primary infection locations are fed into the model, and used to predict where future outbreaks might be expected based on the theory that the natural host will occupy similar ecological locations. Using this approach Peterson *et al.* predicted the geographical distribution of ZEBOV to overlie the distribution of the tropical broadleaf rainforest. This also coincides with the range of the fruit bats described by Leroy *et al.* Using the same model to identify potential hosts produced a dauntingly long list of candidates (Peterson *et al.*, 2004b).

Of great interest were predictions for wider distribution of MARV, mostly in savannah-type regions, including many countries where no cases had ever been seen. One of these was Angola, with a predicted distribution pinpointing Uige province where the MARV outbreak started later in 2004, six months after publication of the model (Centers for Disease Control and Prevention, 2005). At least two MARV cases among miners in Uganda in the summer of 2007 also fall geographically within this model (World Health Organization, 2007). Peterson *et al.* have now updated their model of the geographic distribution to include the Uige province of Angola, and a probably more accurate estimation of the single index case from Zimbabwe (the Sinoia caves). Though the model has limitations, particularly the small number of precise localities in the sample, it predicts a much larger area over which MARV infections should be expected, broadly distributed across the arid woodlands regions of Africa (Peterson *et al.*, 2006).

TRANSMISSION AND RISK FACTORS

With few exceptions, transmission of filoviruses among humans has been the result of lax medical procedures or unwise behaviour (Fisher-Hoch, 1993; World Health Organization, 1978b). Person-to-person spread has been the major mode of transmission in epidemics. But unprotected direct contact with blood or secretions from patients ill with Ebola is the most important factor in determining risk of illness. Other risk factors associated with human-to-human transmission are infection from contaminated materials, particularly sharps, preparation of victims for burial or, occasionally, sexual contact (World Health Organization, 1978a). Injury or injection with a contaminated needle carries the highest risk, and the highest likelihood of death (World Health Organization, 1978b). Close

contact with blood or tissues of infected monkeys is also important, historically in the laboratory (Martini *et al.*, 1968), and currently in the wild (Georges *et al.*, 1996). The virus enters through mucous membranes or skin lesions. Medical personnel who do not take simple, barrier nursing precautions, however, are particularly at risk, as are their uninfected patients who may become exposed during treatment. Indeed, the history of filovirus outbreaks has been one of massive amplification of the epidemic in poorly operated medical facilities (Khan *et al.*, 1999; World Health Organization, 1978b). Outbreaks have been abruptly terminated when blood and/or needle transmission were interrupted (Fisher-Hoch, 1993; World Health Organization, 1978b).

Epidemiological studies in Zaire and Sudan rule out spread through casual contact or by aerosol transmission (World Health Organization, 1978a, 1978b). A formal study of risk factors for virus transmission in the Sudan epidemic in 1979 showed that caring for an ill patient carried a relative risk five times greater than in people with a lesser degree of physical contact. No cases occurred in people who entered the room of an ill patient but had no physical contact (Baron *et al.*, 1983). These data confirm that Ebola is not an air-borne disease.

The most significant risk factor for the monkeys infected in the REBOV epizootic in the Philippines was being an occupant of a gang cage (Hayes *et al.*, 1992). REBOV has been identified at high titre in respiratory secretions in monkeys, and respiratory transmission at close quarters may be a factor in epizootics with this virus. However, the evidence for monkey-to-monkey transmission by reuse of needles for routine procedures, such as tuberculin testing or antibiotic administration cannot be ignored.

The outbreak of MARV in 1967 was caused by infection of individuals handling fresh monkey tissues or contaminated equipment without gloves or other protective clothing (Martini *et al.*, 1968). Otherwise there has only been one reported laboratory-acquired infection (needle-stick) with Ebola virus in 1976 (Emond *et al.*, 1977). Because of its lethal potential, Ebola has been a candidate for biological warfare. Little information is available, but it has been handled extensively in biological research, and further accidental infections may have occurred, specifically in the former Soviet bloc. The key to safe laboratory handling of filoviruses is extreme care in avoiding accidental inoculation.

CLINICAL SPECTRUM

The incubation period for Marburg disease is three to nine days (Martini *et al.*, 1968) and for Ebola virus about

10 days; five to seven days for needle transmission and 6–12 days for person-to-person spread (Baron *et al.*, 1983; World Health Organization, 1978a, 1978b). The incubation period for the Ebola-related virus from Sudan may be slightly longer than for the more lethal ZEBOV strain, and appears to be dose-dependent. The illness-to-infection ratio for Marburg and Ebola viruses is very high. Using molecular techniques a few asymptomatic infections have been observed (Leroy *et al.*, 2000b). Serological studies generally reveal few people who have recovered from ZEBOV or MARV infections, even after a large epidemic (Bausch *et al.*, 2003). SEBOV-infected patients are more likely to survive, and attack rates are lower. REBOV in all individuals documented to have been infected was uniformly asymptomatic (Table 31.1) (Centers for Disease Control and Prevention, 1990e; Miranda *et al.*, 1999).

The human disease caused by the African viruses is dramatic (Figure 31.4) (World Health Organization, 1978a, 1978b). The onset is abrupt, with fever, severe headache (usually periorbital and frontal), myalgia, arthralgia, conjunctivitis and extreme malaise. Sore throat is a common symptom, often associated with severe swelling and dysphagia, but no exudative pharyngitis. A papular, eventually desquamating rash may occur in some patients, especially on the trunk and back, and morbilliform rash has been observed on white skins. In non-human primates petechiae are striking. Gastrointestinal symptoms develop in most patients on the second or

third day of illness, with abdominal pain, and cramping followed by diarrhoeal and vomiting. Jaundice is not a feature of Marburg or Ebola disease. The bleeding begins about the fifth day of illness and is most commonly from the mucous membranes: gastrointestinal tract, gingiva, nasopharynx and vagina. The persistence of vomiting and the onset of any signs of mucosal bleeding carry a high risk of fatal outcome, which is associated with hypovolaemic shock. Bleeding is insufficient to account for death.

Infection in pregnancy results in high maternal fatality and virtually 100% fetal death. Central nervous system involvement has led to hemiplegia and disorientation, and sometimes frank psychosis. Even in convalescence, patients show prolonged weakness, severe weight loss, and in a few survivors serious but reversible personality changes are recorded, namely confusion, anxiety and aggressive behaviour. Ocular involvement is also seen, and may be persistent (Kibadi *et al.*, 1999).

LABORATORY DIAGNOSIS

General

Care should be taken in both drawing and handling blood specimens since virus titres may be extremely high, and the virus is stable for long periods even at room

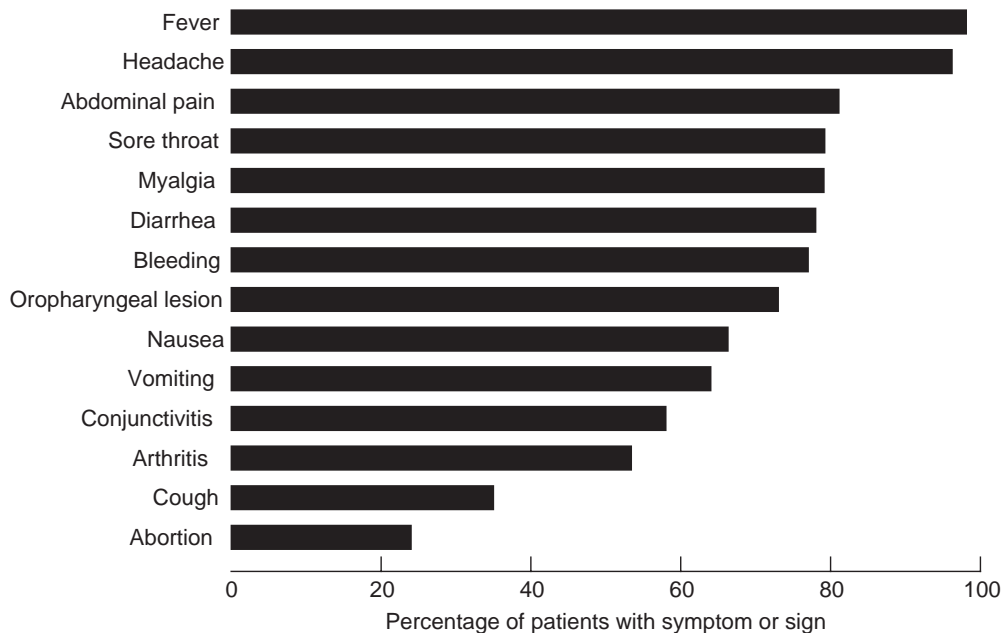


Figure 31.4 Frequency of symptoms and signs in Ebola haemorrhagic fever. (Source: Constructed from published data (World Health Organization, 1978b).)

temperature (Elliott *et al.*, 1982). Gloves should be worn at all times, and discarded directly into freshly made disinfectant. All sharps (instruments, needles, syringes) should be discarded in a puncture-resistant container with a lid, as is recommended for HIV-infected specimens (Centers for Disease Control and Prevention, 1988). In Africa where resources are limited, discard of sharps into freshly made chlorine is recommended. All discarded materials should be burned in a deep pit (World Health Organization, 1985).

Clinical laboratory observations are limited to the Marburg outbreak. No acute-phase investigations were performed during the care of the one Ebola laboratory infection in England. Data from non-human primate studies have been very useful. Thrombocytopenia is invariable, and profound lymphopenia early in disease is followed by marked neutrophilia. Aspartate transaminase (AST) and alanine transaminase (ALT) are raised, but the rise in AST is disproportionately higher than ALT, as was described in the early Marburg cases, (AST : ALT ratios 7 : 1) and is important in distinguishing viral haemorrhagic fevers from hepatitis viruses clinically (Fisher-Hoch *et al.*, 1985; Martini, 1971).

Virus-Specific Diagnosis

For virus isolation, antigen detection and serology a blood specimen should be taken without anticoagulant and serum separated. For PCR assays, separation of buffy coat may be preferred. Specimens may be safely handled for immunological tests by inactivating with γ -irradiation, or heating to 60 °C for 30 minutes (Elliott *et al.*, 1982). Denaturing chemical reagents used in PCR reactions can also be considered to inactivate the virus, though definitive studies are needed to establish this principle.

The indirect IFA is the original basic diagnostic serological test for acute infections evaluated for the diagnosis of human Ebola virus disease (Wulff and Johnson, 1979). Though less sensitive than the ELISA techniques which later superseded it, the IFA has the advantage of being simple enough to perform in the field anywhere a fluorescent microscope is available, with the added advantage that visualization of the infected cells allows experienced observers to make some assessment of specificity. In acute infections a rising filovirus-specific IFA titre (fourfold) in paired serum, or a high IgG titre (>64) and presence of IgM antibody with a clinical illness compatible with haemorrhagic fever are consistent with the diagnosis of filovirus infection (Ksiazek *et al.*, 1992). In the absence of a history of filovirus-like illness, MARV antibody is usually specific, but Ebola virus serology in the absence of a history of recent disease has consistently been plagued by low-titre, nonspecific reactions. ELISA assays using recombinant nucleoprotein (NP) are now the most broadly

reactive and the most sensitive assays (Groen *et al.*, 2003). Use of other proteins as antigens, for example, recombinant VP35, results in a strain-specific assay, but is less sensitive (Ksiazek *et al.*, 1992, 1999).

ZEBOV and MARV are readily isolated from serum, blood or tissue specimens, stored at -70 °C, in Vero E-6 cells. Biological safety level 4 containment facilities are recommended for any procedure such as isolation where virus will be amplified. Blood specimens unrefrigerated for up to 10 days have yielded virus strains. Primary isolation of ZEBOV is more difficult and success may depend on blind passage of cultured cells or guinea-pigs monitored for febrile response. Specimens including throat washing, urine, various soft tissue exudates, semen and anterior eye fluid may also contain virus. Virus identification is made generally by direct immunofluorescence of the tissue culture using monoclonal antibodies or by electron microscopy (Elliott *et al.*, 1993; Wulff *et al.*, 1978). Impression preparations made from a post-mortem liver biopsy may be probed with monoclonal antibodies by IFA for presence of virus antigen which is abundant in liver and spleen. Immunohistochemistry has been widely used for histological studies, but for studies of pathophysiology electron microscopy is much more precise.

An antigen-detection ELISA system in serum has been extensively used, employing monoclonal antibodies (Ksiazek *et al.*, 1992). This assay has been used extensively in human and epizootic outbreaks. One study showed that a positive antigen test was obtained in symptomatic patients, but once symptoms had resolved in survivors, the test became negative (Baize *et al.*, 1999). Most of these assays have been superseded by the PCR, which is now universally used on blood and all other body fluids and tissues. It is rapid, highly sensitive and specific and can be conducted safely in the field (Grolla *et al.*, 2005; Leroy *et al.*, 2000a).

PATIENT MANAGEMENT

Supportive Care

Fluid, electrolyte, respiratory and osmotic imbalances should be managed carefully. Patients may require full intensive care support, including mechanical ventilation, along with blood, plasma or platelet replacement. The maintenance of intravascular volume is a particular challenge but every effort is justified since the crisis is short-lived, and complete recovery can be expected in survivors. Pregnant patients may present with absent fetal movements, and maternal survival may depend on aggressive obstetric intervention. Heparin has been advocated but remains controversial and potentially dangerous. The

only experience has been the therapy of two individuals with Marburg disease in South Africa. However, both of these patients were secondary Marburg infections, and none of 10 known secondary patients with Marburg infection have died (Gear *et al.*, 1975).

Approaches to Antiviral Therapy

The current lack of effective antiviral therapy has led to the development of innovative approaches such as experimental use of RNA interference. Post-exposure prophylaxis with vaccines and molecular approaches have shown some promise in animal models. A single dose of a live-attenuated recombinant vaccine 20–30 minutes after challenge protected four of eight rhesus monkeys (Feldmann *et al.*, 2007). Treatment of guinea-pigs immediately before and after challenge with iRNA targeting the viral polymerase (L) shows some protection (Geisbert *et al.*, 2006). For the future, inhibition of the NP35 suppression of the immune system is a target of interest (Kash *et al.*, 2006), as may be inhibition of apoptosis.

PAST INFECTION AND PERSISTENCE

Humans and non-human primates with documented acute infection with ZEBOV, SEBOV or REBOV seroconvert to high-titre IFA to antigens prepared from all three viruses. Low-titre antibody may be associated with filovirus infection in the past, and also with immunity, but this remains to be demonstrated, though MARV has been isolated from semen seven months after acute infection (Martini, 1973) and from the anterior chamber of the eye two months after acute infection (Gear *et al.*, 1975). A systematic study of the potential of the Ebola filovirus strains to persist in non-human primates failed to detect persistent filovirus. This was despite intensive efforts, including laparotomy to obtain serial specimens, co-cultivation and use of PCR (Fisher-Hoch *et al.*, 1992b).

VIROLOGY

The filoviruses still retain some of the great mysteries of virology. At first filoviruses were thought most to resemble rhabdoviruses, but it is now clear that they form a family of their own, designated *Filoviridae* on account of their filamentous appearance (Kissling *et al.*, 1968). Nucleotide sequence analyses place the family in the order Mononegavirales, which also includes the *Paramyxoviridae* and *Rhabdoviridae* families (Pringle, 1999). Within the *Filoviridae* family are two genera: ‘Ebola-like

viruses’ and ‘Marburg-like viruses’. The ‘Ebola-like’ genus includes the African viruses ZEBOV, SEBOV and CIEBOV and the Asian virus, REBOV. These viruses are of varying pathogenicity in patients and non-human primates, with ZEBOV being the most virulent, and REBOV essentially apathogenic in humans and demonstrating low, but variable pathogenicity in non-human primates depending on the primate species (Fisher-Hoch *et al.*, 1992a). The ‘Marburg-like’ genus includes at present only one strain, MARV, with high pathogenicity in humans and non-human primates. Though still considered a single strain, isolates now include a large number of MARV strains distinguishable genetically, antigenically and phenotypically (Reed and Mohamadzadeh, 2007; Towner *et al.*, 2006).

Filoviruses undergo rapid, lytic replication in the cytoplasm of a wide range of host cells. The nucleocapsids acquire the envelope with its surface projections by a process of extrusion of cell membrane rather than a discrete orderly budding, which may account for the striking pleomorphism of these viruses (Johnson *et al.*, 1977). Filoviruses are among the largest known viruses, with highly variable length (up to 14 000 nm), apparently due to concatamerization (Regnery *et al.*, 1980). The virions are of uniform 80 nm diameter, with a nucleocapsid consisting of a central axis 20–30 nm in diameter, surrounded by a helical capsid 40–50 nm in diameter, with 5 nm cross-striations. A host cell membrane-derived layer with 10 nm projections in regular array surrounds the nucleocapsid and the terminal windings of nucleocapsid at one end of the particle. The virions contain a single negative-strand RNA genome ranging from 4×10^6 (MARV) to 4.5×10^6 Da (EBOV). The RNA is a template for seven polypeptides; an NP, a surface glycoprotein (GP), an RNA-directed RNA polymerase (L) and four other proteins (VP40, VP35, VP30 and VP24) (Elliott *et al.*, 1985).

Aided by the development of reverse genetics, the functions of some of the filovirus proteins are now becoming understood (Volchkov *et al.*, 2001). The surface GP has been studied in most detail. Regions of the GP have been identified which bear antibody and T-cell epitopes (Dowling *et al.*, 2007). The GPs of most ‘Ebola-like viruses’ are cleaved to produce two molecular species, one a soluble truncated protein (sGP) which is secreted from infected cells, and the other the full-length glycoprotein. The sGP is a disulfide-linked homodimer, for which an anti-inflammatory function has been described (Falzarano *et al.*, 2006). Since MARV does not produce an sGP (Feldmann *et al.*, 1999), this molecule is an unlikely candidate for virulence. The ZEBOV GP, when cleaved, yields yet another small, soluble protein, the Δ -protein, whose function is unknown. In all filoviruses

the full-length GP is further proteolytically cleaved to form two heavily glycosylated subunits, GP1 and GP2, which together form the surface spikes seen by electron microscopy. The GP2 is a transmembrane protein and bears a fusion site (Freitas *et al.*, 2007), and has been shown to modify permeability of the plasma membrane (Han *et al.*, 2007). The GP1 is involved in viral attachment and entry, the latter associated with critical conserved sequences (Mpanju *et al.*, 2006). During entry and assembly and budding, viral proteins including GP1 have been shown to associate with lipid rafts on the cell surface, and released viruses, including virus-like particles (VLPs), incorporate raft-associated host molecules (Bavari *et al.*, 2002). Using VLPs, it has also been shown that a heavily glycosylated mucoid domain on the GP is able to stimulate human dendrite cells through NF- κ B and MERK pathways (Martinez *et al.*, 2007).

The VP30, VP35, NP and L proteins are necessary and sufficient for *in vitro* replication. VP30 is a nucleocapsid-associated transcription factor which interacts directly with viral RNA, and is thought to stabilize the nascent molecule (Hartlieb *et al.*, 2007; John *et al.*, 2007). VP35 is capable of targeting several pathways of antiviral interferon systems (Feng *et al.*, 2007; Cardenas *et al.*, 2006). It is an RNA-binding protein which acts as an interferon antagonist. It works by blocking the activation of interferon regulatory factor 3 which is responsible for facilitating interferon-associated gene expression. VP35 has further been shown to block the host iRNA responsible for RNA silencing. This silencing is part of the host innate antiviral activity, and is analogous to the activity of the HIV-Tat protein (Haasnoot *et al.*, 2007). It has also been shown to be capable of blocking α -interferon-induced antiviral activities and inhibition of the β -interferon promoter. The NP is associated with the nucleocapsid, serving as a scaffold (Noda *et al.*, 2002), and binding with VP40 is important for its incorporation into the virion during assembly (Noda *et al.*, 2007). The L protein is the RNA-directed RNA polymerase.

The VP24 protein, an abundant but poorly glycosylated protein, is believed to be a secondary matrix protein. It is associated with assembly and budding, and appears to localize to the plasma membrane and the perinuclear region (Han *et al.*, 2003). It has been shown to be capable of budding from mammalian cells as lipid-bound VLPs (Licata *et al.*, 2003). There is some evidence in a mouse model that VP24 and NP are associated with virulence and may also interfere with the host cell antiviral interferon response (Ebihara *et al.*, 2006).

ANIMAL MODELS

African green monkeys recently imported from Uganda were the source of the original Marburg outbreak (Henderson *et al.*, 1971; Martini *et al.*, 1968), and South East Asian cynomolgus monkeys were the source of the newly described Asian filovirus (Centers for Disease Control and Prevention, 1989; Jahrling *et al.*, 1990). Both are important species imported for medical and other research, and non-human primates have been the most successful animal used for the study of the pathogenesis of filoviruses (Fisher-Hoch and McCormick, 1999; Simpson *et al.*, 1968). The ability of filoviruses to kill guinea-pigs is variable. ZEBOV kills guinea-pigs consistently after several adaptive passages; the Sudan strain and MARV do not. Only the Zaire virus was found to be lethal for suckling mice (McCormick *et al.*, 1983). A lethal mouse model has also been developed and used in protection and pathophysiology studies (Bray *et al.*, 1999).

Rhesus monkeys inoculated intraperitoneally with 10^3 – 10^4 guinea-pig infectious units of ZEBOV become febrile on the third to fifth day after inoculation, and develop a petechial rash on the forehead, face, limbs and chest between the fourth and fifth days. Severe prostration with diarrhoea and bleeding leads to rapid death in almost all animals. Though similar in onset, the disease in non-human primates caused by SEBOV is characterized by lower viraemia, enzyme and immune disturbances, and some survivors, and is not pathogenic in mice (Figure 31.5). Petechiae are rarely seen, and then only in dying monkeys. Viraemias and liver enzymes do not reach the levels seen in ZEBOV filovirus infections, but as the illness progresses severe thrombocytopenia, neutrophilia and lymphopenia developed with very high AST and lactate dehydrogenase (LDH). Monkeys are obviously very sick, including the survivors, but haematological and biochemical parameters return essentially to normal by day 20, and recovery is rapid and complete. Though systematic comparisons have not been made MARV infection in monkeys apparently resembles SEBOV infection. However, African green monkeys experimentally inoculated with the virus soon after the 1967 outbreak all died, regardless of route of inoculation, but progression to death was slowed if the inoculum titre was reduced (Hass and Maass, 1971).

REBOV was first identified in an epizootic in an animal-handling facility, where transmission and a high death rate were observed in cynomolgus monkeys but these animals were co-infected with SHFV so that observations on filovirus pathogenesis are inconclusive (Centers for Disease Control and Prevention, 1989; Jahrling *et al.*, 1990). Using purified REBOV inoculate, free of SHFV, it has been shown that REBOV infected

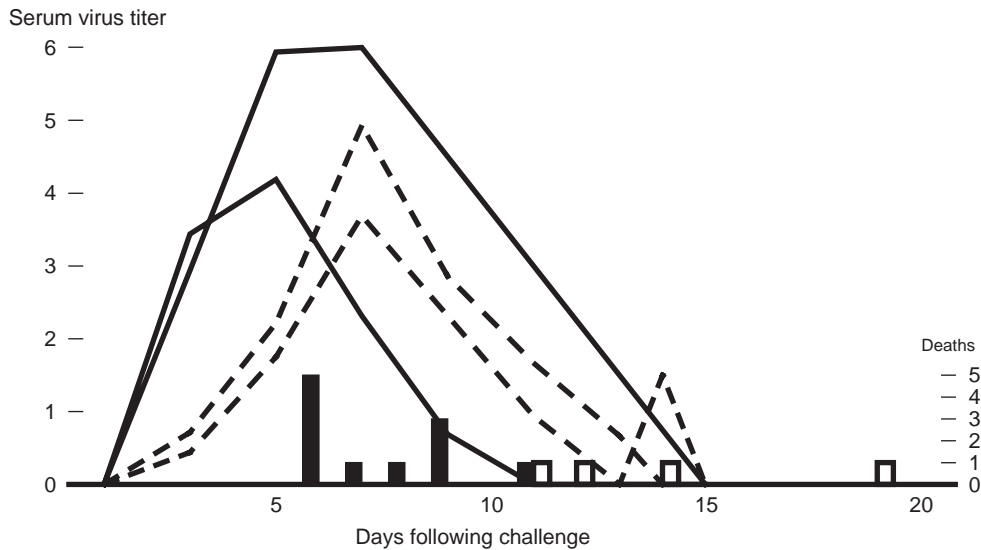


Figure 31.5 Comparison of viraemia and outcome in 31 monkeys challenged with filoviruses. Solid lines denote mean viraemia in monkeys challenged with ZEBOV and dashed lines those infected with REBOV. Numbers of deaths are shown by dark bars for ZEBOV-infected animals and open bars for REBOV-infected animals.

non-human primates have lower viraemia, delayed onset of enzyme and immune disturbances, and lower mortality than those infected with African viruses (ZEBOV or SEBOV) (Fisher-Hoch *et al.*, 1992b). It is clear that the host genetics also contribute, in that African green monkeys are less susceptible to severe or fatal disease due to SEBOV or REBOV than cynomolgus monkeys. ZEBOV infection, however, seems uniformly fatal in all species so far challenged (Fisher-Hoch and McCormick, 1999).

SEROLOGICAL STUDIES

The 'Ebola-like' viruses cross-react immunologically with one other, but not with MARV, which is serologically distinct (Elliott *et al.*, 1985). Serosurveys of village populations in epidemic areas have shown the presence of low-titred Ebola and Marburg antibodies, with antibody prevalence to Ebola virus in Zaire reported as high as 37%, which is at variance with the high mortality (nearly 90%) reported during known outbreaks. The problem is that specificity of responses to 'Ebola like' viral antigens has been a continuing issue, with positive responses, albeit at low titre, in highly improbable populations, such as Alaskan Eskimos (Stansfield *et al.*, 1988). Specificity is not improved by use of Western blot techniques, since nonspecific antibody to several proteins is variably seen. This problem appears to be unique to Ebola virus (EBOV, SEBOV and REBOV), and is not encountered

with MARV. Lack of correlation of serology with history of disease supports the conclusion that the titres are non-specific and results should be interpreted with caution. Possible explanations are cross-reactions with host proteins, or some common probably silent, but as yet unidentified human virus. Though some technical advances have reduced nonspecific reactions they continue to be a problem in interpretation of serological survey data (Elliott *et al.*, 1993).

PATHOGENESIS AND IMMUNOLOGY

Pathogenesis and immunology need to be discussed together since they are inextricably linked, and some of the major manifestations of disease are immunopathological in origin. In humans and non-human primates these processes reflect a complex, poorly adapted host/parasite relationship with extensive disruption of the host immune response in fatal infections (Baize *et al.*, 2000, 2002; Leroy *et al.*, 2001). Other factors such as infecting dose, route of infection and host genetics also undoubtedly play major roles.

At autopsy both Marburg and Ebola-infected humans and primates show widespread haemorrhagic diathesis into skin, membranes and soft tissue. There is focal necrosis in liver, lymph nodes, ovaries and testis. Most prominent are eosinophilic inclusion bodies in hepatocytes (Councilman-like), without significant inflammatory

response (Baskerville *et al.*, 1985). Focal necrosis is observed in many organs, including liver, lymphatic organs, kidney, testes and ovaries, but usually not sufficient to produce organ failure, and there is little infiltration of inflammatory cells in areas of tissue damage. Though REBOV is markedly less pathogenic, virus particles have been observed embedded in the basement membrane of lung alveoli (Ikegami *et al.*, 2002).

Among the long-established facts are that high titres of virus are found in serum and tissues taken at autopsy, and particles may be seen in large numbers with some obvious tropism for reticuloendothelial cells (Baskerville *et al.*, 1985; Fisher-Hoch *et al.*, 1992a). The most profound physiological alteration, invariably associated with death, is shock. Several years before the discovery of cytokines and other cellular messenger profound disruption of biochemical integrity of the endothelial cell was proposed without destruction of the cell itself by lytic replication (Baskerville *et al.*, 1985). More recent studies show that endothelial cells are indeed not primary targets and though permissive for EBOV replication *in vitro*, no significant cytopathic changes are observed, and there is insignificant cell activation. The conclusion is that damage to endothelial cells is likely triggered by immune-mediated mechanisms (Geisbert *et al.*, 2003c). On the other hand, coagulation abnormalities are now thought to involve increase in tissue factor (TF) expression stimulated by macrophage/monocyte activation, and leading to dysregulation of the coagulation cascade (Geisbert *et al.*, 2003b).

Until 2000 very little was understood about the immunology of Ebola virus infections. Dendritic cells, monocytes and macrophages are now recognized as the primary targets (Geisbert *et al.*, 2003a; Hensley *et al.*, 2005b). Filoviruses are also known to utilize a number of host molecules for viral entry such as DC-SIGN and various lectins (Hensley *et al.*, 2005a), and VP35 and VP24 both act as interferon antagonists (Cardenas *et al.*, 2006; Ebihara *et al.*, 2006; Feng *et al.*, 2007). Application of reverse genetics techniques has shown potent induction of TNF- α pathway and NF- κ B genes. Downregulation of many coagulation and acute-phase proteins is also reported, including toll-like receptors (Kash *et al.*, 2006).

Studies in humans are rare and very difficult to do, but intensely revealing. Baize and Leroy have published the only series using specimens from patients collected and processed in Gabon. They have shown the immune system to be playing a very important role in patients in both pathogenesis and protection (Baize *et al.*, 1999). Fatal infections are characterized by monocyte/macrophage activation associated with defective inflammatory and anti-inflammatory responses, culminating in massive intravascular T-cell apoptosis (Baize *et al.*, 1999, 2002;

Leroy *et al.*, 2000b, 2001). In a small number of patients (seven) who were asymptotically infected with ZEBOV, an early, orderly inflammatory immune response was observed. Control of virus replication and elimination of virally infected cells was shown to be associated with a prompt inflammatory response which was rapidly down-regulated. T-cell activation was subsequently associated with clearance of infected cells, followed thereafter by development of IgG antibodies (Leroy *et al.*, 2000b). Interestingly, IgG3 and IgG1 subclasses predominated, and it was hypothesized that these are responsible for containment of persistent virus in privileged sites, such as the uvea and semen.

CONTROL

Containment

It now appears that bat-infested environments and bat guano should be considered potential sources of infection, as should sick or dead apes in the rainforest. Prompt identification of active cases is critical, and is in great part dependent on an accurate and detailed history (Centers for Disease Control and Prevention, 1988). Interruption of person-to-person spread of the virus is essential to control. The most important issue is that of awareness in the medical community that these diseases exist and they may result in extensive nosocomial spread if not recognized early and if appropriate isolation of the patient is not achieved. Thus early institution of safe and orderly care of the ill can be set up with effective surveillance of high-risk contacts and prompt isolation of further cases. This has been shown to ensure rapid control of an epidemic.

The experience from Uganda provided the only unique data showing the value of a national approach to control combined with strong community participation (Okware *et al.*, 2002). This extensive outbreak presented major logistic problems threatening to spread throughout the country. The Ugandans set up a series of task forces at different administrative levels including a District Talk Force in each district of the country. Programmes included public education, mobilization of communities, training for healthcare workers and village volunteers, and timely dissemination of information using radio, mobile phones and foot and motor patrols. Scouts were recruited for active case finding. Isolation wards were set up and a temporary field laboratory. One of the major obstacles was lack of laboratory facilities. Burial teams were established for safe burial, since it soon became clear that the preponderance of female cases was associated with ritual cleansing of the dead. In the Angolan outbreak of Marburg disease, church organizations were successfully used to reach

the community and educate people to avoid spreading the disease.

Contacts

High-risk contact is associated with direct contact with blood or body fluids from acutely infected humans or animals, or sexual contact with a convalescent case. Laboratory accidents must be treated seriously, with careful review of level of risk. If this is thought to be high, isolation for the incubation period (17 days is adequate) and tracing and surveillance of any further potential contacts such as family members are necessary. If the risk is low, simple surveillance of the individual by daily telephone contact for fever during the incubation period suffices. It must be remembered that biocontainment facilities do not protect against injury with needles or other sharps. There is no evidence for or against the use of passive antibody in prophylaxis. Suitably screened and stored material is in any event unavailable.

Hospital Containment

The key to prevention of transmission in both endemic and non-endemic areas has consistently been good hospital and laboratory practice, with strict isolation of febrile patients and rigorous use of gloves and disinfection. Intensive care, surgery and air transport should not be denied. Patient isolators are not recommended since the hazard to contacts is not aerosol, but direct inoculation of virus in blood or other material. Isolators induce loss of manual dexterity and fatigue, inhibit intensive care procedures and communication, do not protect against sharp instrument injury and have no provision for resuscitation. The 1988 Centers for Disease Control and Prevention guidelines for the management of patients with viral haemorrhagic fever recommends routine patient isolation in a single room, preferably but not necessarily with negative air pressure gradient from the hallway, through an anteroom to the patient room (Centers for Disease Control and Prevention, 1988). Staff education and strict supervision, use of gloves, gowns, masks and rigorous disinfection with fresh liquids are mandatory. The recommendations issued for management of AIDS patients are also adequate for containment of filoviruses. Since most cases occur in hospitals in more remote parts of Africa, common sense, and careful use of good techniques and disinfectants by trained staff is the best approach.

Vaccines

There are now candidate vaccines for filoviruses, though the potential target population for a vaccine is small (Bukreyev *et al.*, 2007; Geisbert and Jahrling, 2003; Geisbert *et al.*, 2002; Jones *et al.*, 2005; Sullivan *et al.*, 2003).

Filoviruses do not induce classical neutralizing antibodies, making an effective killed vaccine unlikely. Neutralizing mouse monoclonal antibodies have been produced and shown to protect mice, but they do not protect non-human primates (Oswald *et al.*, 2007). Rodents and primates clearly handle filoviruses quite differently, as is borne out by the failure to protect monkeys of several candidate vaccines which protected mice and/or guinea-pigs (Geisbert *et al.*, 2002). The most promising candidate is currently a vesicular stomatitis virus recombinant expressing EBOV and MARV glycoproteins (Jones *et al.*, 2005). Currently the only vaccine in phase I clinical trials is a adenovirus recombinant vaccine (Martin *et al.*, 2006).

PERSPECTIVE

At the time of writing (August 2007) another large, unconfirmed, outbreak of suspected haemorrhagic fever with more than 100 deaths is reported on ProMed in the DRC near the Angolan border. It is likely that there will be more outbreaks, given the increase in human population density in the endemic area, and the ubiquity of the natural hosts. Nevertheless, there have been recent, major advances in understanding filoviruses. These include the identification of fruit bats as the reservoir, which now allows us to envisage specific control measures, or at least recommendations to avoid bat-infested areas. Bats occupying roofs in larger buildings in Africa can be removed. We are also beginning to understand the highly complex immunopathology of this disease. Because of the high mortality, particularly in non-human primate models, filoviruses are excellent models for understanding such complex processes in small numbers of animals. The introduction of reverse genetics and the identification of functions of the viral proteins also takes us further in understanding how the virus works, and by what means it might be thwarted. Effective treatment and safe vaccines can be anticipated as the outcomes of this knowledge. However, the main obstacle to prevention is the poverty and lack of education of most of the victims, and the degradation and invasion of the habitats of the natural reservoir. We shall need political as well as scientific solutions to control filoviruses.

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Rabies and Other Lyssavirus Infections

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INTRODUCTION

Rabies is a zoonosis of a variety of mammalian species in different areas of the world. The domestic dog is the most important vector, mainly in Asia and Africa, and canine viruses are responsible for >99% of human infections. Sylvatic or wildlife rabies is endemic in Asia, Africa, the Americas, Australia and most of Europe. Classical rabies, genotype I, viruses cause most infections, the remainder being due to the six genotypes of rabies-related lyssaviruses. With one recent exception, the disease has been fatal in unvaccinated people. Despite attempts at intensive care treatment over 30 years, only a few previously vaccinated patients have survived, and all but one had profound neurological deficits. The combination of pre- and post-exposure rabies immunization is 100% effective in preventing this fatal infection.

In English, rabies is also known as hydrophobia, lyssa and mad dog bite; in French, *la rage* or *l'hydrophobie*; in Italian, *la rabbia*; in Spanish *la rabia*; in Portuguese, *a raiva* or *hidrofobia* and in German, *die Tollwut*.

HISTORY

Rabies in dogs and the importance of saliva in its transmission may have been recognized in pharaonic times and in China seven centuries BC (Théodoridès, 1986). Aristotle (322 BC) described rabies in animals but seemed to deny that humans could be infected or could die from the disease. Celsus in 'De medicina' (first century AD) described hydrophobia in afflicted humans and recognized

that the disease was spread by saliva. In the sixteenth century, Fracastoro strengthened the concept of rabies as a contagious disease. A scientific or experimental approach to rabies was delayed until 1793, when John Hunter suggested that the transmission of rabies should be studied by inoculating saliva from rabid animals and humans into dogs and that attempts should be made to inactivate the 'poison' in the saliva. These ideas may have inspired the animal experiments on transmission by Zinke, and Magendie and Breschet (Théodoridès, 1986).

Galtier found that rabbits could be infected with rabies and were more convenient experimental animals than dogs. In 1881, he first demonstrated specific immunization against the disease (Théodoridès, 1986). Pasteur adopted the use of rabbits in his studies of rabies, beginning in the 1880s. He was the first to recognize that the major site of infection was the central nervous system (CNS). Pasteur was able to protect dogs from challenge by immunizing them with a virus attenuated in desiccated rabbit spinal cord and in 1885 he used this as a vaccine for the first time in Joseph Meister and Jean-Baptiste Jupille, boys who had been severely bitten by rabid dogs. The reputation of modified forms of Pasteur's vaccine increased during the first half of the twentieth century, but its efficacy remained uncertain. The bite of a mad dog caused rabies in only about 30% of untreated patients, although with wolf bites the risk was higher. In Iran, treatment of bites by rabid wolves with classical Semple brain tissue vaccine only reduced the case fatality about 10% compared with unvaccinated patients. A dramatic natural experiment in 1954, when a rabid wolf invaded a village and bit 29 people, demonstrated the protective

value of passive immunization in patients with severe bites (Baltazard and Bahmanyar, 1955). This led to a general recommendation that immunoglobulin should be included in rabies post-exposure prophylaxis as well as vaccine.

In 1903 Negri described his diagnostic inclusion body which allowed the post-mortem laboratory diagnosis of rabies. The introduction of the more specific and sensitive immunofluorescence method in 1958 has since replaced Seller's stain for Negri bodies. Remlinger, in 1903, showed that rabies was caused by a filterable agent. It was not until 1936 that the size of the virus was established, and it was first seen by electron microscopy in 1962 (Théodoridès, 1986).

Improvements in Pasteur's vaccine were achieved by Semple and Fermi who killed the virus rather than attenuated it, and by Fuenzalida and Palacios who developed a suckling mouse brain vaccine which carried a lower risk of neuroparalytic complications. Growth of rabies virus in tissue culture was achieved in the 1930s, leading to the development, by Wiktor and his colleagues, of the first tissue culture vaccine for human use. This human diploid cell vaccine (HDCV) was licensed in 1974. Since then other substrates including chick embryo and vero cells have been used to facilitate production and reduce the cost.

CLASSIFICATION

The family *Rhabdoviridae* (*rhabdos* meaning 'rod' in Greek) includes several genera of viruses found in plants, arthropods, fish, birds, reptiles and mammals. Almost all of those known to infect humans belong to two morphologically similar genera: *Vesiculovirus* and *Lyssavirus*. Members of the genus *Vesiculovirus* cause vesiculostomatitis of cattle and horses. In humans, influenza-like symptoms occur occasionally, and two cases of encephalitis have been reported (Quiroz *et al.*, 1988). The genus *Lyssavirus* (*lyssa* meaning 'rage' or 'frenzy' in Greek) comprises seven genotypes: genotype 1 causes classical rabies; genotype 2 Lagos bat virus; genotype 3 Mokola virus in shrews and cats, genotype 4 Duvenhage virus in bats; genotypes 5 and 6 are European bat lyssaviruses (EBLV) found in insectivorous bats and genotype 7 Australian bat lyssavirus (ABLV) in flying foxes (fruit bats) and insectivorous bats. All these rabies-related viruses have been associated with human disease except for Lagos bat virus (see Table 32.1, Figure 32.2 and section on Human infections with rabies-related viruses below). There is serological evidence of rabies-related lyssaviruses in bats in the Philippines, Thailand, Cambodia, Bangladesh and China.

The Lyssavirus genus has been divided into two phylogroups as a result of serological and genetic analyses (Badrane *et al.*, 2001). Phylogroup I includes all genotypes except Mokola virus and Lagos bat virus, which form phylogroup II. All phylogroup I genotypes have caused fatal rabies-like encephalitis in humans, whereas Mokola virus probably caused three known human infections, one of which was a fatal encephalitis without typical features of rabies. Experimentally, phylogroup II viruses are less pathogenic, and there is little if any cross-neutralization with the phylogroup I lyssaviruses. Four as yet unclassified lyssaviruses have been found in bats in the Russian Federation and Central Asian Republics (Table 32.1).

VIRUS STRUCTURE

The bullet-shaped rabies virions measure 180×75 nm. The genome is a nonsegmented strand of negative-sense RNA of 11.9 kb, which bears five structural genes. In the core of the virion the RNA is in a tight helical coil incorporating a nucleoprotein (N) a phosphoprotein (P) and an RNA-dependent RNA polymerase (L), forming the ribonucleoprotein (RNP) complex. A layer of matrix (M) protein covers this cylindrical structure. The outermost lipoprotein envelope is composed of a host-derived lipid bilayer and virus-coded glycoprotein (G) bearing spikes projecting 8.3 nm above the virion surface. Each projection is a trimer of G molecules with a distal knob. The envelope covers all except the flat end of the virion, resulting in the characteristic bullet shape.

The viral nucleoprotein has 450 amino acids, (~57 kDa) and is an integral component of the RNP complex. It is the most conserved protein throughout the Lyssavirus genus, and so its antigens are employed diagnostically both for genus-specific simple immunofluorescent antibody (IFA) techniques and genotype-specific monoclonal antibody and polymer chain reaction (PCR) assays. N protein encapsidates viral RNA, protecting it from ribonucleases, and has a role in regulating RNA transcription. It also bears T helper cell epitopes. The lyssavirus N is phosphorylated, unlike all other rhabdovirus nucleoproteins. Nascent N molecules rapidly aggregate together or combine with molecules of viral P protein, often in large accumulations of filamentous matrix forming the diagnostic Negri bodies (Figure 32.1), which may also include other proteins.

The phosphoprotein has 297 amino acids (~40 kDa), and is the most diverse rabies protein. It exists in different phosphorylated forms and truncated P products which maybe intranuclear. It has previously been known as M1, membrane-associated 1 and NS, nonstructural protein. Its

Table 32.1 The *Lyssavirus* genus

Genotype	Source	Known distribution
Phylogroup I		
1 Rabies virus	Dog, fox, raccoon, skunk, bat and others.	Widespread
4 Duvenhage	Insectivorous bat (<i>Nycteris thebaica</i>)	South Africa, Zimbabwe (very rarely identified)
5 European bat lyssavirus	1a bats, for example, <i>E. serotinus</i> 1b bats, for example, <i>E. serotinus</i>	Northern and Eastern Europe Western Europe (see text)
6 European bat lyssavirus	2a <i>M. dasycneme</i> bats <i>M. daubentonii</i> bats 2b <i>M. daubentonii</i> bat	Netherlands, Germany (see text) UK Switzerland (very rare)
7 Australian bat lyssavirus	Flying foxes (<i>Pteropus</i> sp.) Insectivorous bats	Australia
Phylogroup II		
3 Mokola	Shrews (<i>Crocidura</i> spp.), cats, dog ^a	South Africa, Nigeria, Cameroon, and Ethiopia (rare)
2 Lagos bat virus	Bats, cats, water mongoose ^a , dog ^a . Has not been detected in humans	Africa (rare)
Unclassified		
Irkut	<i>Murina leucogaster</i> bat	<i>Siberia</i>
Aravan	<i>Myotis blythi</i> bat	<i>Kyrgyzstan</i>
Khujand	<i>Myotis mystacinus</i> bat	<i>Tajikistan</i>
West Caucasian bat virus	<i>Miniopterus schreibersi</i> bat	<i>Russia</i>

^aSingle isolates only.

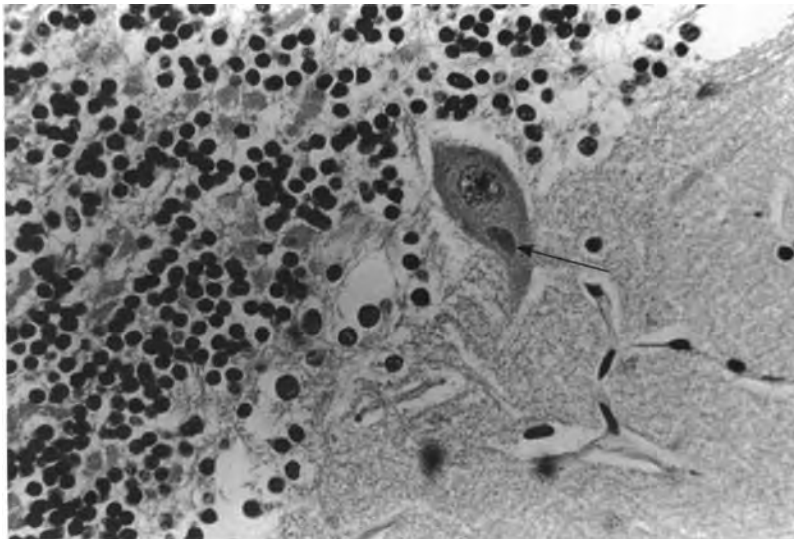


Figure 32.1 A Negri body (arrow) in a cerebellar Purkinje cell. (Source: Courtesy of F.A. Murphy.)



Figure 32.2 World distribution of rabies. Rabies-free areas are white; red indicates terrestrial rabies genotype 1, and countries with only bat lyssaviruses are green. No rabies-related lyssaviruses have been found in the Americas.

functions include binding to nascent N and so preventing its polymerization, and nonspecific binding to cellular RNA. P protein also forms a stabilizing complex with L protein and is a cofactor in genome transcription and replication. P protein has been shown to interact with a light chain (LC8) component of dyenin and myosin V molecular motors. Possible involvement in the intracellular transport of rabies virus fragments remains to be proved (Warrell and Warrell, 2004) (see section on Pathogenesis). The P protein also inhibits interferon transcription and signalling at three different stages (Finke and Conzelmann, 2005; Vidy *et al.*, 2007).

The viral RNA-dependent RNA polymerase is the largest protein (hence the designation L), containing approx 2142 amino acids (~244 kDa). It contains groups of highly conserved residues. This RNA polymerase starts the transcription of the primary genomic RNA. Other functions of L protein include replication, polyadenylation and protein kinase activities.

The matrix protein, M, has 202 amino acids (~25 kDa), and covers the RNP complex. It has several functions including: the compacting of the loose helical core structure; regulation of viral RNA transcription (Finke and Conzelmann, 2005); inhibiting translation of host cell proteins; and interacting with the lipid bilayer and the G protein to enable budding of virus from the cell membrane. M protein can also induce apoptosis experimentally.

The surface glycoprotein is *N*-glycosylated with branched chain oligosaccharides which show microheterogeneity within the virion. It has 505 amino acids (~65 kDa). The external domain of the G molecule is a trimeric spike connecting via a transmembrane anchor to a cytoplasmic domain, which is closely

associated with the M protein. The distal knob on the surface projection is important in pathogenesis as it bears binding sites for cellular receptors, and effects pH-dependent fusion with endosomal membranes. A reversible change in conformation of the protein into an activated hydrophobic state initiates membrane fusion. Single amino acid replacements, notably arginine 333, at antigenic site III on the ectodomain can radically reduce the virulence of the virus (Dietzschold *et al.*, 1983). The G molecule also promotes the passage of virus from cell to cell, is involved in axonal transport, and is essential for trans-synaptic spread (see section on Pathogenesis below). G epitopes are most important as they are the only inducers of protective rabies neutralizing antibodies. They can also induce helper and minimal cytotoxic T-cell responses. At high expression levels, G is associated with induction of apoptosis (Finke and Conzelmann, 2005).

REPLICATION

Entry of virus into a cell occurs via coated pits or by cell receptor-mediated adsorptive endocytosis into an endosome (see section on Pathogenesis below) (Wunner, 2007). The surface viral glycoprotein enables low pH-dependent fusion with the endosomal membrane, uncoating and liberating the nucleocapsid (or RNP complex) into the cytoplasm. Catalysed by the viral L protein, the primary genomic negative-strand RNA is transcribed, beginning with a short leader RNA and followed by 5' monocistronic positive-strand mRNAs and later the full length anti-genome replicative RNA. The amount of

mRNA produced is greatest from the 5' end, in the order of N, P, M, G and L. Protein synthesis proceeds in the cytoplasm on host cell ribosomes. The unstable nascent nucleoprotein and phosphoprotein encapsidate and stabilize the progeny negative-strand genomic RNA by binding to its 5' end, or they may aggregate to form localized masses which accumulate and become inclusions. The viral polymerase is incorporated into the strand of nucleoprotein, which forms a tight helical coil aided by the action of the viral matrix protein. The resulting RNP complex migrates to the cell membrane where the nascent viral glycoprotein has become concentrated and inserted into the lipid membrane. The matrix protein interacts with the cytoplasmic domain of the glycoprotein, and promotes budding of the bullet-shaped virion. The membrane at the flat-ended base bears no G protein. Viral maturation can occur intracellularly by budding through endoplasmic reticulum, as seen especially in neurons (Iwasaki et al, 1985). Unlike vesicular stomatitis virus, rabies virus replication requires the presence of the nucleus in the host cell.

INACTIVATION OF VIRUS AND STABILITY OF VACCINE ANTIGEN

Rabies virus in a phosphate-buffered saline (PBS) solution is rapidly inactivated by heat: at 56 °C the half-life is less than a minute; at 37 °C the half-life is prolonged to about 3 hours in moist conditions and at 4 °C to three or four days. The stabilizing presence of serum or other buffer solutions enhances survival. The lipid coat of the virion renders it vulnerable to disruption by detergents and simple 1% soap solution. About 45% ethanol, iodine solutions (with 1 in 10 000 available iodine), 3% sodium hydroxide, 1% benzalkonium chloride, 3% formalin, neat chloroform and acetone all inactivate the virus (Kaplan *et al.*, 1966). Hypochlorite and glutaraldehyde solutions are suitable for laboratory use with the normal precautions, but phenol is not an effective virucidal compound.

Although benzylkonium chloride and other quaternary ammonium compounds are virucidal experimentally, they are not recommended for cleaning rabies-infected wounds at the concentrations in normal clinical use because they are inactivated by the presence of soap (Kaplan *et al.*, 1966). Soap is recommended as first aid treatment of animal bites.

The potency and immunogenicity of lyophilized cell culture vaccine is retained after tropical ambient temperatures for 11 weeks (Nicholson *et al.*, 1983). In reconstituted liquid form, however, the potency may fall significantly after a week or two. It is therefore unwise to keep an open vial for several days because of the risk

of microbial contamination causing denaturing of antigen, loss of potency and possible infection of the recipient.

EPIZOOTIOLOGY AND EPIDEMIOLOGY

Rabies is transmitted within populations of relatively few species of mammals, principally within the domestic dog population. The vectors of sylvatic, or wildlife, rabies vary in different parts of the world and the virus circulates within the species group, but in South Africa a virus type is related to a geographical area and occurs in several vector species. As a result of the geographical compartmentalization of rabies within a mammalian species, there has been a tendency for adaptation and divergence of virus strains. This is identified by monoclonal antibody tests and genetic analysis. There is occasional overspill between these intraspecies cycles, for example, when a rabid raccoon bites a fox in North America. Transmission outside the cycle occurs more commonly to non-vector mammalian species, for example, when a cow or sheep is bitten by a rabid red fox in Europe, and all human infections.

Rabies is enzootic in terrestrial mammal species in most of the world, but in a few countries rabies-related lyssaviruses are found in bats alone (Figure 32.2). Areas of the world which have been reported to be free of rabies include: Ireland, Iceland, Finland, Sweden, Norway, Portugal, Italy, Greece, the Mediterranean islands (except the Balearic islands, where there is European bat lyssavirus), New Zealand, New Guinea, Japan, Taiwan, Hong Kong islands (but not the New Territories), Singapore, peninsula Malaysia, Sabah, Sarawak, some islands of Indonesia, islands of the Pacific and Indian Ocean (except Madagascar); some Caribbean islands (e.g. Barbados, Bahamas, Jamaica, St Lucia, Antigua and so on); and Antarctica. Some countries are generally free but infected animals occasionally cross their land borders. The epizootiology is constantly changing, so local advice should be sought for detailed information.

Rabies is spread between mammals by bites; by contamination of scratches or intact mucosal membranes by virus-laden saliva; by ingestion of infected prey; transplantally; and possibly by inhalation of aerosols (in heavily populated bat caves). The principal reservoir or vector species in different geographical areas are listed below.

Europe

- Red fox (*Vulpes vulpes*)—Germany, Poland, Hungary, Croatia, Serbia, Slovenia, Romania and then eastwards to include the Russian Federation.

- Arctic fox (*Alopex lagopus*)—northern Russian Federation.
- Raccoon dog (*Nyctereutes procyonoides*) (not an established reservoir)—the Baltic States, Poland, Belarus, Ukraine and the Russian Federation.
- Wolf (*Lupus lupus*)—from Poland east to the Russian Federation
- Domestic dog (*Canis familiaris*)—dominant vector in Turkey. Cases also found in the Baltic States, Poland, Slovakia south to Croatia and eastwards to the Russian Federation. These are likely to be due to infection from foxes or other wildlife.
- Insectivorous bats—harbour the rabies-related European bat lyssaviruses (Amengual *et al.*, 1997). Transmission to terrestrial mammals has very rarely been found; to date only in five sheep in Denmark and a stone marten in Germany.
- Genotype 5, EBLV-1a—found mainly in serotine bats, *Eptesicus serotinus*, in the Netherlands, Denmark, Germany, Poland, Hungary, the Russian Federation and a single case in France. (Isolates also from the Slovak Republic and Czech Republic but typing not available. Seropositive bats found in Belgium.)
- EBLV-1b—usually found from the Netherlands down to Spain. The host is *E. serotinus* in the Netherlands, France and a single bat in Poland; also several bat species in Spain, and rarely in unidentified bats in Germany.
- Genotype 6—rare. EBLV-2a was isolated eight times in the United Kingdom: from a Scottish patient in 2002 and from seven infected Daubenton's bats (*Myotis daubentonii*) first from Newhaven in 1996, then from Lancashire, Surrey, Oxfordshire and Shropshire. The seroprevalence in that species is 2–3% (Fooks *et al.*, 2006). EBLV-2a was found in *Myotis dasycneme* (pond bats) in the Netherlands; also rarely from Germany and a doubtful isolate in the Ukraine.
- EBLV-2b was isolated from *M. daubentonii* in Switzerland and from the Swiss or Finnish human case (see section on Human infections with rabies-related viruses below and Table 32.1).

Middle East

- Dominant vectors are the fox and the wolf. Dogs maybe infected from wildlife.
- Other vectors include golden jackals (*Canis aureus*) in Israel.

Asia

- Domestic dog (*C. familiaris*) is the dominant vector.

- Frugivorous and insectivorous bats in the Philippines, Cambodia, Thailand, Bangladesh and China are seropositive for lyssavirus.

Africa

- Domestic dog (*C. familiaris*) rabies is predominant throughout the continent and in Madagascar.
- Black backed jackals (*Canis mesomelas*)—southern Africa
- Yellow mongoose (*Cynictis penicillata*)—southern Africa
- Bat-eared fox (*Otocyon megalotis*)—southern and East Africa
- Frugivorous and insectivorous bats—Duvenhage virus very rarely found (see section on Human infections with rabies-related viruses below).

For more on Africa see Nel and Rupprecht, 2007.

North America

- Arctic fox (*A. lagopus*)—Alaska, north-western Canada
- Red fox (*Vulpes fulva*)—western Canada and north-eastern United States.
- Grey fox (*Urocyon cinereoargenteus*)—Texas, Arizona.
- Striped skunk (*Mephitis mephitis*) and other species—Texas, central United States, California, also western Canada.
- Raccoon (*Procyon lotor*)—eastern coastal states of United States from Florida to the Canadian border, and adjacent regions of Canada.
- Insectivorous bats—in all continental states of United States, e.g. Mexican free-tailed bat (*Tadarida brasiliensis mexicana*), red bat (*Lasiurus borealis*), big brown bat (*Eptesicus fuscus*), hoary bat (*Lasiurus cinereus*), silver-haired bat (*Lasionycteris noctivagans*), eastern pipistrelle (*Pipistrellus subflavus*).

Caribbean

- Vampire bat—Trinidad, Tobago and Isla de Margarita.
- Mongoose (*Herpestes* species)—Puerto Rico, Granada, Cuba, Dominican Republic and probably Haiti.

Central and South America

- Domestic dog rabies—in Mexico and areas of Central and South America, except the Southern Cone.

- Grey fox (*Urocyon cinereoargenteus*)—in Colombia, Mexico.
- Vampire bat (*Desmodus* species)—causing bovine paralytic rabies from Mexico south to the northern parts of Argentina and Chile.
- Insectivorous bats—widespread

Australia

- Flying foxes or fruit bats (*Pteropus* species) and rarely insectivorous bats in Eastern coastal regions harbour ABLV (Warrilow, 2005) (see section on Human infections with rabies-related viruses below, Table 32.1 and Figure 32.2).

Vampire bat rabies has serious economic effects for farmers. The bats feed on the blood of large mammals, particularly cattle, transmitting, in the process, a form of paralytic bovine rabies. The disease causes an estimated loss of 50 000 head of cattle each year in Brazil. Outbreaks have been reported from Ecuador and Peru.

All bat rabies in the Americas is due to genotype 1 virus, whereas bats in the rest of the world harbour only the rabies-related lyssaviruses: EBLV, ABLV or Duvenhage virus.

Cyclical epizootics of rabies, such as the fox epizootic in Europe, result from an uncontrolled increase in the population of the key reservoir species. This epizootic started in Poland at the end of the Second World War and spread to France. In Western Europe it has been controlled by vaccination (see section on Control below).

Many other species of domestic and wild mammals may develop rabies due to infection by known local vector species.

INCIDENCE OF HUMAN RABIES

The true global incidence of human rabies has been obscured by underreporting, and is not reflected in official figures. Canine rabies virus is the cause of more than 99% of human infections. A large study in Asia and Africa (Knobel *et al.*, 2005) estimated an annual mortality of 55 270 or 1.38 per 10 000 population. Data suggest that only 3% of human cases are recorded by central health authorities and 80% occur in rural areas. The highest annual mortality is 20 000 in India, 2–3 per 100,000 population, and deaths rates are high in Bangladesh and Pakistan. Human cases have been mounting for a decade in China, with a reported increase of 30% in one year, to 3209 cases in 2006. Rabies encephalitis is often misdiagnosed; among 26 children dying of presumed cerebral malaria in Malawi, 3 (11%) proved to have rabies infection at post-mortem (Mallwela *et al.*, 2007).

In Latin America, mortality from canine rabies is reported from Brazil, El Salvador, Mexico, Bolivia, Haiti and Venezuela (Schneider *et al.*, 2007). In the United States an average of two to three deaths occur annually. More than 80% are indigenous infections and 90% of these are due to bat rabies viruses, usually the silver-haired bat or the eastern pipistrelle type. Less than 40% of patients had recognized a bat bite. It has been suggested that rabies is underdiagnosed in the United States (Messenger *et al.*, 2002).

In Europe 62 human deaths have been reported in the last six years, mainly from Russia and also Ukraine, Eastern European states plus imported cases in Western Europe. In Great Britain 10 people have died of rabies since 1980s, nine of whom were infected abroad. After a century of freedom from indigenous rabies, a man died of EBLV-2a infection in Scotland in 2002.

PATHOGENESIS

Entry of Virus into the Nervous System

Bites by rabid animals may inoculate virus-laden saliva through the skin into muscle. Local replication of virus in striated muscle occurs experimentally and might account for the long incubation periods often observed (Charlton *et al.*, 1997). Virus has been detected at local motor or sensory nerve endings (Murphy, 1977). Direct invasion of neurons has also been shown after peripheral inoculation. The mechanism of infection from superficial skin lesions inflicted by bats is unknown.

The rabies virus infects a great variety of cell types *in vitro* and *in vivo*, and viral attachment has been demonstrated to several types of cell surface receptor including carbohydrates, phospholipids and sialylated gangliosides. This binding is not specific and there is no evidence yet that these receptors are important *in vivo*, but it might explain the diversity in the tissue culture cell lines that will support rabies infection.

Specific binding occurs at neuromuscular junctions where it co-localizes with the nicotinic acetylcholine receptor (Jackson, 2007b; Lentz *et al.*, 1982). Binding at this post-synaptic site is competitive with cholinergic ligands, including the snake venom neurotoxin alpha bungarotoxin, which shows sequence homology with rabies virus glycoprotein. The varied susceptibility of different species to rabies infection could be related to the quantity of nicotinic acetylcholine receptors in muscle tissue (Baer *et al.*, 1990).

Viral attachment occurs *in vitro* to the neural cell adhesion molecule (NCAM), found on susceptible cell lines (Thoulouze *et al.*, 1998). *In vivo*, NCAM is in

neuronal membranes especially at presynaptic terminals. Its presence enhances infection, but is not essential. Another *in vitro* G protein-binding site is to the neurotrophin receptor p75 (p75^{NTR}) (Langevin *et al.*, 2002) found on selected neurons. Again it contributes to but is not essential for infection, suggesting that a variety of receptors are involved in rabies, perhaps at different stages of the pathogenesis. Virus entry via coated pits or receptor-mediated endocytosis into a vesicle occurs and pH-dependent fusion of the viral glycoprotein with the endosomal membrane may release the RNP complex into the cytosol.

Transport of Virus to the Brain

Rabies antigen can be found along the nervous pathways from a peripheral site of infection towards the brain during the incubation period. The migration of virus from a wound in a peripheral nerve to the CNS occurs within motor axons via the retrograde fast axonal transport system (Finke and Conzelmann, 2005; Jackson, 2007b). Its progress can be blocked by sectioning nerves or by metabolic inhibition with locally applied colchicine, which disrupts the axon microtubular system. The rate varies experimentally but an *in vitro* estimate is 50–100 mm per day; it can be considerably faster *in vivo*. Viral transport is not affected by the presence of extracellular neutralizing antibody (Tsiang, 1993). Since rabies virus has strictly retrograde axonal movement, it has been used as a tracer to identify neural pathways (Ugolini *et al.*, 2006). Attachment to the dyenin axonal motor molecule is assumed to be essential for this retrograde transport, but the means of viral binding remains speculative (Warrell and Warrell, 2004). The rabies P protein associates with LC8, a component of a cytoplasmic dynein LC, suggesting a means of attachment of the viral RNP complex to the retrograde dyenin axonal motor (Jacob *et al.*, 2000; Raux *et al.*, 2000). Further studies show that binding of viral proteins to the LC8 molecule is not essential for pathogenesis (Mebatsion, 2001), and that the viral G protein alone promotes retrograde microtubular transport (Mazarakis *et al.*, 2001). Viral replication is intracytoplasmic. Interneuronal spread is dependent on the presence viral G protein (Etessami *et al.*, 2000; Wickersham *et al.*, 2007) and budding of virus at synapses directly into an adjacent axon is the most likely mechanism (Iwasaki *et al.*, 1985). Contact with the extracellular environment is thereby avoided.

Effect of Infection on the Brain

Rabies progresses rapidly through the spinal cord and brain, with massive viral replication and accumulation of

viral proteins resulting in inclusion formation and eventually Negri bodies (see section on Virus structure above). Infected glial cells have rarely been observed in humans, and they are not a significant route of dissemination of virus. Infection of the limbic system affects the behaviour of the host which, in a vector species, increases the chance of transmitting the disease.

The areas of maximum inflammatory change in the human brain do not correlate with the distribution of Negri bodies. Electroencephalogram (EEG) evidence of pathology is greater in animal infections with attenuated virus than virulent street strains (Tsiang, 1993) and neuronal death is not a prominent feature at post-mortem. Rabies infection may have surprisingly little visible pathological effect on the brain. Pathogenic strains of virus cause less viral glycoprotein expression and neuronal apoptosis than avirulent ones, so that apoptosis only appears at a terminal stage of infection (Dietzschold *et al.*, 2001).

The cause of the gross neuronal dysfunction of living cells remains elusive but it may be associated with altered activity at a variety of neurotransmitter-binding sites (Jackson, 2007b; Tsiang, 1993). Rabies infection of neurons alters cellular gene expression. Although infection downregulates host cell gene expression overall, a few genes are upregulated, not only in infected cells, but probably also in uninfected neurons, including ones associated with synaptic vesicle function (Prosnjak *et al.*, 2001; Dhingra *et al.*, 2007). The depletion of metabolic pools may limit cell survival (Dietzschold *et al.*, 2001). Changes in the functional expression of some channels, and attenuation of inhibition of others, have been shown *in vitro* (Iwata *et al.*, 2000). The suggestions that nitric oxide might have neurotoxic or beneficial effects remain unproven (Jackson, 2007b).

Centrifugal Spread of Virus from the Brain

There is a final phase of diffuse centrifugal spread by axonal transport of virus from the brain by many nerve pathways including the autonomic nervous system. Virus has been isolated from human tissues such as peripheral nerve, skeletal and cardiac muscle, kidney, lung, skin, salivary, lacrimal and adrenal glands (Helmick *et al.*, 1987). Rabies antigen has been detected in nerves and ganglia adjacent to these organs and the gastrointestinal tract, and extraneurally in tongue tissues, often without an inflammatory reaction (Jackson *et al.*, 1999; Metzger and Feiden, 1991). Viraemia has very rarely been detected, only in animals, and is not thought to be involved in pathogenesis or spread. Virus is shed from human salivary and lacrimal glands, respiratory tract and rarely in urine (Helmick *et al.*, 1987) and possibly in milk.

The predominant site of replication changes as the virus reaches the salivary gland. In contrast to neural cells

there is profuse production of extracellular virus from acinar cells. Viral budding occurs into the gland lumen or intercellular canniculae with secretion into the saliva where it is available to infect a new host.

IMMUNOLOGY

Immune Responses to Rabies Virus

Following a rabid bite, no immune response can be detected until after the development of symptomatic rabies encephalitis. Viral antigens are not recognized by the immune system during the incubation period. Rabies neutralizing antibody, which is only induced by glycoprotein epitopes, is protective against rabies following challenge in animals. *In vitro*, this antibody impairs viral attachment and penetration of cells, and reduces cell-to-cell spread of virus. Although the presence of neutralizing antibody is the best available indicator of protective immunity, the level does not correlate with protection in all experiments, so other immune mechanisms are also involved. N protein-induced immunity can contribute to protection experimentally. N antigens are more cross-reactive with other strains, including rabies-related viruses, than G antigens. There is evidence that N acts as a weak superantigen, but any effect on the course of infection remains elusive. An immunosuppressive effect of rabies infection has been shown experimentally to rabies antigens and also unrelated viral antigens (Camelo *et al.*, 2001). Rabies P suppresses interferon induction (Finke and Conzelmann, 2005; Vidy *et al.*, 2007). CD8⁺ T cells do not seem to have a significant rôle in rabies encephalitis.

Survival of infection in animals is associated with a strong immune response, shown by cell membrane expression of G, neutralizing antibody induction, and upregulation of MHC class II mRNA expression in the CNS early in infection (Irwin *et al.*, 1999). Infected neurons undergo T cell-mediated apoptosis. Paralytic disease develops with subsequent neurological deficit in survivors (Lafon, 2005).

During infection of mice by virulent virus, immune CD3T cells migrate into the CNS, but they are destroyed on contact with infected neurons, due to Fas ligand-mediated apoptosis (Lafon, 2005). Neurons remain intact. Infected human brains show remarkably little apoptosis, which is in keeping with experimental findings.

Response in Human Encephalitis

In unvaccinated humans with encephalitis, neutralizing and other antibodies appear in serum about seven days after the first symptom, and later in the cerebrospinal fluid (CSF) (Anderson *et al.*, 1984). Patients may die before

these antibodies are detectable, but in patients whose lives are prolonged by intensive care antibody titres may rise to very high levels. A low level of rabies-specific IgM is sometimes detectable in serum and CSF, but it does not appear earlier than the IgG response (Warrell *et al.*, 1988).

A minimal lymphocyte response is detected histologically in brain, blood and CSF (Warrell *et al.*, 1976), and there is no pleocytosis in 40% of patients in the first week of illness, or in 13% in the second week (Anderson *et al.*, 1984). Evidence of cell-mediated immunity by specific lymphocyte transformation tests was found in six of nine furious encephalitis patients, but not in seven with paralytic disease (Hemachudha *et al.*, 1988).

It has long been recognized that the rabies incubation period is shorter than average in patients who were inadequately vaccinated and develop rabies. Although high levels of neutralizing antibody are protective, low levels can accelerate the terminal phase of the illness, resulting in the 'early death' phenomenon (Prabhakar and Nathanson, 1981). The effect can be produced in mice by adoptive transfer of antibody or immune B lymphocytes during the incubation period.

Interferon- α prevents rabies viral replication *in vitro*, but only very low levels have been found in the serum and CSF of one-third of patients with encephalitis (Merigan *et al.*, 1984). Mice die of rabies despite high levels of interferon- α and - β in the brain, and high doses of intravenous and intrathecal interferon- α after the onset of symptoms of human rabies encephalitis have not proved to be effective therapy in humans (Merigan *et al.*, 1984; Warrell *et al.*, 1989). In contrast, the early appearance of interferon- γ in infected rodent brain was associated with survival or delayed mortality (Koprowski and Dietzschold, 1997).

Immune Response to Rabies Vaccine

Despite the energy seen in patients with encephalitis, rabies virus proteins are highly immunogenic when given as vaccine. Neutralizing antibody usually becomes detectable in serum between 7 and 14 days after starting a primary course of vaccine. Antibody production is accelerated if the dose of tissue culture vaccine is given in multiple sites, either by increasing the intramuscular dose or by dividing a single dose between several intradermal sites (Suntharasamai *et al.*, 1987; Turner *et al.*, 1976). The early IgM component of the antibody response does not protect against rabies experimentally, has low affinity for viral antigens and is confined to the vascular system. It is unlikely to be important in immediate post-exposure prophylaxis (Turner, 1985).

No antibody or T lymphocyte function test predicts protection against disease, but the level of neutralizing

antibody induced by rabies G protein gives the best correlation available, and should be used to evaluate vaccines. The cumbersome mouse serum neutralization test has been replaced by tissue culture methods. The rapid immunofluorescent focus inhibition test (RIFFIT) (Smith *et al.*, 1996) takes 24 hours. The similar fluorescent antibody virus neutralization (FAVN) test (Cliquet *et al.*, 1998), which is easier to read and automate, takes 40 hours. The results are expressed in International Units (IU/ml), compared with an International Standard serum. An arbitrary minimum neutralizing antibody level of 0.5 IU/ml indicates unequivocal seroconversion and is taken to be the minimum adequate response after a pre- or post-exposure course of vaccine.

Other antibody tests employing killed virus antigen are more convenient but detect a variety of non-neutralizing antibodies, whose titres show less correlation with protection from disease. A commercially available enzyme-linked immunosorbent assay (ELISA) method gives the best correlation with neutralization to date (Feysaguet *et al.*, 2007). Cell-mediated immune responses following vaccination do not correlate with neutralizing antibody titres.

The amount of antibody induced by a vaccine depends on host factors. Lower titres were found in people over 50 years old. Immunosuppression due to drugs, human immunodeficiency virus (HIV), cirrhosis or other disease can also impair the response. Genetic effects have been demonstrated in animals, and 3% of people only produce low antibody levels (Strady *et al.*, 2000).

ROUTES OF INFECTION

Human infection usually occurs by inoculation of virus-laden saliva through the skin by the bite of a rabid dog or other mammal. Broken skin and intact mucosae can admit the virus, and scratches, abrasions and previous open skin lesions can be contaminated with infected saliva. Intact skin is an adequate barrier to the infection. The following are very unusual routes of human infection:

- Human to human transmission: This has only been documented in recipients of transplanted tissue. Six virologically proven cases have been reported in which infected corneas were transplanted from donors who had died of unsuspected rabies. Two further cases resulted in clinically diagnosed rabies in India. Seven organ transplant recipients recently succumbed to a mysterious encephalitis in Texas and in Germany. Liver, kidney, pancreas and even an iliac artery graft transmitted rabies virus from two donors aged 20 and

26 years old, who both died of undiagnosed neurological disease. Subsequent histories elicited a previous bat bite in the United States and contact with a dog in India (Burton *et al.*, 2005; Rabies Bulletin Europe, 2005). Rare reports of infection by human bite or kissing (Fekadu *et al.*, 1996) have been persuasive, but infection from another source could not be excluded. Despite these anecdotal cases, thousands of people dying of rabies have been nursed in poverty-stricken homes, yet their relatives in intimate contact with saliva and tears have not developed rabies. This indicates that the infection is not easily transmitted from human to human.

- Inhalation: This has been reported from the United States where there were two laboratory accidents involving the inhalation of fixed virus during vaccine preparation (Centers for Disease Control and Prevention, 1977; Winkler *et al.*, 1973). Air-borne rabies virus was assumed to be the cause of death of two people who had visited caves densely populated with insectivorous bats in Texas 50 years ago. Aerosol transmission of rabies virus was demonstrated in caves experimentally, presumably from infected bats' nasal secretions and possibly urine (Winkler, 1975). However, no similar human cases have been reported, and infection can also be due to unnoticed physical contact with a bat (Gibbons, 2002; Messenger *et al.*, 2002).
- Vaccine-induced rabies ('*rage de laboratoire*'): Incomplete inactivation of virus in human vaccine should no longer be a problem but in the worst incident 18 people developed paralytic rabies in Fortaleza, Brazil in 1960.
- Transplacental infection: This has occurred in animals but only once documented virologically by antigen detection in brain tissue of a mother and infant in Turkey. Many women with rabies encephalitis have been delivered of healthy babies.
- Oral infection: This has been shown in animals, although high titres are needed. The transmission of rabies from mother to suckling infant via the breast milk has been suspected in at least one human case and is said to occur in animals. However, there have been no documented cases of transmission of rabies by ingestion of milk from an infected animal. Boiling and pasteurization inactivate rabies virus. Transmission of virus is theoretically possible from ingestion of raw milk, but it is not a criterion for post-exposure immunization. Nevertheless, in the United States people who had drunk milk from rabid cows were given rabies prophylaxis (Centers for Disease Control and Prevention, 1999).

CLINICAL FEATURES OF RABIES IN ANIMALS

In domestic dogs, the incubation period ranges from 5 days to 14 months, but is usually 3 to 12 weeks. It is under four months in 80% of cases. Prodromal symptoms include change in temperament, fever and, as in many humans, intense irritation at the site of the infecting bite. The familiar picture of a 'mad dog' with furious rabies is seen in only 25% of infected animals. The commoner, paralytic or dumb presentation is less dramatic and more dangerous as it may not be recognized. The clinical features of furious canine rabies include irritability, convulsions, dysphagia, laryngeal paralysis causing an altered bark, hypersalivation, trembling, snapping and extreme restlessness, causing the animal to wander for miles. Dogs with furious rabies attack and swallow inanimate objects, often breaking their teeth and injuring their mouths in the process. Dogs with paralytic rabies may be reclusive and exhibit paralysis of the jaw, neck and hind limbs, dysphagia and drooling of saliva. Virus may be excreted in the saliva seven days before the appearance of symptoms and the animal usually dies within 7 to 10 days of onset. Among other species, signs are usually furious in horses, cats, mustelidae and viverridae, and usually paralytic in foxes and bovines. Although rabid animals are commonly unable to swallow, they do not exhibit hydrophobia.

Recovery From Infection and Chronic Rabies in Dogs, Vampire Bats and Other Mammals

Rabies is not a universally fatal disease in mammals. The pathogenicity of rabies viruses can vary greatly when tested in another mammal species. Dogs, cats, bats and most often mice have recovered from experimental infection with street rabies virus. It is conceivable that a low dose of virus inoculated in the wild could immunize without causing disease. Although the fox is one of the most susceptible species to rabies, about 3% of animals survive the infection and become immune. Animals of several species have been found to be seropositive and so are assumed to have recovered from rabies. These include the natural rabies vectors: mongooses in Granada; foxes and raccoons in Alabama; hyenas in Tanzania; insectivorous bats in America and, very rarely, stray or unvaccinated dogs in Ethiopia and Nigeria (Ogunkoya *et al.*, 1990; Sérié and Andral, 1962; Warrell and Warrell, 2004); similarly, rabies-related lyssaviruses in bats in Europe, Australia and across South East Asia.

Vampire bats have long been considered chronic carriers of rabies virus, and some bats carried rabies in their saliva for more than two months before death; but there is now doubt about the validity of these reports (Jackson, 2007b). Some bats recover from infection but there is no evidence of excretion of virus following recovery

and experimental inoculation has failed to induce a carrier state.

The repeated shedding of rabies virus in the saliva of apparently healthy dogs is a cause for great concern. This chronic infection is apparently exceptionally rare (Jackson, 2007b), but it has been reported in an Indian dog, and in Ethiopian and Nigerian dogs. Thorough searching in other areas has not revealed any chronically infected animals. The incidence of chronic rabies is presumed to be so low that it has not influenced recommendations for human post-exposure treatment.

In Tanzania 37% of spotted hyenas were found to be seropositive and rabies was detected by reverse transcription polymerase chain reaction (RT-PCR) in 13% of saliva samples from seropositive animals, and occasionally from brain. Attempts at virus culture failed, and infection was not associated with disease or decreased survival. The virus strain was distinct from those of other local species and may be of low virulence. It is assumed that prolonged and perhaps repeated infection occurred with a strain of low virulence (East *et al.*, 2001).

Infection of apparently healthy bats by EBLV-1 (genotype 5) has been found in wild *Myotis* and other species of Spanish insectivorous bats and bats in a Dutch zoo, with an antibody prevalence up to 20% in some populations. EBLV-1 was detected by RT-PCR in brain and other tissues of healthy-looking bats (Wellenberg *et al.*, 2002) and in oropharyngeal swabs of live bats (Echevarria *et al.*, 2001). To date there has been no isolation of virus from these asymptomatic persistently infected bats.

CLINICAL FEATURES IN HUMANS

Incubation Period

The extreme incubation range recorded is four days to 19 years (Gavrila, Iurasog and Luca, 1967): a deep shoulder bite by a rabid dog may have inoculated virus directly into the brachial plexus, explaining the shortest reported incubation period of four days. It is between 20 and 90 days in more than 60% of cases however, and much longer incubation periods of four and six years have been well documented in immigrants to the United States (Smith *et al.*, 1991). Others have reported incubation over one year in from 4 to 6.8% of cases, but in those studies the possibility of a second more recent exposure could not be excluded (Lancet, 1991).

Incubation periods tend to be shortest with severe bites on the face, head and neck, especially in children and in experimental animals injected with large doses of virus. Average incubation for bites on the head and neck are 25–48 days; for the extremities 66–69 days; for the

upper limb 46 and for the lower limb 78 days. However, the virus may remain quiescent until activated by some kind of stress, which has been shown experimentally and suspected clinically (Smith *et al.*, 1991). The incubation appears to be shorter in patients who have received (unsuccessful) post-exposure treatment than in those who have not (Hattwick, 1974).

Prodromal Symptoms

A vague feverish illness associated with a change in mood may precede, by up to about a week, the appearance of definite signs of rabies encephalomyelitis. The patient becomes anxious, agitated, apprehensive, restless, irritable and tense and may suffer from nightmares, insomnia, loss of concentration and depression. Physical symptoms include vague malaise, anorexia, headache, other aches and pains, weakness, tiredness, fever, chills, sore throat and other symptoms suggestive of upper respiratory tract infection, influenza or gastroenteritis. This wide range of misleading symptoms has encouraged inappropriate referral to psychiatrists, otolaryngologists, gastroenterologists and other specialists. Itching, tingling, burning, pain, numbness or some other paraesthesia at the site of the healed bite wound is experienced by about half the patients and may be associated with trembling, fasciculations, muscle contractures or weakness of the bitten limb. Most of the patients infected by corneal transplants noticed pain behind the grafted eye. Although local paraesthesia is suggestive of imminent rabies encephalomyelitis, this symptom is also surprisingly common in healthy victims of animal bites who fear that they are developing rabies. It might be the result of trauma by the bite or by vigorous local infiltration of rabies immunoglobulin. Pruritis is, in the author's experience, by far the commonest local prodromal symptom. Neuritic pain is also reported. Itching may be so intense that the patient excoriates large areas of skin by scratching.

Clinical Presentations of Rabies Encephalomyelitis

Human rabies can take two clinical forms. In the more familiar type, furious or agitated rabies, the brainstem, cranial nerves, limbic system and higher centres bear the brunt of the infection, while in dumb or paralytic rabies the medulla, spinal cord and spinal nerves are principally involved. The predominantly but not exclusively paralytic picture seen in human victims of vampire bat-transmitted rabies and other evidence from experimental rabies infection in animals suggests that the virus strain or size of infecting inoculum may contribute to the pattern of CNS infection. Host factors may also modify the pattern of the disease; the frequency of the paralytic form of rabies varies from species to species (it is common in bovines

but rare in cats) and paralytic rabies may be more likely to develop in those who have received antirabies vaccine (Hattwick, 1974).

Many different clinical features and patterns of presentation of human rabies have been described over the past 100 years and, since the recognition of strains of classical rabies lyssavirus genotype I and rabies-related lyssaviruses, clinicians have been interested in attempting to associate distinctive clinical patterns with these various viruses. This is premature. Even the use of the term 'classical rabies' for a clinical presentation has become misleading as it has been applied to the furious form of the disease (Jackson, 2007a), but also to both furious and paralytic forms to distinguish them from hypothetical atypical patterns (Hemachudha *et al.*, 2002).

Furious (Agitated) Rabies

This is the more familiar and probably the commoner presentation in humans. Without intensive care most patients with furious rabies die within a week of their first prodromal symptom and within a few days of developing hydrophobia. This short and hectic clinical course is characterized by hydrophobia, aerophobia and periods of extreme excitement alternating with lucid intervals, features of autonomic system dysfunction and finally by unconsciousness and complete paralysis. Hydrophobia (from the Greek 'dread of water') is a reflex series of forceful jerky inspiratory muscle spasms provoked by attempts to drink water and associated with an inexplicable terror (Figure 32.3). Driven by thirst to confront this terror, the patients attempt to drink but, even before the liquid has reached the lips, a rapid succession of violent inspiratory gasps is provoked. forcible contractions of the diaphragm depress the xiphisternum and contractions of the accessory muscles of inspiration, particularly the sternocleidomastoids, are visible during these spasms. There is usually no evidence of laryngospasm and most patients deny pain in the neck or retrosternal region. A draught of air on the skin produces a similar reflex response—'aerophobia'.

Initially, the spasms (also known as 'phobic spasms') affect mainly the inspiratory muscles, but a generalized extension response may be produced ending, not infrequently, in opisthotonos and generalized convulsions with cardiac or respiratory arrest. The hydrophobic response may be reinforced by unpleasant consequences of the inspiratory spasms, such as aspiration of water up the nose or into the trachea. However, the first attack may occur without preceding difficulty in swallowing and with no opportunity for the establishment of a conditioned reflex. The hydrophobic response may eventually be produced merely by the sight, sound, mention or thought of water; by touching the palatal mucosa; by splashing water on the skin; by loud noises, bright lights or by attempts to speak.



Figure 32.3 The terror of furious rabies in an 18-year-old Nigerian, bitten on the temple by a dog. After 13 days he developed classical hydrophobic spasms. Periods of lucidity were interspersed with episodes of extreme agitation, violent struggling, shouting and terrifying hallucinations. (Source: Courtesy of D.A. Warrell.)

There may be associated excitement, agitation and aggression and many patients develop generalized convulsions and die during a hydrophobic spasm.

Cardiac tachyarrhythmias and ECG evidence of myocarditis may be observed during this phase of the disease (Jackson *et al.*, 1999; Warrell *et al.*, 1976). There is transient hypertension during the hydrophobic spasms and the violent inspiratory spasms may result in pneumothorax (Warrell *et al.*, 1989). Pneumomediastinum may result from alveolar rupture or tears in the lower oesophagus. If the patient survives this phase of the disease and sinks into coma, hydrophobia is replaced by a variety of respiratory arrhythmias (cluster or Biot's breathing and Cheyne—Stokes respiration) with long apnoeic periods.

Pathophysiology of Hydrophobia

There is some clinical evidence that the inspiratory spasms of hydrophobia may be an exaggeration of reflexes which normally protect the respiratory tract from foreign bodies (Warrell *et al.*, 1976). These include sneezing, coughing, the neonate's aspiration reflex and perhaps the immersion reflex, all of which have a powerful inspiratory contraction as their main motor effect. In furious rabies there is a selective brainstem encephalitis which damages cells in the region of the nucleus para-ambiguus (where the inspiratory motor neurons are situated) and in the limbic system. These lesions could disinhibit the respiratory tract protective reflexes and explain the associated terror of hydrophobia. The responses could be reinforced by conditioning, resulting in the heightened reaction to a variety of sensory stimuli which provoke spasms.

Other Features of Furious Rabies

Alternating phases of extreme arousal and calm, lucid intervals are typical of furious rabies. Physical findings include meningism, cranial nerve lesions (especially III, VII, VIII), upper motor neuron lesions, muscle fasciculations and involuntary movements. However, conventional neurological examination may fail to disclose any abnormality unless a hydrophobic spasm is observed. Evidence of autonomic stimulation includes lacrimation, hypersalivation and excessive sweating, piloerection, high fluctuating body temperature and blood pressure, pupillary abnormalities and Horner's syndrome. Involvement of the amygdaloid nuclei and limbic system may explain the increased libido, priapism and spontaneous orgasms/ejaculations in a small minority of patients. These symptoms are reminiscent of Klüver—Bucy syndrome which can be induced experimentally in rhesus monkeys and by surgical treatment for temporal lobe epilepsy in humans (following bilateral temporal lobectomy). Evidence of an extensive axonal neuropathy was found in two patients with furious rabies in the second week of intensive care. The normal progression of furious rabies is to coma and death within a week of the first symptom, but some patients have been kept alive for several months in intensive care units. In these cases a variety of other complications can develop (Table 32.2).

Paralytic Rabies

Paralytic rabies is less likely to be recognized than furious disease, and this contributed to its being apparently less common in humans. The patients may become literally dumb ('*rage muette*'), because their laryngeal muscles

Table 32.2 Complications of rabies

Cardiovascular	Arrhythmias, hypotension and cardiac failure
Respiratory	Asphyxiation (hydrophobic spasm) Pneumonia Respiratory failure Pneumothorax, Pneumomediastinum
Neurological	Adult respiratory distress syndrome Convulsions Diabetes insipidus Syndrome of inappropriate anti-diuretic hormone Hypo- or hyper-pyrexia Cerebral oedema
Gastrointestinal	Stress ulcers Mallory—Weiss syndrome Haemorrhage

are paralysed, but the term ‘dumb rabies’ also describes the quieter symptoms and more insidious and protracted clinical course exemplified by its other French name, *rage tranquille*.

There was a famous outbreak of paralytic rabies in Trinidad involving 20 people, mostly children, between 1929 and 1931. After prodromal fever and headache, burning, tingling, numbness, cramp or weakness developed in one foot followed by a gradually ascending hypoaesthesia and paralysis, eventually affecting respiratory and deglutitive muscles. Constipation, urinary retention, high fever and profuse sweating were common. Some patients survived for three weeks and only one showed terminal hydrophobic spasms. Vampire bats were eventually implicated. Paralytic rabies was seen in patients with post-vaccinal rabies (due to defective inactivation of vaccine) and in two patients who inhaled a vaccine strain of virus.

Neurological signs include quadriparesis with predominant involvement of proximal muscles, loss of deep tendon reflexes and progressive paralysis. In most patients sensation is intact, but in some there is evidence of ascending myelitis with loss of pinprick and joint position sense and fasciculations. Myoedema has been claimed to be a distinguishing feature of paralytic rabies (Hemachudha *et al.*, 2002). However, this transient local mounding of muscle in response to percussion, which was used historically to lateralize pulmonary tuberculosis, is unlikely to be a helpful sign as it is associated with a variety of pathological conditions (wasting, myxoedema, hyponatraemia) and may also be found in healthy people (Jackson, 2007a). Fasciculation in the muscles becoming involved in the ascending paralysis was mentioned

in the original description of paralytic rabies by Gamaleia in 1887. Cranial nerve involvement may result in ptosis, external ophthalmoplegia, paralysis of the tongue and deafness. Most patients with paralytic rabies do not experience hydrophobia, but in a few it may be represented by some terminal respiratory spasms. The course of the disease is less acute and stormy than furious rabies. Even without intensive care, patients may survive for up to 30 days before they succumb to bulbar and respiratory paralysis.

Differential Diagnosis of Rabies and Infections by Rabies-related Viruses

In a rabies endemic area, the diagnosis of furious rabies may be obvious in a patient with hydrophobic spasms who has a history of being bitten by a dog within the previous few weeks or months. However, exposure to a mammal bite or scratch may not be remembered, as in the majority of cases of genotype 1 insectivorous bat-transmitted rabies in the United States (Noah *et al.*, 1998). Although hydrophobia is a pathognomonic sign of furious rabies, it may be misdiagnosed as a laryngopharyngeal or even a psychiatric problem. Some of the reported misdiagnoses and differential diagnoses of furious and paralytic rabies are listed in Table 32.3. Rabies phobia, an hysterical response to the fear of rabies, differs from true rabies in its shorter incubation period which may be only a few hours after the bite, exaggerated aggressive and dramatic

Table 32.3 Differential diagnosis of rabies

Furious rabies	Hysterical pseudo-hydrophobia (Cephalic) tetanus Other brain stem encephalitides (e.g. complicating serum sickness) Other causes of muscle spasms: (e.g. phenothiazine dystonia, tetany, strychnine poisoning) Delirium tremens Porphyria Cerebrovascular accident, epilepsy
Paralytic rabies	Post-vaccinal encephalomyelitis Paralytic poliomyelitis Other causes of acute ascending paralysis (e.g. acute idiopathic inflammatory polyneuropathy—Guillain-Barré syndrome) Drug abuse Cercopithecine herpesvirus (B virus) encephalomyelitis (after monkey bites) Creutzfeldt—Jakob disease

symptoms and its excellent prognosis. Severe (cephalic) tetanus, involving the cranial nerves, is distinguished by its shorter incubation period, the presence of trismus, the persistence of muscular rigidity between spasms and the absence of pleocytosis.

In the few remaining countries where nervous tissue vaccines are still used, post-vaccinal encephalomyelitis (PVE) is the commonest differential diagnosis of paralytic rabies. In PVE, symptoms usually develop within two weeks of the first dose of vaccine, but no clinical or laboratory features reliably distinguish the two conditions while the patient is still alive, save for the absence of demonstrable rabies virus in skin biopsies, saliva or CSF. Cercopithecine herpes virus (B virus) encephalomyelitis, transmitted by monkey bites, has a much shorter incubation period than rabies (three to four days). Vesicles may be found in the monkey's mouth and at the site of the bite. The diagnosis can be confirmed virologically and the patients treated with acyclovir.

Clinical Investigations in Human Rabies

Haematological and Biochemical Tests

Routine haematological and biochemical tests are initially normal, apart from neutrophil leukocytosis. Later, hypernatraemia or hyponatraemia may reflect diabetes insipidus or the syndrome of inappropriate antidiuretic hormone (ADH) secretion respectively. In a majority of patients there is a lymphocytic pleocytosis and sometimes an elevated protein concentration in the CSF. Hypoglycorrhachia has been reported (Roine *et al.*, 1988).

Neurological Investigations

The EEG may be normal or may show the expected wide range of nonspecific changes, including evidence of seizure activity. Electromyography has revealed evidence of primary axonal neuropathy.

Most reported computed tomographic (CT) scans in rabies have been normal, especially in the early stages. Hypodensities of the basal ganglia and of cortical lesions have been described.

Reports of magnetic resonance imaging (MRI) (Jackson, 2007a) have mainly been in patients with paralytic rabies although, surprisingly, both paralytic and encephalitic rabies patients are said to show a similar distribution of ill-defined, mildly T2-weighted hyperintensity of the brainstem, hippocampus, hypothalamus, deep and subcortical white matter and deep and cortical grey matter (Hemachudha *et al.*, 2002). Minimal gadolinium enhancement, indicating mild inflammation, may be seen in the later stages of the clinical course. Similar changes in the

spinal cord, suggestive of myelitis, might indicate a diagnosis of paralytic rabies. None of the MRI changes reported to date would be diagnostic of rabies infection.

Recovery from Rabies

Animals of several species have recovered from rabies (see above). Humans with paralytic rabies can survive for several weeks, especially with intensive care, but the illness progresses relentlessly. Six patients over the last 40 years have been claimed as survivors of rabies encephalitis. Five received some rabies vaccine before the onset of symptoms. No virus or viral antigen was detected in any patient and so the diagnoses were based on finding high rabies neutralizing antibody levels in the CSF.

Two of the patients were treated post-exposure with rabies vaccines of nervous tissue origin. In Argentina, in 1972, a 45-year-old woman was bitten by her clinically rabid dog and began a course of suckling mouse brain vaccine 10 days later (Porras *et al.*, 1976). Three weeks after the bite she had paraesthesiae of the bitten arm, with tremors, myoclonic spasms, ataxia and other signs of cerebellar dysfunction. She recovered but, following booster doses of rabies vaccine, relapsed twice. Clinical features included hypertonia, tetraparesis, dysphonia, dysphagia, varying levels of consciousness and a cardiac conduction defect, with slow resolution over a year. The CSF and serum neutralizing antibody levels were very high. The clinical features typical of rabies were the apparently subjective paraesthesiae of the bitten limb, the cardiac conduction defect (if it was a new finding) and the high-antibody level. Cerebellar signs have rarely been reported in rabies. A possible diagnosis of post-vaccinal encephalitis cannot be excluded.

The second patient was a six-year-old boy in Ohio, USA in 1970, who was bitten on the thumb by a proven rabid big brown bat (*E. fuscus*). He began treatment with duck embryo vaccine four days later (Hattwick *et al.*, 1972). Twenty days after the bite he developed a meningeitic illness progressing to encephalitis with unilateral weakness maximal in the bitten arm. Focal seizures, paralysis, cerebral oedema and coma ensued, lasting more than a week. He also had an atrial arrhythmia. Prolonged intensive care resulted in complete recovery in six months. The CSF and serum rabies antibody levels were high. Features suggesting rabies were the dominant signs in the bitten limb, a cardiac arrhythmia and the high antibody level. Now, over 30 years later, the patient lives a normal life.

The third case was a 32-year-old laboratory worker in New York in 1977 who was thought to have inhaled an aerosol of a fixed strain of rabies (SAD) virus (Centers for Disease Control and Prevention, 1977). He had had pre-exposure prophylaxis with duck embryo rabies vaccine, with a neutralizing antibody titre of 1 : 32 six months

before exposure. Fever, encephalitic symptoms, spastic hemiparesis, myoclonus, impaired consciousness and respiratory arrest developed over two weeks, and the rabies neutralizing antibody titres were high in the serum and CSF. A gradual improvement was followed by onset of a personality disorder and dementia. Two and a half years later he still had profound neurological deficits.

A Mexican boy severely bitten on the head by a proven-rabid dog was given a course of vero cell vaccine, starting the following day, but no rabies immunoglobulin (RIG) treatment (Alvarez *et al.*, 1994). Nineteen days later he developed encephalitis with fever and convulsions. Intracranial hypertension and coma ensued. He improved over three weeks, and reacted to painful stimuli, but quadriplegia persisted, he became blind and deaf, and eventually died within four years (Jackson, 2007a). High titres of rabies antibody appeared in the CSF and the serum. A second boy with similar clinical features survived at least nine months.

Another case of survival was reported from India (Madhusudana *et al.*, 2002). A six-year-old girl was severely bitten by a stray dog which died four days later. The wound was not cleaned but she was given purified chick embryo cell (PCEC) rabies vaccine (see section on Human rabies prophylaxis below) on days 0, 3 and 7 after the bite. Sixteen days later she would not drink and developed fever, hallucinations and impaired consciousness which progressed to coma with excessive salivation and focal seizures. Rabies antibody titres rose to very high levels in serum and CSF. After three months of coma, improvement began very slowly but after 18 months she had spasticity, tremors and involuntary limb movements, and she died after two years. Neurological illnesses following tissue culture rabies vaccines has been reported rarely (see below) but have been relatively mild.

The most recent and surprising report is reminiscent of the second case. A 15-year-old-girl, bitten on a finger by a bat in Wisconsin in 2004, had no rabies prophylaxis (Willoughby *et al.*, 2005). A month later she developed tingling and numbness of the bitten hand. Neurological signs including V1th nerve palsy, slurred speech, nystagmus and tremors, with lethargy, fever and hypersalivation. MRI and magnetic resonance angiography (MRA) scans were normal, but she had a mild lymphocytic pleocytosis. Rabies antibody was detected on the sixth day of illness, but no virus or antigen was detected. Treatment comprised coma induction, ketamine, ribavirin and amantadine. The neutralizing antibody rose to high levels. She was extubated after a month, and made a slow motor recovery over two years. She has returned to independent normal life, albeit with some neurological deficits. The effect of multiple antiviral treatments cannot be evaluated in a single case. Her symptoms were of paralytic rabies with evidence of

an early immune response. Her treatment possibly maintained her vital functions until the spontaneous specific immunity eliminated infected neurons.

This case is similar to the second patient, the boy from Ohio. Both were infected by American bat rabies viruses which differ from canine rabies experimentally: infection is slower to evolve and progress; virus replication is not restricted to neurons and histopathological changes are milder with very little apoptosis. This suggests that the genotype 1 bat viruses are less pathogenic, and could explain the earlier induction of immunity.

Rabies virus was not isolated nor was antigen identified in any of these patients. Diagnostic samples were taken when there was already a high titre of antibody, probably masking virus or antigens. For the three patients given tissue culture vaccines, the term 'survival' is more appropriate than 'recovery', as all had profound residual neurological deficits. Severe impairment of nervous function was irreversible before the infection was controlled, presumably by the immune response.

Human Infections with Rabies-related Viruses

Mokola (Lyssavirus Genotype 3)

Two cases of Mokola virus infection occurred in girls admitted to University College Hospital, Ibadan in 1968 and 1971. The first was a three and half year old who presented with a febrile convulsion, and had a temperature of 105 °F and a sore throat. She recovered. Virus was isolated from CSF, but no specific complement fixing antibodies were detected. A similar strain of Mokola, isolated from a shrew, was being handled in the laboratory at the same time (Familusi and Moore, 1972). The second case was a six-year-old girl admitted after six days of feverish illness. Initially she had a cough and vomited but later became drowsy, confused and weak. She died three days after admission. Mokola virus was isolated from brain tissue, and a Coxsackie A virus isolated from a rectal swab. (Familusi *et al.*, 1972). A laboratory worker recovered from a mild Mokola virus infection (Crick, 1981).

Duvenhage (Lyssavirus Genotype 4)

This virus was named after a 31-year-old white South African man who had been bitten on the lip by a bat of unknown species (Meredith *et al.*, 1971). One month later he noticed headache, dizziness and aching in the neck and back. He began to sweat profusely, suffered involuntary spasms of the face and limbs, became progressively more confused, irritable and aggressive, had nightmares and noticed difficulty in swallowing. He had typical hydrophobia, agitation, aggressive episodes and

had involuntary spasms of the neck and back. He died within a week.

In 2006, another infection appeared in South Africa in a 77-year-old man, scratched on the face by an insectivorous bat. He received no treatment and 27 days later developed classical features of rabies encephalitis. He died after 14 days of illness, and Duvenhage virus was isolated from saliva and brain tissue (Paweska *et al.*, 2006). A third patient, a 34 year old unvaccinated dutch woman visiting Kenya was scratched on the nose by a bat. She died of a rabies-like encephalitis after 14 days of illness, and Duvenhage virus was identified by PCR of skin biopsy.

European Bat Lyssaviruses

Genotype 5, EBLV-1 Three human deaths from rabies following bat bites have been reported from Russia in 1977 and 1985 (Selimov *et al.*, 1989) and in 2002 from the Ukraine. None of them received rabies vaccine. One month after being bitten on the hand, a 15-year-old girl became unwell with fever, anxiety and paraesthesia of the bitten hand. She developed an acute ascending paralysis, encephalitis and myocarditis and died five days later. An 11-year-old girl was bitten on the lip by a bat in Belgorod. Three weeks later she became unwell with pain in the bitten cheek, weakness and drowsiness. She developed typical furious rabies and died six days later. No rabies antigen was detected in brain impression smears by the immunofluorescent test, but virus was isolated in suckling mice and proved to be an EBLV-1a. A 34-year-old Ukrainian developed clinical rabies with hydrophobia six weeks after a bat bite, but there was no laboratory confirmation (Botvinkin *et al.*, 2005).

Genotype 6, EBLV-2 Two human infections due to EBLV-2 virus have been documented. An unvaccinated 30-year-old Swiss zoologist visiting Finland who studied bats had been bitten several times by bats in Malaysia and Switzerland over five years (Roine *et al.*, 1988). Then, in southern Finland, he was bitten by a sick Daubenton's bat (*M. daubentonii*) and 51 days later developed numbness of the palm of his right hand and pain in the neck radiating to his right cheek. He became unable to walk, was feverish, weak with numbness of the right arm and neck and retrosternal pain. Myoclonic twitching of the feet, trismus and typical hydrophobic symptoms of furious rabies ensued. Delirium, muscle spasms, convulsion, respiratory failure and diabetes insipidus followed. He died 23 days after his first symptoms. No rabies antibody was detected, but EBLV-2b was isolated from brain tissue. As EBLV-2b has only been found in Switzerland, it is possible that his infection originated there.

The second patient, a 55-year-old unvaccinated bat conservationist, had been bitten by bats on several

occasions in Scotland, most recently in Angus in 2002 on the left hand, about four months before his illness. He developed pain, paraesthesiae and diminished sensation in the left arm. He received nonsteroidal anti-inflammatory drugs and, five days later, had acute haematemesis attributable to these drugs. He was feverish, disinhibited, dysarthric, with nystagmus and ataxia. Touch sensation was diminished over the left arm. There was no meningism or pleocytosis. He became suddenly confused, aggressive and agitated and deteriorated with a pneumothorax, respiratory failure, decreased consciousness and hypersalivation. Neither aerophobia nor hydrophobia was observed. He became comatose. There was electrophysiological evidence of a peripheral, predominantly motor, axonal neuropathy. He died 19 days after the first prodromal symptom (Nathwani *et al.*, 2003). EBLV antigen was identified ante-mortem by RT-PCR, and from the brain post-mortem by immunofluorescence and isolation of EBLV-2a.

Australian Bat Lyssavirus (ABLV) (Lyssavirus Genotype 7)

First discovered in Australian bats in 1995, ABLV is more closely related to genotype 1 rabies than any of the other rabies-related lyssaviruses.

There have been two reported human fatalities from ABLV infection in Queensland. The first was a 39-year-old woman who cared for bats and had been scratched by bats during the previous 30 days. After a few days of left shoulder pain, dizziness, vomiting, headache, fevers and chills, she developed arm weakness, ataxia, cerebellar signs, slurred speech, diplopia, dysphagia, bilateral facial palsies, progressive quadriplegia and a fluctuating level of consciousness. She died 20 days after the start of her illness. Rabies antibody was detected and the CSF was PCR-positive (Samaratunga *et al.*, 1998). The insectivorous bat (*Saccolaimus* spp.) variant of ABLV was isolated from post-mortem brain.

The second case was a 37-year-old woman who had been bitten on her left hand by a flying fox or fruit bat (*Pteropus* spp.) (Hanna *et al.*, 2000). Twenty-seven months later she presented at hospital with a five-day history of fever, vomiting, anorexia, pain around the left shoulder girdle, paraesthesiae of the dorsum of the left hand, sore throat and difficulty in swallowing. She was feverish, unable fully to open her mouth, was drooling saliva and had difficulty speaking. There was increased muscle tone, painful spasms provoked by examination of the throat. She also had a neutrophil leukocytosis. She became agitated with dysphagia, dysphonia and increasingly severe frequent muscle spasms which were uncontrollable by medication, and she died 19 days after the first symptom. ABLV was detected by PCR in saliva. A post mortem

revealed diffuse pancarditis, and virus was isolated from the brain.

DIAGNOSIS

Intra Vitam Diagnosis of Human Rabies Encephalitis

The laboratory diagnosis of rabies is rarely attempted in developing countries. However, confirmation of the diagnosis is possible within a day or two and is important to guide the appropriate management of the patient, relatives and staff, prevent unnecessary investigations and allow characterization of the virus (Table 32.4). Routine haematological and biochemical tests are likely to be normal initially, but a plasma neutrophil leukocytosis may be present. A mild CSF pleocytosis is only seen in 60% of patients in the first week (Anderson *et al.*, 1984).

The diagnosis can be made by virus isolation, identification of antigen or, in unvaccinated people, antibody detection, and taking serial samples for all tests will enable most rapid detection of the infection. The success of tests depends on the quality of sample-taking. For example, a throat swab is no substitute for a saliva sample.

Isolation of Rabies Virus

Culture of the virus from saliva, throat, tracheal or eye swabs, brain biopsy samples, and rarely the CSF is most successful during the first week of illness (Anderson *et al.*,

1984) and in seronegative patients. The isolation of virus is best attempted in murine neuroblastoma cells, which yields results in only a few days, whereas suckling mouse inoculation takes one to three weeks.

Antigen Detection

The most rapid diagnosis of rabies during life can be made by direct IFA identification of antigen in a skin biopsy (Noah *et al.*, 1998; Trimarchi and Nadin-Davis, 2007). A full-thickness biopsy, preferably taken with a disposable biopsy punch, must include the bases of hair follicles. It is taken from a hairy area, usually the nape of the neck, and in addition near the original bite wound, if there is an adjacent proximal hairy area. Vertical frozen sections through hair follicles indicate rabies antigen in the nerve twiglets around the base of the follicles, in a characteristic pattern (Bryceson *et al.*, 1975) (Figure 32.4). Careful controls of specificity are needed. For a reliable result, more than 20 sections including hair follicles are compared with sections stained with an adsorbed negative control conjugate. The method is 60–100% sensitive (Blendon *et al.*, 1986; Warrell *et al.*, 1988), and false positives have not been reported. Antigen detection by IFA in the corneal smear test is too insensitive to be useful (Anderson *et al.*, 1984; Warrell *et al.*, 1988) and false positives have occurred. Brain biopsy samples are ideal for antigen detection (see section on Animal diagnosis below) or culture, but biopsies are very rarely indicated in life.

Table 32.4 Human rabies diagnosis

Sample	Aim	Test
Intra vitam		
REPEAT until a diagnosis is made		
Skin punch biopsy ^a	Antigen detection	IFA test on frozen section RT-PCR
Saliva, tears, CSF	Virus isolation	Tissue culture Mouse inoculation test
Serum	Antigen detection	RT-PCR
	Serology	Antibody test Unvaccinated, test immediately Vaccinated, save for comparison later
CSF	Serology	Test immediately with serum
Post-mortem		
Brain ^a : Needle biopsy (see text) or brainstem and cerebellum	Antigen detection	IFA test on impression smear
	Virus isolation	RT-PCR Tissue culture Mouse inoculation test
Serum	Serology	Antibody test

^aCollect in small moistened container, keep cool during transport. Do not freeze. IFA, immunofluorescent antibody; RT-PCR, reverse transcription polymerase chain reaction; CSF, cerebrospinal fluid.

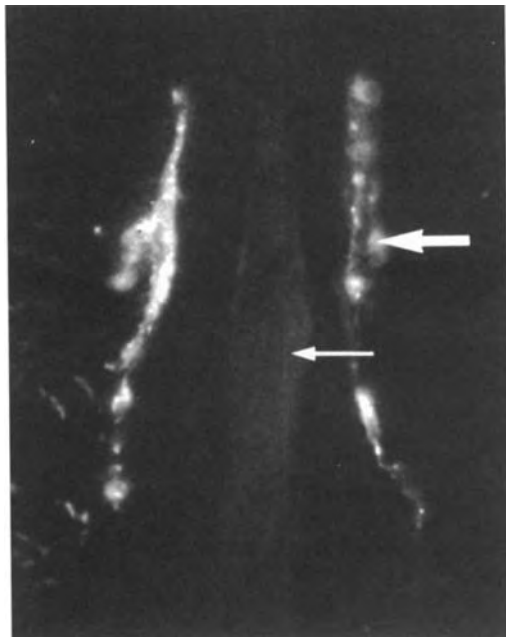


Figure 32.4 Vertical section through a skin biopsy, shows a hair (small arrow) surrounded by its follicle. The bright fluorescence (large arrow) indicates rabies antigen in neurons surrounding the follicle, and is a rapid means of diagnosing rabies during life. (Source: Copyright M.J. Warrell.)

RT-PCR techniques differ between laboratories, but they have become a popular means of diagnosis and genetic identification of rabies strains. Rabies RNA may be found in saliva, CSF, skin biopsy tissue and urine. Nested PCR techniques enhance the sensitivity. Real-time PCR methods are being evaluated (Trimarchi and Nadin-Davis, 2007).

Antibody Detection

In unvaccinated patients, the diagnostic appearance of rabies antibody, using any specific technique, often occurs during the second week of illness, but it may take longer. Antibody may be detectable in the CSF a few days later (see section on Immunology above). A low level of rabies-specific IgM has been detected in the serum and occasionally in the CSF of rabies patients, but it did not appear earlier than IgG, and IgM has also been found in post-vaccinal encephalitis patients (Warrell *et al.*, 1988). In vaccinated people, very high levels of antibody in the serum, and especially in the CSF, have been considered diagnostic (Hattwick *et al.*, 1972).

Post-mortem Diagnosis in Humans

Post-mortem diagnosis can be made by any of the above tests but rabies virus is most readily detected in the brainstem and cerebellum. Brain tissue can, however, be obtained without a full post-mortem examination. Needle necropsies are taken with a Vim—Silverman or other long biopsy needle via the medial canthus of the eye through the superior orbital fissure; via the nose through the ethmoid bone; by an occipital approach through the foramen magnum or through burr holes or open fontanelles in children. The IFA test for antigen takes about 3 hours to perform on brain impression smears. Viral isolation from brain by inoculation of murine neuroblastoma cells or suckling mice may be successful even if the IFA staining is negative. A retrospective diagnosis using formalin-fixed brain specimens is possible by protein digestion and antigen detection by IFA or enzymatic techniques. Genomic and mRNAs can also be analysed by *in situ* hybridization (Trimarchi and Nadin-Davis, 2007; Warner *et al.*, 1997). Rabies-related viruses may give a weak or negative reaction in the IFA test.

Diagnosis in the Biting Mammal

Suspect rabid animals should be euthanased immediately and their brains tested for rabies infection (Trimarchi and Nadin-Davis, 2007; World Health Organization, 1997). In practice this is often impossible if the animal escapes, or if laboratory facilities are not available. Observation in captivity is potentially dangerous and uncertain. Animal brain samples should include brainstem and cerebellum, but tissue can be obtained without craniotomy, via the occipital foramen. The diagnosis can be confirmed by a direct IFA test on acetone-fixed brain impression smears. A rapid enzyme immunodiagnosis kit will detect rabies antigen in a suspension of brain tissue. This is useful for laboratories without a fluorescence microscope, although the method is slightly less sensitive than the IFA test. No single laboratory method is sufficiently accurate for this crucial diagnosis. The IFA test has been found to be about 2% less sensitive than virus isolation in tropical dog rabies endemic areas, while false positive results are rare. Culture of virus should be attempted in antigen-negative samples. The sensitivity of detection, especially on decomposing samples, can be further increased by RT-PCR, and genotyping can also identify the vector species and geographical origin of the rabies or rabies-related virus. The diagnosis can also be made on formalin-fixed brain specimens (see section on Diagnosis in humans above).

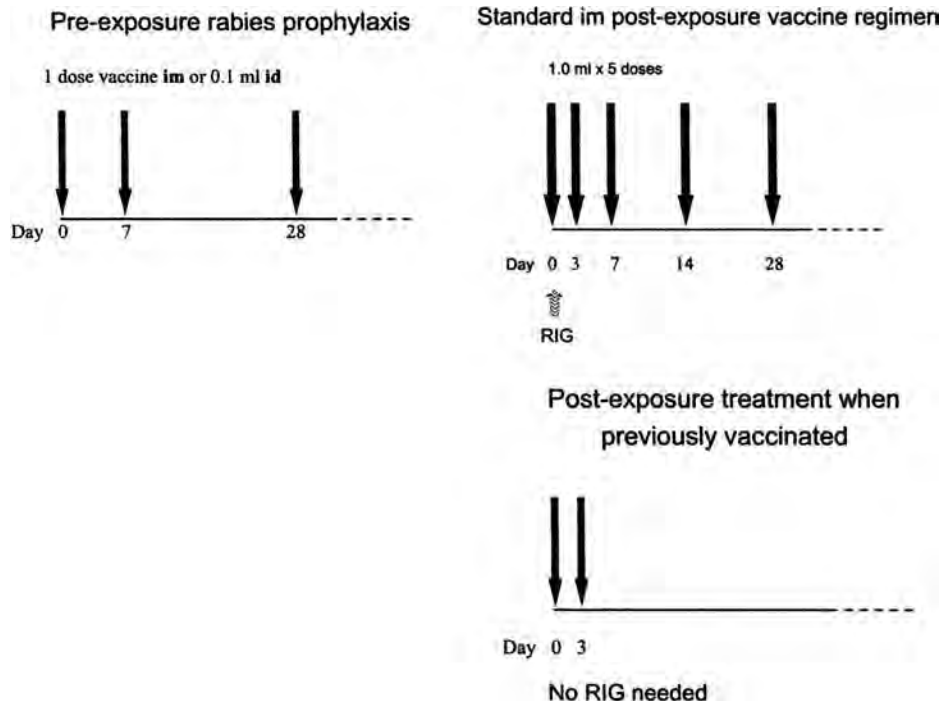


Figure 32.5 Rabies vaccine regimens. Pre-exposure: the final dose may be advanced towards day 21 (see text). RIG, rabies immune globulin; id, intradermal; im, intramuscular.

MANAGEMENT OF HUMAN RABIES

The mortality from rabies in patients infected by canine rabies viruses is 100%. Despite many attempts at intensive care treatment over 35 years, only two patients have recovered, and both were infected by bat bites (Hartwick *et al.*, 1972; Willoughby *et al.*, 2005). Their survival may have been due to the reduced pathogenicity of the American bat rabies viruses (see section on Recovery from rabies above). Intensive care may be appropriate for similar patients who are seropositive at presentation. Life can be prolonged, but many complications arise (see Table 32.2). Heavy sedation and analgesia should be given to relieve the agonizing symptoms. Ketamine, an anaesthetic agent and a noncompetitive antagonist of the *N*-methyl-D-aspartate (NMDA) receptor, has specific antirabies activity (Lockhart *et al.*, 1992), but the human therapeutic dosage is unlikely to give adequate concentrations, and it proved ineffective therapeutically in mice (Jackson, 2007a, 2007b). Immunosuppressive drugs including corticosteroids, rabies immunoglobulin, antiviral agents such as vidarabine, cytosine arabinoside, ribavirin (Centers for Disease Control and Prevention, 1984; Warrell *et al.*, 1989) and interferon- α (Merigan *et al.*, 1984; Warrell *et al.*, 1989) have not proved useful.

For canine rabies virus infections, until any new specific therapy has been evaluated, palliative care is recommended. Patients and their relatives should be advised that although intensive therapy may prolong life there can be no expectation of survival without severe permanent neurological disabilities (Jackson *et al.*, 2003).

PATHOLOGY

Rabies causes an acute nonsuppurative meningoencephalomyelitis, usually accompanied by diagnostic intracellular inclusions called Negri bodies (Figure 32.1). These are masses of dense eosinophilic material in neuronal cytoplasm. Electron microscopy shows they are composed of disorganized filaments in an amorphous matrix, largely consisting of rabies ribonucleoprotein. The inclusions described by Negri contained a basophilic inner body which may include fragments of cellular organelles and occasional virions, probably mechanically trapped by the fusion of smaller inclusions. Negri bodies are found in about 75% of patients and are most numerous in the hippocampus, Purkinje cells, medulla and ganglia (Perl, 1975). Inclusions with no internal structure were called 'lyssa bodies' by Goodpasture.

These contain the nucleoprotein alone, and are usually smaller than Negri bodies.

By the time the patient dies, cerebral congestion with some petechial haemorrhages is usual, but without gross oedema (Tangchai *et al.*, 1970). Inflammation with perivascular mononuclear cell infiltrate is common, with neutrophils seen only at an early stage. Neuronophagia, microglial reaction, ganglion cell degeneration, foci of demyelination and perineural infiltrates (Babes' nodules) (Perl, 1975) are less frequent. All changes may be widespread and are most pronounced in the grey matter of the brainstem and spinal cord and in paralytic rabies, spinal cord pathology may predominate. Children often show meningeal inflammation (Perl, 1975). The degree of histopathological change in rabies encephalitis may depend upon the strain of virus, and varies from absence of any inflammation (Iwasaki *et al.*, 1985; Tangchai *et al.*, 1970) to complete disruption of neuron structure in a patient treated with intensive care for several weeks. Peripheral nerve changes include axonal degeneration of myelinated and unmyelinated nerve fibres with leukocyte infiltration and degeneration in dorsal root ganglia, especially in the region of the site of the bite.

Extraneural changes include focal degeneration of salivary and lacrimal glands, liver, pancreas, adrenal medulla and lymph nodes and ocular tissues. An interstitial myocarditis with round cell infiltration has been described (Metze and Feiden, 1991; Warrell *et al.*, 1976) and tissue in the myocardium is also affected, which may account for associated cardiac arrhythmias.

HUMAN RABIES PROPHYLAXIS

Rabies Vaccines

Two rabies vaccines are now licensed for use in the United Kingdom and United States: HDCV (Sanofi Pasteur) and PCEC vaccine (Rabipur[®] Novartis). Both are in 1 ml dose vials. Elsewhere, purified vero cell vaccine (PVRV) (Verorab, Sanofi Pasteur) is widely available. The single-dose vial contains 0.5 ml.

Pre-exposure Prophylaxis

No rabies deaths have been reported in those given pre-exposure prophylaxis with post-exposure boosting. Pre-exposure immunization is indicated for residents of or visitors to areas where dog rabies is endemic, and all those at occupational risk of contact with a rabid animal or rabies virus, in quarantine facilities, customs departments, zoos, laboratories or hospitals. Those staying in rural areas of foreign countries where rabies is enzootic in other

mammals (foxes, jackals, wolves, coyotes, mongooses, bats, etc.) should seek advice about the risk.

Subsequent post-exposure treatment will be simplified and much cheaper after a prophylactic course. Pre-exposure immunization is especially important for children, and the high cost of vaccine is the only constraint to widespread immunization. Official recommendations on the need for pre-exposure rabies vaccine use a risk assessment analysis to define a length of stay in the endemic area, but as rabies is a fatal disease and a primary vaccine course is only required once, prophylaxis should be encouraged for all those visiting endemic areas.

Pre-exposure Vaccine Regimens

One dose of HDCV, PCEC or PVRV is given intramuscularly into the deltoid on days 0, 7 and 28 (the last dose can be advanced towards day 21 if necessary, but the antibody level may wane more rapidly) (Figure 32.5). An economical but effective alternative, recommended by the World Health Organization (1997, 2005) is to give the vaccine intradermally if more than one person is to be immunized. The dose of 0.1 ml of any of these vaccines is injected intradermally over the deltoid to raise a papule. If the injection is too deep, withdraw the needle and repeat the procedure. Opened ampoules should be stored in the fridge and used the same day because they do not contain preservatives and are not registered as multi-dose vials (World Health Organization 1997). The level of antibody may be lower following intradermal vaccination, but the quality of the secondary immune response to a booster dose is similar for intradermal and intramuscular injections. Chloroquine antimalarial chemoprophylaxis may inhibit the induction of rabies antibody after intradermal vaccination, so the larger dose must be given intramuscularly. All vaccinees must keep a record of their immunisation.

Booster Doses of Vaccine

A booster dose intramuscular or intradermal one year later enhances and prolongs the immune response, which lasts more than 10 years in 96% of people (Strady *et al.*, 1998). The neutralizing antibody level after three years has been used to decide whether to give a booster dose every 3 years, or every 10 years (Strady *et al.*, 2000). If no serology is available, or affordable, doses may be given every 5 to 10 years to those at continued risk of infection. In the United States, frequent serology and boosters are recommended only for those at high risk, but no boosters are given after the primary course for travellers assuming that vaccine will be available promptly after exposure (Centers for Disease Control and Prevention, 2008). A neutralizing antibody test should be performed six monthly for rabies

laboratory staff and when appropriate to avoid unnecessary treatment. A booster dose is given if the titre is <0.5 IU/ml. Measurement of antibody levels after vaccination is necessary only if immunosuppression is suspected, for example, in patients with acquired immune deficiency syndrome (AIDS). Those with a low CD4⁺ count may fail to produce antibody, even to repeated doses of vaccine.

Post-exposure Treatment

Post-exposure treatment is in increasing demand especially where dog rabies is endemic. According to World Health Organization data, over 13 million people are treated with vaccine annually, but many other patients remain untreated.

Any bite or close contact with an animal in a rabies endemic area is a potential exposure to rabies virus. Evaluation of the risk of infection depends on the history and clinical features of both the patient and the biting animal, and information about the local vectors and epidemiology of rabies.

The Site of Infection?

The virus gains access through any bite, scratch or contamination of broken skin or mucous membrane by animal's saliva, but intact skin is an adequate barrier against infection. The risk of infection is greatest from bites on the head, neck and hands, and multiple bites carry a higher risk than single bites. Other routes of infection are very rare.

The Animal Species and Behaviour?

An unprovoked attack by a known local rabies vector (see section on Epidemiology above), or an animal which may have been in contact with a vector species, suggests a high risk of exposure. Abnormal behaviour, either excitability or partial paralysis, is typical of rabies. Wild animals can appear unusually tame. Vaccination of domestic animals is not always protective. About 20% of proven rabid dogs in Bangkok had been vaccinated.

To Confirm Exposure

Every effort should be made to obtain a virological diagnosis, by examining the animal's brain for rabies antigen, and other tests if appropriate (see section on Diagnosis of biting animal above).

If rabies exposure is suspected or proven, post-exposure prophylaxis must be started as soon as possible. The greater the delay in starting treatment the greater the risk of virus entering a peripheral nerve where it becomes inaccessible to immune attack. If in doubt vaccinate; it is worth giving prophylaxis even if months have elapsed. Treatment can be stopped or converted to a pre-exposure regimen if the cat or dog is alive 10 days after the bite. The recommended criteria for treatment are shown in Table 32.5.

Table 32.5 The decision to give post-exposure treatment^a

Category	Type of contact with a suspect or confirmed rabid domestic or wild ^b animal, or animal unavailable for observation	Recommended treatment
I	Touching or feeding of animals	None, if reliable case history is available
II	Nibbling of uncovered skin Minor scratches or abrasions without bleeding Licks on intact skin	Administer vaccine immediately Stop treatment if animal remains healthy throughout an observation period ^c of 10 days or if animal is killed humanely and found to be negative for rabies by appropriate laboratory tests
III	Single or multiple transdermal bites or scratches. Contamination of mucous membrane by animals' saliva	Administer rabies immunoglobulin ^d and vaccine immediately. Stop treatment if animal remains healthy throughout an observation period ^c of 10 days or if animal is killed humanely and found to be negative for rabies by appropriate laboratory tests

^aThis table is a simplification of the WHO recommendations (World Health Organization, 1997, 2005).

^bExposure to rodents, rabbits and hares seldom, if ever, requires specific antirabies treatment.

^cThis observation period applies only to dogs and cats. Other domestic and wild animals suspected as rabid should be killed humanely and their tissues examined using appropriate laboratory tests. An exception may be made for animals of threatened or endangered species.

^dIf previously vaccinated against rabies, RIG is probably not needed, see text.

Primary post-exposure treatment For those who have not had a previous course of vaccine, the treatment consists of three parts: wound treatment, active immunization with vaccine and passive immunization with RIG.

Treatment of wounds The recommendation for all animal bite wounds is immediate vigorous washing, scrubbing and flushing with soap or detergent and water. This can be 50% effective in preventing rabies experimentally. Local infiltration, proximal to the wound, with procaine hydrochloride 1% in saline for pain may also have some antiviral action (Kaplan and Cohen, 1962). Then apply either 70% ethanol or povidone iodine. Avoid or postpone suturing the wound. Tetanus prophylaxis may be needed. Consider giving a prophylactic antimicrobial agent for serious bites or those on the hands (co-amoxycylav, doxycycline or erythromycin for dog or cat bites).

Vaccine regimens A course of five intramuscular injections of HDCV or PCEC vaccine are given into the deltoid on days 0, 3, 7, 14 and 28 (Figure 32.5) (Centers for Disease Control and Prevention, 2008; World Health Organization, 2005). Economical intradermal post-exposure treatment has been used for 15 years in Asia (see below). Although an intradermal regimen may have some advantages, it is not officially recommended in Europe and North America where full intramuscular treatment is freely available. If immunosuppression is suspected, due to diseases such as AIDS or to drugs, the antigenic stimulus might be increased by doubling the initial dose of intramuscular vaccine (one intramuscular dose into each deltoid) or by dividing a whole single dose between multiple intradermal sites (see below). A shortened course of intramuscular treatment, requiring four instead of five doses of tissue culture vaccine, is occasionally used outside the United Kingdom. Two doses are injected on the first occasion, one into each deltoid muscle, then one dose is given on days 7 and 21 (Vodopija *et al.*, 1988), but the antibody level wanes more rapidly than with the conventional regimen (Lang *et al.*, 1998; Suntharasamai *et al.*, 1987). Pregnancy is not a contraindication to rabies vaccination.

Rabies immune globulin (RIG) Rabies immune globulin should be given with every primary post-exposure treatment. It is most important for severe exposure to infection: bites on the head, neck or hands or multiple bites. Passive immunization provides some protection for the 7–10 days before vaccine-induced immunity appears. RIG apparently neutralizes virus in the wound. The dose of 20 units/kg body weight of human RIG (or 40 units/kg equine RIG, outside Europe and North America) should be infiltrated deep under and around the wound. If this is anatomically impossible, for example, in a bitten finger, give the rest by intramuscular injection at a site remote

from the vaccine, but not into the gluteal region. For multiple bites the RIG can be diluted two- or threefold in saline to ensure infiltration of all wounds. The recommended dose of RIG must not be exceeded as this will impair the immune response to the vaccine. Serum sickness has not been reported with human RIG. As there are problems of production and supply of RIG worldwide, efforts to find alternative products continue, including the use of a mixture of monoclonal antibodies.

Post-exposure treatment in previously vaccinated patients Treatment of animal bites, including immediate wound cleaning, is always urgent. Provided that a complete pre- or post-exposure course of a tissue culture vaccine has been given previously, or if a serum rabies neutralizing antibody level of >0.5 IU/ml has been recorded, an abbreviated course of only two doses of vaccine may be used. It is injected intramuscularly into the deltoid on days 0 and 3 (Figure 32.5). RIG treatment is not necessary (Centers for Disease Control and Prevention, 2008; World Health Organization, 2005) If there is any uncertainty about past immunization, the full post-exposure regimen and RIG must be used.

How effective is post-exposure treatment? The untreated mortality from proven rabid dog bites in India was 35% and 57% in separate studies more than 35 years ago. Optimal modern post-exposure treatment started on the day of the bite in healthy recipients is practically 100% effective. 'Failures of treatment' are due to not delivering the three components correctly and promptly, or failure of the patients' immune response. Examples are: if there is delay in starting treatment; failure to clean the wound; failure to complete the course of vaccine; injections of vaccine or RIG into the buttock; failure to infiltrate the wound with RIG, or immunosuppression by drugs, HIV, cirrhosis or other illness. There has been no suggestion that treatment has failed because the potency of the recommended vaccines was low; nor that the vaccine virus strain did not protect against the infecting virus. Deaths have been documented very rarely following complete prompt treatment with modern products (Hemachudha *et al.*, 1999; John and Patnaik, 2005). Nevertheless if wounds are extensive and severe, especially from wolf bites, primary post-exposure treatment cannot ensure survival.

Efficacy against rabies-related viruses Tissue culture vaccines do afford some protection against European bat lyssaviruses (genotypes 5 and 6), but they are probably less effective than they are against the genotype 1 rabies strains and ABLV (genotype 7). Vaccine is less protective against Duvenhage (lyssavirus genotype 4) and gives

little or no protection against Mokola virus (genotype 3) (Badrane *et al.*, 2001; Nel, 2005).

Side Effects of Tissue Culture Vaccines

There is very wide variation in the incidence of side effects in different groups of recipients. Mild erythema and pain at injection sites are reported in 7–64% of HDCV recipients, and local irritation is more common (13–92%) after intradermal injections. Generalized symptoms of headache, malaise and fever occur in 3–14%, (World Health Organization, 1997) and up to 3% reported a ‘rash’ distant from the injection site. There are similar data for PCEC and PVRV vaccines.

Rare case reports of neurological illness temporally associated with tissue culture vaccine treatment have described a Guillain—Barré-like syndrome (Bernard *et al.*, 1982; Chakravarty, 2001; Bøe and Nyland, 1980; Knittel *et al.*, 1989) a relapsing mild hemiplegia (Tornatore and Richert, 1990) or in two patients, symptoms restricted to an arm (Gardner, 1983). The incidence of neurological disease after these rabies vaccines is no more than after other commonly used vaccines.

Late booster doses of HDCV were followed, 3 to 13 days later, by a systemic allergic reaction in 6% of vaccinees in the United States (however, reaction rates were not evenly distributed between the groups of volunteers) (Dreesen *et al.*, 1986). The urticarial rash, angioedema and arthralgia responds to symptomatic therapy. It is possibly caused by an IgE-mediated reaction to β -propiolactone-modified vaccine components (Warrington *et al.*, 1987). Repeated booster doses of vaccine for people at continued risk of infection can be avoided by serological testing to confirm the need.

Human RIG has a very low reaction rate (Suwansrinon *et al.*, 2007).

What to do if Bitten in a Rabies Endemic Area

If bitten by a mammal in a rabies endemic country (or if any direct contact with a bat in the Americas), immediately wash the wound thoroughly. Seek advice on the local epidemiology. If there is a risk of rabies infection, start post-exposure treatment without delay. Tissue culture vaccines are too expensive for worldwide use, and so nervous tissue vaccines, Semple (sheep brain) or suckling mouse brain vaccines, are still produced in several developing countries. Their potency is variable and they are associated with allergic encephalomyelitis. Nevertheless, if only locally produced animal brain vaccine is available it might be reasonable to begin treatment immediately, and change to one of the European vaccines as soon as possible. If RIG is not available initially, it should be given up to seven days after starting vaccine. Equine RIG,

usually used in Asia and Africa, causes reactions in 1.8% and serum sickness in 0.7%, but anaphylaxis is exceptional (Suwansrinon *et al.*, 2007). If the risk of rabies exposure seems high it is worth curtailing a holiday to seek a recommended vaccine and RIG.

Two other post-exposure treatment regimens are recommended by the World Health Organization (1997). They are economical multi-site intradermal regimens which use only 40% of the amount of vaccine needed for the standard intramuscular course. The eight-site regimen gives an accelerated antibody response: on day 0 use a whole 1 ml vial to inject about 0.1 ml of PCECV or HDCV intradermally at eight sites (deltoids, thighs, suprascapular, lower anterior abdominal wall), on day 7 give 0.1 ml intradermally at four sites (deltoids, thighs) and on days 28 and 91, 0.1 ml intradermally at one site (Warrell *et al.*, 1985). This regimen is not suitable to use with PVRV, which contains 0.5 ml per ampoule.

The two-site intradermal post-exposure regimen has been used widely in Asia, usually accompanied by RIG. It was designed for use with PVRV (Chutivongse *et al.*, 1990), with an intradermal dose of 0.1 ml per site. If PCECV or HDCV (1.0 ml ampoule) are used, each intradermal dose must be 0.2 ml. On days 0, 3 and 7, give one intradermal dose at two sites (deltoids); on days 28 and 91 give one intradermal dose at one site (deltoid).

The two intradermal regimens use the same amount of vaccine, and aseptic techniques are essential when sharing ampoules. Opened ampoules should be used the same day. A comparative study shows that the eight-site method induces neutralizing antibody more rapidly and to higher levels than the two-site regimen, which is important when RIG is not available (Madhusudana *et al.*, 2001).

Difficulties with the current intradermal regimens have inhibited their use. A new variation, a four-site intradermal method has proved at least as immunogenic as the standard intramuscular regimen (Warrell 2008). On day 0, a whole ampoule of vaccine is divided between four intradermal sites (deltoids, thighs), 0.1/0.2 ml (depending on the vaccine used) is given intradermally at two sites on day 7 and one site on days 28 and 90. It is applicable with vaccine of any volume, has all the advantages of the eight-site method, uses the same amount of vaccine, and could replace both the present regimens, simplifying the confusing range of options.

CONTROL OF ANIMAL RABIES

The ability to control animal rabies depends on: the prevalence and host range of rabies in wild and domestic mammals in the region; the effectiveness of the local rabies surveillance network; the mode of transmission of

infection; the suitability of animal rabies vaccines; the social and cultural attitudes of the population to the vector species and the financial resources to implement a control programme.

Canine Rabies

Enzootic rabies is widespread in domestic and stray dogs. Muzzling, vaccinating and restricting the movement of owned dogs and reducing the number of strays was effective in eradicating rabies from some islands and peninsulas. In several large cities in South America intense mass vaccination programmes were dramatically effective in reducing the incidence of canine rabies and eliminating human disease (Schneider *et al.*, 2007). The size of urban dog populations, as with wild species, is determined by availability of food, water and shelter. Attempts to eliminate stray dogs by shooting and poisoning are difficult, unpopular and inefficient as fertility increases, restoring the numbers. Oral rabies vaccines for stray dogs have not proved very effective or easy to distribute; but methods of clearing rubbish to reduce the food supply, with parenteral vaccination and fertility control of strays can be successful in localized areas (Reece and Chawla, 2006). The support of the residents is essential. The role and influence of traditional methods of rabies prophylaxis must be taken into account and tactfully overcome by discussion and education.

Vaccination of owned dogs and cats should be mandatory in these areas. Facilities for viral diagnosis, disease surveillance and pre- and post-exposure vaccination of humans are also necessary. Countries free of rabies should prevent reinvasion by controlling the importation of mammals and by enforcing strict quarantine regulations from areas with canine rabies.

Sylvatic (Wildlife) Rabies

Attempts to reduce populations of vector species such as foxes and skunks have not been effective in the long term. Live attenuated rabies virus vaccines and a live vaccinia recombinant vaccine expressing rabies glycoprotein proved immunogenic by the oral route for foxes and raccoon dogs, and they are apathogenic in other local species. Since 1978, these oral rabies vaccines, disguised in baits, have been distributed over the countryside by hand or by aeroplane. The massive repeated control campaigns in 14 countries have proved very successful in Western Europe. Fox rabies has been eliminated in seven countries (Cliquet and Aubert, 2004). Long-term vaccination and surveillance are planned to prevent resurgence. In North America vaccinia recombinant vaccines have also been used to eliminate coyote rabies, and for control in raccoons and foxes. Raccoon rabies has been spreading north

along the eastern coastal states of the United States, and into Ontario despite an intensive campaign of oral vaccination. New vaccines are being developed for skunks, mongooses and jackals. Despite much work on developing DNA vaccines for rabies, none has yet proved suitable for use in animals or humans. Experimental plant-derived oral vaccines have induced rabies immunity. A single human vaccinia infection from a recombinant rabies vaccine has been reported in a pregnant woman with a chronic skin condition, epidermolytic hyperkeratosis (Rupprecht *et al.*, 2001).

In Latin America, vampire bat rabies is a major cause of death in cattle, with disastrous economic consequences. Specific control methods include vaccination of cattle or treating them with anticoagulants, to which the bats, but not cattle, are highly sensitive.

No attempts have been made to control rabies in some vector species despite their potential to infect humans. Insectivorous bats in North America and Europe are examples, due to their inaccessibility. Practical measures to avoid contact with bats, and a low threshold for giving post-exposure treatment are the only means of preventing human infection (Centers for Disease Control and Prevention, 2008). In rabies endemic areas, people should be educated to avoid unnecessary contacts with wild and domestic carnivores and to seek medical advice immediately if they are bitten or scratched.

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Papillomaviruses

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INTRODUCTION

The genus *Papillomavirus* is one of the two genera of the family *Papovaviridae*. However, the viruses belonging to this group are quite different from those of the other genus, *Polyomavirus*, both in genome size, organization and pathogenesis.

The papillomaviruses (Latin: *papilla* = 'nipple'; *oma* = 'tumour') produce in their host benign skin tumours (papillomas), containing variable amounts of infectious virus. The lesion has been recognized since the fifth century BC. Common hand and plantar warts are the most frequent skin papillomas of humans and until recently the viruses responsible have generated little clinical or scientific interest since the typical lesions were a cosmetic nuisance and the viruses were not thought to be involved in serious disease. However, 25 years ago the first papillomavirus isolated from invasive carcinoma of the cervix was described (Durst *et al.*, 1983) and since then papillomaviruses have been linked with other squamous cell carcinomas (SCCs) of mucosal and cutaneous epithelia. This has stimulated interest in these viruses, resulting in significant advances in our understanding of the natural history and pathogenic process.

CLASSIFICATION

At present papillomaviruses are classified into five supergroups: A (genital human papillomaviruses (HPVs)), B (associated with epidermodysplasia verruciformis (EV)), C (ungulate fibropapillomaviruses), D (bovine papillomaviruses (BPVs) causing true papillomas) and E (animal

and human cutaneous papillomaviruses). There are 11 groups under supergroup A, 2 under B and C and one group under both D and E. The classification results from the deduced evolutionary relationships among the papillomaviruses as judged by the sequence similarity of their genomes. For example, A9 contains the commonly isolated virus HPV-16 and the related viruses HPV-31, -33, -35, -52 and -58. Table 33.1 breaks down the classification. Note that two of the animal viruses, rhesus monkey and pygmy chimpanzee papillomaviruses, are grouped with the human genital isolates in groups A9 and 10, respectively.

PHYSICAL AND CHEMICAL PROPERTIES

Structure

The capsids of the papillomaviruses have icosahedral symmetry containing 72 capsomeres with a diameter of 52–55 nm (Figure 33.1).

Genome

The HPV virion contains a double-stranded DNA molecule of 5×10^6 Da molecular weight with an average of 7900 bp. The DNA, when in the virion, has a supercoiled circular configuration.

The molecular organization of the papillomavirus genome is well conserved between viruses of various species and an example of one of the common genital isolates is shown in Figure 33.2a. In this figure the genome is represented as a linear molecule with the boxed areas indicating the open reading frames (ORFs)

Table 33.1 Classification of the papillomavirus subfamily

Supergroups																
Group A											B		C		D	E
1	2	3	4	5	6	7	8	9	10	11	1	2	1	2	1	1
32	3	61	2a	26	30	18	7	16	6b	34	5	4	B1	E1	B3	1a
42	10		27	51	53	45	40	31	11		8	48	B2	D1		41
	28		57	69	56	39	43	33	13		9	50			B4	63
	29				66	59		35	44		12	60				Co
						68		52	55		14	65			B6	Ro
							70	58	P1		d					Cr
								67			15					
								69			17					
								R1			19					
											20					
											21					
											22					
											23					
											24					
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											48					
											49					

R, rhesus monkey papillomavirus; P, pygmy chimpanzee papillomavirus; B, bovine papillomavirus; Cr, cottontail rabbit papillomavirus; Co, canine oral papillomavirus; D, deer papillomavirus; E, elk papillomavirus; Ro, rabbit oral papillomavirus.
Source: Adapted from the Los Alamos National Laboratory HPV database, <http://hvp-web.lanl.gov/>

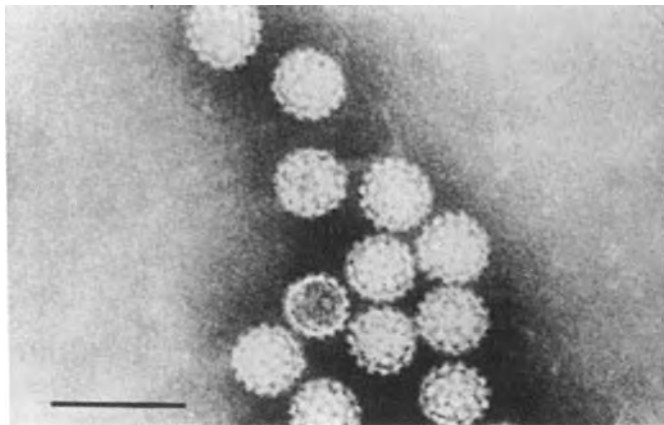
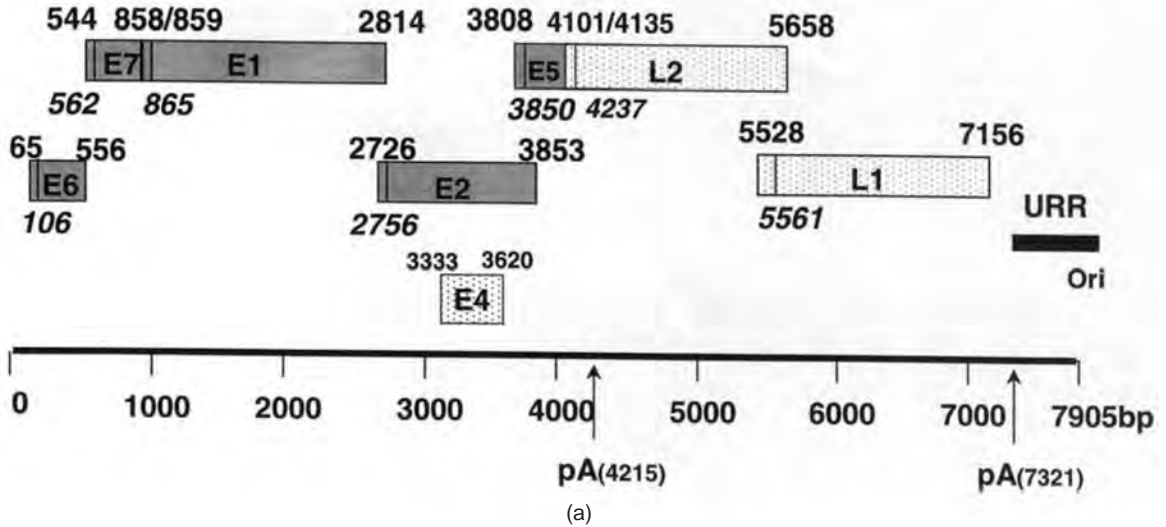


Figure 33.1 Electron micrograph of human papillomaviruses from a genital wart (negative stain $\times 154\,000$; bar = 100 nm). (Source: Courtesy of Dr J.D. Oriel.)

and for convention it is divided into two areas coding for early (E) and late (L) proteins. Because of the overlapping nature of the ORFs, the mRNAs transcribed are complex and it is not always clear which proteins

each transcript codes (Figure 33.2b). All the ORFs are transcribed from the same strand and so are read in the same direction, remembering that transcription is in the 5' to 3' direction. HPV-16 codes for 5 early and



Early Transcripts

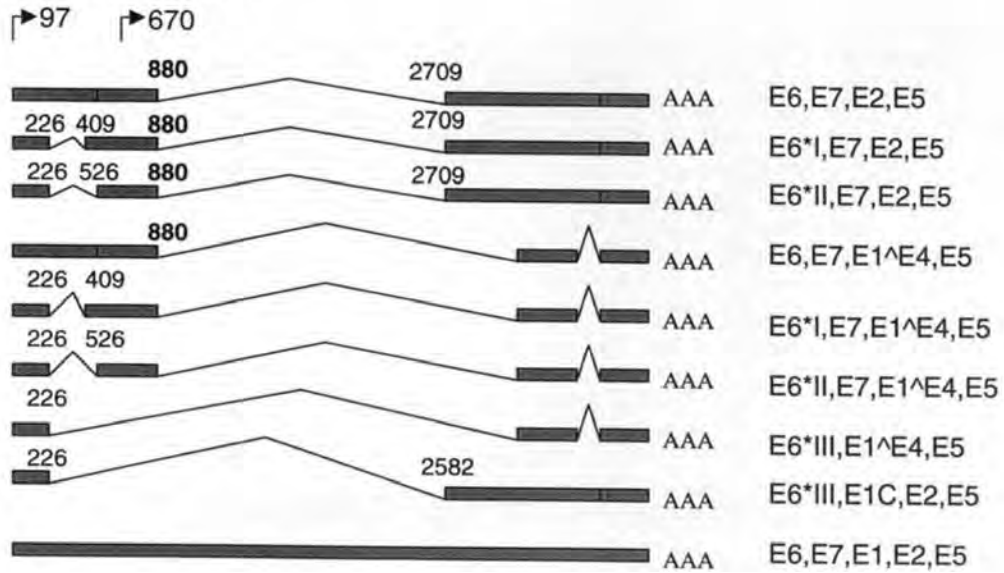


Figure 33.2 (a) Schematic of the HPV-16 genome organization. The early genes are solid grey and the late genes are speckled. The base pairs of the open reading frames are shown and the ATG start codon is shown in italics. E4 is spliced to the N-terminal of E1 (E1^{E4}) since it has no ATG of its own. The poly(A) + signals for the early and late mRNA are shown (pA). The origin (Ori) of replication and the upstream regulatory region (URR) or the main early promoter region are indicated. (b) Determination of mRNA species detected in many cases by RT-PCR, so some of the 5' and 3' ends are uncertain. E4 is always expressed as a function of E1^{E4}.

Table 33.2 Molecular weights of HPV-16 proteins

Protein	Molecular weight (kDa) ^a
E1	70
E2	45
E4	17
E5	8
E6	16
E7	11
L1	55
L2	50

^aMolecular weight on SDS-PAGE gels may be different, e.g. E7 runs at 15–16 kDa because of charged amino acids in the N-terminus of the protein, but codes for a protein of 11 kDa and L1 runs at 75 kDa.

3 late proteins. There is an upstream regulatory region which contains the major early promoter at base 97 and enhancer elements for transcription of early genes and also contains the origin of replication. A late promoter for the transcription of the capsid protein mRNA has been identified and lies in the E7 ORF at 670, although start sites for some of the late transcripts may initiate at different bases around this nucleotide.

Viral-Coded Proteins

There are five proteins coded for by the early region of the HPV genome and three late proteins. Because there are differences in the pathogenesis of various HPV types this is reflected in some of the functions of the early proteins. For brevity, therefore, a short summary of the functions of HPV-16 (Figure 33.2) early (E prefix) and late (L prefix) proteins is presented and their molecular weights are shown in Table 33.2.

Three of the early proteins, E6, E7 and E5, have properties that are consistent with the virus having to stimulate the infected cells into S phase so that the viral DNA has the cell's replicative machinery available for propagation of the genome. It has to be remembered that the virus is attempting to replicate in cells that are programmed to differentiate and so will have little or no replicative enzymes available to the virus. Both E6 and E7 are important for the efficient immortalization of human keratinocytes and have functions that disrupt the normal control of G₁ to S phase progression. E6 has been shown to bind a number of other cellular proteins (McMurray *et al.*, 2001), but the biological consequences of the binding to many of these proteins is unclear. For instance, E6 has also been shown to bind to a Ca²⁺-binding protein called E6BP, which has homology to a cellular protein ERC-55 of unknown function (Chen *et al.*, 1995), but the biological outcome

of this interaction is unknown. There are two proteins interactions of E6, which have been shown to be important for the pathogenesis of the high-risk, cancer-inducing viruses. E6 has been shown to bind the human p53 protein and cause its rapid degradation through the ubiquitin proteolysis pathway (Scheffner *et al.*, 1990). The viral protein also binds to proteins with a PDZ domain, such as membrane-associated guanylate kinase (MAGUK) homologues, which are found at the membrane and are thought to be involved in intra- and intercellular interactions (Thomas *et al.*, 2001). Mutants of E6 unable to bind and degrade p53 or PDZ-binding proteins are unable to cooperate with E7 to immortalize primary human keratinocytes and have reduced ability to replicate (Lee and Laimins, 2004; McMurray and McCance, 2004; Shai *et al.*, 2007).

E7 binds another cellular protein, the retinoblastoma gene product (pRb, (Dyson *et al.*, 1989)) and de-represses the inhibitory activity of Rb for transcription factors that are important for expression of genes whose products are essential for DNA synthesis. Repression of transcription may be due to the binding by Rb of a histone deacetylase protein (Brehm *et al.*, 1998), which functions to condense chromatin and restrict the access of transcription factors to DNA. Whether E7 competes for the binding site of the deacetylase on Rb or has an effect on the enzymatic activity of the deacetylase is unclear at present. Both p53 and pRb are negative regulators of the cell cycle and so interference with their normal function may allow cells to divide in an uncontrolled manner. E7 has also been shown to bind to other cellular proteins such as various members of the AP-1 family of transcription factors and upregulate their activity (Antinore *et al.*, 1996) as well as some members of the basal transcription machinery such as TATA-binding protein (TBP) (Phillips and Vousden, 1997).

E1 and E2 are involved in the replication of the HPV genome (see section on Viral replication below). E2 has additional functions in that it can positively and negatively regulate transcription from the early promoter. The full-length E2 protein contains a transactivation domain at the 5' end and a DNA-binding domain in the 3' half, and the active complex is a dimer. Negative regulation of the HPV early promoter is due to the fact that one of the E2-binding sites lies next to the TATA box of this promoter and so E2 binding sterically inhibits binding of the TATA-binding protein and therefore inhibits initiation of the early transcripts.

E5 is a membrane-associated hydrophobic protein with transforming activity for rodent fibroblasts. The E5 protein inhibits the acidification of endosomes (Straight *et al.*, 1995) by binding to the smallest subunit (16 kDa)

of the vacuolar ATPase, a multi-component proton pump (Conrad *et al.*, 1993). In human keratinocytes this results in the delay of the epidermal growth factor receptor (EGFR) degradation and a hyper-stimulated cell. Since the EGFR is the major growth factor receptor on keratinocytes, this activity may be important for stimulating cells into S phase for viral DNA replication. E5 can transform rodent fibroblasts and in the presence of the epidermal growth factor there is an increase in the efficiency of transformation (Leechanachai *et al.*, 1992).

The late proteins L1 and L2 are the major and minor capsid proteins, respectively, of the virion. The DNA and amino acid sequences are highly conserved between HPV types, especially in the L1 protein. The amino acid homology can be as high as 76% between HPV-16 and -33 (group A9). However, even within groups of papillomaviruses type-specific conformational epitopes predominate, so it is possible to differentiate between types on a serological basis (see section on Serology below).

The mRNA for the E4 protein is spliced, with the 5' portion coded for by the first few base pairs of E1 and then spliced to the E4 ORF. This is referred to as E1^E4. The message and protein are detected late in infection, although it has the prefix of an early gene. E1^E4 interacts with cellular microfilaments, causing them to collapse (Doorbar *et al.*, 1991), but the motive for this interaction is not clear. It has been suggested that this might allow the virions to be more easily released from the differentiated keratinocyte after transfer to a new susceptible host. It has also been shown to arrest cells in G₂/M by modulating cyclin/kinase complexes (Davay *et al.*, 2005; Knight *et al.*, 2006).

There are host coded histone proteins H2a, H2b, H3 and H4 associated with the viral DNA within the virion.

SEROLOGY

The expression of the major capsid protein, L1, in yeast and insect cells results in the folding and production of virus-like particles (VLPs). This has permitted serological studies on the relatedness of HPVs and to determine serological responses in infected patients (Kirnbauer *et al.*, 1992). Virus-like particles can also be produced which contain both the major (L1) and minor capsid protein (L2). The latter appears to stabilize the icosahedral structure and also has been shown to possess neutralizing epitopes. Antiviral antibodies have been produced to virions of HPV-11 and -16 and these antibodies recognize virus-like particles of the L1 protein from HPV-11 and -16, respectively (Rose *et al.*, 1994), but do not

cross-react. Therefore, virus-like particles are recognized by antibodies raised to infectious virus particles, and vice versa. The antibodies to virus-like particles or virions detect conformational epitopes, unlike earlier serological studies, where disrupted particles, or fusion proteins of L1 or peptides of regions of L1 were used either as targets or to raise antibodies to the L1 protein, and produced extensive cross-reactivity. This cross-reactivity was so complete that antibodies to disrupted BPV-1 particles recognized linear epitopes in most HPV types. Therefore, it was only with the production of virus-like particles that the serological differences between HPVs, even those closely related, became apparent. For example, within one group, say A9, containing HPV-16, -31 and -33, polyclonal antibodies only react well with the type to which the antibodies were raised, although some cross-reactivity has been observed. The level of cross-reactivity is very low and is probably not biologically relevant for cross-protection. This does limit the spectrum of cross-protection induced by the HPV types contained in the vaccine (see section on Vaccination below). Monoclonal antibodies to virions or virus-like particles have shown that there are predominantly type-specific epitopes, although in closely related viruses such as HPV-6 and -11, where L1 is are over 80% homologous at the amino acid level, cross-reactive epitopes have been observed. While there is little cross-reactivity at the antibody level, there appears to be more at the T-cell receptor level as lymphoproliferative assays show some cross-reactivity between types such as HPV-6 and -16.

VIRAL REPLICATION

Infection of tissue culture cells with papillomavirus particles and subsequent propagation of infectious virus has not been achieved. The major problem is that HPV virion production depends on terminally differentiating epithelial cells and by their nature these cells do not grow *in vitro*. However, it has recently been possible to transfect human keratinocytes, the natural host cell, with HPV-31 DNA, select stable cell lines containing episomal copies of HPV-31 DNA, and then differentiate the cells using the raft culture system and show that virus particle production occurs in a few cells in the upper most part of the differentiated epithelium (Frattini *et al.*, 1996). HPV-11 DNA has also been replicated in keratinocytes by the same laboratory, although because HPV-11 is unable to immortalize human keratinocytes the cells senesce and so the replicating DNA is lost over time. The distribution of the virus particles is the same as that observed in infected epithelium (Figure 33.3). The level of virus production is very

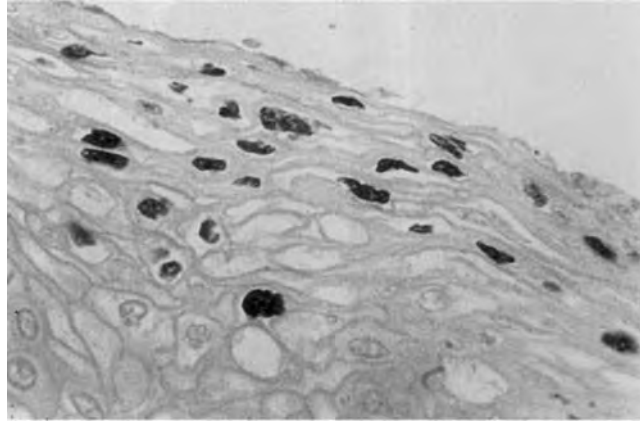


Figure 33.3 A section of biopsy containing CIN 1, showing nuclear staining of HPV-16 L1 antigen using a monoclonal antibody, followed by alkaline phosphatase-tagged anti-mouse secondary antibodies. Note that the dark-staining nuclei are found in the outer, differentiated part of the epithelium.

Table 33.3 HPV types isolated from patients with the genetic disease epidermodysplasia verruciformis (EV) and associated diseases

HPV type	Lesions
5	Warts/SCC
8	SCC
9	Warts
12	Warts
14	SCC
15	Warts
17	Warts/SCC
19	Warts
20	Warts/SCC
21	Warts
22	Warts
23	Warts
24	Warts
25	Warts
36	Warts
46	Warts
47	Warts

SCC, squamous cell carcinoma. HPV types 38 (melanoma), 41 (SCC) and 48 (SCC) were isolated from immunosuppressed patients.

This table was assembled with the help of Dr C. Wheeler.

low and not enough to produce virus particles for serological or infectivity studies. However, propagation of HPV types has been successful in a nude mouse kidney capsule system (Kreider *et al.*, 1985) and in human skin grafts onto the epithelium of severe combined immunodeficient (SCID) mice (Bonnez *et al.*, 1998). In the former system human foreskin tissue fragments are mixed with a viral

suspension of HPV and then the tissue is transplanted under the kidney capsule of a nude mouse. Over the next 60 days the tissue fragments grow and produce infectious HPV virus. Both HPV-11 and -16 have been propagated in this animal model, but, the system is obviously complex, requiring skills and animal facilities not available to all. In the alternative method, the tissue fragments are placed under the skin of the immunocompromised SCID mouse and so the growth of the tissue can be monitored. While the surgical skills are less demanding, the latter model is not a routine one.

The origin of replication of HPV-11 and -31 (Frattoni and Laimins, 1994; Lu *et al.*, 1993) has been mapped extensively and the region is conserved in other HPV types. The mapping has been achieved by cloning the origin region into a bacterial vector and transfecting mammalian cells along with plasmids expressing the E1 and E2 proteins of the respective virus. The E1 and E2 proteins are the only HPV-specific products necessary for the replication of the origin-containing plasmid. The origin region of the DNA has been mapped to a region at the 3' end of the upstream regulatory region (Figure 33.2a) and both E1 and E2 have specific DNA-binding sites at the origin. In addition to binding at the origin, E1 and E2 can bind together and E1 has been shown to have ATPase and helicase activities, which are typical of viral proteins, such as SV40 large T antigen, that are involved in the initiation of viral DNA replication.

NATURAL HISTORY OF HPV INFECTIONS

HPVs infect and replicate in squamous epithelium on both keratinized and mucosal surfaces. Most people are

infected with certain cutaneous viruses, such as the ones causing hand and foot warts (HPV types 1, 2, 3, 4), during childhood and adolescence. A small group of individuals with the rare autosomal recessive disease epidermodysplasia verruciformis (EV) harbour a number of virus types not often isolated from normal people (Table 33.1, groups B1 and 2). Two common presentations in EV patients are multiple warts, which may be so numerous as to produce coalescent areas and dry, scaly flat lesions. These may be red or heavily pigmented and although not having a wart-like appearance, may contain many of the unusual types of HPV in groups B1 and 2. SCC develop in nearly one-third of patients with EV, usually in areas of skin exposed to the sun (face, neck and hands being most commonly affected); HPV-5 and -8 are commonly found in these lesions while types 14, 17 and 20 (Table 33.3) account for a smaller number. While the viruses found in EV patients are not isolated from normal individuals, some types, such as 5 and 8, have been detected in SCC in allograft recipients. The disease EV is rare but since some of the EV-associated HPVs have been isolated from transplant patients this suggests that these viruses must be circulating in the community, infecting normal individuals perhaps without any associated clinical lesions.

As mentioned earlier, there are mucotropic HPVs, which mostly infect the genito-urinary tract. Common isolates like HPV-6 and -11, which cause benign condyloma, can also infect the oral cavity, particularly the larynx. Infection of the larynx is rare, but it usually requires several episodes of surgery or laser treatment to remove recurrent lesions. Frequent recurrences may be due to the fact that only the visible lesions are treated, but healthy-looking areas of mucosal tissue may harbour HPV genomes. The oral cavity is also infected with other HPV types associated with oral warts and hyperkeratosis.

Transmission is thought to occur from one epithelial surface to another in exfoliated cells containing infectious virus, rather than free virus particles. Therefore direct contact is the most efficient way to transmit infection. Direct contact through sexual intercourse is the most important mode of transmission of genital viruses. However, an infected mother may transmit virus to her neonate during delivery through the birth canal. This route of infection is thought to be a major cause of laryngeal warts in babies and young children. It is possible that other modes of transmission occur for the genital viruses, since a number of studies have detected DNA in the genital and perianal regions of young children. However, the routes of transmission, apart from those mentioned above, are unclear.

Infection of the genital mucosa is common and in the United Kingdom involves hundreds of thousands of new cases each year, usually among sexually active

individuals. The 18- to 30-year-olds have the highest incidence (cf. common hand warts). It is estimated that a sexually active woman has a 70–80% chance of being infected with a genital HPV type over her lifetime. Most of these infections will lead to transient disease which will spontaneously regress with the virus removed by the immune response. It is the women who have a persistent infection with high-risk HPV types that are at most risk of developing clinical disease and eventually cancer if the lesion is not detected in time. The transmission rate between partners was first described back in the 1970s when Oriel (Oriel, 1971) showed that 64% of partners of individuals with genital warts developed warts. Subsequent studies have shown that up to 90% of male partners have shared common HPV types with their partner's cervical isolates. Therefore, transmission of HPV types is common in a relatively stable partner setting. However, it is still unclear how frequent transmission is during casual sex with different partners.

As mentioned above, infection with certain of the genital HPV types increases the risk of malignant disease, especially of the cervix, and in the next section there will be a discussion of the pathogenesis of the genital HPV types.

PATHOGENESIS

HPVs cause benign and malignant changes in epithelial cells. It is the latter property that will be dealt with in this section since certain HPV types are the most important component of the aetiology of genital cancers and worldwide cervical cancer is one of the most common causes of cancer-related death in women.

Oncogenic Potential of Papillomaviruses

Until recent epidemiological and laboratory-based studies, most of the evidence for an oncogenic potential of HPVs came from research with animal papillomaviruses. Work in the 1930s showed that the cottontail rabbit papillomavirus (CRPV) produced benign tumours in this animal, its natural host, and that in 25% of cases these benign tumours would become malignant after 12 months. Benign tumours produced in domestic rabbits became malignant more frequently and within a shorter time. Also, application of hydrocarbons or tar produced a higher and more rapid malignant conversion in both animal species. The viral DNA was detected in both the benign and the malignant lesions. These results suggested that the CRPV produced the benign lesion, but other factors, genetic and environmental, were necessary for production of malignant disease. More recently, oesophageal, intestinal and bladder papillomas produced by BPV-4 were

shown to become malignant when cattle were fed on a diet of bracken. In this case the BPV-4 DNA was detected only in the benign lesion and was not detectable after malignant conversion. Recently a rhesus monkey papillomavirus type 1 has been isolated from a lymph node metastasis of a penile carcinoma. This virus is sexually transmitted and is associated with both penile and cervical cancers. This animal virus may serve as a good, if expensive model, to investigate the natural history of papillomavirus infections.

In humans, one-third of patients with EV develop squamous cell carcinoma, usually in sun-exposed areas. Over 30% of these lesions contain HPV DNA, most commonly types 5 or 8. This suggests that given the right environmental or genetic conditions, benign HPV-associated lesions may develop into carcinoma. Other evidence of a helper function associated with malignant conversion concerns laryngeal papillomas, where a high rate of malignant disease was observed in patients who were treated with X-irradiation some 50 years ago. With the increase in the number of allograft recipients there has been an increase in reports of SCC at many different sites. The cutaneous cancers are associated with those viruses isolated from similar lesions in EV patients and the genital cancers harbour the viruses found in similar cancers in immunocompetent individuals. The increased incidence of disease in immunosuppressed individuals, such as transplant patients, suggests that the immune response is important in controlling infectious and tumour development.

It is the genital papillomaviruses that are responsible for the most common HPV-associated cancer and these types will be discussed in more detail in the following sections.

Genital Cancers Associated with HPV

While the association between the genital cancers, especially cervical cancer, is now known to be causal rather than casual, not everyone infected with an oncogenic HPV type will develop cancer. In fact, it appears that people can be infected and have disease which spontaneously regresses, or perhaps have no obvious lesions resulting from infection. It is not clear why the outcome from infection can be so varied, but the immune response may play an important role as well as other less well-described factors, such as genetic background and response of the epithelial cells to infection.

HPV Infection and the Normal Cervix

It has been observed for some time that HPV DNA could be detected in cervical cells from women with a normal cervix as assessed by normal cytology and absence of a visible lesion upon colposcopic examination. What

is not clear, however, is whether the cervix epithelium is really normal or if there are micro-lesions present which harbour the virus. So far, no histologically normal section of the cervical epithelium has been shown to harbour the DNA of a high-risk HPV type, although viral DNA has been detected in cells from an apparently normal cervix. These infected cells probably emanate from undetectable micro-lesions. The rate of detection of HPV DNA in cervical cells varies dramatically depending on the method used to detect the DNA and the age and demographics of the group studied. Using polymerase chain reaction (PCR), longitudinal studies have shown a very high lifetime risk (70–80%) of HPV infection. However, there is variation in isolation rate depending on the age and lifestyle of the individuals studied. For instance, in one of the earliest studies of young women between the ages of 18 and 25, up to 46% had detectable HPV DNA by PCR in epithelial cells from a normal cervix over a four-year period (Bauer *et al.*, 1991). HPV types 16 and 18, which are commonly found in malignant disease, accounted for about one-third of the viruses detected. This infection rate has been observed in other populations of young, sexually active women. While similar studies on large populations of males have not been carried out, it is clear from smaller studies that males are infected at similar rates, a finding to be expected with a sexually transmitted disease. Therefore, a large number of young women may be infected with HPV types which cause malignant disease. It is clear that while many individuals will develop transient premalignant disease, only a small number will ever develop malignant disease. However, recognizing those at subsequent risk is not possible at present. HPV detection decreases in older women (>40 years of age) and is usually in the 5–10% range. While the frequency of infection is lower in older women, it has been shown that they are more likely to have underlying disease.

HPV Infection and the Abnormal Cervix

The most common genital lesion caused by HPV infection is the genital wart (condylomata acuminata). HPV-6 and -11 are the predominant types associated with these lesions, which are benign and where the rare malignant conversion has only been documented in patients with an underlying immune deficiency. These warts are distributed throughout the female genital tract on the cervix, vaginal wall, vulva, perivulval and perianal region. In males the lesions are found on the penis, scrotum and perianal region.

The premalignant lesions associated with HPV can occur on the same sites as described above for warts, although the cervix is where malignant conversion is most often observed. The premalignant lesions of the cervix

Table 33.4 Genital HPV types and their associated risk of cancer

Low risk	6
	11
	40
	42–45
	53–55
	57
	59
	61
	67
	69
	71
	74
	82
	High risk
18	
31–35	
51–52	
56	
58	
61	
66	
68	
70	
73	

are called intraepithelial neoplasia and are graded according to the Bethesda system (Kurman *et al.*, 1994) as low-grade squamous intraepithelial lesions (LSILs) or high-grade squamous intraepithelial lesions (HSIL). HPV-6 and -11 are found in LSIL, while the oncogenic types HPV-16 and -18 are found in all grades and in malignant disease. Table 33.4 gives a breakdown of the high and low risk of HPV types. The premalignant cervical lesions occur almost entirely on the transformation zone, the metaplastic zone between native squamous epithelium of the exocervix and the columnar epithelium of the endocervical canal, and are white in appearance after the addition of 5% acetic acid to the surface of the epithelium. Invasive cancer arises from these areas of HSIL and malignant cells migrate up into the uterus and out to local lymph nodes. HPV-16 is the most common virus found in intraepithelial and malignant disease, with a detection rate of 70% in cases of HSIL in Germany and the United Kingdom. Worldwide this type has been isolated in 50–60% of cases of invasive cancer of the cervix.

HPV-6 and -11 have not been found in malignant disease of the cervix, but have been isolated from locally invading lesions of the vulva such as verrucous carcinoma. However, when the genomes of the viruses were sequenced it was found that there were duplications in the long control region, which may be associated with the

change in pathogenesis. The HPV types and associated lesions are shown in Table 33.5.

There is a difference in the state of the HPV-16 DNA in premalignant and malignant lesions. In LSIL and HSIL the HPV-16 DNA is free and unintegrated, while in the majority of malignant cells the DNA is integrated. While integration is random within the chromosomes of cells, the HPV genome is usually integrated in the E1 or E2 regions of the DNA, resulting in the retention of the expression of the E6 and E7 proteins, which appear essential for the malignant phenotype. It is not clear how important integration is for the development of invasive cancer, since in a minority of cases the viral DNA is episomal in malignant cells, but continued expression of E6 and E7 appears necessary.

Malignant disease of the penis, while rare in developed countries, is much more common in developing parts of the world. HPV-16 and -18 were detected in over 50% of penile cancers in Brazil (McCance *et al.*, 1986) where in one area of the north-east of the country the incidence of penile cancer is 10 times that seen in Europe. Again in malignant disease of the penis the viral DNA is integrated into the host cell chromosomes in the majority of cases.

While HPV types have a role in the aetiology of cervical cancer, there are certainly other factors involved that act with the virus to produce invasive disease, since not everyone infected and exhibiting premalignant lesions will develop cancer. It is estimated that up to 25% of women with LSIL will progress to HSIL if not treated, while most will regress over a three-year period. However, in older women (>40 years of age) infection and persistence are associated with more serious underlying disease and so they are a group to be closely monitored (McCance, 1998). The co-factors involved have not been delineated, although smoking and use of oral contraceptives (>5 years) may be such components.

Persistence Infections

Epidemiological evidence suggests that HPVs can persist in squamous epithelium without producing clinically obvious lesions. Up to 50% of allograft recipients develop cutaneous warts within a year after transplant, this proportion being high when compared with the incidence in age-matched controls. This suggests that transplanted patients experience either new infections or reactivation of persistent virus, the latter being supported by the finding of HPV DNA sequences in biopsies of normal areas of larynx from individuals who have had episodes of laryngeal papillomas. As the recurrence rate of laryngeal warts is high, this suggests that the virus is capable of persisting somewhere in the larynx, respiratory tract or oral cavity without producing recognizable lesions—an inapparent infection.

Table 33.5 Genital human papillomavirus types and the site of associated lesions^a

HPV type	Associated lesion	Site
HPV 6a-f	Condylomata acuminata	Vulva Vagina Cervix Penis
		Shaft Prepuce Urethral meatus
		Perianal
		Larynx
	LSIL/HSIL ^b	Cervix
	VIN I-III ^c	Vulva
	PIN I-III ^d	Penis
11a, b	Condylomata acuminata	Vulva Cervix Perianal
		Larynx
	LSIL/HSIL	Cervix
	PIN I-III	Penis
16	Condylomata acuminata	Vulva, cervix and penis
	LSIL/HSIL	Cervix
	VIN I-III	Vulva
	PIN I-III	Penis
	Bowenoid papulosis	Vulva and penis
	Malignant carcinoma	Cervix, vulva and penis
18	LSIL/HSIL	
	Malignant carcinoma	Cervix and penis
31	LSIL/HSIL	Cervix
	Malignant carcinoma	
30	LSIL/HSIL	Cervix
33	LSIL/HSIL	Cervix
	Malignant carcinoma	
34	LSIL/HSIL	Cervix
35	LSIL/HSIL	Cervix
	Malignant carcinoma	
39	LSIL/HSIL	Cervix and penis
	PIN	
	Malignant carcinoma	
40	LSIL/HSIL	Cervix and penis
42	LSIL/HSIL/VIN	Cervix and vulva
43	LSIL/HSIL	Cervix
44	LSIL/HSIL	Cervix
45	LSIL/HSIL	Cervix
	Malignant carcinoma	
51	LSIL/HSIL	Cervix
	Malignant carcinoma	
52	LSIL/HSIL	Cervix
	Malignant carcinoma	
53		Normal cervix
54	Condyloma	Penis
55	Bowenoid papulosis	Penis
56	LSIL/HSIL	Cervix
	Malignant carcinoma	
57	Intraepithelial neoplasia	Oral cavity, cervix
58	LSIL/HSIL/Malignant carcinoma	Cervix

Table 33.5 (Continued)

59	VIN	Vulva
61	VIN	Vulva
66	LSIL/HSIL	Cervix
67	Malignant carcinoma	Cervix
	Intraepithelial neoplasia	
68	LSIL/HSIL	Cervix
	Malignant carcinoma	
69	Intraepithelial neoplasia	Cervix
70	Condyloma	Vulva
	Malignant carcinoma	
71	VAIN ^e	Vagina
74	VAIN	Vagina
82	HSIL	Cervix

^a Adapted from De Villier (1989) and updated with the help of Dr C. Wheeler.

^b Low-grade squamous intraepithelial lesion and high-grade squamous intraepithelial lesion of the cervix.

^c Vulvar intraepithelial neoplasia.

^d Penile intraepithelial neoplasia.

^e Vaginal intraepithelial neoplasia.

Also, the viruses associated with the lesions in EV patients are not normally found in lesions from immunocompetent individuals, yet EV is such a rare disease that these patients cannot circulate the viruses amongst themselves. It would seem that these viruses are circulating in the normal population, causing inapparent infections. This is supported by the fact that viruses isolated from warts and SCC in immunosuppressed individuals are often the same types observed in EV patients.

In addition, during pregnancy, genital warts can appear on the vulvar epithelium and then disappear post partum. It is not known if this is an hormonal effect, or due to perturbations in the immune response that may accompany pregnancy, or the result of acquiring a recent infection from her partner. In none of the above situations is there any direct evidence as to which cells harbour the virus. The basal epithelial cells are the most likely site, although there is a considerable turnover of cells. However, not all cells in the basal epithelium have the same capacity to divide, so the viral DNA may be sequestered in quiescent basal cells, which when they subsequently divide may activate replication and produce lesions.

DIAGNOSIS

Apart from the familiar hand and verruca (plantar) warts, the clinical appearance of papillomavirus infections varies considerably, from the scaly flat lesions on cutaneous epithelium of individuals with EV to the aceto-white flat lesions on the cervix. The reader is referred to specific papers for details of the clinical presentation of genital

lesions (Walker *et al.*, 1983). This section will deal with the laboratory diagnosis of HPV, in particular the genital isolates.

Culture Methods

Although several efforts have been made, no easily amenable cell type has been capable of supporting replication with production of infectious papillomavirus particles. The nude mice kidney capsule model or the SCID mouse subcutaneous system has been used to propagate some HPV types, but they cannot be considered *in vitro* systems and are not amenable to most virus laboratories. Also, the raft system (McCance *et al.*, 1988) only produces low amounts of infectious virus and the technique itself is rather cumbersome. There is therefore no readily available simple culture system for HPV propagation.

Serological Methods

Recently, with the advent of yeast and baculo virus-produced virus-like particles, it has become possible to detect antibodies by an enzyme-linked immunosorbent assay (ELISA) technique in the serum of patients infected with HPV. At present there is a limited number of HPV types that have been used as antigens in these assays, but the initial results suggest that antibodies are detected in approximately 50% of infected individuals, which is less sensitive than DNA-based detection methods. The reasons for the low positivity rates in infected people is unclear, but it has to be remembered that the virus is confined to the stratified epithelium of the genital tract and few cells in a lesion support viral particle production.

In addition, the mature viral particles are only produced in the outer layers of the epithelium, where the immune response is at its least effective. Serological assays may not therefore be very sensitive for diagnostic or screening purposes.

Polymerase Chain Reaction (PCR)

The most sensitive method for the detection of HPV infection is by PCR. This is a powerful technique which amplifies a specific piece of DNA from a small amount of template. The advantages of this method are (i) the extreme sensitivity; (ii) the versatility, in that it can be used to detect more than one type of HPV when degenerate primers are used; it is, in fact possible to detect even unknown HPV types (i.e. ones that have not been cloned and sequenced); (iii) it is possible to test large populations. The major disadvantage stems from the false sensitivity of the method whereby it is possible to amplify contaminating sequences and so have false positives. This was a major problem in the beginning, but now that it is recognized investigators are more careful and include strict controls.

Several sets of partially or degenerative primers have been used to detect HPV types in the DNA extracted from lesions, and are directed to the L1 ORF. The first described, MY09/MY11 (Bauer *et al.*, 1991) has been improved (PGMY09/PGMY11) and used with a filter containing 27 HPV types (Gravitt *et al.*, 1998). This means that the PCR products can be directly hybridized to the filter, allowing for a rapid method to detect specific HPV types. Other sets such as GP5⁺/GP6⁺ (de Roda Husman *et al.*, 1995) and SPF-10 (Kleter *et al.*, 1998) have also been used successfully to detect multiple HPV types in clinical samples. The main difference between the sets of primers is that some detect certain HPV types better than others, but since these types are of low frequency in the populations studied, in reality there is little difference in the sensitivity of detection between the sets (van Doorn *et al.*, 2002). None of the PCR sets have been licensed for use as diagnostic tools, although the hybrid capture method described next has been approved for diagnostic use in the United States.

Hybrid Capture Method

This method is commercially available as a kit from Digene Diagnostics (Maryland, USA), and uses type-specific RNA probes to detect viral DNA in samples. The method is not as sensitive as PCR, but does not have the problems of false positives associated with PCR since it does not rely on the PCR-type amplification of the signal. In practice, although not as sensitive as PCR, the method is sufficiently sensitive to detect HPV DNA and has been

shown to be more sensitive than cytology in detecting HPV from lesions diagnosed as atypical squamous cells of unknown significance (ASCUS), LSIL and HSIL. Cytological smears diagnosed as ASCUS are difficult to interpret, since after biopsy more serious disease may be detected and HPV typing has been shown to be helpful in these situations. This test detects 16 mucosal types, whereas PCR can theoretically detect all HPV types.

TREATMENT

Although in most cases warts are a cosmetic nuisance and will eventually disappear spontaneously, they are notoriously difficult to treat. However, since premalignant lesions, especially on the cervix, may lead to malignant disease, treatment to eliminate disease is important. This section will concentrate on the treatment of genital areas as others (Bunney, 1982) have dealt with the treatment common hand and plantar warts.

Podophyllin

Podophyllin is a resin mixture obtained from the roots of the American mandrake (*Podophyllum* sp.) and is an irritant on cutaneous and mucous surfaces. It is an antimetabolic agent and should be used with care. It is painted carefully onto the surface of genital warts and should remain for no longer than 6 hours and then be washed off. It is poorly adsorbed by cutaneous surfaces and so has a limited effect. Several treatments are required and the continual inflammation produced can lead to fibrosis of the areas treated, without getting rid of the lesions. Podophyllin is even less effective in treatment of plantar warts and should never be used for treatment of hand warts.

Cryotherapy

Liquid nitrogen (-190°C) and dry ice (solid carbon dioxide, -50°C) can be applied to warts to produce local destruction of the lesion. Care should be taken to limit application to the lesion and not surrounding areas, as this will lead to pain and blistering of the healthy area. Cryoprobes are used to apply these cryogenics to the cervix.

Electrodiathermy

This is used in treating mucosal lesions such as those on the cervix to destroy the diseased tissue by heat.

Laser Evaporation

The carbon dioxide laser has been used to treat lesions on mucosal surfaces (cervix and vaginal wall) (Singer and

Walker, 1985) as well as cutaneous lesions. The success rate of laser treatment of the cervix is very good, with cure rates of 85–90%. However, its efficacy with cutaneous lesions has not as yet been adequately assessed. The difficulty with these lesions is in knowing how deep to vapourize to eliminate diseased tissue.

Loop Electrosurgical Excision Procedure (LEEP)

This is a relatively new procedure for the removal of cervical lesions and makes use of a heated loop, which very precisely removes the complete lesion. One advantage over laser evaporation is that the lesion is removed intact and the tissue can be used for pathological staging without having to biopsy the lesion first. In addition, the margins of the lesion are intact and so the extent of lesion removal can be assessed. Loop electrosurgical excision procedure (LEEP) also allows the lesion to be used for other studies, such as HPV typing.

Surgery

Curettage of common warts is not a common mode of treatment. In any case, not all warts are suitably sited for surgical removal. Furthermore, if all the abnormal tissue is not moved, small islands of warts can recur around the site of the initial lesion.

Interferon

Interferon has been used to treat recurrent laryngeal warts and cervical neoplasias. In the former cases, tumour load was first reduced by surgery or by CO₂ laser, interferon then being given parenterally. Although recurrences were rare within two years after starting the interferon course, it was necessary to maintain patients on interferon to prevent new lesions. The expense of this regime and possible side effects associated with interferon administration are drawbacks to this method of treatment. Cervical pre-malignant lesions have also been treated with interferon, but variable results have been reported.

Imiquimod

Imiquimod is an immune-response modulator that has shown some success in the treatment of external genital warts which are usually recalcitrant to treatment. The cure rate using a 5% cream is between 35 and 52% in females and the side effects are usually local skin reactions such as erythema, oedema and erosion. The drug is a synthetic imidazoquinoline that binds to the toll receptors TLR-7 and -8 and stimulates the innate immunity to produce a range of cytokines such as interferon- α (IFN- α), IFN- γ , tumour necrosis factor- α (TNF- α), interleukin 1 (IL-1), IL-8, IL-10 and IL-12. These in turn activate T-cell responses to the virus.

VACCINATION

Two vaccines, one from Merck & Co called Gardasil and the other from GlaxoSmithKline called Cervarix have been developed. Both vaccines are based on the same concept, but with different adjuvants and covering different combinations of HPV types (Wheeler, 2007).

Nature of the Vaccine

Both vaccines contain the major capsid protein L1, produced in either yeast (Gardasil) or insect cells (Cervarix). The L1 protein folds into an icosahedral structure called a virus-like particle in an identical fashion to the viral capsid and so produces a protective immune response to conformational epitopes on the virus particle. Gardasil contains the L1 protein from the high-risk types HPV-16 and -18 and also the low-risk types HPV-6 and -11, while Cervarix contains only the high-risk types HPV-16 and -18. The adjuvants used also differ, with Gardasil containing the conventional alum (aluminium hydroxyphosphate sulfate) while Cervarix contains a new adjuvant called ASO4 (aluminium hydroxide and 3-deacylated monophosphoryl lipid), which the manufacturers suggest may increase the cross-protective nature of the vaccine so that immune responses are raised to other closely related types not in the vaccine. Cross-protection remains to be proved in long-term vaccine trials.

Vaccine Trials

Both vaccines are given intramuscularly as three doses at zero, two and six months for Gardasil and zero, one and six months for Cervarix in a volume of 0.5 ml. To date the combined phase II and III for both vaccines show an efficacy of 100% for HPV-16 and -18 associated cervical intra-epithelial neoplasia (CIN)-2 or -3 and 100% efficacy for the Gardasil for protection against HPV-6- and -11-associated genital warts (Harper *et al.*, 2006; Villa *et al.*, 2005). The antibody titres to L1 are much higher in vaccinees than in people infected by the normal route of transmission. These results are impressive but the combined follow-up is a median of only 21 months, so longer term follow-up is required to confirm these statistics. Safety is good with no serious side effects and only some minor soreness at the site of inoculation. Also, initial impressions are that women who became pregnant during the vaccination period have similar side effects to the women receiving placebo, that is, they have no added risk.

Age to Vaccinate

Data from the trials suggest that the vaccine does not protect people who have already acquired the infection

and so it is important that vaccination is carried out in an age group that is unlikely to have become sexually active. In both the United States and United Kingdom approximately 25% of females are sexually active at 15 years of age and over 70% by 18 years (Wheeler, 2007). In addition, nearly half the women between the ages of 19 and 21, and 10% of 13- to 15-year-olds, report having had four or more sexual partners in their lifetime and HPV acquisition after the start of sexual activity is quite rapid. Therefore, it is important to vaccinate young prepubescent women before sexual activity is likely. At present the recommendation in the United States and United Kingdom is to vaccinate girls of 11 years of age. There are no plans to give the vaccine to males, although they are an important part of the natural history of HPV infections.

The cost of the vaccine course (three doses) will be in the range of £350 in the United Kingdom or \$350 in the United States. While it may have little effect on the incidence of malignant disease in the developed world it could have a major impact in third world countries where 80% of the cancer cases worldwide are found. However, at the price given above, the vaccine will have very limited exposure there and some arrangement with the manufacturers and these developing countries will need to be implemented to have the desired result.

Duration of Protection

While the median follow-up so far is 21 months, a small proportion of the vaccines have been followed for up to five years with no perceptible waning of their antibody titres (Villa *et al.*, 2005). Therefore, early indications are that repeat vaccinations may not be needed in the medium term and it may be that the HPV vaccine will have a similar schedule to the hepatitis B vaccine. One potential problem is that while HPV-16 and -18 make up 70% of the case of cervical cancer worldwide, the other 30% are caused by viruses not contained in the vaccine. There is a theoretical possibility that the other HPV types may become more prevalent once competition from HPV-16 and -18 wanes.

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The Human Polyomaviruses

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CLASSIFICATION AND DETECTION

In the past, the human polyomaviruses were grouped with papillomaviruses, polyomaviruses and SV40, the so-called vacuolizing agent, in the family *Papovaviridae*. Molecular studies made clear that Simian virus 40 (SV40) is highly related to the other polyomaviruses, and with growing knowledge on the genomic structure and function of proteins the previous subfamily is now classified as independent family of *Polyomaviridae*. The classical human viruses are now grouped as polyomavirus hominis (huPyV) type 1 (BKV) and type 2 (JCV). Although mouse polyomavirus was detected almost 50 years ago, the existence of primate polyomaviruses was not realized until the 1960s, with the detection of SV40 in monkeys. Ten years later, JCV and BKV were described. JCV was isolated from brain tissue of a patient with progressive multifocal leukoencephalopathy (PML) in human fetal brain cultures, and BKV grew in cell cultures after inoculation with urine from a renal transplant recipient (Padgett and Walker, 1976; Zu Rhein, 1969).

VIRION STRUCTURE AND COMPOSITION

Polyomaviruses are non-enveloped viruses with icosahedral capsids about 40 nm in diameter containing three virus-encoded proteins—VP1, VP2 and VP3. The shell surrounds a DNA molecule that is stabilized by cellular histones in a chromatin structure. From studies on SV40 and murine polyomavirus it is known that 360 molecules of the major capsid protein VP1 are associated with approximately 30–60 molecules of each of the minor capsid proteins VP2 and VP3. The icosahedron is composed of

72 pentamers. Each consists of five VP1 molecules and one molecule VP2 or VP3. VP1 is the most related capsid protein among the three primate viruses. BKV can be propagated in tissue culture, whereas a cell line for the effective propagation of JCV has not yet been found. Virus preparations contain at least two kinds of particles. *In vitro* passage of polyomaviruses at high multiplicity is accompanied by the generation of considerable amounts of empty capsids. In addition, defective viral genomes containing deletions, duplications and rearrangements of viral genetic information can be encapsidated, if they are in the appropriate size limits. *In vivo* the amount of empty shells is considerably lower, pointing to a highly effective virus growth under natural conditions. Complete virus particles form a band at a density of 1.34 g ml^{-1} in CsCl_2 equilibrium density gradients, whereas empty capsids have a density of about 1.29 g ml^{-1} (Figure 34.1).

Recombinant JCV VP1 self-assembles into pentameric capsomers, and under appropriate conditions, these molecules will further assemble into virus-like empty capsids (VLPs) (Chang *et al.*, 1997). Calcium ions are required for capsid stability and disulfide bonds exist between capsid proteins, as reducing agents are required to disassemble virus particles. The human polyomaviruses are able to haemagglutinate erythrocytes at high virus concentrations. Each virus also has distinct antigenic epitopes and can be distinguished by neutralization and haemagglutination inhibition assays.

VIRUS LIFE CYCLE

One of the first steps in the viral life cycle is the adsorption of the virion to the cell surface. In view of the

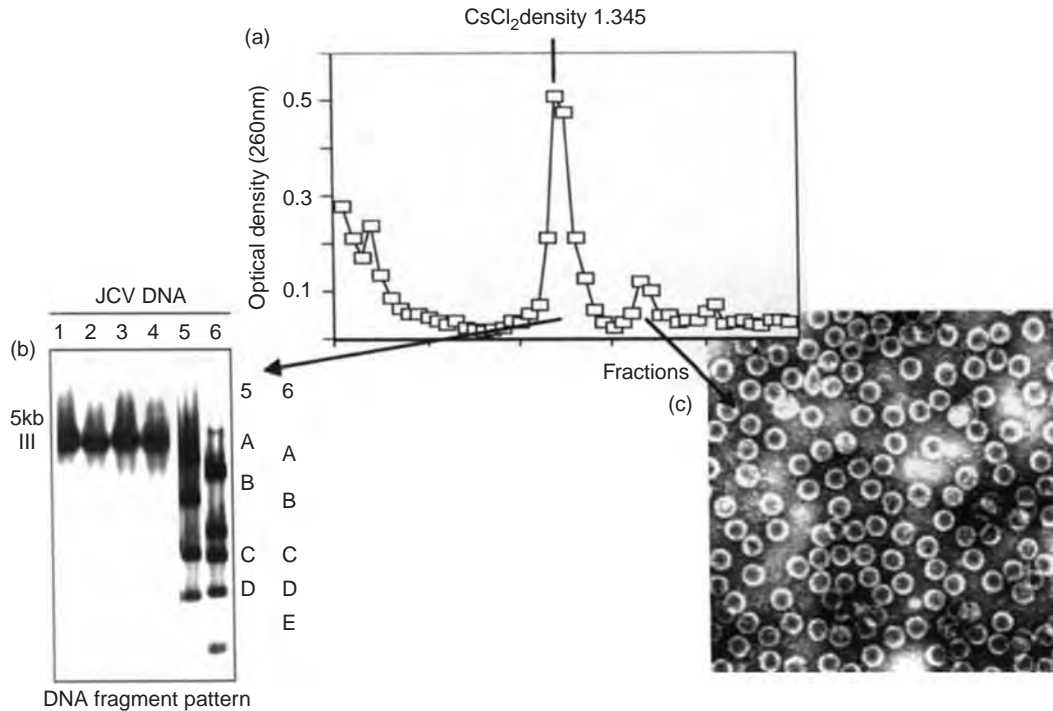


Figure 34.1 JCV purification out of PML brain tissue. (a) Optical profile of the centrifugation with banding of virus particles in two bands; intact virus particles at a density of 1.345 g ml^{-1} of CsCl_2 . (b) Restriction endonuclease cleavage of DNA extracted from the major peak of the gradient followed by Southern blotting and JCV-specific radioactive hybridization. (1–4) Cleavage with single cut enzymes *EcoRI*, *BamHI*, *HpaI*, *PstI* generating linearized form III DNA at about 5 kb in length. (5,6) Cleavage with single and more cut enzymes in combination (*BamHI/PvuII*, *BamHI/HindIII*) generating DNA fragments A–D and A–E. (c) Electron micrograph of negatively stained JCV icosahedral virus particles of the small virus peak representing empty particles without encapsidated viral DNA.

stringent cell specificity of JCV for glial cells the initial experiments were based on the assumption that the high number of gangliosides in the central nervous system (CNS) made these types of molecules good candidates for a JCV receptor. It was demonstrated that JCV VLPs bind to glycolipids and glycoproteins containing sialic acids and require $\alpha(2,6)$ -linked sialic acid bound to a protein for successful infection. However, binding to the respective sialic acid alone was not sufficient to promote virus infection. Recently, the proteinaceous part of the receptor molecule in glial cells was characterized as the serotonergic receptor 5HT_{2A}. The two components are present in the most prominent target cells *in vivo*, glial as well as kidney epithelial cells and are important for initial attachment. Interestingly, endothelial cells, which did not express 5HT_{2A}, were infected by JCV *in vitro*, and produced a virus yield comparable to that in glial cell cultures suggesting additional mechanisms of entry.

JCV binding to infected cells is followed by activation of the MAP kinase ERK1 and ERK2 pathway, signalling

events required for downstream steps of entry and infection. JCV requires proper assembly of clathrin-coated pits for rapid entry into the target cell. Following entry, the virus co-localizes with the clathrin marker transferrin in endosomes clarifying that JCV utilizes clathrin-mediated endocytosis for entry. This is in line with the fact that 5HT_{2A} is internalized in the same way. After entry, polyomaviruses are transported generally through the cytosol to the nucleus. For such directional intracellular transport viruses depend on the active cytoskeletal transport machinery. During early JCV infection intact microtubules are necessary as well as the intact intermediate filament network for productive infection. This is probably attributed to actin participation in assembly of the clathrin machinery rather than direct involvement of actin filaments in viral trafficking (Chapagain *et al.*, 2007; Elphick *et al.*, 2004).

Early studies on BKV receptors demonstrated a role for sialic acids, galactose and polysialylated gangliosides in BKV attachment. Recent experiments suggest that

$\alpha(2,3)$ -linked sialic acids attached to an *N*-linked glycoprotein are sufficient for viral infection. A coreceptor as described for JCV has not yet been identified for BKV, however, BKV apparently does not share protein receptor specificity with the other primate or the murine polyomaviruses. In addition, the gangliosides are also used as BKV receptors, but similar to the sialic acid components, the gangliosides used, GD1b and GT1b, differ from the molecule used by JCV (GT1b). The gangliosides and $\alpha(2,3)$ -linked sialic acids independently mediate BKV infection, suggesting that BKV might use alternate receptors or even entry pathways in different cell types. Because of the high structural similarity among the major capsid proteins, the BKV sialic acid receptor is believed to interact with the same BC loop domain of VP1 as utilized by JCV.

Following attachment, a relatively slow internalization phase requires 2–4 hours for completion. In contrast to JCV, BKV appears to use caveolae-mediated endocytosis as entry pathway. In biopsy material, BKV particles were located within invaginations of the cytoplasmic membrane morphologically consistent with caveolae. Although the steps are not yet clarified, it is clear that an acidification step as well as a tyrosine phosphorylation signal is needed/necessary for BKV infection. Microtubules are involved in the transporting process, probably allowing virus vesicles to shuttle along the microtubule track. The infection is microtubule-dependent for about 10 hours post infection. Although actin disruption does not interfere with BKV transport to perinuclear sites, the dynamic of microfibre reorganization is an important factor, possibly facilitating the transport process. Further trafficking to the nucleus is dependent on co-localization of BKV with CT-B between 4 and 6 hours post infection, suggesting that BKV passages through the Golgi by the endoplasmic reticulum (ER) after fusing BKV vesicles to stacked tubules continuous with the Golgi and the ER. Alternatively, BKV may bypass the Golgi and accumulate in the ER. Electron microscopic examination revealed that vesicles containing BKV fuse with ER-like structures and possibly Golgi organelles and then accumulate at perinuclear regions (Dugan *et al.*, 2007). Uncoating of polyomaviruses is believed to occur inside the nucleus.

Polyomavirus expression is essentially divided into three major stringently controlled phases. After expression of the regulatory early protein large T antigen (TAg), early post infection, TAg initiates replication of viral DNA. Shortly after onset of DNA replication, virus multiplication enters the late phase, which includes expression of late mRNAs. Translation of the late regulatory agnoprotein and the production of viral capsid proteins is followed by virion assembly. Regulated by complex

interactions of viral promoter elements with cellular factors and the viral regulatory proteins, late genes are expressed efficiently only after DNA replication. In contrast, early genes continue to be expressed at late stages of infection, than serving as viral transcription factors. The functions of TAg and agnoprotein are controlled by consecutive phosphorylation events. However, tropism of huPyV is predominantly to resting, differentiated cells. In order to produce enzymes necessary for virus growth, they have evolved mechanisms to overcome cell cycle growth arrest. A majority of the cell cycle-stimulating activities are induced by TAg. Consequently, expression of TAg can lead to transformation of a cell from a normal to a growth-deregulated state. The best-studied cellular growth-promoting and -inhibitory proteins interacting with TAg are the tumour suppressor proteins retinoblastoma binding protein (pRB), p53 and their downstream factors (White and Khalili, 2005).

In comparison to other polyomaviruses, JCV has a long lytic life cycle with an early transcription and DNA replication phase lasting about five days followed by continuing initiation of late RNAs for 15–20 days. In contrast is the high amount of virus produced and the fast progression of JCV infection *in vivo*. This discrepancy was solved recently by the discovery that JCV infection is able to interfere with programmed cell death (Pina-Oviedo *et al.*, 2007). JCV infection is able to induce the expression of a member of the inhibitor of apoptosis family of proteins, survivin. Survivin is normally expressed during embryonic development and is silenced in adult tissue. Dysregulation of suppression results in reduced cell death and abnormal cell viability. In glial cells high levels of survivin were expressed as early as day 5 post infection. Preliminary results suggest that this is an early event mediated by JCV TAg and replaced by proapoptotic measures late in infection, when production of late proteins increases and the late agnoprotein might be involved in the suppression of pro-survival signalling (Merabova *et al.*, 2008).

Viral auxiliary proteins have diverse effects on different stages of infection including transcription, viral assembly and the release of viral particles. The late leader regulatory protein agnogene plays an important role by modulating viral gene transcription and DNA replication. Agnoprotein may interact with the viral key regulatory protein TAg, which is expressed early during infection. However, in contrast to the other viral proteins it is not only found in the nucleus but is also localized in cytoplasmic compartment with high concentrations in the perinuclear area, particularly in the late stages of infection. Agnogene of BKV has intracellular localization similar to that of JCV. Since proteins smaller than 60 kDa cannot overcome passive diffusion within cytosol without interactions with associated

molecules, it was assumed that agnogene-binding proteins play a role in its cytoplasmic localization. JCV agnogene inhibition impairs the viral maturation pathway. The first JCV agnogene-binding protein characterized was the cytoskeleton microtubule protein tubulin. Then interaction with the fasciculation and elongation protein FEZ1 was described. FEZ1 is a protein that plays an essential role in kinesin-mediated transport pathways. Agnogene appears to block the association of FEZ1 with microtubules, thereby inducing the dissociation of FEZ1. Whether this is a potentially protective function by inhibiting the release of progeny virus from JCV-infected cells or promotes an effective intracellular translocation of progeny virus is not clear (Khalili *et al.*, 2005; Suzuki *et al.*, 2005).

The mechanism underlying JCV virion assembly include the synthesis of major and minor capsid proteins in a defined ratio from alternatively spliced mRNA molecules. Unlike the bipartite BKV nuclear localization signal (NLS), JCV has a weak monopartite NLS. JCV VP1 needs VP2/3 for proper import, suggesting that assembly of pentamers occurs in the cytoplasm. The capsid proteins are translocated to the nucleus in a cooperative manner and accumulate at the dot-shaped nuclear domain ND10. It is likely that capsid assembly at this site is associated with DNA replication followed by formation of intranuclear viral inclusions. The newly packaged virion progeny is thought to be released by lytic rupture, however, electron microscopical studies report secretion of virions from the plasma membrane of intact cells (Clayson *et al.*, 1989).

JCV has a stringent cell specificity and replicates efficiently *in vitro* only in human glial cells (Walker and Padgett, 1983), whereas BKV can be grown in a wide range of human cell types. With the upcoming knowledge on virus host interactions related to the viral life cycle, it becomes more and more likely that cell specificity of both human polyomaviruses is regulated by a large number of mechanisms that might additionally vary from cell type to cell type.

Molecular Structure of the Genome

The genomic structure is highly related among the primate polyomaviruses. The supercoiled, circular double-stranded DNA of the human viruses is about 5100 bp in length. The genome is divided into two regions encoding multiple overlapping genes. Each DNA strand carries about one half of the genetic information. Early and late mRNAs are synthesized bidirectionally from opposite strands of the genome. Protein-coding sequences consist of open reading frames for the early and late proteins. The noncoding region directs activity and specificity of virus multiplication. It is divided into two regulatory segments with a single origin of DNA

replication (ORI) and the transcriptional control elements within the promoter region (Dörries, 1997; Kim *et al.*, 2001) (Figure 34.2).

The coding sequences exhibit high DNA sequence homology. Homology between the human viruses is greater in all proteins than between JCV and SV40 (69%). JCV DNA shares between 83 and 59% amino acid homology with BKV in the cases of large TAG and agnogene respectively. The rates are even higher in functionally active regions of the virus genes. The early region codes for the tumour or TAGs. Both human polyomaviruses encode two major proteins, small t (tAg) and large TAG, so-called on the basis of size. The multifunctional TAG directly controls the virus life cycle and interacts with key cellular regulatory circuits. Functional activities include effects on nuclear localization, on viral DNA replication by direct DNA binding, helicase activities and binding to DNA polymerase alpha that initiates DNA replication. Interaction with cellular tumour suppressor proteins is associated with cellular transformation and TAG as a transcription factor is known to be crucial for the regulation of early and late gene transcription. Many of the activities depend on the TAG viral chaperone domain, which interacts with cellular chaperones to orchestrate functions that require the rearrangement of multiprotein complexes.

The tumour antigens are generated from a common pre-mRNA molecule by one alternative splicing event leading to identical N-termini and different C-terminal DNA sequences. Recently, three additional early virus proteins, T'135, T'136 and T'165, were characterized in JCV-infected cells. All T proteins use the first alternate splice donor site, leading to the same 132 N-terminal amino acids. For T' proteins a second splice donor site is combined with alternate acceptor sites. Whereas T'165 shares the C-terminus with TAG, T'135 and T'136 have unique ends encoded by an alternate reading frame. Expression of the proteins is modulated during the virus life cycle and appears to be related to TAG-mediated DNA replication and transcriptional control. T' proteins may utilize the shared tumour suppressor and chaperone-binding domains to enhance DNA replication by differential interaction with pRB and related proteins. Significant differences in splicing pattern in JCV-transformed cells and increased expression of the '17 kDa' species suggested correlation with transforming activity of JCV. Additionally, it has been proposed that the apoptotic function of TAG might be negatively influenced by T' proteins (Frisque *et al.*, 2006; Kim *et al.*, 2001).

The late region of the viral genome encodes two minor proteins, VP2 and VP3, and a major capsid protein, VP1. Late mRNAs are generated from a common precursor by alternative splicing. The sequences of the minor

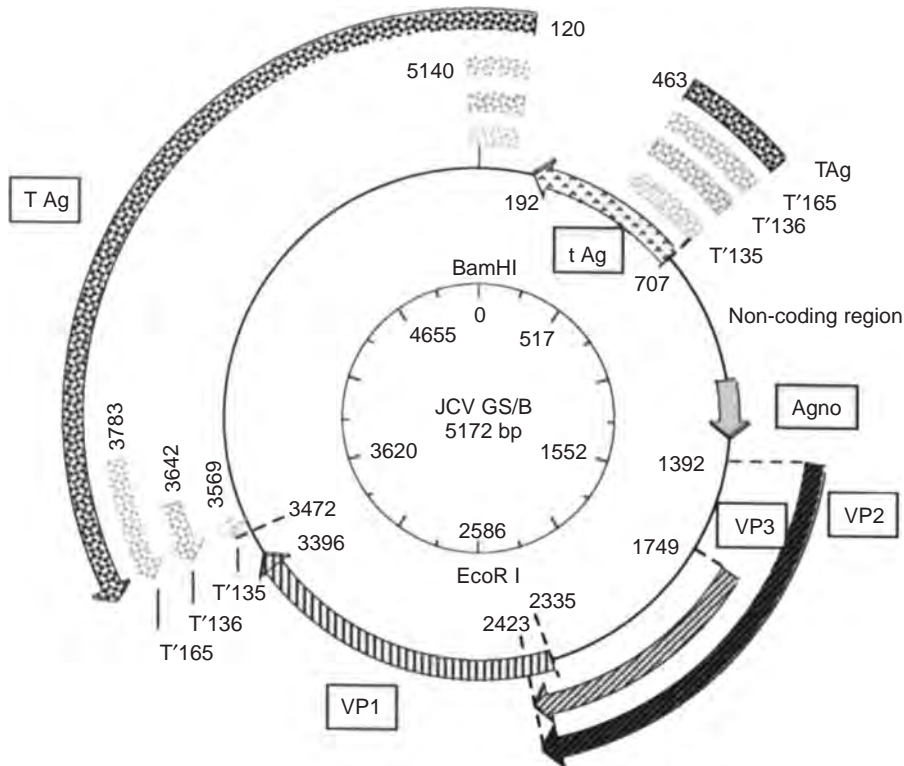


Figure 34.2 Circular genomic map of the human polyomavirus JC subtype GS/B. Coding DNA segments are represented by shaded arrows. Numbers designate the first nucleotide of the start and stop codons for the proteins (Loeber, G. and Dörries, K. (1988) DNA rearrangements in organ specific variants of polyomavirus JC strain GS. *Journal of Virology*, **62**, 1730–1735). Position of splice donor and acceptor sites are indicated. The position of the unique *Bam*HI and *Eco*RI restriction sites are indicated on the inner circle.

proteins VP2 and VP3 are overlapping. VP2 contains the entire VP3 sequence at its C-terminus and an additional sequence of approximately 400 amino acids at its N-terminus. In contrast, the major protein VP1 is generated by an alternative reading frame. The three primate polyomaviruses all encode the nonstructural regulatory agnoprotein in an open reading frame in the late leader region. The protein is probably not essential for virus multiplication but takes part in the orchestration of efficient progeny virus production. It is produced late in the virus lytic cycle and physically and functionally interacts with the early key regulator TAg and the cellular transcription factor YB-1. Recently, it became obvious that agnoprotein plays an important role in the viral life cycle by modulating viral gene transcription and DNA replication. Likewise, it is involved in the morphogenesis of virus particles, the localization of capsid proteins to the nucleus, intracellular vesicular transport, and appears to deregulate cell proliferation by association with tumour suppressor p53 (Khalili *et al.*, 2005) (Table 34.1).

The non-coding part of the genome is framed by the start codons for early and late genes. The ORI is located between the TATA box and the initiation codon for the early genes. This segment includes the conserved binding sites for large TAg. Corresponding to similar DNA replication strategies, the polyomavirus ORI DNA segment is highly conserved in sequence and structure. In contrast, the promoter region to the late side of the control region reveals extensive differences. Heterogeneity of structure and sequence of individual transcription factor binding sites is reflected in the divergent cell type specificity and activity of transcription among the huPyV types (Dörries, 1997).

Control of Viral Gene Expression

DNA Replication

Regulatory mechanisms leading to DNA replication are closely related among the primate polyomaviruses. This is reflected in an ORI that is constructed by protein-binding

Table 34.1 Human polyomavirus early and late proteins

Protein	BKV		JCV	
	Size (amino acids)	Mol. wt (kDa)	Size (amino acids)	Mol. wt (kDa)
<i>Early</i>				
tAg	172	20	172	20.2
TA _g	695	90	688	79.3
T'135	NR	NR	135	17
T'136	NR	NR	136	22
T'165	NR	NR	165	23
<i>Late</i>				
Agno	66	8	71	8
VP1	362	46 ^a	354	39.6
VP2	351	40.5 ^a	344	37.4
VP3	232	30.5 ^a	225	25.7

^aMolecular weight varies considerably between laboratories. NR, not reported.

elements with comparable sequence and spacing requirements. Bidirectional replication takes place in the presence of the core ORI and TA_g, proceeding from the ORI elements with the TA_g-binding sites terminating at a site about 180° from the initiation site. The features shared by all ORI regions are an inverted repeat on the early side, a GC-rich palindrome in the centre and an AT sequence on the late side of ORI. All core ORIs contain these elements and replication studies on the human polyomaviruses confirmed that besides TA_g binding, site II and the inverted repeat are essential for DNA replication. Whereas the presence of flanking sequences stimulates DNA replication, the transcriptional control region (TCR) does not affect DNA replication activity directly. Interestingly, the efficiency of DNA replication directed from JCV ORI is substantially lower than that from BKV ORI. TA_g produced in each of the viruses varies considerably in its ability to support replication from homologous or heterogeneous origins. It is likely that differences of ORI sequences or spacing among the primate polyomaviruses are responsible for these observations (Kim *et al.*, 2001) (Figure 34.3).

Transcriptional Expression

The transcriptional control region (TCR) of the human polyomaviruses is composed of a great number of different regulatory protein-binding motifs. Promoter activity is mediated bidirectionally and often independently from the binding motifs by multiple interactions of transcription factors and associated cellular and viral proteins stimulating basal, cell type-specific, and, in response of external stimuli, induced functions of the viral promoter (Kim

et al., 2001; Manley *et al.*, 2006; Merabova *et al.*, 2008; Monaco *et al.*, 2001).

The JCV control region is artificially dissected in the ORI domain and following four TCR subdomains (A–D) in each of which a clustering of early and late interactive promoter sites is observed. Outside the TCR domain on the early site of ORI a binding sequence is located for the potent transcriptional enhancer nuclear factor-κB (NF-κB) and a potential binding site for a member of the nuclear factor of activated T cells (NFAT1) family. NF-κB is constitutively expressed in B lymphocytes and can be retained in the cytoplasm by an inhibitor κB protein. This binding can be released through stimulation by a number of agents acting as tumour promoters, such as phorbol-myristate-acetate (PMA) or inflammatory cytokines. It results in the transport of NF-κB to the nucleus, where the binding to the consensus DNA sites exerts activity. Upon PMA treatment both JCV promoters are responsive to NF-κB induction leading to increased JCV activity. In addition, tumour necrosis factor alpha (TNF-α) is able to enhance binding of NF-κB to the JCV κB site. Similarly NFAT1 is responsive to TNF-α and interleukin 2 (IL-2). This stimulated the idea that activation of the transcriptional control *in vivo* is dependent on the expression of inducible factors involved in immunologically regulated signalling pathways to modulate JCV expression (Figure 34.4).

It has been shown that interference with κB function in late gene expression decreased, but did not abolish, activity. This suggested that additional TCR elements may confer inducibility to the JCV late promoter coming into effect during the persistent state of infection. The GGA/C-rich sequence (GRS) motif within the ORI might also interact with the κB motif. The GRS is similarly inducible by PMA and inflammatory cytokines. The responsive region interacts with the novel GGA/C-binding protein GBP-i. GBP-i is induced in a wide range of cell types; thus it could play a role in mediating JCV activation at all suspected sites of persistence. Comparable to the NF-κB class of proteins, the GBP-i complexes probably represent a combinatorial assembly of various protein species, which is changed upon induction. Duality of function could involve the basal transcriptional machinery and other transcription proteins associated with late promoter activity. One potential factor is transforming growth factor-β (TGF-β) acting through the GRS and the NF-1 sites. In such a model the status of the viral promoter could be modulated through the GRS and variable interaction of cytokine induced proteins. Although these cytokines may use different signal transduction pathways for protein activation, in consequence, the nuclear milieu will contain the factors leading to expression by interaction with a promoter containing the respective contact sites.

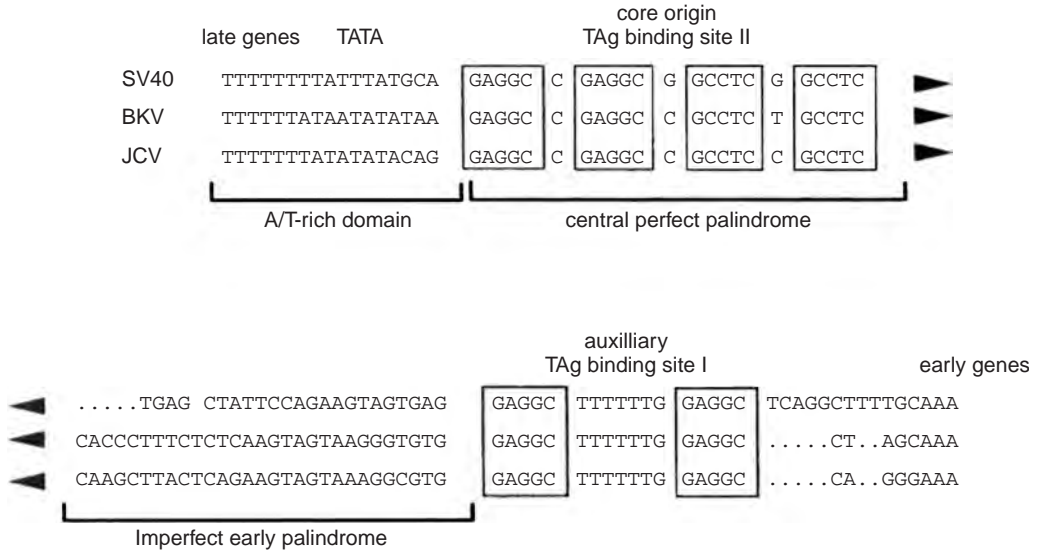


Figure 34.3 Alignment of the DNA sequence encompassing the origin of DNA replication in the noncoding genomic region of the primate polyomaviruses SV40, BKV and JCV. All sequences are given in the sense of the early coding strand. TAG consensus pentanucleotide recognition sites common to all virus strains are in boxes.

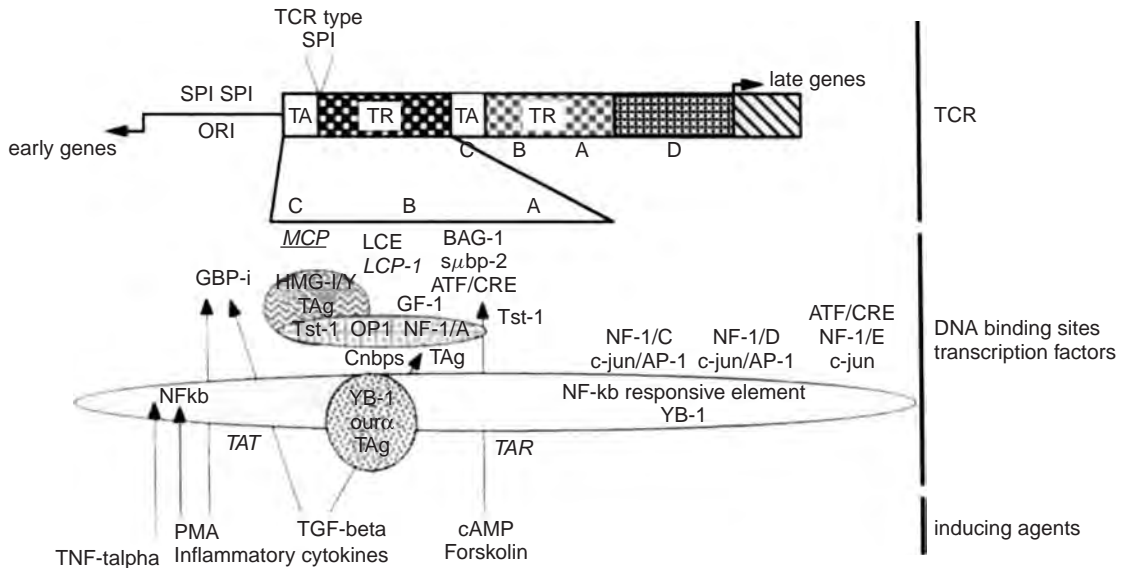


Figure 34.4 JCV TCR with protein-binding sites and interaction of cellular and viral transcription factors. The transcriptional control region (TCR) structure of prototype JCV Mad-1 is shown between the start codons (arrows) for early and late genes. ORI, origin of DNA replication; TA, TATA box; TR, tandem repeated promoter elements. Similar shading represent identical DNA sequences (see legend to Figure 34.5); A–D are dissected promoter domains. MCP, minimal core promoter; LCE, lytic control element. Binding sites and the respective proteins are indicated below the promoter domains. Filled circles indicate interaction of transcription factors. *TAT*, *TAR* are HIV-1-related transactivation domains. Arrows indicate interaction of inducing agents with the respective proteins. The position of the SP1-binding site in JCV TCR type II genomes is indicated at the top of the figure.

Most JCV TCR subtypes contain SP1-binding sites around the TATA box activating early promoter activity. The JCV early minimal core promoter (MCP) in domain C binds the glial-specific transcription factor Tst-1/SCIP/Oct-6, a member of the POU-domain protein family. It is an intrinsically weak transcriptional regulator and relies on viral or glial cell-specific coactivators. TAG has been identified as viral coactivator that stimulates the function of Tst-1 synergistically by direct interaction and activates the high mobility group proteins HMG-I/Y as cellular coactivators. Additional crosstalk was assumed with the glial cell-specific late transcriptional silencer OP-1 that contacts downstream of the Tst-1 binding. Another cellular nucleic acid-binding protein, Cnbps, negatively regulated the JCV early promoter, and the TAG-binding protein p53 is discussed as a transcriptional silencer. The OP-1 motif also interacts with the adjacent NF-1 site. OP-1 and NF-1 form a composite element that increases JCV early activity and reduces JCV late activity. In the same region the JCV lytic control element (LCE) interacted with pur α and YB-1. The YB-1 family are responsive to a wide variety of stimuli including stress signals, drug and IL-2 treatment in T cells and activate transcription from both the viral early and late promoter. It was proposed that pur α , YB-1 and TAG are also involved in the transition of JCV early-to-late promoter activity.

The B-domain in the early orientation contributes positively to expression in glial cells and is also important for transcriptional activation of JCV late genes. The central motif responds to glial factor 1 (GF-1) and appears to be a part of the human Sp1/2 factor. GF-1 protein expression is most abundant in brain tissue. Interestingly, the amount of GF-1 is higher in kidney cells than in other cell types. Therefore it is thought that the level of GF-1 in kidney cells could be responsible for the ability of JCV to replicate in urogenital tissue. Pronounced high-affinity NF-1-binding sites (NF-1A/B) are closely associated with overlapping binding sites for nuclear factor Jun as well as cyclic AMP-responsive element (CRE) and a site resembling the sequence for the activator protein AP-1. NF-1 sites are involved in basal as well as in glial cell-specific modulation of JCV early and late promoters, they are affected by TAG transactivation and BAG-1, a novel factor, interacts with anti-apoptotic Bcl-2 protein (Devireddy *et al.*, 2000). Cell-specific activity in glial cells includes the CRE overlapping with the AP-1 site. The second messenger cAMP and forskolin substantially increase JCV early gene expression. Activity is mediated by a CRE-binding protein (CREB). Importantly, the mechanisms of induction for the factors are through different signal transduction pathways. This combination allows a highly flexible JCV transcriptional re-

sponse to a large number of environmental signals. In domain A a transactivator element associated with HIV-1 TAR homologous sequences and Tst-1-binding sites has been identified. The D domain is close to the start codon of the late proteins spanning binding sites for NF-1, c-jun, YB-1 and an NF- κ B-responsive region. YB-1 in this position is believed to be a transcriptional coactivator for regulation from the NF- κ B site. NF- κ B/rel subunits modulate JCV late promoter activity at multiple levels. This provides an exquisite control mechanism over constitutive and induced NF- κ B activity (Sweet *et al.*, 2002).

The BKV promoter region has been less well characterized. However, similar to the JCV promoter region the so-called noncoding control region (NCCR) of BKV also consists of the conserved ORI region with a true palindrome, two inverted repeats and an AT-rich region and is dissected in subdomains designated by letters P, Q, R and S. They harbour a string of conserved promoter-binding sites for transcription factors NF-1 and Sp1, which are required for early transcription. There are AP-1-binding sites, believed to be important for basal BKV activity, and there are potential NF- κ B and C/EBP β -binding sites immediately upstream from the early initiation codon. The interaction of an NF- κ B and the C/EBP β transcription factors cooperatively stimulate BKV early expression. Exogenous agents activate BKV early transcription by the adenylate cyclase pathway. They signal through a cAMP-responsive element, CREB, and phorbolsters mediate activity probably through a putative responsive element in the early region. In the late region the oestrogen responsive element (ERE) and the nonconsensus glucocorticoid response element (GRE)/progesterone response element (PRE) overlap the NF1 and p53-binding sites immediately upstream. The major late promoter (MLP) sequences harbour multiple NF1 sites. Usually NF1 factors act as transcriptional activators, however, here they are also involved in repression of transcription, probably because NF1 binding is involved in early to late switch of promoter activity (Gorrill and Khalili, 2005; Kraus *et al.*, 2001; Moens and Rekvig, 2001).

Heterologous Transactivation of Virus Transcription

Additional to activating effects of cellular transcription factors, viruses are thought to influence human polyomavirus expression through the direct or indirect interaction of a heterogeneous gene product. In general, transactivation can occur at any step of protein synthesis, beginning with the initiation of transcription and ending with post-translational modifications. In the case of JCV and BKV, promoter activity and DNA replication were affected. The question whether HIV-1 may transactivate human polyomaviruses has been discussed since it

became clear that PML is one of the most prevalent opportunistic infections and new BKV-induced syndromes were described in patients with AIDS. The HIV-1-encoded transregulatory protein Tat has been found to be a potent activator of the JCV late promoter. Tat is a transcriptional activator and an essential component for the establishment of a productive HIV-1 infection. In the JCV promoter at least two responsive elements, the TAT element in the ORI domain and the TAR element, have been identified. Transactivation occurs *in vitro* at the level of transcription by HIV-Tat induction of the JCV promoter. Limited expression of HIV-1 proteins has been detected in astrocytes, indicating that astroglial cells might be a site of co-infection. Co-infection of astrocytes by JCV and HIV-1 *in vitro* altered HIV-1 gene expression and JCV gene expression was slightly increased in HIV-1 replicating cells. The effect was due to interaction of JCV agnogene and Tat protein. Changes in subcellular localization of both proteins as well as a negative impact on the interaction of Tat with its target DNA, TAR and critical upstream transcription factors suggested that agnogene may utilize different pathways to interfere with the transcriptional activation of the long terminal repeat (LTR) by Tat, thereby controlling the level of JCV as well as HIV-1 gene expression in astrocytes (Kaniowska *et al.*, 2006). In oligodendrocytes HIV-1 expression is regularly low, but biologically active Tat protein is secreted by the HIV-1-infected cell and could affect neighbouring cells by transcellular or receptor-mediated interaction. In polyomavirus-infected cells transcription from Tat responsive promoter elements was induced. Alternatively, after super-infection of already infected cells Tat could directly interact with the respective promoter elements. Recently, it became clear that the activation of BKV by HIV-1 transcriptional regulatory proteins is dependent on interaction with the NF- κ B-binding site and a pentanucleotide motif, the so-called BKVE-TAR site, at the early site of the promoter. In contrast, BKV TAg is able to transactivate the HIV-1 LTR (Gorrill *et al.*, 2006).

Transactivating effects of another retrovirus, human T-cell lymphotropic virus type I (HTLV-I), is modulated by interaction with the regulatory protein Tax. Transactivation is also postulated for herpes viruses. Cytomegalovirus (CMV) infection is often activated in immuno-impaired patients and common target tissues are kidney, lung, the CNS and lymphoid organs. Increased JCV DNA replication is mediated *in vitro* by the human CMV immediate-early transactivator 2 (IE2). Similarly, it was found that BKV TAg is able to induce the expression of CMV early gene expression (Dörries, 2001). Human herpes virus type 6 (HHV-6) infection was co-localized with JCV in PML lesions. HHV-6 establishes persistent

infection in brain, urogenital tract, lung, liver or peripheral blood cells and often occurs after transplantation. Obviously, huPyVs and HHV-6 have a wide range of target organs in common. Due to putative transactivation mechanisms of other herpes viruses, a comparable interaction of HHV-6 with the human polyomaviruses is possible.

Although the mechanisms of huPyV transactivation by heterogeneous viruses *in vivo* are at present not completely understood, the increasing number of HIV-1 patients with active polyomavirus infections argues for a potential role of concomitantly infecting viruses for the activation of polyomavirus infections and the pathogenesis of polyomavirus-associated disease.

Genomic Heterogeneity of Viral Subtypes

Analysis of JCV isolates from all over the world revealed that a large number of JCV subtypes (at present 14) exist. Comparable variations in the BKV and the SV40 genome pointed to a general role of genomic heterogeneity among the polyomaviruses (Dörries, 1997; Knowles, 2001). Throughout the JCV genomes numerous single-base changes were observed and the TCRs exhibit extensive structural differences. Single-base mutations in coding genes do not affect the reading frames and most are silent, having no effect on the amino acid composition of the proteins. In contrast to BKV, JCV DNA does not exhibit sufficient sequence variation to generate different serotypes. Therefore genotypes are exclusively defined by their DNA sequence. The biological significance of the changes remains uncertain. Although the role of the major capsid protein VP1 and its mutations at the receptor binding groove has been intensively studied, the possible involvement of protein alterations in viability or cell specificity of viral subtypes has not yet been clarified.

Of all the viral genes, VP1 shows the greatest degree of variation, whereas the agnogene is the most conserved. The clustering of mutations on the terminal end of VP1 and TAg proteins provides the tool for V-T subtyping of JCV genotypes from different regions of the world. It is used for virus transmission studies and is thought to be a marker for population migration. At present, major V-T genotypes are assigned to different geographic regions by creation of a phylogenetic tree from isolates all over the world. Three separate JCV genotypes 1 were found in individuals of European origin. Type 2, the so-called European/Asian type, was traced in a wide region extending from Europe to western and eastern Asia. Additional types (types 3 and 6) were dominantly found in African states and in Afro-Americans. Type 7 is located in South East Asia and southern China populations. In Asia most territories are dominated by type 2A, which is closely related

to the Japanese types Cy and My. Type 8 is a related clade in the South Pacific region. The relationships among genotypes is still under debate and it can be assumed that the number of true genotypes will increase as more populations are tested for their JCV strains (Agostini *et al.*, 2001).

Changes within the noncoding region of JCV are used for classification of three major TCR subtypes: class I, class II and the archetype. All major JCV variants isolated so far can be grouped into those basic types. The extensive heterogeneity of the JCV TCR gave rise to the hypothesis that the rearrangements might be involved as a virulence factor in the pathogenic process. It was assumed that JCV TCRs may change from a basic persisting subtype in peripheral organs to a virulent virus type growing efficiently in glial cell (Dörries, 1997). The variable TCR elements are constructed of segments with a high degree of sequence conservation that can be deleted or duplicated and rejoined to new units in individual subtypes. The junction between these domains are variable in length and preferentially used as breaking regions thus serving as a source of rearranged sequence pattern. Type I DNA

contains two TATA sequences by inclusion of the TATA box in the repeated element; type II DNA has only one TATA sequence and a 23-bp insertion, which includes a potential enhancer core sequence followed by extensively rearranged repeats. The archetype with a single promoter element and a 66-bp insertion does not exhibit any major repetitions (Figure 34.5).

Genomic comparison of JCV populations revealed that a peripheral archetype virus may invade affected tissue. Duplicated promoter elements may subsequently be generated at activated states of infection. More virulent subtypes may outgrow others in heterogeneous TCR populations, which could be disseminated from the periphery to the brain during persistent infection resulting in the homogeneous populations found in PML tissue. Immune impairment does not play a major role in the induction of rearrangements and it is not cell type dependent. At present, it is rather likely that a number of stable TCR subtypes may develop, which might than be less sensitive to rearrangements than others. Nevertheless, it also remains open whether TCR heterogeneity is a determining factor in virus growth rate or is related to pathogenicity. Along

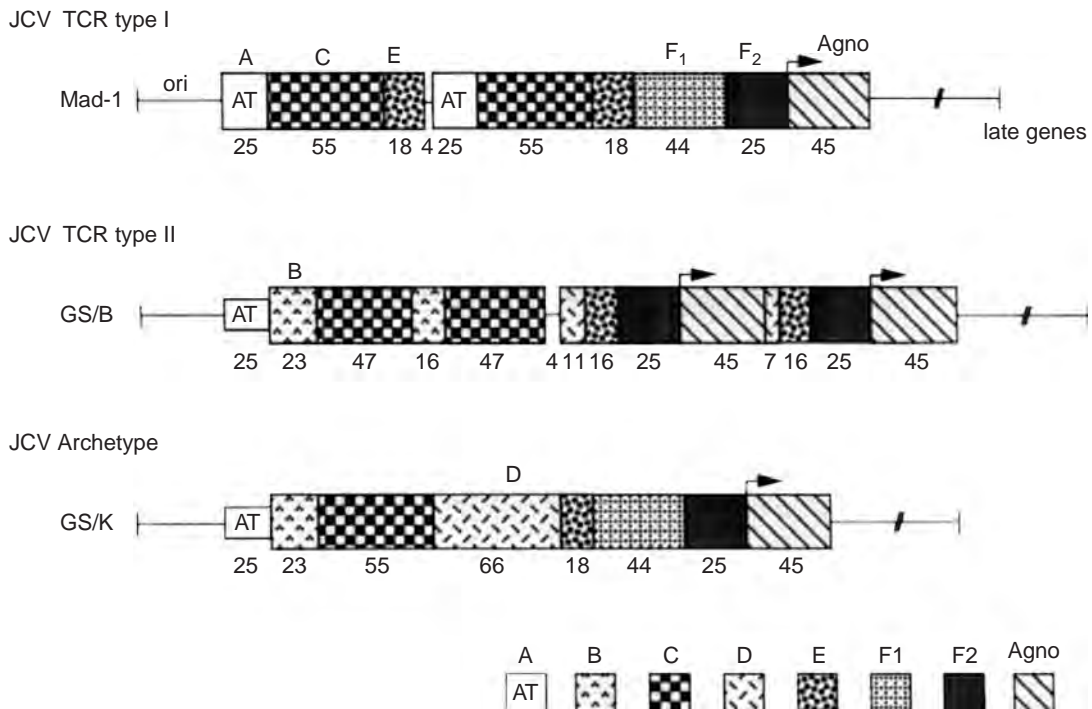


Figure 34.5 Structure of JCV transcriptional control region (TCR) subtypes. JCV TCR subtype I: American JCV prototype Mad-1; TCR subtype II: European JCV prototype GS/B. Archetype: European isolate JCV GS/K. The sequence is divided into seven boxed segments (A, B, C, D, E, F₁, F₂) differing in number and/or length. A represents the TATA box. The initiation codon for the putative agnogene is indicated by an arrow. Segment length is given in base pairs; AT, TATA box; Ori, origin of DNA replication.

these lines, in some PML patients the European/American genotype 2 was found in combination with PML type TCRs. In consequence, it was asked whether a specific genotype might constitute a pathogenic strain. However, genotype 1 and 2 can be linked to both archetypal and PML-type TCR elements in patients with PML and persistently infected individuals. Since geographic clustering of JCV TCR type II genomes cannot be excluded, it has to be further evaluated whether a specific type of JCV genotype/TCR subtype combination is dominantly associated with PML (Agostini et al., 2001; Elsner and Dörries, 1998).

Heterogeneity of BKV genomes with diverging sequences in coding regions and the TCR were regularly described. A number of different isolates and serological groups corresponding to BKV genotypes were detected. Nucleotide substitutions responsible for antigenic diversity are located in the N-terminal quarter with the BC1 and BC2 loops of the VP1 major capsid protein with changes in the amino acid sequence and potential implications for the secondary and tertiary structure of the protein. At present, BKV isolates are classified into four subtypes by genotyping and serological methods (Knowles, 2001). Subtype I is the most prevalent subtype. Based on DNA sequence mutations it has been further classified into four subgroups, assigned geographically to Africa, South East Asia, north-eastern Asia and Europe. It is thought that BKV prototype I probably emerged in Africa and might then have diverged into subgroups with population spread to other continents. Similarly, other BKV subtypes can be divided in a variable number of subgroups that can be associated with different geographic regions. Whether this is due to population isolation or to genetic drift is not yet clear. A number of attempts to associate BKV subtypes with clinical conditions or a particular group of patients remains debatable.

The TCR has been sequenced in a large number of BKV isolates. Comparable to the JCV promoter, the BKV archetype mediates the interaction with a large number of cellular transcription factors. TCR genetic variations among naturally occurring BKV isolates are due to point mutations, duplications, deletion and rearrangements of TCR regions. Changes in the biological activity of TCR variants from diseased and healthy individuals have been described, but an unquestionable association of certain TCR variants with BKV-associated diseases has not been found. The organ in which severe rearrangements may occur has not yet been defined, but a higher variability of genomic diversity appears to rise in the background of progressive disease, linking changes to higher activity of replication and its activating factors rather than to an organ-specific function. BKV and JCV have in common that the biological role of the genetic diversity of subtypes

as well as that of the heterogeneous TCRs is not yet fully understood (Cubitt, 2006).

STATE OF HUMAN POLYOMAVIRUS INFECTION

Primary Infection

Primary contact with the human polyomaviruses is generally asymptomatic. It usually occurs during childhood or early in adulthood, leading to lifelong infection in the immunocompetent host. Viruses normally undergo an initial replication cycle in cells proximal to the site of entry and prior to viraemia. The route of infection has not yet been defined, although BKV seroconversion has been associated with tonsillitis, upper respiratory tract disease, mild pyrexia and transient cystitis. Investigation of nasopharyngeal aspirates and saliva by polymerase chain reaction (PCR) occasionally demonstrates the presence of BKV DNA, but subsequent virus isolation is often unsuccessful. In contrast, urinary excretion is observed at high levels in young children early in infection. This was supported by studies on sewage in regions all over the world, revealing that human polyomaviruses are distributed to fresh waters. BKV and JCV DNA at high concentrations suggested not only urine but also human excrement as a source of viral excretion. The finding of BKV in stool and rectal swabs of young children confirmed that BKV in the gastrointestinal tract could also be a source of BKV exposure and the high frequency of transmission early in childhood.

BKV-specific IgM antibody in umbilical cord blood or in the sera of children less than two weeks of age suggests prenatal infection. This was supported by PCR detection of BKV DNA in maternal and fetal tissue. In addition, BKV DNA in male and female genital tissue on a background of a low prevalence of JCV DNA pointed to a possible sexual transmission. Family studies of BKV subtype distribution have revealed that BKV transmission is common outside the family. This is in contrast to JCV and suggests a higher transmission efficiency of BKV. In addition it almost excludes vertical transmission as a general mode of BKV transmission. Matching molecular fingerprints in both the donor and transplant recipient additionally confirmed the kidney as a source for post-transplant infection. At present the alimentary tract is proposed as the more likely port of entry (Figure 34.6).

Despite the knowledge that JCV infection is widespread in the population, almost nothing is known about how JCV is transmitted in humans. Urinary excretion is a common event, although direct infection of B lymphocytes and tonsillar stroma favours the respiratory tract. However, quantitative analysis of JCV

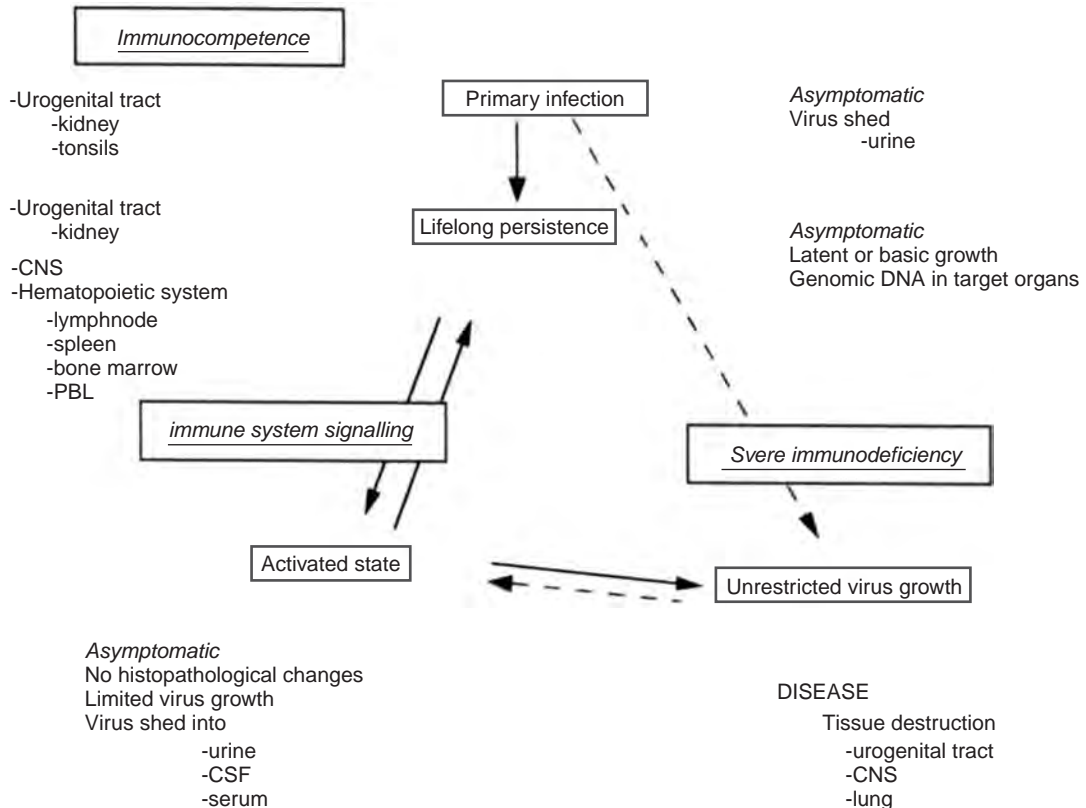


Figure 34.6 Course of human polyomavirus infection.

in saliva and oropharyngeal fluid showed that JCV DNA is only rarely present. This makes saliva and oropharyngeal fluid an unlikely source of transmission during latency. So far there is no evidence for JCV infection of the fetus. JCV-specific IgM antibodies were not detected in the cord blood of infants whose mothers had a rise of anti-JCV haemagglutination inhibition antibody titres during pregnancy. Detection of JCV in the colon suggested the lower gastrointestinal tract as a possible entry site. JCV is also distributed to fresh water by faecal contamination. Genomic typing of urinary strains in Japanese families revealed transmission within families and outside the family group. Since children have many opportunities to come into contact with urinary JCV infection, it is likely that urinary excretion is a prominent source of JCV in the human population (Berger *et al.*, 2006; Knowles, 2006).

Persistent Infection

The discovery of BKV came from the observation of cytological abnormalities in the urinary sediment of a kidney

transplant patient. Later studies revealed that BKV is an urotheliotropic virus affecting the epithelia of the renal calyces, renal pelvis, ureter and urinary bladder. It can be detected in about 20% of randomly selected autopsy samples. Analyses of prostate biopsies revealed virus infection of glandular epithelium in 60% of asymptomatic tissue, comparable to the rate of infection in the bladder. Sperm gave an incidence of 95% and cervix and vulvar tissue an incidence of 70%. In contrast, glandular tissue was free of JCV virus and in sperm 21% of the samples were positive for JCV DNA. Recently, BKV as well as JCV were detected in ocular tissue. The high rate of viral DNA in asymptomatic tissue and semen suggests that these sites might be involved in polyomavirus persistence (Berger *et al.*, 2006; Knowles, 2006).

JCV was first detected in the urinary tract of immunosuppressed individuals and pregnant women. PCR analysis suggested that the number of affected individuals closely parallels the percentage of people with serological evidence of contact with JCV. Thus the kidney is one

site of JCV infection. JCV DNA was detected more often in kidney tissue, renal pelvis and ureter than BKV, whereas BKV was predominantly located in urinary bladder samples. This characterized the renal tract as a site of persistence. The higher detection frequency in the renal medulla indicates that epithelial cells lining collective tubules are the major targets and more often subject to activation processes than other cells in the renal tract. JCV is also frequently present in the upper and lower intestinal tract, infecting the oesophageal and gastric mucosa at 76–100% normal biopsies. This demonstrates that the virus is highly prevalent in the human upper and lower gastrointestinal tract representing a new site of JCV persistence.

The dissemination of the human polyomaviruses to a wide range of organs led to the hypothesis of a possible haematogeneous spread in the host. This was supported by an early report on polyomavirus particles in lymphocytes of immunocompetent children and induced studies on a general role of lymphoid cells in the establishment of polyomavirus persistence. In tonsils BKV DNA was associated with the lymphoid tissue of Waldayer's ring, indicating involvement of lymphocytes in polyomavirus infection. Lymphoid interaction of BKV was further supported by a stimulatory effect of virus infection on human lymphocytes in cell culture, and the demonstration of specific BKV receptors on the surface of peripheral blood cells. Interestingly, only a small number of cells carried receptors for BKV. They were discussed as B lymphocytes. Nevertheless, virus protein expression was restricted to less than 1% of the cells, pointing to an attenuated replication in lymphocytes. Although the virus is able to attach and penetrate into monocytes, expression remained negative. However, the treatment of monocyte cultures with BKV antisera was followed by antibody-dependent enhancement of virus replication. Therefore it is conceivable that circulating monocytes or tissue resident macrophages in the normal individual might be permissive for polyomavirus infection.

The presence of full-length BKV DNA in leukocytes of persistently infected individuals confirmed the involvement of BKV in peripheral blood infection (Dörries, 1997). A large body of analyses has been performed, concentrating on the search for virus DNA in peripheral blood mononuclear cells (PBMCs) by PCR. The results have been highly variable, giving incidences of no presence of BKV DNA in 60% of bone marrow transplantation (BMT) patients, none in between 10% and 100% of HIV-1 patients and no virus in a group of systemic lupus erythematosus (SLE) patients. Similarly, presence of BKV DNA in 53–94% of healthy individuals contrasted a finding of no virus DNA, respectively. These discrepancies might be due to geographical localization, group selection and

technical differences, however, the amount of BKV DNA was estimated to be low, compared with that of JCV in PBMCs. Taken together, there is a body of evidence for a regular lymphotropism of BKV in the host.

Haematogeneous spread of JCV to the CNS in PML was suspected in early reports because of the multifocal distribution of JCV foci in PML. Occasional involvement of spleen and lymph node led to the assumption that lymphoid cells might also be involved in virus spread. This was confirmed by demonstrating JCV DNA and capsid protein in a small number of B lymphocytes in bone marrow and spleen of patients with HIV/PML. CD4/CD8⁺ T lymphocytes were not affected. The presence of JCV-infected PBMCs in perivascular parenchyma and in Virchow—Robin spaces further supported the haematogeneous route of entry into the brain for JCV. Although JCV DNA could not be detected in the spleen of patients with HIV-1 and controls, the detection rate of 40% in patients with PML suggests that virus DNA may accumulate in lymphoid organs. The thesis of lymphocytes as a reservoir and a vector for dissemination of JCV closed a gap in our understanding of viral pathogenesis. The role of JCV infection in lymphoid cells was further clarified by the detection of tonsillar B lymphocytes and tonsillar stroma cells as host cells for JCV infection. Cultivation of tonsillar cells followed by demonstration of JCV DNA established the susceptibility of tonsillar cells. These findings strongly not only suggest the involvement of the tonsils in primary infection but also argue for a persistent polyomavirus infection in tonsillar cell types.

The role of lymphoid cells in JCV persistence and pathogenesis was studied more intensively by asking whether JCV DNA is present in circulating peripheral blood cells in patients who are immunocompromised. The infection rates in peripheral lymphocytes of patients with PML were the highest at about 30–100%, irrespective of the underlying disease, the affected cell type, the number of infected cells or the virus load. This pointed to a regular involvement of lymphoid cells in the disease process. The rate of positive cells in risk group patients without evidence of PML was between 10 and 60%. Detection of JCV DNA in PBMCs of healthy people at similar rates demonstrated that replication leading to the detection of JCV DNA in PBMC is independent of the type of immune impairment. *In situ* hybridization added further support to the idea that the human polyomaviruses are regularly associated with PBMCs. In most PBMC samples the concentration of virus-specific DNA was estimated in the range of less than 1 genome equivalent in 20 cells (Dörries, 2006).

Further attempts to characterize virus—cell interaction addressed the question for the cellular target of JCV in

lymphoid tissue or peripheral blood. PCR analyses suggested that JCV was not associated with B cells and haematopoietic precursor cells alone. It became clear that unsorted blood cells from a normal individual might be negative for JCV, whereas sorted populations from the same patient amplified JCV DNA. This corresponds to a low virus load in PBMCs. JCV DNA could be detected by PCR in B and T lymphocyte populations, in granulocytes, in monocytes or in all cell types of a given blood sample. However, the most prominent cell population affected was the granulocyte. A cell type that may not only be specifically associated with JCV infection, but is also involved in phagocytosis. The susceptibility of haematopoietic precursor cells for virus infection was mediated by enhanced expression of the transcription protein nuclear binding factor NF-1. The expression of high levels of JCV receptor molecules on B cells and a low level replication of JCV in human B lymphocytes *in vitro* additionally suggested the involvement of those cell types in natural JCV infection. Investigations on JCV expression in haematopoietic cells suggests a preferential latent state of virus infection in PBMCs that might be activated by immune modulatory events in PML. Clearly, more experiments are necessary to understand the role of JCV DNA in PBMCs for viral persistence and possible consequences for the disease process. Although the suggestion that haematopoietic cells form a reservoir for the virion in the diseased brain might be questionable, it is conceivable that JCV-infected lymphoid cells may act as a vector for JCV CNS invasion and dissemination during persistent infection.

The pathogenic question, whether PML results from cytolytic invasion of the CNS under severe immunosuppression or as a consequence of a preceding persistent infection, remains controversial (Dörries *et al.*, 2003; Eash *et al.*, 2004). Although JCV can easily be demonstrated in disseminated areas of PML autopsy material, in non-PML adults JCV DNA sequences were rarely detected. JCV obviously has no topographical preference, because dissemination patterns are comparable to those in PML. JCV DNA molecules in brain tissue were exclusively episomal. The amount of virus-specific DNA in asymptomatic individuals was in an estimated range of 1–100 genome equivalents per 20 cells or lower, thus confirming that it is restricted to isolated cells and most likely represents chronic infection and not the early stages of disease.

BKV-associated CNS disease (Vallbracht *et al.*, 1993) demonstrates that BKV replicates occasionally in the CNS. Attempts to detect BKV in asymptomatic brain tissue in different groups of patients often were not successful. Although considerably less frequent than JCV, isolation of full-length BKV genomes from CNS tissue and sequencing of new genomic BKV DNA subtypes was

in line with a persistent type of BKV infection in the CNS (Elsner and Dörries, 1998; White *et al.*, 1992).

The findings prove that human peripheral polyomavirus infection is associated with subclinical virus entry into the CNS, probably long before the development of clinically overt PML. Although the cell type or types targeted by the virus are not yet characterized, productive *in vitro* JCV replication in microvascular endothelial cells indicates that JCV may also infect endothelial cells, thereby crossing the blood—brain barrier (Chapagain *et al.*, 2007). Irrespective of the route, virus activation during persistence may lead to an increased number of infected cells and a higher detection frequency in cases of severe immunosuppression. This being in line with the assumption that impairment of immune competence favours involvement of the CNS in polyomavirus infection.

Concomitant JCV and BKV Infection

Double infection of JCV and BKV was established after organ transplantation in HIV-1 infected patients, in pregnancy and in immunocompetent individuals by urinary excretion or antibody rise against both viruses. Molecular detection of JCV- and BKV-specific DNA confirmed that concomitant persistence frequently occurs in kidney tissue. Extensive homologies of the genomic structure, similarities of virus spread and state of infection very early started the discussion on possible other sites of dual JCV and BKV infection. After PBMCs were found to be a target of polyomavirus infection, the presence of JCV and BKV DNA in blood cells of the same individual was not surprising. Concomitant infection was evidenced molecularly in healthy and immunosuppressed individuals. Although BKV was not expected to invade the CNS at a high rate, the cell specificity of BKV is less stringent than that of JCV, therefore several laboratories investigated dual infection in the CNS. Cloning from CNS gene libraries and PCR revealed frequent BKV dissemination to the brain. Indirectly, BKV infection of the human CNS was confirmed by the report of a subacute BKV-associated meningoencephalitis in a patient with AIDS. The physical state and genetic complexity of BKV genomes was comparable with that of JCV DNA, however, the low concentration of brain-derived BKV-specific PCR products suggests a considerably lower activity of BKV in the CNS than that of JCV. This gives strong evidence for concomitant polyomavirus infection in the CNS and demonstrates that not only JCV but also BKV is neurotropic in the human host.

Asymptomatic Activation of Infection

Transient polyomavirus viraemia probably occurs at the time of primary infection; however, in most instances

the presence of virus in the urine is due to activation processes. JCV DNA was detected in the urine of about 30% of American and 60% of Japanese and European non-immunocompetent individuals. Incidence of excretion was dependent on age, with lower rates in the young, gradually increasing in older age. Although BKV shedding is frequently lower than that of JCV, BKV follows the early peak of primary infection in early childhood with a higher incidence of viruria. Viruria declines until the third decade of life and then increases gradually with age reaching 44% as the highest level in the ninth decade. In contrast, JCV reaches more than 70% in the same age group. Pregnancy is among the most common conditions that have been linked to viral activation. Incidence of viruria as detected by periodic cytological examination was about 3–7% in case of JCV, and up to 54% in case of BKV. The onset of viruria was late in the second trimester and during the third trimester of pregnancy. In excretors, once it was established virus shedding continued intermittently to term and then ceased in the post-partum period. The detection rates parallel high or rising antibody titres in a comparable study population, and therefore probably represent the true rate of activated persistent infections. Renal transplant recipients experienced viruria at a considerably higher rate. Duration periods may last over month to even years. In bone marrow transplant recipients most viruria was due to BKV with an incidence of up to 87% in the post-transplant period. Although these findings point to a significant role of the immune state for the level of polyomavirus expression in the kidney, an enhancement of urinary excretion by HIV-1-induced immunosuppression was not detectable. Similarly, PML patients do not necessarily have concomitant JCV viruria. Aggressive chemotherapy does not increase virus frequency, and in other immunosuppressive diseases viruria can be intermittent with sparsely distributed infected cells in cytological positive urine, similarly pointing to a rather low rate of virus production. Because of the high frequency of JCV and BKV excretion throughout adulthood, the authors suggest that JCV infection regularly may not be in a latent but in a productive persistent state. Since most data were accumulated by less sensitive methods, it is conceivable that virus expression is permanently maintained at a basic level (Dörries, 2006; Zhong *et al.*, 2007).

Activation at other sites of persistent infection is less intensively examined. This is due to reduced virus expression in asymptomatic transient activation states and the low virus load in persistently infected healthy individuals. In patients with HIV/PML mononuclear cells in PML tissue have been characterized expressing JCV-specific proteins. Thus it appears likely that a JCV infection in PBMCs is activated under as yet unknown circumstances. Besides the close association of viral DNA with PBCs,

circulating JCV DNA was detected in cell-free plasma. The rate of viraemia varied from no virus present to 4% in patients with SLE or control groups including healthy individuals. Virus load is in the range of 5×10^2 copies per μg DNA. In transplant patients between 0 and 28% of individuals had JCV viraemia alone. The virus load was in the range of $10^3 - 10^4$ genomes per ml serum. In HIV-1-infected patients rates from 4 to 23% were reported. Although no correlation with immunodeficiency could be calculated, the incidence of detection appeared to be slightly higher in patients with PML. Often the presence of JCV DNA in PBCs and free circulating virus was not correlated, suggesting that either JCV-carrying cells are quickly eliminated or there is source for JCV in plasma other than infected PBCs (Dörries, 2006).

Free circulating BKV is routinely detected in patients with BKV-associated diseases; in healthy individuals viraemia was reported in up to 27.6%. Viraemia does not normally exceed a virus load of about 10^2 viral genome copies per ml in individuals without BKV disease. In contrast, detection rates in prospective studies of renal transplant recipients were reported to be up to 75%, the viral load was considerably higher, ranging from 10^3 to 10^6 viral genome copies per ml and detection of BKV in plasma closely follows the detection in urine. In addition, BKV load decreased not only in urine but also in plasma after successful therapy. This indicates that activated BKV infection in the kidney is related to virus load in plasma and may therefore contribute to the circulating virus load (Hirsch *et al.*, 2005).

The increasing incidence of JCV DNA in brain tissue with age and in patients with malignancies point to viral activation in the CNS. The detection of virus-specific protein in a limited number of glial cells in non-PML brain and PCR amplification of JCV DNA in the cerebrospinal fluid (CSF) of the same patient group similarly points to asymptomatic JCV activation in persistently infected brain tissue. JCV DNA was detected in the CSF in immunocompetent individuals with different neurological symptoms and/or patients with HIV-1 infection with highly divergent results (0.22–100%). Nevertheless, in the high-risk groups active infection appears to be more frequent than in immunocompetent individuals. Although an influence of the immunosuppressive state as defined by the $\text{CD4}^+/\text{CD8}^+$ T lymphocyte ratio in AIDS patients was not found, the role of immune modulatory mechanisms for JCV activation became clear when JCV DNA was detected at varying rates in patients with multiple sclerosis or those receiving different immunomodulatory or anti-inflammatory therapies. Although the amount of virus-specific DNA is considerably lower in persistent

CNS infection, it is similarly distributed as typically described in PML tissue. This is in line with rare and transient activation events. In addition, replication is probably severely restricted and thus remains limited to few isolated cells. In cases of extensive immuno-impairment, however, it is likely that virus activation leads to an increasing number of infected cells. Whether a lifelong accumulation of JCV in the CNS might eventually contribute to uncontrolled cytolytic virus growth and PML can probably only be answered in an appropriate animal model (Dörries, 2006).

BKV DNA in the CSF was first detected in immunosuppressed patients with BKV-associated CNS disease (Vallbracht *et al.*, 1993). Consequently, the presence of BKV in CSF is similarly indicative for an active BKV infection as it is in case of JCV infections. Studies on patients with PML occasionally reveal the presence of BKV DNA in CSF, pointing to a possible persistence and asymptomatic activation of BKV in the CNS. Although co-infection of both polyomaviruses is widely accepted, it can be anticipated that BKV dissemination to the CNS and shedding to the CSF is generally a rare event.

If the current knowledge is summarized, it must be assumed that activation of BKV and JCV infection is species-specific and as a result of immune system alterations induced by pregnancy, older age, malignant tumour growth or AIDS. Some of those individuals may undergo sporadic activation as a consequence of their genotypes or of incidental transactivation events by other viruses. In general, however, virus growth appears to be dependent on the impairment of immunological control resulting in a differentially regulated activation pattern in target organs. Although related mechanisms are so far unknown, the higher frequency of deficiencies correlated with T-cell function in patients with PML confirms deficient cellular immunity as a major virulence factor.

Polyomavirus-associated Diseases

Activation of huPyV infection may either represent transient asymptomatic events or pathological processes. The induction of fatal disorders, however, is almost exclusively observed under long-lasting severe impairment of the immune system and immunomodulatory therapies increase the incidence of polyomavirus-associated disease, thereby confirming a close relationship of virus growth and immunomodulation. BKV is a urotheliotropic virus almost exclusively linked to urogenital tract diseases. Mild courses are reported in primary BKV infection, whereas BKV disease is associated with persistent BKV infection. Virus-associated pathogenic effects are observed in polyomavirus-associated nephropathy (PVAN)

and ureteral stenosis as complications of renal transplantation, as well as in haemorrhagic cystitis after BMT and human stem cell transplantation (HSCT) (Table 34.2).

Haemorrhagic cystitis is a late-onset complication and a common cause of prolonged hospitalization, morbidity and occasional mortality in patients undergoing bone marrow or HSCT. Recipients of autologous tissue are at a particular risk. Its incidence varies from 7 to 68% of BMT cases. The pathogenesis of post-engraftment haemorrhagic cystitis includes a large number of risk factors, with the activation of BKV infection being the most common and consistent risk factor. It is characterized by painful haematuria due to haemorrhagic inflammation of the urinary bladder mucosa resulting in damage to the bladder epithelium and blood vessels. The manifestations vary from microscopic haematuria to severe bladder haemorrhage. Haemorrhagic cystitis is divided into four categories according to severity of haematuria from grade 1 to grade 4. In most cases haemorrhagic cystitis resolves spontaneously, but in some cases BKV excretion may continue, followed by severe manifestation of haemorrhagic cystitis until death from extensive chronic graft-versus-host disease (GVHD).

The association of BKV with haemorrhagic cystitis is not only determined by the large amounts of virus ($>10^{12}$ copies per ml of urine) but also by the pattern of viruria. Two patterns of excretion can be observed. In about 50% of patients BK viruria occurred at about two to three weeks post transplantation. Interestingly, in this group all patients with haemorrhagic cystitis had a peaking of BKV viruria (more than 3-log increase over baseline level), which invariably preceded the onset of haematuria by a median of 29 days. Moreover at the time of haematuria, BKV viruria was already on the decline or had reached basic levels. In a second pattern, a persistent low level BK viruria without peaking occurred and in this group none developed haemorrhagic cystitis. Whether BK viraemia is associated with the risk of developing haemorrhagic cystitis and can be used as a second disease marker remains disputable. Recently, elevated pretransplant BKV-specific antibody titres of the recipient were reported as an additional discriminating factor for haemorrhagic cystitis (Wong *et al.*, 2007). The definition of risk factors in addition to serial quantification of BKV urinary load is an important aim for the development of early antiviral therapeutic strategies.

The emergence of PVAN during the last decade is well documented by the increase of prevalence rates from 1% in 1995 to about 8% in 2003. The majority of cases occur within the first year after renal transplantation, but approximately a quarter of cases are diagnosed later. Allograft loss due to PVAN ranges from 10% to $>80\%$ of cases, but may be lower in centres with active screening

Table 34.2 Human polyomavirus-associated diseases

Patients	JCV infection		BKV infection	
	Disease	Cell type involved	Disease	Cell type involved
Immunocompetent	None observed		Mild respiratory disease Mild pyrexia Transient cystitis	
Immunocompromised Urogenital system	Nephropathy	Tubular interstitial cells	Nephropathy (PVAN) Ureteral stenosis Haemorrhagic cystitis	Epithelial cells Fibrocytes Bladder epithelial cells
Lung	None observed		Interstitial pneumonitis	Epithelial cells Pneumocytes Endothelial cells Fibrocytes
CNS	Progressive multifocal leukoencephalopathy (PML)	Oligodendrocytes Astrocytes Rare inflammatory infiltrates	Subacute meningoencephalitis	Epithelial cells Fibrocytes Endothelial cells Astrocytes
	Immune reconstitution inflammatory syndrome (IRIS)	Same as in PML and pronounced inflammatory infiltrates		Ependymal cells Peripheral blood mononuclear cells
	Granule cell neuropathy (GCN)	Granule neurons		
	Meningitis	Cell type not defined		
	Meningoencephalitis	Cell type not defined		

and intervention programmes. Molecular studies indicate that most of the cases of PVAN are caused by BKV. Only in less than 3% of cases JCV has been detected. Clinical features may mimic graft rejection or drug toxicity, but histopathological examination almost always demonstrate BKV infection. BKV-associated pathologic lesions have a characteristic appearance with streaky fibrosis of the renal medulla and circumscribed cortical scars. Microscopically, sclerosed glomeruli, atrophic and necrotic tubules and interstitial fibrosis associated with mononuclear cell infiltration can be observed. Fibrocytes and tubule cells are often enlarged with intranuclear BKV inclusions extruding from the epithelial layer into tubular lumen causing marked denudation of basement membranes. Polyomavirus-induced tubular injury is viewed as a major morphological correlate for allograft dysfunction. The pathogenesis of PVAN involves the interaction of multiple risk factors. Intense immunosuppression is perceived as a major risk factor. With few exceptions, PVAN has been diagnosed in patients receiving a maintenance ther-

apy consisting of three of four drug classes (calcineurin inhibitors, antimetabolites, mTOR inhibitors and corticosteroids). The majority of patients received combinations containing tacrolimus or mycophenolate mofetil (MMF), which were used concurrently in >50% of the cases. An increased risk for BKV replication and PVAN has been reported for tacrolimus—MMF—corticosteroid combinations (Hirsch *et al.*, 2006; Roskopf *et al.*, 2006).

Strong evidence for BKV-induced systemic and CNS disease originally came from patients with AIDS. Histopathological evaluation revealed an association of BKV with affected lung, kidney, and CNS tissue. The major pathological findings were tubulointerstitial nephropathy, interstitial desquamative pneumonitis and subacute meningoencephalitis. In the kidney, alterations principally consisted of focally accentuated tubular necroses. Virus was detected in epithelial cells along the entire nephron. Alterations in the lung were characterized by intra-alveolar aggregates of desquamated pneumocytes with virus in exfoliated pneumocytes, in epithelial and

in smooth muscle cells of the bronchioli. Occasionally, isolated endothelial cells in the lung carried virus protein. A common feature in both organs was focal interstitial fibrosis with a mild inflammatory response. Whereas fibrocytes were infected, inflammatory cells were free of virus products (Vallbracht *et al.*, 1993).

In the CNS no pathological changes apart from mild inner atrophy were described. Mononuclear cells indicating chronic inflammation loosely infiltrated thickened fibrotic leptomeninges. In cortex and adjoining white matter oedematous tissue alterations were found. Reactive astrocytes were localized in the outer layers of the cerebral cortex. The ventricular system exhibited focal degeneration of its ependyma and spongiform destruction of subjacent brain tissue. The choroid plexus disclosed fibrosis of stroma and atypical epithelial cells. In some areas necrosis and exfoliation of the plexus epithelium occurred. Small infiltrates were seen in association with the lesions. The dominant target cells of BKV infection were fibroblasts of the loose reticular connective tissue, endothelial and smooth muscle cells of blood vessels, astrocytes and infiltrating macrophages in pons and medulla oblongata. Epithelial cells in the choroid plexus and astrocytes of the subependymal brain tissue were additionally infected. The only glial cell type involved in BKV-associated CNS disease was the astrocyte, whereas oligodendrocytes and nerve cells were not affected. The detection of a BKV-associated disease affecting kidney, lung and the CNS is in line with the involvement of those organs in BKV persistence.

In contrast to BKV, JCV was never observed as an aetiological agent in urogenital tract or lung disease. The only and most prominent disease associated with JCV is the CNS disorder PML (Berger and Major, 1999). Although SV40 was described as the cause of PML in three American and Japanese patients, it later became clear that most probably JCV was the only agent responsible for the disease. PML is a demyelinating disorder occurring as a late complication of pre-existing systemic diseases that impair immunological competence. Several years of treatment precede PML in diseases such as systemic rheumatic diseases, lupus erythematosus, chronic asthma, sarcoidosis and chronic polymyositis. The use of more potent immunomodulatory and immunosuppressive therapies adds to risk of increasing numbers of PML cases in as yet unrelated basic disorders. Before the AIDS era malignant proliferative diseases were the dominant basic disorders in PML in about half of the cases. In HIV-1-infected patients a steadily increasing number of cases was associated with AIDS prior to the introduction of highly active antiretroviral therapy (HAART). Although longer PML survival times can be observed under HAART, the rate of PML patients remains stable at about 5% of patients with AIDS.

Table 34.3 Causes of JCV activation and induction of PML prior to the AIDS epidemic and immunomodulatory therapies

Conditions associated with activation of persistent infection	PML (% of cases ^a except AIDS)
Age	None
Pregnancy	None
Inflammation	None
Transplantation	Rare
Diabetes	Rare
Lymphoproliferative disease	62.2
Myeloproliferative disease	6.5
Carcinomatous disease	2.2
Immune deficiency states	16.1
Granulomatous/inflammatory disease	7.4

^aSource: From Brooks, B.R. and Walker, D.L. (1984) Progressive multifocal leukoencephalopathy. *Neurologic Clinics* 2(2), 299–313.

In addition, a higher frequency of PML can be observed in correlation with immunomodulatory therapies and is probably due to higher JCV activation rates (Table 34.3).

The onset of classic PML is often insidious. In the earliest manifestation multiple pinhead-sized demyelinating lesions are described beneath the cortical ribbon. New small foci are continuously added in neighbouring tissue as the growth centres of new lesions. This concept of the histologic evolution of the disease is supported by the clinical evolution: the onset might be gradual, but each new functional impairment becomes progressively more severe. Once clinical signs appear, the disease usually progresses steadily. Early neurologic symptoms regularly indicate multiple disseminated lesions in the brain. Extent and topography of lesions correlate well with duration and symptomatology of the illness (Table 34.4).

The pathognomonic feature of the disease is the striking alteration of oligodendrocytes with eosinophilic inclusion bodies in the peripheral rim surrounding the zone of myelin loss. The central area is composed of reactive astrocytes including giant cells in mitosis and astrocytes resembling malignant cells of pleomorphic glioblastomas. The basic cause of tissue destruction is a cytolytic JCV infection of the oligodendrocyte resulting in loss of myelin, tissue breakdown and impairment of brain function. Studies on the mechanisms involved revealed the absence of apoptosis-related changes in the nuclei of oligodendrocytes in areas adjacent to virus particles. The lack of apoptotic markers in PML suggested non-apoptotic cell death and necrosis as the major mechanism of oligodendrocyte lytic destruction. Recently, it was detected that expression of the normally dormant anti-apoptotic protein survivin is induced by JCV infection, probably leading to

Table 34.4 Development of PML lesions*I Early lesion*

Altered oligodendrocytes in areas of demyelination
 Nuclei enlarged (2–3×)
 Regular chromatin pattern is lost
 Nuclei tint deeply with basophilic dyes
 Irregular basophilic nucleic inclusions
 Seldomly activated pleomorphic microglia
 Pinhead size (mm) lined up the cortical ribbon

II Aging lesion

Reduced number of altered oligodendrocytes in the centre of demyelination
 Shrinkage of nuclei at the rim of lesions
 Widely distributed larger foci with coherent tissue

III Late lesion

Altered oligodendrocytes at the peripheral advancing zone surrounding the area of myelin loss
 In the centre
 Very sparse or absent oligodendrocytes
 Singly scattered reactive hypertrophic astrocytes
 Giant astrocytes in mitosis
 Astrocytes resembling cells in pleomorphic glioblastomas
 Forming irregular necrotic patches (cm)

inhibition of apoptosis during JCV infection. As a consequence, increased survival of JCV-infected glial cells would also increase the risk of rapid disease progress, whereas release could be through necrosis or lysis of cells (Seth *et al.*, 2004). Viral expression products and virions are found in nuclei and cytoplasm of the oligodendrocytes. Bizarre astrocytes may contain JCV; however, it appears likely that astrocytes represent a semi-permissive cell type mediating reduced JCV expression or, alternatively, being sensitive to virus transformation.

In contrast to classic PML, in patients with AIDS fronto-parietal and grey matter involvement increased. However, there are cases reported with only one lesion present. An inflammatory reaction with perivascular mononuclear cell infiltrates is often observed. Mononuclear cells in the Virchow—Robin spaces occasionally contain JCV-specific DNA and capsid antigen, and in the subcortical white matter adjacent to blood vessels the density of infected cells appears to be increased. JCV-infected cells have also been detected beneath the ependymal layer. This indicates a close topographical association between the infected cells and the ventricular system (Mazlo *et al.*, 2001).

The duration of disease after the onset of neurological symptoms is reported to be on average four to six months (Berger and Major, 1999). However, there have been cases of more than 12 months, with an intermit-

tently progressive or subclinical course over more than years. Those cases are more often found in combination with HIV-1 infection under HAART therapy. Since the introduction of HAART the definition of HIV/PML cases has had to be adapted to reflect changes in the disease pattern probably resulting from HAART-induced immune reconstitution in about one half of the patients. For these groups a new terminology is proposed for disease outcome. It is based on diagnostic criteria for definite, probable and possible PML with a definition of PML outcome based on disease activity. Disease progression can be classified as 'active' or 'inactive' based on clinical, radiological, virological and pathological criteria. Patients in 'active PML' have a high diagnostic evidence of disease activity throughout the course of the illness over a few months. Patients with 'inactive PML' lack evidence for disease activity. Stabilization may occur at any time of disease progression. Usually it occurs in a few months after first diagnosis and there is virtually no progression over years of follow-up even following withdrawal of HAART (Cinque *et al.*, 2003).

Although HAART influences the outcome of PML often positively, there are cases described of new onset or clinical worsening of PML shortly after initiation of HAART. This occurs in a setting of recovery of the immune control as described by increase of CD4⁺ T cells and decrease of HIV-1 viral load. It is usually associated with an inflammatory reaction in brain lesions and has been called immune reconstitution inflammatory syndrome (IRIS). Usually the resolution of the inflammatory phase results in clinically improvement. Another syndrome, JCV granule cell neuronopathy (GCN), is distinct from PML. In an HIV-1-infected patient JCV caused a lytic infection of cerebellar granule cell neurons associated with cerebellar symptoms. Furthermore, a case of JCV meningitis and a meningoencephalitis occurred in patients with systemic lupus erythematosus and an immunocompetent girl (Koralnik, 2006). These cases demonstrate that new courses of persistent and activated JCV infection can be expected and only the use of standardized terminology in the description of clinical and biological studies will allow us to understand the mechanisms of pathogenesis and therapeutic intervention.

Oncogenicity of the Human Polyomavirus

Cancer is thought to progress through multiple stages and with each step cells with increasing malignant phenotype may arise. Progression can be driven by mutations in tumour suppressor genes or oncogenes; it can also be dependent on newly acquired transforming proteins. These genes encode proteins regulating genome stability, cell cycle proliferation or apoptosis. The polyomaviruses with the large TAg provide a prototype of viral oncogenes.

The first 121 amino acids are the shortest fragment to induce transformation. Here TAg interferes with two key tumour suppressor proteins that regulate cell cycle progression, pRB and p53. Activation leads to elevated steady state levels of p53 ending in a block of the cell cycle, and inactivation of pRB family members promotes proliferation. In addition, p53 protection against DNA damage by inhibiting cell cycle progression and apoptosis activity is prevented. Binding to β -catenin and its translocation to the nucleus enhances expression of genes such as c-myc and cyclin D1 involved in promotion of the cell cycle. JCV TAg also binds to insulin receptor substrate 1 (IRS-1), inducing its translocation to the nucleus. This interferes with Rad51-dependent DNA repair and appears to be involved in the activation of the apoptosis inhibitor survivin. JCV attempts to prevent cells from apoptosis by activating the survivin pathway and this has been demonstrated not only in clinical samples from PML but also in glioma cell lines. Alternative splice variants of JCV TAg yielding truncated proteins have been shown to interact differentially with proteins of the retinoblastoma family, thereby possibly contributing to cell transformation. Small tAg is probably not essential for the viral life cycle, however, it is likely that tAg is an oncoprotein that stimulates multiple growth-promoting pathways. It binds to the negative regulator protein phosphatase 2A (PP2A), a key regulator with multiple functions in cellular signalling including cell cycle progression and transformation, and tAg is associated with the molecular chaperone CDC37 which functions in G₁/S cell cycle transition and activation of the Ras-MAPK pathway. The late leader protein agnogene may have profound effects on the virus—cell interaction in the absence of other viral proteins. This includes dysregulation of cell cycle progression, irregular response of the cells to DNA damage, increased chromosome damage and impaired DNA repair. Besides the role of the regulatory proteins in the tightly controlled replication cycle, it can be assumed that they are able to transform cells by disrupting cellular signalling pathways. They also have a mutagenic effect on cellular DNA and promote karyotype instability. Since elimination of mutated cells is impaired, it appears likely that these functions may contribute to the development of neoplasia in a nonpermissive setting (Frisque *et al.*, 2006; Khalili *et al.*, 2005; White and Khalili, 2006).

The discussion of a possible oncogenicity of a huPyV in humans had its origin not only in the oncogenic potential of the early proteins, but also in the bizarre pleomorphism of astrocytes within lesions of PML. This was described as a hallmark of PML in the initial description of the disease and later oligodendroglioma and multifocal glioblastoma were reported, corresponding topographically to the demyelinated lesions in PML. Thus onco-

genicity of JCV and BKV was examined almost from the beginning in experimental animal systems. Developing tumour types reflected the cell type specificity of the viruses. Corresponding to the specificity of JCV for glial cells predominantly CNS tumours were observed in cerebrum, cerebellum, in the brainstem and in the spinal cord. Mesenchymal tumours within cerebral meninges were classified as malignant meningiomas. Ependymomas are the dominant type of intraventricular tumours, and occasionally choroid plexus tumours developed. The most common neoplasms were medulloblastomas followed by malignant astrocytomas, glioblastoma multiforme, neuroectodermal tumours and pineocytomas. Regularly, the virus genome was integrated, JCV TAg was expressed and rescue of JCV from tumour tissue was occasionally successful.

The oncogenic potential of BKV appears to be less pronounced. The most prominent cell types affected by BKV infection in humans—epithelial cells, fibrocytes, ependymal cells, astrocytes and endothelial cells—are reflected by different tumour types in experimental animal models. Choroid plexus papillomas and papillary ependymomas were observed alone, whereas sarcomas and ependymomas were also associated with insulinomas, osteosarcomas and tumours of the intestine. In the tumours BKV DNA is either integrated in the cellular genome or in a free episomal state. Most cells express nuclear TAg and virus can often be rescued by cell fusion with permissive cells. The spectrum of BKV-induced tumour types is comparable in all sensitive animal species and it is thought that human polyomaviruses might also be responsible for the respective tumour types in their natural host. However, despite the oncogenicity of these viruses in animal systems, association with human tumours remains controversial.

TAg expression was analysed immunohistologically in medulloblastomas, oligodendrogliomas, astrocytomas, glioblastomas, ependymomas, choroid plexus and urinary tract tumours. The results were inconsistent. Antibody titres to TAg in sera from tumour-bearing patients exhibited no significant difference to sera from normal people. JCV DNA sequences have been detected so far in up to 57% of oligodendrogliomas, 75% of various astrocytomas, 60% of glioblastomas, 83% of ependymomas and 87% of medulloblastomas. Examination of the p53 level in tumours expressing TAg suggested close association of both proteins within the tumour cell. Recent analyses of individual tumour cells by laser capture microdissection revealed the presence of JCV DNA sequences from the early, late and control region in addition to TAg and agnogene expression. No capsid protein was observed, thereby excluding productive infection and enforcing the role of JCV in tumour development (Pina-Oviedo *et al.*, 2006; White and Khalili, 2005).

Human polyomavirus DNA and protein has also been detected in lung tumours. However, virus was also present in normal tissue and it remains unclear whether this represents a persistent type of infection. Search for viral DNA in B-cell lymphomas is similarly controversial and needs to be further clarified. In gastrointestinal tumours JCV DNA and proteins have been detected. Most colorectal cancers are characterized by chromosomal instability and it is hypothesized that JCV TAG might be responsible for this effect. Since JCV DNA was detected at similar rates in normal tissue, the colon is accepted as a new site of persistence. Although no evidence of an excess risk for colorectal cancer in JCV- and BKV-seropositive individuals was detected in a prospective study, the molecular findings are in line with a potential role of JCV in the development of gastrointestinal tumours (Shin *et al.*, 2006; Zheng *et al.*, 2007).

BKV DNA was only occasionally detected in a series of human tumour types. This included brain tumours, osteosarcomas, insulinomas and Kaposi's sarcoma, pancreas and various urinary tract neoplasms. Analyses of the state of BKV DNA revealed that mostly episomal virus DNA and virus could be rescued. This is indicative of a persistent state of infection, rather than an association with transforming activities, although BKV DNA could also be present in the integrated state. TAG expression in neuroblastomas and its interaction with p53 has also been described. Recently, a potential role of BKV in prostate and bladder cancer was discussed. Whereas a possible association of BKV with bladder cancer remains open, analyses of prostate cancer tissue and precursor lesions have demonstrated the presence of BKV DNA at higher frequency than in normal tissues. In addition TAG co-localizes with p53, thus underlining a potential role of BKV in prostate cancer (Das *et al.*, 2004; Moens and Rekvig, 2001). Detection of virus DNA in tumour tissue in organs also harbouring persistent virus makes a differentiation of persistent infection and transformation difficult. Furthermore, it cannot be ruled out that polyomaviruses interact synergistically with other factors to induce malignant growth in human cells. In that case, the presence of virus DNA in tumour tissue could be a consequence of an event occurring early in the history of tumour development, when the cell cycle might be deregulated by TAG and its interaction with cellular factors. After genetic alterations are established, the virus genome and transcription products might be dispensable. In such a setting the role of huPyV remains uncertain and a more detailed analyses will be required to answer the question whether or not JCV and BKV are involved in human tumorigenesis.

DIAGNOSTIC EVALUATION OF POLYOMAVIRUS-ASSOCIATED DISEASE

Diagnosis of PML by Biopsy

The only disease linked to JCV is PML. The classic method of PML diagnosis involves neurological evaluation and neuro-imaging of the brain followed by definitive laboratory diagnosis on biopsy material. Topographical selection of samples for stereotactic biopsy at the outer rim of lesions is followed by the identification of virus products and typical cellular changes in glial cells. The extraordinary multiplication rate of the virus in diseased tissue allows detection of viral nucleic acids and proteins by classical immunohistological methods. Molecular detection of JCV by PCR in biopsy samples is usually confirmed by histopathology. Essential pathological criteria for the term 'active PML' in AIDS are the presence of JCV-infected glial cells, bizarre astrocytes and lipid-laden macrophages in the context of demyelination. In contrast, 'inactive PML' is characterized by the presence of demyelinated areas without JCV proteins by immunohistochemistry. Regression of astrocytic and oligodendroglial changes, disappearance of macrophages and JCV proteins have already been described in biopsy-proven PML in the pre-AIDS era (Cinque *et al.*, 2003). In the asymptomatic state of infection virus DNA is regularly not detectable by diagnostic PCR analysis of single brain samples. If the infection is activated in immuno-impaired individuals, the virus load might increase and could then be detectable. However, compared with the thousands of genome equivalents present in PML tissue the amount of virus is considerably lower. Consequently, in cases with doubtful results quantitative PCR analysis of any type differentiates a persistently activated and a PML-associated JCV infection. At present, a combination of stereotactic biopsy and PCR techniques ensures a rapid diagnosis of PML with the highest sensitivity and specificity available.

Demonstration of Polyomavirus DNA in CSF

In view of the higher frequency of PML in AIDS patients and the future development of immunomodulatory therapies, less-invasive methods with a comparable detection rate in early diagnosis are frequently used. Although the concentration of JCV in affected tissue is extraordinary high, virus load in CSF is considerably lower and qualitative and quantitative PCR are the only reliable techniques available for virus detection. PCR on CSF often caused divergent diagnostic results. Reports on the test specificity vary from 10 to over 90%. In particular, the sensitivity of qualitative test systems reduced the prognostic significance of the technique. High-sensitivity PCR on the CSF of high-risk HIV-1 patients without PML resulted in JCV

amplification and an increasing number of false-positive PML diagnoses. This finding can be explained by asymptomatic activation of JCV CNS infection in states of immunocompromise. Whether this is indicative of an early state of disease or might represent a timely restricted activation is not yet known. In PML, CSF samples were tested negative in autopsy verified cases. Serial sampling revealed that early in disease the virus load can be low, often almost undetectable, and may increase in the late stages of disease. In these cases repeated CSF sampling at time intervals depending on the clinical and radiological progression of the disease is an essential factor for virus detection. Nevertheless, there are cases that remain negative even in the late stages of disease. Course of disease history is comparable to that of patients shedding JCV either continuously or intermittently into the CSF. However, the virus load appears to be dependent on the individual state of cellular immunity. This is further confirmed by long-term surviving PML patients, whose virus load was first diminished considerably and was eventually eliminated by improvement of the immune state by therapeutic intervention (Eggers *et al.*, 1999).

Modern quantitative PCR techniques allow specific detection ranges over a 7-log dynamic range (McNees *et al.*, 2005). This was the basis for therapeutic monitoring in cases where the immunological state could be improved. It became clear that in classical PML cases the virus load may vary between 1.5×10^2 and 324×10^6 . A correlation of virus load with the volume of CNS lesions could not be established, whereas survival time appears to be inversely related to virus load. In HIV/PML patients on HAART, analysis of sequential CSF samples is even more complicated, at present leading to an increased prevalence of undiagnosed PML cases even after analyses of multiple CSF samples. In patients with diagnoses other than PML, virus load is regularly very low in comparison with virus load early in disease. This argues for the analyses of sequential samples in cases of doubt (Alvarez-Lafuente *et al.*, 2007).

In view of the asymptomatic activation of JCV persistent infection under immunosuppression and the individual responses to immune modulatory therapy in risk group patients, PCR must be understood as a supporting diagnostic tool. The definition scheme for PML is based on criteria for 'definite PML' involving (i) progressive uni- or multifocal neurological disease, (ii) typical magnetic resonance imaging (MRI) lesions associated with (iii) typical brain biopsy features with confirmation of JCV-specific products. 'Probable PML' represents cases with typical clinical and imaging findings, with amplification of JCV DNA in CSF, but without bioptic diagnosis. Absence of histological confirmation and JCV demonstration in

CSF in a setting of PML typical clinical and radiological findings leads to a diagnosis of 'possible PML'. If either pathological diagnosis can be performed, or JCV can be detected in CSF in serial CSF samples this may later classify for a 'definite PML' diagnosis. Incidentally discovered white matter lesions or JCV amplification in CSF without active clinical or radiological findings should not be considered as representative of PML (Cinque *et al.*, 2003).

Diagnosis of BKV-associated diseases

Diagnosis of BKV-associated diseases is based predominantly on detection of viraemia, viruria or histopathological demonstration of BKV in tissue. In case of haemorrhagic cystitis first diagnosis is based on clinical symptoms. However, demonstration of BKV involvement in the disease process is complicated by asymptomatic activation of BKV infection in 50–100% of patients, whereas only 5–40% of them develop haemorrhagic cystitis. Qualitative detection of BKV in patients with haemorrhagic cystitis can be performed by PCR, immune histology or electron microscopy on urine or BKV-infected decoy cells. However, the presence of BKV alone is not indicative of haemorrhagic cystitis. This problem was resolved by the introduction of quantitative detection methods. In contrast to 10^4 and 10^5 DNA copies per ml of urine in asymptomatic patients, haemorrhagic cystitis-indicative virus loads were determined as 10^8 – 10^{12} BKV DNA copies per ml of urine. With a virus load of more than 10^7 DNA copies per ml the risk of developing haemorrhagic cystitis increases considerably. In viraemic patients the risk has been reported to increase with a virus load of $>10^4$ DNA copies per ml plasma. This is indicative of activated BKV infection preceding clinical manifestation. In addition, it was found that there is a predictive correlation of high virus load and acute GVHD before the onset of haemorrhagic cystitis. Since haemorrhagic cystitis is sometimes fatal, this provides the tool for early diagnosis and strongly argues for a monitoring scheme and early intervention for patients at risk (Bogdanovic *et al.*, 2004).

PVAN is most commonly recognized after allograft biopsy due to renal dysfunction in the advanced stages of PVAN. Therefore a screening scheme has been developed for patients at higher risk of developing PVAN allowing early detection and reduction of PVAN-induced renal complications. This includes viral quantification by PCR in serum and urine and urine decoy cell monitoring as a noninvasive method of PVAN diagnosis and monitoring (Hirsch *et al.*, 2005). Detection of mRNA in urine with a cut-off value of 6.5×10^5 BKV mRNA copy number per ng of total RNA was found to be predictive for PVAN. Similarly, persistent decoy cell shedding greater than 10 cells per cytospin has been found to be associated

with PVAN. This is recommended for monitoring at three-month intervals post transplant and every six months thereafter in addition to more intense monitoring during treatment of rejection episodes. In case of a positive screening assay and possible PVAN, quantitative assays are used to determine threshold values of $>10^7$ BKV DNA copies per ml of urine and a value of 10 000 copies per ml in serum. Persisting polyomavirus loads above these thresholds for >3 weeks is highly suggestive of PVAN and allograft biopsy is recommended.

The definitive diagnosis of PVAN requires a histological identification of intranuclear inclusion bodies in affected cells. Alterations seen by light microscopy are not pathognomonic for PVAN. BKV association can be determined by immunohistochemistry for viral proteins, by *in situ* hybridization to identify BKV DNA or by electron microscopy to demonstrate polyomavirus particles morphologically. However, because of the focal nature of PVAN and the possibility of sampling error there is consensus that negative results cannot rule out PVAN with certainty. In cases with significant BKV replication above threshold levels additional studies are recommended. The resolution of PVAN following successful intervention is diagnosed based on the disappearance of viral cytopathic changes in cortex and medulla and absence of polyomavirus in plasma or urine. On the basis of highly variable patient-related and organ-specific risk factors all patients at risk of PVAN have in common a highly active state of BKV replication. Therefore screening for polyomavirus replication may allow earlier intervention with reduced allograft loss.

POLYOMAVIRUS-SPECIFIC IMMUNE RESPONSE

Humoral Immune Response

Determination of the virus-specific humoral immune response concentrates primarily on analyses of virus-specific antibodies and their dynamic titre changes in acute disease. The predominant polyomavirus subgroup-specific antigenic sites on the major capsid protein VP1 are accessible only after disruption of virions, in virus-infected cells or on purified VP1 protein. Consequently, virus-specific antisera against intact virus particles are species-specific. Species-specific antibodies can be distinguished from one another by neutralization and haemagglutination inhibition (HAI) assays with good correlation between virus-specific HAI, neutralizing antibody titres and quantitative PCR. Cross-reactivity between recombinant JCV and BKV virus-like particles used as antigen in enzyme-linked immunoassays (EIAs)

is negligible (Viscidi and Clayman, 2006). With the use of this more sensitive technique it became clear that the majority of prediagnostic samples in a case—control study do not change polyomavirus antibody status over a period of 15 years, however, absolute levels of antibodies to JCV and BKV decreased over time for 70% and 80% of individuals respectively (Rollison *et al.*, 2006). An increase in antibody levels appears to be related to activation processes either linked to immune modulatory events or disease. This is confirmed by the detection of higher antibody levels in AIDS patients with JCV or BKV viraemia (Engels *et al.*, 2005).

The prevalence of BKV-specific antibodies in sera is about 50% by the age of three years, and nearly all individuals are seroconverted by the age of 10 years. The incidence of JCV antibodies is about 50% during adolescence and more than 50% by adulthood. The rates differ slightly according to demographic data, geographical distribution and economic status. In contrast to JCV antibody titres, the range of BKV-specific antibody titres is strongly associated with age, titres decreasing linearly at a rate of 8.7% per 10 years (Knowles, 2006). In pregnant women rising titres indicate an incidence of active infection in more than 25% mediated by immunomodulation during pregnancy. The first polyomavirus-specific IgM assays were performed with BKV antigen. The prevalence of BKV-specific immunoglobulin type IgM in children was consistent with age distribution of primary BKV infection. Occurrence of BKV IgM in the range of 5% in sera from healthy blood donors is consistent with the finding that BKV activation is uncommon in healthy adults. Thus it is possible that cross-reactive IgM antibodies might be in the minority.

In organ transplant patients a BKV-specific humoral immune response is regularly mounted despite the immunosuppressive therapy regimens. BKV-specific antibodies were analysed in sera of renal transplant patients and their donors. The titres of donors predicted frequency, magnitude and duration of post-transplant viraemia. In recipients a temporal correlation between development of antibodies against BKV and clearance of the virus was defined, suggesting that recipients with a higher antibody level may have a better prognosis. Besides the enhanced antibody levels in viruric and viraemic donors and recipients, IgM and IgA antibodies were detected as well. Whereas IgM was rarely found in normal donors, IgA antibodies were demonstrated in 23% of donors and recipients with enhanced titre levels in activated infection. Median levels were higher in viraemic patients, thereby confirming that viraemia follows viruria and represents higher activation status. Since serologic changes were not observed in patients with chronic renal failure on dialysis, the serologic status in BKV-associated diseases

might play a role in future diagnostic strategies (Randhawa *et al.*, 2006).

JCV-specific IgG antibodies in normal people and age-matched patients with various tumours exhibited similar geometric mean titres. Since antibody levels are not influenced by age and titres in patients with malignancies remain stable even under multidrug therapy or immunotherapy, it can be assumed that JCV infection is not markedly influenced by these diseases or associated therapies. However, recently it became clear that patients with non-Hodgkin lymphoma who were originally seropositive developed increased JCV antibody levels in the course of disease, suggesting that a low-level activation during tumour development might be accompanied by increasing antibody titres (Rollison, 2006). Equally, under PML, the range of JCV-specific serum and HAI titres can often not be distinguished from those in the general population. This is explained by the severe basic illnesses abrogating a normal antibody increase; however, because of the fast course of PML modest titre changes might not be measured even in the modern EIA systems (Viscidi and Clayman, 2006). JCV-specific IgM antibodies were detected in 15% of healthy blood donors. In the same group almost all sera contained HAI antibodies, suggesting that blood donors harbour the virus in a persistent state. The high prevalence of JCV infections in adults led to the assumption that presence of IgM is frequently associated with JCV activation in the healthy. In about half of the PML patients a rise of IgM antibodies with increasing levels was observed under progression of the neurological illness. At present, the high prevalence of IgM-positive sera in healthy people does not allow a correlation of IgM presence and acute disease (Knowles, 2006).

In early studies the CSF was usually found to be unremarkable in PML, and JCV HAI titres were only rarely detected. Thus the humoral immune response has been regarded as essentially unhelpful for diagnosis in the past. However, recently a slight increase in CSF protein was reported in about one-quarter of PML patients, an elevated IgG-albumin index in about one-fifth, and a slight pleocytosis in other patients, further analyses revealed moderate blood–brain barrier impairment in PML patients. JCV-specific intrathecally synthesized HAI antibodies and oligoclonal bands were detected in CSF of 67% confirmed PML cases and changes of HAI titre in the CSF were observed under PML treatment. Thus JCV intrathecally produced HAI antibodies appear to be suggestive of active JCV multiplication within the CNS. This was further confirmed by EIA analyses with recombinant JCV VP1 capsid protein as antigen. An intrathecal immune response was detected in 78% of PML patients versus 3% of controls. Detection of JCV-specific oligoclonal bands was slightly less sensitive. Interestingly, a JCV-specific

intrathecal immune response against JCV VP1 evolved in HIV-1 patients during therapy. At present, it appears likely that a response evolves over time in the majority of PML patients (Weber *et al.*, 2001).

The range of titres in individual PML patients appears to be highly dependent on the state of the disease and may additionally reflect the type of underlying disease with characteristics differently affecting parameters of the immune system. Analyses of virus-specific, intrathecally produced antibodies and oligoclonal bands are well matched in PML versus non-immunosuppressed cases. It can be assumed that a mild intrathecal immune response with the presence of oligoclonal JCV-specific antibodies indicates intrathecal growth of JCV. It remains to be determined whether the finding of intrathecally produced JCV-specific antibodies and oligoclonal bands is indicative of acute disease and PML only, or might also be characteristic for a persistent activated state under immune impairment of other diseases.

Cellular Immune Response

JCV activation occurs in the context of immune impairment, and humoral immunity obviously is not able to control JCV spread. Therefore from the early beginnings breakdown of cellular immunity was suspected to play a major role in the pathogenesis of polyomavirus diseases. However, studies on the immune response were limited and did not contribute to a general understanding of polyomavirus-associated mechanisms of immune control. Recently, it was found that the major histocompatibility complexes class II and I were expressed at high levels in PML lesions, indicating an intact antigen presentation. In HIV/PML patients immune reconstitution by HAART therapy is related to a rise in helper CD4⁺ T-lymphocyte counts as well as a drop in HIV-1 load and clinical improvement of JCV-related neurological symptoms. However, about 50% of HIV/PML patients do not respond to HAART despite reduction of HIV-1 load and rise in CD4⁺ counts. Therefore it appears likely that the CD4⁺ T-cell subset does not play an important role in PML development.

Studies on cytotoxic CD8⁺ T lymphocytes revealed the presence of JCV-specific circulating cells directed against JCV proteins in long-term survivors of PML. Compared with other persistent viral infections, JCV-specific cytotoxic T lymphocytes (CTLs) were found at considerably lower frequencies. Comparison of the CTL responses among PML patients demonstrated that the frequency of JCV-specific CD8⁺ T cells is related to the outcome of disease. A strong response was detected in most patients with a prolonged disease course, whereas the response in classic PML patients or HIV/PML fast progressors was almost not detectable. Most of these cells, which have

been shown to control virus replication and disease progression, were directed against epitopes on the JCV VP1 capsid protein. Analyses of the frequency of CTLs in the PBMC population of healthy individuals demonstrated a broad range from one in 100 000 to one in 2494 PBMCs. These values are comparable to those in HIV/PML patients with a favourable outcome. In addition, it could be demonstrated that JCV-specific CD8⁺ cells co-localize with lymphocytic infiltrates and JCV-infected glial cells in PML lesions, suggesting that an active ongoing recruitment of CD8⁺ T cells and viral antigen-specific retention could occur. These data confirm that the JCV-specific cellular immune response plays an important role in the control of disease progression. In addition, it is conceivable that JCV-specific CD8⁺ T cells are able to control the spread of JCV even in immunosuppressed individuals. Therefore this cell population may provide a useful diagnostic and prognostic tool in the therapeutic management of these patients (Koralnik, 2006).

TREATMENT OF POLYOMAVIRUS-ASSOCIATED DISEASES

Treatment of huPyV infections concentrated for years on PML. Different treatment regimens have been proposed on the basis of molecular findings and small series of patients. However, randomized therapeutic trials have been introduced only recently and the observation of stabilized PML or even remission highlights the inadequacies of early anecdotal reports suggesting the value of specific therapies. Nucleoside analogues have proven to interfere with viral DNA synthesis in virus infections, and several compounds have been tried in the treatment of PML. Cytosine arabinoside (ARA-C, cytarabine) has been used and various degrees of improvement reported. Recently acquired molecular data showed that cytosine- β -D-arabinofuranoside suppressed JCV replication in tissue culture, supporting the potential interference of ARA-C with PML. However, the study on the efficacy of ARA-Cy by the AIDS Clinical Trials Group, trial 243, showed no benefit. In contrast, in a small group of non-AIDS PML patients the treatment stabilized PML in 36% of the patients for one year. Other analogues such as adenosine arabinoside (ARA-A, Vidarabine), iodo-desoxyuridine or zidovudine similarly do not appear to have an effect in the treatment of PML. The use of HAART in patients with HIV-related PML is associated with disease stabilization. Prolonged survival in about 50% of the patients is associated with decrease in JCV load. The survival rate significantly increased in patients receiving a protease inhibitor-containing regimen.

The effect of HAART on PML is believed to be the result of improved immune defence. However, in half of the patients PML progressed despite virological and immunological response to HAART. Failure to respond to HAART as demonstrated by high plasma viral loads has been associated with poor PML prognosis and a course of disease similar to that in the absence of antiretroviral therapy. Nevertheless, the use of HAART significantly improved survival time, although the prognosis for HIV/PML patients is still severe.

Cidofovir diphosphate, a structural analogue of deoxycytidine triphosphate, is known to be a potent virus inhibitor. It demonstrated a significant inhibitory effect *in vitro* and was therefore introduced as a potential drug in PML treatment. Anecdotal reports pointed to clinical improvement and follow-up studies reported beneficial effects, either alone or in combination with HAART in AIDS-associated PML. However, the efficacy of the drug remains controversial. The use of immunomodulatory agents in PML therapy is based on the findings of extended survival in patients with improvement of immunological competence. Alpha-interferon has established efficacy in the treatment of other polyomavirus-associated diseases, and prolonged survival has been reported. In contrast, other studies reported no significant enhancement in survival time. Similar discrepancies were found with the combination of adenine arabinoside and β -interferon or transfer factor revealing no efficacy, whereas interferon alone was associated with modest improvement in the clinical picture and on MRI. Recently, an effect on survival after IL-2 treatment of a lymphoma patient after BMT was contradicted in another patient. In addition, treatment with low-dose heparin sulfate was suggested to prevent seeding of JCV to the CNS by activated lymphocytes. Other agents have been tried either alone or in combination. However, unequivocal effective therapy of PML as yet remains elusive, and further investigations are needed to decide which of the different approaches might be effective for the treatment of PML (Cinque *et al.*, 2003; Roskopf *et al.*, 2006).

There is currently no standardized treatment available for patients diagnosed with BKV-associated disease. The most common treatment strategy is to reduce or discontinue immunosuppressive drugs and treatments if possible. The management of haemorrhagic cystitis is generally symptomatic. This approach is designed to rescue viral replication, but may be associated with risks such as acute rejection and graft loss in renal transplant recipients, so patients must be monitored closely. Several case reports have described the successful use of antiviral agents such as vidarabine, ribavirin and cidofovir, however, they were used on a small number of patients

and are still not defined as a standard therapeutic option. Based on these reports, cidofovir may be effective for treatment of haemorrhagic cystitis in the setting of BKV and should be considered for patients who fail conventional methods for controlling haemorrhagic cystitis. With vidarabine therapy the viral inclusion bodies in urinary sediments disappeared. If viruria recurred it could be successfully cleared with another course of vidarabine, thus it appears to be a safe alternative for treatment of haemorrhagic cystitis (Gorczyńska *et al.*, 2005; Roskopf *et al.*, 2006).

Prophylactic and therapeutic interventions for PVAN are limited since antiviral drugs with specific activity directed against the viral life cycle are not available for prevention or treatment of PVAN. Antiviral agents used with anecdotal success include cidofovir, leflunomide, quinolone antibiotics and intravenous immunoglobulin. The efficacy of these strategies is unclear, because it was always accompanied by reduction of immunosuppression. This allows reconstitution of immune responsiveness to BKV and may lead to stabilization and prevention of graft loss. Different regimens have been developed for patients with progressive PVAN as potentially effective therapies, however, it was found that therapeutic regimens were most successful in the early stages of disease. Recently, this was verified by a prospective study on monitoring and the impact of reduction of maintenance immunosuppression on the BKV viral load kinetics and PVAN. In all viraemic patients the treatment resulted in clearance of viraemia and prevented development of PVAN without increasing the rate of acute rejection or causing graft dysfunction. It was also observed that reduction of viral load correlated with improved BKV-specific cellular immunity. Reduction of drug doses may result in improvement or stabilization of PVAN, however, uniformly accepted threshold values for drug levels have not yet been identified. The impact of antilymphocyte preparation may depend on the type of agent used and the specific clinical situation. Avoidance or early cessation of steroids in calcineurin inhibitor-based regimens may reduce the incidence of PVAN or positively influence the course of PVAN, where steroid treatment for acute rejection was followed by decreased maintenance immunosuppression. Recent studies suggest that a brief increase in immunosuppression followed by subsequent decrease may be the best way to manage rejection episodes occurring at or after PVAN diagnosis (Hirsch *et al.*, 2006; Vats *et al.*, 2006).

Although a number of drugs and therapeutic regimens for the treatment of polyomavirus diseases were associated with some beneficial effect, an established antiviral therapy is not available. The high prevalence of these viruses in humans and their pathogenic potential makes

intensive analyses of viral activation mechanisms and the development of specific drugs essential aims for the near future.

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Human Parvoviruses

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INTRODUCTION

Parvoviruses are, as their name suggests, small viruses (from the Latin *parvum*, meaning 'small'), with a single-stranded DNA genome. The *Parvoviridae* family consists of two subfamilies, the *Densovirinae* and the *Parvovirinae*: the *Densovirinae* are all viruses of insects and the *Parvovirinae* are viruses of vertebrates (Tattersall *et al.*, 2005). The *Parvovirinae* is further subdivided into five genera based on the replication pattern, sequence homology and transcription map (Table 35.1). At least four different types of parvovirus are known to infect humans, of which human parvovirus B19 (B19V) is the best studied.

The genus *Parvovirus* contains a wide range of viruses of mammals and birds, some of which cause major diseases in their animal hosts, but some, first isolated as contaminants of cell cultures, have unknown primary hosts. No member of this family is known to infect humans.

B19V was initially discovered in the serum of an asymptomatic blood donor (coded 19 in panel B) as a cause of false-positive results in counter-immunoelectrophoresis tests for the detection of hepatitis B virus surface antigen (Cossart *et al.*, 1975). Originally the virus was classified with the autonomous parvoviruses, but is now the type member of the *Erythrovirus* genus. Subsequently, related parvoviruses have been identified in cynomolgus monkeys, pig-tailed macaques and rhesus macaques. These erythroviruses share with B19V up to 60% homology, similar genome organization and similar biological behaviour in natural hosts.

The dependoviruses have been described in a number of mammalian and avian species. To date, at least nine different primate dependoviruses have been described

(Gao *et al.*, 2004), and adeno-associated viruses (AAVs) 1, 2, 3, 8 and 9 are common human infections (Gao *et al.*, 2004). Although AAV DNA has been detected in a wide range of tissues, including some fetal abortion tissues, none of the dependoviruses have been definitively linked with disease in either humans or animals.

Bocaviruses are viruses that infect the respiratory and gastrointestinal tract of young animals. Recently sequences of a human bocavirus (HuBoV) have been identified in respiratory samples of Swedish children with lower respiratory tract infections (Allander *et al.*, 2005). Subsequent studies have identified HuBoV DNA sequences in children in many different parts of the world, but often associated with other pathogenic viruses. However, there is increasing evidence that the virus is pathogenic, and especially associated with wheezing and respiratory disease in young children (Allander *et al.*, 2007). The viral sequences have also been identified in faecal samples from children (Vicente *et al.*, 2007), and it is unclear if this is the virus responsible for the parvovirus-like particles that have been described in human faecal specimens.

Using similar methods to those used to identify HuBoV, a fourth group of parvoviruses, Parv4 (Jones *et al.*, 2005), has also recently been identified. The viral sequences are commonly found in pooled serum samples (Fryer *et al.*, 2007), and in bone marrow and lymphoid tissue of intravenous drug users and people with haemophilia (Manning *et al.*, 2007). However, whether the virus is pathogenic is unclear and, as with HuBoV, currently there have been no published studies of antibody seroprevalence. The virus has not been grown in culture, and preliminary studies of the transcription map suggest that Parv4 does not group with any of the other parvovirus genera.

Table 35.1 The *Parvovirinae*

Subfamily	Genus	Virus	Host	
<i>Parvovirinae</i>	<i>Parvovirus</i>	Canine parvovirus—CPV	Dogs, foxes	
		Mice minute virus—MVM	Mice	
		Porcine parvovirus—PPV	Pigs	
		Feline parvovirus—FPV	Cats	
	<i>Dependovirus</i>	Adeno-associated virus 1–6—AAV	Humans, primate	
		Avian adeno-associated virus—AAAV	Birds	
		Canine adeno-associated virus—CAAV	Dogs	
		Bovine adeno-associated virus—BAAV	Cattle	
		Parvovirus B19—B19V	Humans	
	<i>Erythrovirus</i>	Simian parvovirus—SPV	Primates	
		Rhesus parvovirus—RhPV		
		Pigtailed macaque parvovirus—PtPV		
	<i>Bocavirus</i>	Bovine bocavirus	Cows	
		Canine bocavirus	Dogs	
		Human bocavirus—HBoV	Humans	
	<i>Amdovirus</i>		Aleutian mink disease virus	Minks
	Unclassified		Parv4	Humans

HUMAN PARVOVIRUS B19 (B19V)

Structure

In electron micrographs of negatively stained preparations parvovirus B19V appears as non-enveloped, icosahedral particles with a diameter varying from 18 to 25 nm, and often both ‘full’ and ‘empty’ capsids are visible (Figure 35.1). The infectious particles have a buoyant density of $\sim 1.41 \text{ g ml}^{-1}$ in caesium chloride gradients, whereas the empty particles have a density of 1.31 g ml^{-1} .

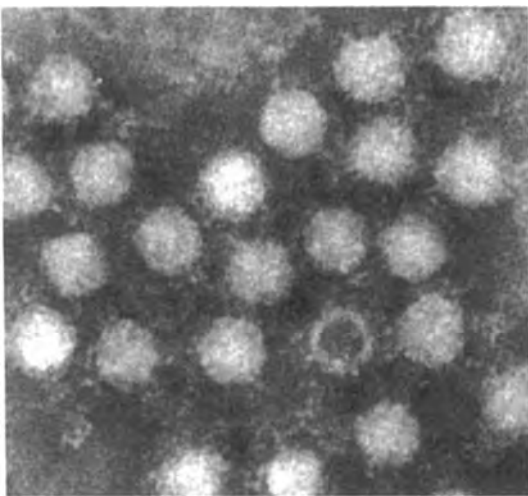


Figure 35.1 The appearance of B19V particles by immune-electron microscopy.

The particles do not contain lipids or carbohydrates. As with all parvovirus particles, the infectious particle is stable over a wide range of pH and resistant to lipid solvents, although B19V is not quite as resistant to heat as other parvoviruses (Blumel *et al.*, 2002). B19V can also be inactivated by formalin, β -propiolactone, oxidizing agents and γ -irradiation.

Genome Organization

Parvovirus virions consists of a linear single strand of DNA with a molecular weight of approximately 1.8×10^6 . In contrast to the autonomous parvoviruses that preferentially encapsidate single-stranded DNA of negative polarity, parvovirus B19 encapsidates both positive and negative strands with equal frequency. The genome of B19V is one of the largest among the parvoviruses, comprising 5596 nucleotides (nt), and with long (365 nt) imperfect palindromic sequences at both the 5' and 3' termini. These segments of the DNA fold back on themselves to form hairpin loops which are stabilized by hydrogen bonding. There is some mismatching in the middle of the hairpin, leading to two slightly different sequence configurations (referred to as the flip and flop configurations).

In contrast to most other parvoviruses, there is a single strong promoter at the far left side of the genome (map point 6, or p6) which regulates the synthesis of all nine of the different viral transcripts (Figure 35.2 (Ozawa *et al.*, 1987)). The single nonspliced transcript encodes the nonstructural (NS) protein and, by a combination of different splicing events, the other eight encode the two capsid proteins (VP1 and VP2) and two smaller proteins

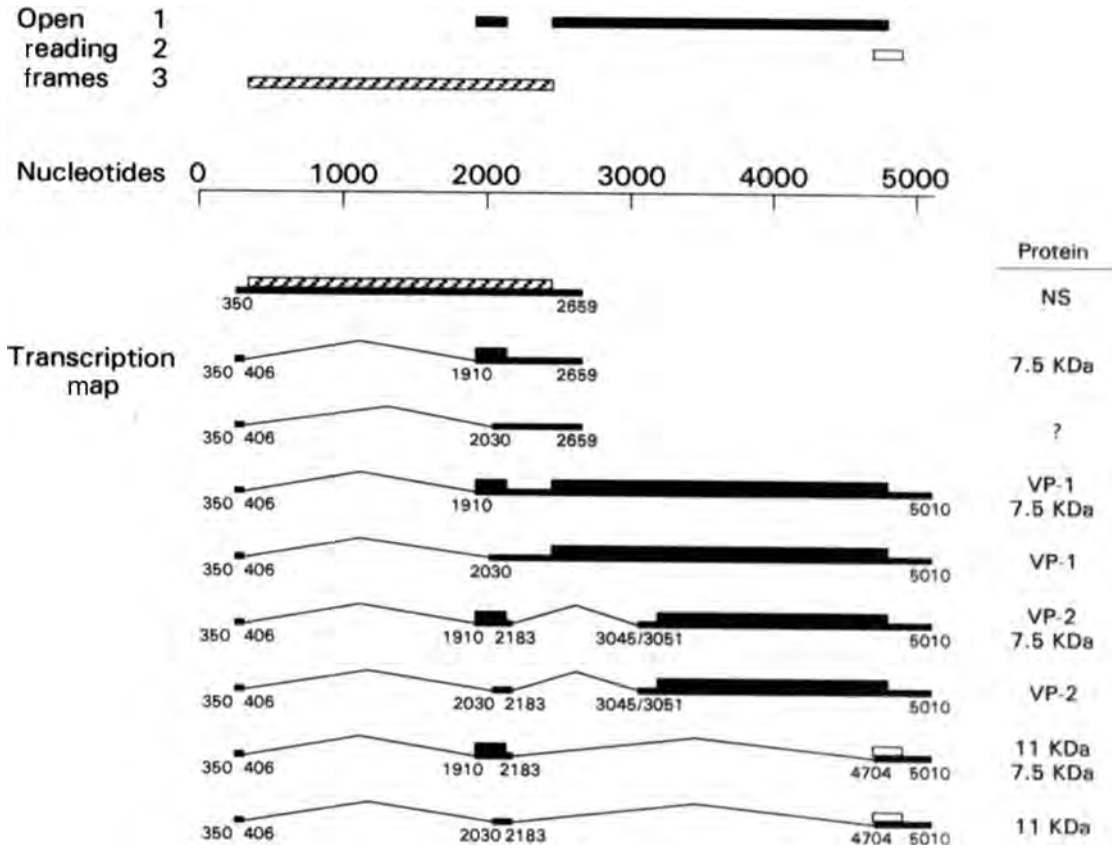


Figure 35.2 Transcription map of parvovirus B19V.

(7.5 and 11 kDa proteins). Until recently, the length and structure of the hairpin sequences thwarted attempts to produce a full-length clone of the virus. The virus has now been cloned, and knockout studies of different regions of the virus have allowed elucidation of the critical roles of the different proteins (Zhi *et al.*, 2006).

Viral Proteins

The only unspliced transcript encodes the NS protein, a 78 kDa phosphoprotein. The protein has DNA-binding properties, adenosine and guanosine triphosphatase activity, nuclear localization signals and is essential for B19V infectivity and DNA replication (Zhi *et al.*, 2006). In addition, expression of the NS protein causes host cell death through triggering caspases and induction of apoptosis.

There are two structural proteins in B19V, VP1 (84 kDa) and VP2 (58 kDa), which differ only in that VP1 has an additional 227 amino acids at the N-terminus (VP1u). The capsid of B19V is composed of 60 capsomers: VP2 is the major capsid protein, with only ~5% VP1 in the infectious particle. Expression

of VP2 capsomers alone will self-assemble to form recombinant empty capsids that resemble B19V particles morphologically and antigenically (Kajigaya *et al.*, 1991). The major cellular receptor for B19, globoside or P antigen (Brown *et al.*, 1993), binds to VP2. The VP1 unique region (VP1u) is known to be important in generating neutralizing antibodies (Saikawa *et al.*, 1993), has phospholipase activity (Zadori *et al.*, 2001), and is critical for the production of infectious virus. Structural studies of B19V have been undertaken for B19V using both crystal diffraction and cryo-electron microscopy studies. They indicate that as seen for other parvoviruses, B19V has a central structural core of eight anti-parallel β -sheets, but the large loops that give the prominent spikes on the threefold axis of canine and feline parvoviruses (FPVs) are absent from B19V capsids (Kaufmann *et al.*, 2004).

Virus Variation

Although generally there is <5% sequence difference between different isolates of B19V, sequences with ~10%

difference have been identified, and the sequences are divided into three different genotypes (Servant *et al.*, 2002). The predominant genotype identified is genotype 1, with only limited reports of the identification of genotypes 2 and 3. Most studies in blood and blood products have failed to identify genotypes 2 and 3 (Baylis, 2008). However genotype 2 and 3 have been identified in bone marrow and tissues, and one study from Finland suggested that genotype 2 may have been more prevalent in the 1960s (Norja *et al.*, 2006). Genotype 3 infection also appears to be much more common in parts of Africa (Candotti *et al.*, 2004).

Although there are three genotypes, there are only limited differences between the amino acid sequence of the different viruses. Antibody and cross-neutralization studies indicate that there is only a single B19V serotype.

PATHOGENESIS

B19V was first identified in a sample being tested for hepatitis B (Cossart *et al.*, 1975), and for a number of years following its discovery B19V appeared to be associated with, at most, a mild, nonspecific, febrile illness accompanied by self-limiting leukopenia. In the early 1980s the central role of B19V in the aetiology of aplastic crisis in chronic haemolytic anaemias was identified and then, in 1983, erythema infectiosum (fifth disease) began to emerge as the common manifestation of B19V infection. Subsequently the virus has been shown to be responsible for a significant percentage of cases of hydrops fetalis and to cause chronic infection in the immunocompromised. It is now clear that the pathogenesis of B19V-associated disease involves two components. The first is due to the lytic infection of susceptible dividing cells and the second is dependent upon interaction with the immune response.

Volunteer Studies

Studies of experimentally infected volunteers have allowed the kinetics of infection to be elucidated. In both cases volunteers (9 and 3 respectively) were infected by intranasal inoculation of a saline solution containing approximately 10^8 virus particles. The volunteers were followed daily and clinical, biochemical, haematological and viral studies undertaken (Figure 35.3).

Viraemia was first detected from day 6 and reached a peak at days 8–9 at about 10^{11} particles per ml, a level comparable to that seen in natural infections in blood donors and patients with aplastic crisis (Anderson *et al.*, 1985). Virus was also detected in throat swabs and gargles at the time of viraemia only. Virus was not found in urine or faecal samples. On days 6–8 the volunteers showed

the typical symptoms of a viraemia, with headache, myalgia, and chills, associated with pyrexia. These features are now known to be due to the production of inflammatory cytokines (Isa *et al.*, 2007). During the viraemia reticulocyte numbers fell to undetectable levels, recovering 7–10 days later, and there was a consequent temporary fall in haemoglobin of about 1 g dl^{-1} in a normal individual. Lymphopenia, neutropenia and thrombocytopenia also occurred though slightly later, with the lowest numbers being recorded 6–10 days after inoculation and not so consistently as the changes in reticulocyte numbers and haemoglobin concentration.

In the second study bone marrow morphology was also examined at intervals (Potter *et al.*, 1987). On day 6 post inoculation the marrow appeared normal. However at day 10 there was an almost total loss of erythroid precursors at all stages of development. The erythroid progenitor cells (BFU-E) from bone marrow and peripheral blood were reduced at this time. The myeloid compartment of the bone marrow appeared normal, but myeloid precursors from peripheral blood were reduced as soon as two days from the onset of the viraemia.

The viraemia resolved as the patients developed a detectable antibody response (Figure 35.3) and a second phase of the illness began in most of the volunteers at day 15–17 (Anderson *et al.*, 1985), as the IgM response peaked and IgG became detectable, characterized by pruritis followed by the development of a fine maculopapular cutaneous eruption. The rash extended over the limbs and was accompanied by arthralgia and a mild arthritis. The rash was present for two to four days and the joint symptoms for four to six days. In the second study none of the patients developed the second phase symptoms. It may be significant that in the second study all (3/3) the volunteers were male and that joint symptoms in natural B19V infection appear to be much less frequent in male patients.

All the volunteers who received $>10^6$ virus particles in the inoculum, and were B19V antibody negative (IgG <0.3 au (arbitrary unit); equivalent to <0.3 IU ml^{-1}) developed viraemia and an antibody response to the virus. Volunteers with a positive B19V IgG (IgG <2 IU ml^{-1}) did not become infected. One of the pilot volunteer patients had 'equivocal' levels of IgG and appeared to develop a modified infection, with minimal viraemia and a boosting of his immune response.

In both of the studies, viraemia was monitored by the relatively insensitive dot-blot hybridization technique. Studies with polymerase chain reaction (PCR) have now shown that B19V DNA can be detected for many weeks or months following acute infection (Lefrere *et al.*, 2005), and can probably be detected in tissue for the rest of life (Norja *et al.*, 2006).

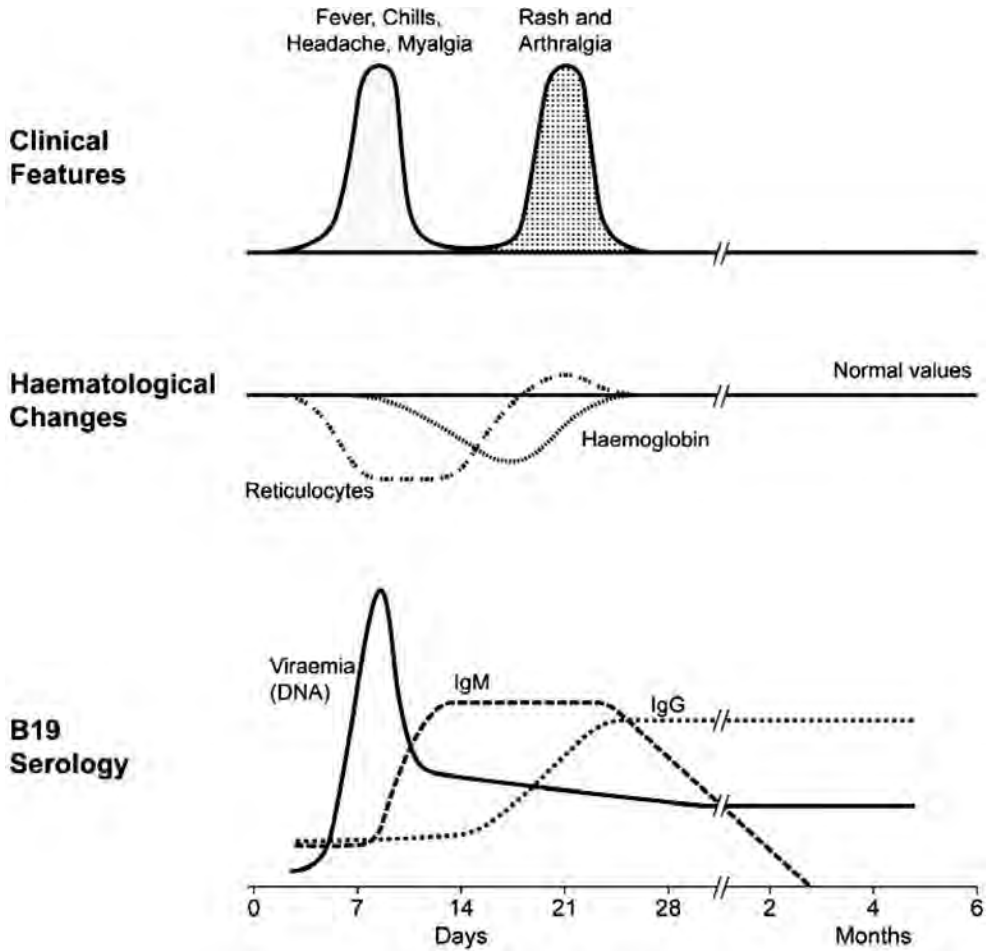


Figure 35.3 Virological, haematological and clinical events during B19V infection of volunteers.

Cell Tropism

Parvovirus B19, like the other autonomous parvoviruses, depends on host cell factors or functions only present in cells transiting the late S- or early G₂-phase of mitosis for replication. However, B19V also has a very narrow target cell range and can only be propagated in human erythroid precursor cells. When bone marrow or peripheral blood cells are cultured to develop erythroid colonies in the presence of B19V, the formation of colonies is inhibited. For erythroid cells from bone marrow, susceptibility to parvovirus B19V increases with differentiation; the pluripotent stem cell appears to be spared and the main target cells are the CD36-positive BFU-E and CFU-E cells (cells capable of giving rise to erythroid colonies *in vitro*) and erythroblasts.

Erythroid progenitors from a variety of sources have been used to study *in vitro* viral replication,

including human bone marrow, fetal liver and peripheral blood. B19V will also replicate in a limited number of erythropoietin-dependent megakaryocytic cell lines including UT7/Epo and KU812Ep6. However the yield of virus from all these cell lines is poor, and they cannot be used as a source of antigen for diagnostic tests.

Erythroid specificity of parvovirus B19V is in part due to the tissue distribution of the primary virus' cellular receptor, globoside, also known as blood group P antigen (Brown *et al.*, 1993). P antigen is found on erythroid progenitors, erythroblasts and megakaryocytes. Purified P antigen (globoside) blocks the binding of virus to erythroid cells and these cells can be protected from infection by preincubation with monoclonal antibody to globoside. P antigen is also present on endothelial cells, which may be targets of viral infection involved in the pathogenesis of transplacental transmission, possibly

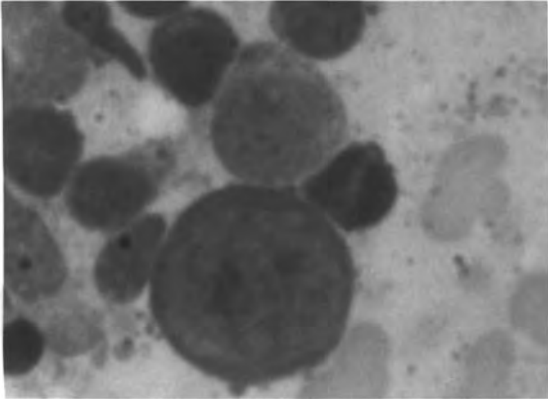


Figure 35.4 Giant pronormoblast, typically seen in the bone marrow and occasionally in the peripheral blood of patients with B19V infection.

vasculitis and the rash of fifth disease, and on fetal myocardial cells. Rare individuals who genetically lack P antigen on erythrocytes are resistant to B19V infection, and their bone marrow cannot be infected with B19V *in vitro* (Brown *et al.*, 1994b). However, erythroid specificity may also be modulated by specific erythroid cell transcription factors. At least two other antigens have been proposed as co-receptors for B19V, integrin and the KU80 autoantigen.

The virus is cytotoxic, producing a cytopathic effect with characteristic light and electron microscopic changes. Infected cultures are characterized by the presence of giant pro-normoblasts (Figure 35.4), 25–32 μm in diameter, with cytoplasmic vacuolization, immature chromatin and large eosinophilic nuclear inclusion bodies. By electron microscopy virus particles are seen in the nucleus and lining cytoplasmic membranes, and infected cells show marginated chromatin, pseudopod formation and cytoplasmic vacuolation, all typical of cells undergoing apoptosis. However, not all the giant pro-normoblasts contain virus particles, and part of the cytopathy probably reflects the expression of the cytotoxic NS protein. Megakaryocytopoiesis is also inhibited by B19V parvovirus *in vitro* but this is in the absence of virus replication and almost certainly due to the NS cytotoxicity.

Immune Response

The pattern of B19V disease is strongly influenced by the immune response. Bone marrow depression in parvovirus infection occurs during the early viraemic phase and under normal conditions is terminated by a neutralizing antibody response. The pathogenesis of the rash in erythema infectiosum and polyarthralgia is almost certainly immune-complex mediated. In volunteer studies,

the rash and joint symptoms appeared when the high titre of viraemia had dropped significantly and coincident with a detectable immune response (Anderson *et al.*, 1985).

Persistent B19V infection is the result of failure to produce effective neutralizing antibodies by the immunosuppressed host. Antibodies to parvovirus, as determined in immunoassays or enzyme-linked immunosorbent assay (ELISA), are not present in most patients, but a pattern of antibody response suggestive of early infection (weak IgM antibody and IgG antibody directed to VP2 only) may be found in patients with congenital immunodeficiency. Confirming the role of the humoral response appears to be dominant in controlling human parvovirus infection, administration of commercial immunoglobulins can cure or ameliorate persistent parvovirus infection in immunodeficient patients: fifth disease symptoms can be precipitated by treatment of persistent infection with immunoglobulin (Frickhofen *et al.*, 1990).

The infected fetus may suffer severe effects because red blood cell turnover is high and the immune response deficient. During the second trimester there is a great increase in red cell mass. Parvovirus particles can be detected by electron microscopy within the haematopoietic tissues of liver and thymus. B19V DNA and capsid antigen have been detected in the myocardium of infected fetuses (Porter *et al.*, 1990), and there is evidence that the fetus may develop myocarditis, compounding the severe anaemia and secondary cardiac failure. By the third trimester, a more effective fetal immune response to the virus may account for the decrease in fetal loss at this stage of pregnancy.

Role of the Cellular Immune Response

There have only been a limited number of studies on the role of the cellular immune response in controlling B19V infections. B19V infection induces both a CD4 and CD8 proliferative response, with a CD4 response primarily to capsid proteins and a CD8 response to the NS protein. The CD8 response is prolonged for many months even after an acute infection (Norbeck *et al.*, 2005), and suggests a previously unrecognized role for the control of B19V infection.

EPIDEMIOLOGY

Parvovirus B19 is a common infection in humans, and serological studies indicate that infection is worldwide (Kelly *et al.*, 2000), with infections occurring in all populations, apart from some isolated groups in Africa and Brazil. In temperate climates infection occurs throughout the year but outbreaks are more common in late winter, spring and the early summer months. These outbreaks

of infection are often centred on primary schools, where up to 40–60% of the school may be clinically affected by the rash illness of erythema infectiosum (EI). During these outbreaks, susceptible adults (parents and teachers of cases) frequently become infected.

In addition to seasonality, the virus exhibits longer term cycles: in Jamaica, peaks of incidence (monitored as cases of aplastic crisis) occur every three to four years. In the United Kingdom, the cycle seems somewhat longer, with peaks occurring every four to five years.

Clinical observations of the frequency of cases of EI among different age groups reflect the serological profile of the population; antibodies are most commonly acquired between the ages of 4 and 10 years, after which the frequency continues to rise, but more slowly. By age 15 years approximately 50% of children have detectable IgG. Infection also occurs in adult life, so that more than 75% of those over 45 have detectable antibody (Vyse *et al.*, 2007). Women of child-bearing age show an annual seroconversion rate of 1.5% (Koch and Adler, 1989). Studies in many different countries (United States, Scandinavia, France, Germany, Japan) all show similar patterns.

Case-to-case intervals, determined by the time elapsing between acquisition and excretion of the virus, are independent of the type of disease. Volunteer studies predict case-to-case intervals of 6–11 days, which accords well with case-to-case intervals observed both in outbreaks of EI and in aplastic crisis. Transmission between siblings is common, and in families where more than one child is affected by chronic haemolytic anaemia all susceptibles should be monitored for two weeks if one develops an aplastic crisis.

Transmission

Although spread from respiratory tract to respiratory tract is the common route of transmission of B19V, the high-titre viraemia that occurs during infection can lead to transmission by blood and blood products, and documented transmissions are reported in the literature. As noted earlier (and Figure 35.3), low levels of B19V DNA can be detected in blood for many months following acute infection, and the reported prevalence of B19V DNA in blood varies widely depending on the sensitivity of the assay being used, and on the presence of local epidemics of infection in the community, but with sensitive assays can be ~0.5% (Brown and Simmonds, 2007). The significance of low levels of B19V being transfused into immunocompromised seronegative individuals is not known.

B19V DNA can be detected in plasma pools and blood products. Although plasma pools invariably contain parvovirus B19V DNA for many years it was considered that the high titre of neutralizing antibodies present in

plasma pools would prevent transmission of infectious virus. It is now known that infusion of plasma pools containing high titre virus ($>10^7$ IU ml⁻¹) can transmit infection, and therefore in Europe and the United States, all human plasma has to be tested and shown to contain $<10^4$ IU ml⁻¹ B19V DNA.

CLINICAL FEATURES

The consequences of B19V infection range from the wholly asymptomatic to serious and potentially fatal conditions in a minority of the population that is particularly predisposed. The spectrum of clinical consequences of infection depends in part on the natural variation in symptomatology that occurs, and in part on recognizable host factors (Table 35.2).

Minor Illness

Combined clinical and laboratory studies of infection in children, in whom B19V infection is most common, have indicated that about half of all infections are asymptomatic. Nonspecific respiratory tract illness is the next most common consequence of infection. This can be mild or severe enough to mimic influenza and these respiratory tract illnesses coincide with the viraemic phase of the illness.

Rash Illness/Erythema Infectiosum

Erythema infectiosum is most common in children aged 4–11 years and was probably first described by Robert Willan in 1799. In 1905 Cheinisse classified it as the ‘fifth rash disease’ of the classical exanthema of childhood, and this name ‘fifth disease’ remains in use to this day. The

Table 35.2 Disease manifestations and persistence of parvovirus B19V infection in different host populations

Disease	Acute/ chronic	Host
Fifth disease	Acute	Normal children
Polyarthropathy syndrome	Acute/ chronic	Normal adults
Transient aplastic crisis	Acute	Patients with increased erythropoiesis
Persistent anaemia	Chronic	Immunodeficient/ immunocompromised patients
Hydrops fetalis/ congenital anaemia	Acute/ chronic	Fetus (<20 wk)

link between B19V infection and EI was made in 1983 (Anderson *et al.*, 1984), and human parvovirus B19 is now known to be the only aetiological agent for EI.

EI had been well described by clinical investigators prior to the discovery of B19V. Infection is characterized by a nonspecific prodromal illness which often goes unrecognized. Fifth disease may be associated with symptoms of fever, coryza, headache and mild gastrointestinal symptoms (nausea, diarrhoea). The exanthem occurs in three stages. Approximately 18 days after acquisition of the virus, and two to five days following the prodromal stage of the infection, the classic slapped cheek eruption appears, a fiery red rash on the cheek, accompanied by relative circumoral pallor (Figure 35.5). The edges of the involved areas may be slightly raised and there is relative circumoral pallor. At this stage the appearance may be suggestive of scarlet fever, drug sensitivity or other allergic reactions, or collagen vascular diseases. One to four days later the second stage appears, an erythematous maculopapular rash on the trunk and limbs. This rash is initially discrete but spreads to involve large areas. As this eruption fades it produces a typical lacy or reticular pattern. This third stage of the exanthem is highly variable in duration and maybe transient, or recurrent over one to three or more weeks with periodic evanescence and recrudescence. The rash can be exacerbated by exercise, emotion, hot baths or sunlight. In addition, there may be great variation in the eruption's appearance; from a very faint erythema that is easily missed to a florid exanthema,

and is often pruritic in adults, especially on the soles of the feet. While classic cases of EI are easy to recognize clinically, especially during outbreaks, the wide variation in the form of the rash may make individual cases hard to distinguish from other viral exanthema, including rubella.

Rarely the rash may be purpuric, or resemble Henoch–Shonlein purpura. In most B19V infections the platelet count is normal but thrombocytopenia has been recorded. Occasionally other dermatological presentations are seen, including vesicopustular rash, purpuric glove and stocking syndrome, and erythema multiforme.

Joint Involvement/Arthropathy

In children, B19V infection is usually mild and of short duration. However, in adults, and especially in women, there may be arthropathy in ~50% of patients (Woolf, 1990). It is now also clear with the widespread application of B19V diagnostic tests that the joint involvement may occur in the absence of any evidence of a rash.

In adults the most common presentation is with a sudden onset of symmetrical arthralgia and even frank arthritis affecting the small joints of the hand. Proximal interphalangeal and metacarpophalangeal joints are most often affected, followed by wrists, ankles, knees and elbows. Shoulders, cervical spine and lumbar spine as well as the hips may also be involved. There may be pain and stiffness in the joints, which may be accompanied by minor swelling or synovitis. Resolution usually occurs within a few weeks, but persistent or recurring symptoms



Figure 35.5 Child with characteristic slapped cheek appearance of fifth disease.

can continue for years (Reid *et al.*, 1985). In children the joint involvement may be asymmetrical and symptoms seem more severe than in adults and may be of longer duration.

In the absence of a history of rash, the symptoms may be mistaken for acute rheumatoid arthritis, especially as prolonged symptoms do not correlate with serologic studies, such as the duration of B19V IgM response, or persistent high-titre viraemia. In addition B19V infection can be associated with transient autoantibody production including transient rheumatoid factor. In one large study of patients attending an 'early synovitis' clinic in England, 12% had evidence of recent infection with B19 (White *et al.*, 1985). Three patients would have fulfilled the American Rheumatism Association's diagnostic criteria for definite rheumatoid arthritis. B19V infection should be considered as part of the differential diagnosis in any patient presenting with acute arthritis. In contrast to rheumatoid arthritis, B19V infection is not generally associated with joint destruction. However, differentiation between early rheumatoid arthritis and B19V arthropathy is important, as immunosuppressive therapy prescribed for rheumatoid is not indicated in parvovirus B19V infection.

The role of parvovirus B19V in the aetiology of chronic arthritis is unclear. Parvovirus B19V DNA can be found in synovial tissue of many patients, including children with arthritis. However B19V DNA can also be detected in synovial tissue in patients without arthropathy, and in one controlled study although B19V DNA was indeed detected in synovial tissue of 28% of children with chronic arthritis, but also found in 48% of non-arthropathy controls (Soderlund *et al.*, 1997), indicating that PCR-detectable DNA may persist in synovial tissues, as in other tissues for months/years/life.

It has been postulated that B19V is involved in the initiation and perpetuation of rheumatoid arthritis leading to joint lesions (Takahashi *et al.*, 1998), but these results have not been reproducible by other groups. In addition, in one study of long-term follow-up none of 54 patients with B19V-associated arthralgia reported persistence of joint swelling or restricted motion, and no evidence of inflammatory joint disease was found (Speyer *et al.*, 1998). Recent data suggest that rheumatoid arthritis may be triggered by a number of different pathogens in a susceptible individual. B19V infection may be one of the possible triggers, especially in children with juvenile rheumatoid arthritis (Lehmann *et al.*, 2003), and may also lead to a flare in disease activity.

Transient Aplastic Crisis

Transient aplastic crisis (TAC) is the abrupt cessation of erythropoiesis characterized by a fall from steady-state values of haemoglobin concentration, disappearance of

reticulocytes from peripheral blood and the absence of red blood cell precursors in the bone marrow. It is classically seen in patients with haemolytic anaemia, where the B19-induced cessation of erythropoiesis lasts five to seven days and patients present with symptoms of worsening anaemia, namely fatigue, shortness of breath, pallor, lassitude, confusion and sometimes congestive cardiac failure. The event is serious in most patients and occasionally it is fatal. Blood transfusion is required in the acute phase but after about one week the bone marrow recovers rapidly. There is a brisk reticulocytosis and the haemoglobin concentration returns to steady-state values.

TAC was the first clinical illness associated with B19V infection. When stored sera from 600 children admitted to a London hospital were examined six children had evidence of recent B19V infection (either antigenaemia or seroconversion). All were Jamaican immigrants with sickle cell disease presenting with aplastic crisis. There was a reduced haematocrit, and evidence of aplastic crises on their bone marrow (Pattison *et al.*, 1981). Studies since then have shown that more than 90% of all cases of aplastic crises in patients with chronic haemolytic anaemia are due to B19V infection. Most cases occur in children under the age of 20 but adults who remain susceptible to the virus infection may have B19-associated aplastic crises in later life.

B19V-associated aplastic crises are not confined to patients with sickle cell anaemia, but have been described in a wide range of patients with underlying haemolytic disorders, such as hereditary spherocytosis, thalassaemia, red cell enzymopathies such as pyruvate kinase deficiency and autoimmune haemolytic anaemia. Aplastic crisis can also occur under conditions of erythroid 'stress', such as haemorrhage, iron deficiency anaemia and following kidney or bone marrow transplantation. Acute anaemia has been described in normal patients and a drop in red cell count (and reticulocytes) was seen in healthy volunteers (Anderson *et al.*, 1985), but usually there is sufficient haematopoietic reserve, and this is not clinically apparent.

Although they have an ultimately self-limiting disease, patients with aplastic crisis can be severely ill. Symptoms include not only the dyspnoea and lassitude of worsening anaemia, but the patient may develop confusion, congestive heart failure, severe bone marrow necrosis cerebrovascular complications and the illness can be fatal. Aplastic crisis may be the first presentation of an underlying haemolytic disease in a well compensated patient.

Community-acquired aplastic crisis is almost always due to parvovirus B19V and should be the presumptive diagnosis in any patient with anaemia due to abrupt cessation of erythropoiesis as documented by reduced reticulocytes and bone marrow appearance. In contrast to patients

with EI, those with TAC are often viraemic at the time of presentation with concentrations of virus as high as 10^{14} IU (genome copies) ml^{-1} , and the diagnosis is readily made by detection of B19V DNA in the serum. As the B19V DNA is cleared from the serum, B19V-specific IgM becomes detectable. Typical TAC is readily treated by blood transfusion. It is a unique event in the patient's life, and following the acute infection immunity is lifelong.

TAC and B19V infection in haematologically normal patients is often associated with changes in the other blood lineages. There may be varying degrees of neutropenia, and thrombocytopenia. Transient pancytopenia is less common. Haemophagocytosis, which can occur after many different viral infections, has been noted in acute and persistent B19V infection.

B19V Infection in Pregnancy

Animal parvoviruses are known to cause congenital infections in a variety of animals, leading to fetal loss in rodents and pigs, and to congenital infections in cats and dogs, and B19V is no different, with B19V infection during pregnancy possibly resulting in either miscarriage or the development of hydrops fetalis. Following fetal loss, the clinical features of the fetuses are similar, with evidence of leukoerythroblastic reaction in the liver and large pale cells with eosinophilic inclusion bodies and peripheral condensation or marginatum of the nucleus. B19V DNA can be detected by dotblot, PCR or *in situ* hybridization, and parvovirus particles seen by electron microscopy (Field *et al.*, 1991).

However, an adverse fetal outcome is not typical following maternal B19V infection, and the timing of maternal infection is also crucial. In a prospective British study of over 400 women with serologically confirmed B19V during pregnancy, the excess rate of fetal loss was confined to the first 20 weeks of pregnancy and averaged only 9%, with a risk of hydrops fetalis of 2.9% following maternal infection between weeks 9 and 20 (Miller *et al.*, 1998). Transplacental transmission of B19V infection has been estimated at $\sim 33\%$, and discordant infections in twin pregnancies have been recorded. Transplacental transmission can occur even in the absence of maternal symptoms and there may be significant delay between maternal infection and development of hydrops, with intervals ranging from 2 to 17 weeks. No abnormalities were found at birth in the surviving infants, even when there was evidence of intrauterine infection by the presence of B19V IgM in the umbilical cord blood. There were also no long-term sequelae of B19V infection in the 129 children followed for over seven years, although three children were reported to have developmental delay.

B19V probably causes 10% of all cases of non-immune hydrops. Non-immune hydrops fetalis is rare (1:3000 births) and in approximately 50% of cases the aetiology is unknown. In a study of 55 cases of non-immune hydrops, the majority were due to cardiovascular or chromosomal abnormalities, but B19V infection was diagnosed in 14% of the cases (Ismail *et al.*, 2001).

Congenital Malformations

Although sporadic case reports have noticed an association between genitourinary abnormalities, cerebral abnormalities and ocular malformations, the abnormalities reported are all relatively common and without a control population it is difficult to interpret the abnormalities as being due to B19V. No systematic studies have shown evidence for congenital abnormalities following B19V infection. In the two British studies only one case with congenital malformation seen (a ventral septal defect) and it was concluded that the risk of congenital malformations due to B19V infection was less than 1%. In a similar study in the United States, again no increase in adverse long-term outcomes was observed. Thus there is no evidence to date that B19V causes birth defects, although it should be remembered that the sample sizes in the studies have been too small to detect a rare defect, that is, one with a rate of 1% or less. Nevertheless there is no reason to recommend termination of pregnancies complicated by laboratory-proven B19V virus infection.

Infants born with chronic anaemia following a history of maternal B19V exposure and intrauterine hydrops have been described. In three such cases two at birth were found to have hypogammaglobulinaemia, and viral DNA was present in the bone marrow, although absent from the serum (Brown *et al.*, 1994a). One child died but the other two remained persistently anaemic in spite of immunoglobulin therapy. In a related study of bone marrow from children with Diamond-Blackfan anaemia, B19V DNA was found in 3/11 marrow smears, all from children who underwent remission of their anaemia. Thus intrauterine B19V infection may be responsible for some cases of congenital anaemia, although the incidence is probably rare.

Pure Red Cell Aplasia

A chronic B19V infection resulting in pure red cell aplasia occurs in immunocompromised individuals who have failed to produce neutralizing antibody to the virus. Chronic infection has been reported in a wide variety of immunosuppressed patients, ranging from patients with congenital immunodeficiency, acquired immunodeficiency syndrome (AIDS), lymphoproliferative disorders and transplant patients. The stereotypical presentation

is with persistent anaemia rather than immune-mediated symptoms of rash or arthropathy. Patients have absent or low levels of B19V-specific antibody (Kurtzman *et al.*, 1989b) and persistent or recurrent parvoviraemia as detected by B19V DNA in the serum. Bone marrow examination generally reveals the presence of scattered giant pronormoblasts. Administration of neutralizing antibody in the form of human normal immunoglobulin often leads to a fall in virus titre, reticulocytosis and in some cases rash illness presumed to be due to immune complexes (Kurtzman *et al.*, 1988).

In less severely immunosuppressed patients (i.e. systemic lupus erythematosus on steroid therapy) prolonged anaemia following B19V infection has also been described. However in these patients there was a spontaneous, albeit delayed, development of antibodies, and viraemia resolved without therapy. Presumably such patients represent one end of the spectrum of disease manifestations of B19V in patients with a compromised immune system.

Atypical Presentations

A wide variety of symptoms and diseases have been associated with parvovirus disease (Table 35.3 and see Török, 1997), generally as case reports or limited small series of patients. Determining the role of B19V in these diseases is often difficult: the diseases are rare and B19V may not be the only cause. In addition, with sensitive PCR-based assays B19V DNA can be detected in bone marrow and other solid tissues from healthy individuals for years following acute infection. When the disease is rare, large multicentre trials may be required to substantiate or disprove the causal relationship. Many of these

Table 35.3 Atypical disease presentations of parvovirus B19V

Infection in the healthy host
Neurological disorders/encephalitis
Myocarditis
Hepatitis
Glomerulonephritis
Haematological
Thrombocytopenia
Neutropenia
Pancytopenia
Haemophagocytosis
Rheumatic diseases
Chronic fatigue syndrome
Fibromyalgia
Myositis
Vasculitis
Kawasaki disease
Polyarteritis nodosa

diseases have an autoimmune component associated with their pathogenesis (Lehmann *et al.*, 2003), and B19V is one of many of the putative causes or triggers.

LABORATORY DIAGNOSIS

Aplastic crisis is the only one of the clinical syndromes that can be assumed, with some accuracy, to be due to B19V infection. Even so, in patients with chronic haemolytic anaemia moderate to severe degrees of hypoplasia may be associated with systemic bacterial infection or marrow-suppressive drugs, and anaemia in the immunocompromised may have many other causes. Illnesses associated with maculopapular rashes with or without joint involvement also have a multiplicity of causes and only a minority of cases of hydrops fetalis will prove to be due to B19V infection. Thus accurate diagnosis of B19V infection depends upon specific laboratory tests.

Specimens

Serum is the principal specimen used for the laboratory diagnosis of B19V infection. This approach is suitable for virus detection in cases of aplastic crisis, persistent infection in immunosuppressed patients and persistent fetal infection. The detection of specific IgM antibody in serum is the cornerstone of the diagnosis of rash illness, and is often valuable in cases of aplastic crisis. Standard blood specimens are all that are required and no special arrangements are needed for transport to or storage in the laboratory. It should be remembered that viraemic samples may contain high titres of infectious virus, and care should be taken when handling samples, especially those taken early in the course of an aplastic crisis, to ensure that seronegative individuals in the laboratory are not infected (cases of laboratory-acquired infection have been described).

B19V studies can also be performed on tissue samples. DNA can be extracted from fresh, frozen or fixed samples for detection of parvoviral DNA. Detection of B19V by either immunohistochemistry or *in situ* hybridization can also be performed on formalin-fixed, paraffin-embedded tissue, so that standard pathology protocols can be used for dealing with bone marrow or fetal tissue.

Specific Antibody Detection

Standard solid-phase enzyme-labelled immunoassays (ELISAs) are the preferred method for the serological diagnosis of recent B19V infection, although immunofluorescent and Western blot assays are commercially available. Due to the inability to grow B19V in standard

cell culture systems, antigen used in these assays is recombinant proteins expressed in insect, yeast or bacterial cells, or linear peptide.

The best assays to detect recent infection with B19V are IgM capture or indirect assays, using recombinant viral-like particles as antigen. The commercial assays based on viral-like particles have excellent sensitivity and specificity. Sera containing high test : cut-off optical densities are associated with recent infection, but low positives can be seen in autoimmune diseases, and in patients with other rash-like illness. However, high concentrations of anti-B19 IgM usually appear within three to four days of the onset of symptoms and antibody can be detected in over 90% of cases by the third day of TAC or at the time of rash in EI. IgM antibody remains detectable for two to three months following infection (Figure 35.3). In patients presenting with symptoms of aplastic crisis, and occasionally in rash-like presentations specific IgM may not appear until 7–10 days after the onset. Therefore if a negative or equivocal result for anti-B19V IgM is obtained with a serum taken within 10 days of the onset of the illness, the serum should be tested for B19V DNA and/or a second specimen to be taken about 14 days after the onset of symptoms should be requested. Specific IgM is detectable for two to three months after acute infection. Interpretation of equivocal low concentrations of anti-B19V IgM in sera taken after this time can be difficult. However, B19V DNA generally remains detectable in serum for several months following infection, and low B19V IgM can be confirmed by the detection of B19V DNA. Alternatively tests to look for low-avidity antibody to the Vp1u, or the detection of antibody to linear epitopes to B19V VP2 proteins can be used to try and determine the time of infection. These assays are especially helpful in determining timing of possible infection in pregnancy (Enders *et al.*, 2005).

Evidence for previous infection is by detection of B19V IgG, preferably by indirect assay. For greatest sensitivity assays using recombinant viral-like particles should be used. B19V IgG is usually present by the seventh day of illness and probably is lifelong thereafter. As more than 50% of the population have IgG antibody to B19V infection, detection of B19V IgG in a single sample is not useful for the diagnosis of acute infection, but can be used to document seroconversion.

In 1995 the WHO Expert Committee on Biological Standardization recommended that an international standard (IU ml⁻¹) be set for anti-parvovirus B19V IgG testing. The international standard chosen correlates well with the original au scale in the earlier literature. In the 12 months following infection patients usually have relatively high (>50 IU ml⁻¹) concentrations of anti-B19V IgG and the finding of such concentrations supports the

diagnosis of infection during that time. However, there is marked individual variation in the maximum amounts of specific IgG that can be demonstrated in a patient, so that the finding of only low values (>20 IU ml⁻¹) does not exclude recent infection. Previous infection (and therefore immunity in most individuals) is indicated by the detection of >5 IU ml⁻¹ of anti-B19V IgG. However, a negative result cannot be taken to be synonymous with susceptibility.

There is a poor humoral immune response in immunocompromised patients with chronic parvovirus infection and only low concentrations of anti-B19V IgG (and IgM) can be detected in these patients. None of the detectable antibody neutralizes virus infectivity and this is taken to be an essential component of the pathogenesis of the chronic infection. The diagnosis of B19V infection in these patients is dependent on the detection of viral antigen or DNA.

Virus Detection

The culture of B19V in erythroid progenitor cells remains a research procedure and is not used in the routine diagnosis of B19V infections. Instead B19V detection relies on the detection of either viral antigen or viral DNA. Although ELISA- and haemagglutination-based assays have been developed to detect B19V antigen this is generally in the context of blood screening, and they are not used for B19V diagnosis. Instead, detection of viral DNA by quantitative PCR is the mainstay of detection of B19V. A variety of different primers and probes have been described, the most sensitive assays are capable of detecting 1–10 virus particles. Quantitation of the viral load is critical, as low levels of viral DNA (<10⁴ IU (genome copies) ml⁻¹) can be detected for months, or even years after acute infection.

The significance of low levels of B19V DNA in tissue or bone marrow can be confirmed by looking for evidence of viral antigen (by immunohistochemistry) or for evidence of replicating virus by detection of viral RNA.

The diagnosis of B19V infection in a fetus also depends upon the detection of viral DNA (or RNA). Maternal infection will have occurred some weeks previously and maternal serum may therefore be B19V-specific IgM negative. In most instances the fetus has also been found to be specific IgM negative but there is frequently a persistent viraemia. Therefore the diagnosis is best made by detection of B19V in fetal blood samples by the techniques described above. Equally virus can be detected in fetal tissues taken at autopsy from which DNA has been extracted or detected by immunohistochemistry or *in situ* hybridization on formalin-fixed, paraffin-embedded tissue sections. Fixed material can also be used for electron microscopy, but scattered B19V particles within cells

may be more difficult to recognize due to the large number of ribosomes of similar size, often necessitating immuno-gold techniques.

TREATMENT AND PREVENTION

There is no specific antiviral chemotherapy for B19V infection. Symptomatic relief of troublesome joint symptoms may be required for B19V-associated arthralgia, and blood transfusion may be necessary in the acute phase of aplastic crisis.

The only specific treatment for B19V infection is the intravenous administration of human immunoglobulin in cases of persistent infection in the immunocompromised patient. Human normal immunoglobulin preparations are a good source of neutralizing antibodies to B19V virus since at least half the adult population have been exposed to the virus. Controlled trials have not been done but on an empirical basis the administration of 0.4 g kg⁻¹ body weight per day for 5–10 days has proved effective in reducing viraemia and allowing the haemoglobin to return to near-normal values (Frickhofen *et al.*, 1990; Kurtzman *et al.*, 1989a). Patients sometimes relapse months later but they have been shown to respond to repeat courses.

Prevention of disease by isolating susceptible individuals is impractical because infections may be asymptomatic or subclinical, and even symptomatic individuals are most frequently infectious before any sign of illness. Theoretically, susceptible individuals with chronic haemolytic anaemia (or immunocompromised children) could be temporarily protected by the administration of human immunoglobulin but to date this has not been put into practice.

Many animal parvovirus infections are prevented in animals by vaccination, and the prospects for a B19V parvovirus vaccine are good. The immunogen will be recombinant capsid rather than attenuated or killed virus, due to the difficulty of cultivating B19V *in vitro* and the potential dangers of inadvertently modifying, for the worse, the host range of B19V by selection *in vitro*. Baculovirus-produced B19V capsids induce neutralizing antibodies in experimental animals (Kajigaya *et al.*, 1991), even without adjuvant. The presence of VP1 protein in the capsid immunogen appears critical for the production of antibodies that neutralize virus activity *in vitro*, and capsids with supra-normal VP1 content are even more efficient in inducing neutralizing activity (Bansal *et al.*, 1993), and sera from human volunteers immunized with this type of candidate vaccine had neutralizing antibody titres equal to or higher than those observed after natural infection. Phase I trial results appear promising, and phase II trials are planned. However, the targets for such a vaccine remain to be

determined. Should only patients at high risk of severe or life-threatening disease, such as sickle cell patients be protected? Or, in view of the wide variety of disease manifestations affecting all strata of the population should a universal vaccine policy be pursued?

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Human Retroviruses

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INTRODUCTION

Retroviruses are important human pathogens, which have also played several key roles in medical advances. It is thanks to the retroviral enzyme reverse transcriptase (RT) that complementary DNA (cDNA) is made, allowing the production of recombinant proteins as drugs; we also could not perform reverse transcription polymerase chain reaction (RT-PCR) assays to quantitate the load of many viruses of medical importance without the viral enzyme. If oncogenes had not been discovered and characterized in animal retroviruses, our understanding of the molecular basis of human cancer would have developed much more slowly. Finally, the use of retroviruses as vectors for gene transfer has had a significant impact on clinical gene therapy. This chapter introduces retroviruses both as pathogens and as medical tools, and briefly covers the retroviruses in humans other than the well-known pathogens human immunodeficiency virus (HIV) and human T-lymphotropic virus (HTLV). These retroviruses and their associated pathologies are discussed in detail in Chapters 37 and 38.

Retroviruses occur in numerous vertebrate species and are associated with a diversity of diseases: malignancy, autoimmune disease, immunodeficiency syndromes, aplastic and haemolytic anaemias, bone and joint disease (osteopetrosis and arthritis) and neuropathy (Table 36.1). Historically, veterinary diseases such as bovine leukosis and sheep pulmonary adenomatosis were recognized to be transmissible in the mid-nineteenth century. In the early years of the twentieth century, swamp fever in horses, and leukaemia and sarcomas in chickens were first identified as having a viral aetiology. Retroviruses were later found to be associated with malignant disease in mice, cats, ruminants, primates and other hosts,

including fish. A comprehensive text covers most aspects of the molecular biology of animal and human retroviruses (Coffin *et al.*, 1997). There is also a recent critical review of all the claims for association of retroviruses with human diseases (Voisset *et al.*, 2008).

The first genuine human retrovirus to be characterized was the human T-lymphotropic virus type 1 (HTLV-1) in 1980 (Poiesz *et al.*, 1980) while HIV-1 was first reported in 1983 (Barre Sinoussi *et al.*, 1983) just two years after the recognition of acquired immune deficiency syndrome (AIDS) as a new disease entity.

There are also numerous cases of zoonotic infections of humans by retroviruses maintained in animal reservoirs, for example, foamy viruses. HIV-1 and HIV-2 had zoonotic origins in the twentieth century, while HTLV-1 and HTLV-2 became human-to-human infections much earlier. However, new simian-to-human infections of HTLV-1-related viruses still occur.

RETROVIRUS REPLICATION AND GENOMES

Retroviruses are a single taxonomic group of RNA viruses that encode the RNA-directed DNA polymerase reverse transcriptase. Upon infection, this enzyme catalyses the synthesis of a double-stranded DNA genome. The provirus subsequently becomes integrated into host chromosomal DNA and serves as a template for viral genomic and messenger RNA transcription by the host cell's RNA synthetic and processing systems (Figure 36.1). Other special features of retroviruses include a diploid RNA genome, a high frequency of intermolecular recombination between related viruses and the ability to acquire host genes which encode functions responsible for neoplastic

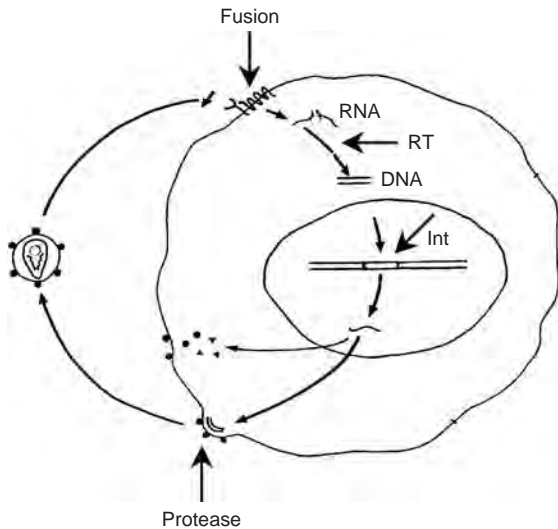


Figure 36.1 Simplified replication cycle of a retrovirus. The virion containing two RNA genome copies enters the virus via a specific cell surface receptor complex. The single-stranded RNA genome is converted into a double-stranded DNA provirus by the virion enzyme reverse transcriptase (RT). The provirus inserts into host chromosomal DNA in the same orientation as the original virion RNA. Transcription of RNA from the integrated DNA provirus is mediated by cellular RNA polymerases, and this RNA serves both as messenger RNA for the synthesis of viral antigens and as genomic RNA, which becomes packaged into progeny virions budding from the cell surface. The straight arrows indicate steps in replication that can be blocked by antiretroviral drugs.

transformation (oncogenes). Moreover, there are numerous endogenous proviruses in the normal cellular DNA of all vertebrates examined (including humans), which represent 'fossil' infections by retroviruses of the germline of the hosts' ancestors and which are passed from generation to generation in a mendelian manner (Weiss, 2006).

All retroviruses carry at least three genes with the genome structure in the order 5' LTR-*gag-pol-env*-3' long terminal repeats (LTRs). *Gag* encodes a precursor protein which is cleaved to yield three or four structural core and matrix proteins; *pol* encodes the RT, protease and integrase, synthesized from a *gag-pol* precursor; while *env* encodes a precursor cleaved to form the two envelope proteins, surface (SU) and transmembrane (TM). The core proteins are often named by molecular weight (e.g. p24 and p17 of HIV). The outer SU protein is glycosylated and is known as gp120 (e.g. for HIV with an approximate molecular weight of 120 000) or gp70

(e.g. gamma-retroviruses such as murine leukaemia virus (MuLV)). The anchored TM protein, to which the SU protein is bound, is glycosylated in some retroviruses (e.g. gp41 of HIV) but not others (e.g. p15E of C-type oncoviruses). The DNA proviral genome is capped by LTRs at both ends and the RNA form has a polyadenylate tract at the 3' end, like mRNA. The LTRs are the sites for integration of the DNA provirus with cellular DNA, and contain important promoter and enhancer sequences that bind cellular and viral proteins regulating viral gene expression. Integration is an obligatory step in the retroviral life cycle. The main steps in replication that are targets for antiretroviral drugs are indicated in Figure 36.1.

TAXONOMY

The *Retroviridae* used to be divided into three subfamilies: the *Oncovirinae*, which include those with oncogenic potential; the *Lentivirinae* or slow viruses, including HIV and the prototype maeda-visna virus (MVV) of sheep which causes progressive wasting disease, pneumonia and degeneration of the central nervous system; and the *Spumavirinae* or foamy viruses, which have not been shown to be pathogenic in any species. Retroviruses are now reclassified into seven distinct genera by dividing oncoviruses into five, as they are only distantly related by genome sequence and morphology (Figure 36.2 and Table 36.2). More generally, retroviruses are divided into those with 'simple' genomes, having *gag*, *pol* and *env* genes and perhaps one other gene, and those with 'complex' genomes. Members of the latter group possess regulatory genes, such as *tat* and *rev* of HIV and *tax* of HTLV, and accessory genes, such as *nef*, *vif* and *vpr* of HIV.

HUMAN AND ZONOTIC RETROVIRUS INFECTIONS

At least five groups of retroviruses have been reported as human infections.

1. Human immunodeficiency virus types 1 and 2 are the lentiviruses that cause AIDS. Before the term HIV was coined in 1986, HIV was called lymphadenopathy virus (LAV), HTLV-3 or ARV. HIV and AIDS are covered in Chapter 38.
2. Human T-cell lymphotropic virus types 1 to 4 (HTLV-1, etc.) are deltaviruses (note the nomenclature HTLV-3 to avoid confusion with a previous name for HIV). Type 1 causes adult T-cell leukaemia and neurological disease, and is reviewed in

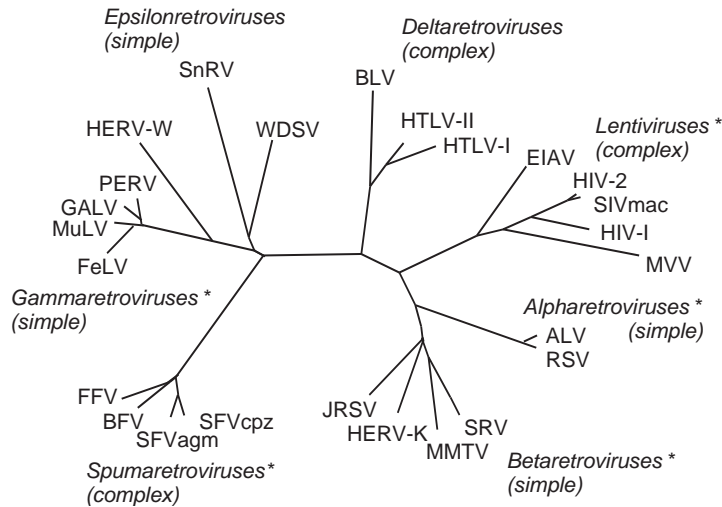


Figure 36.2 Phylogenetic tree of the seven main genera of retroviruses. Abbreviations beginning with H are human viruses. The * denotes genera that include endogenous viral genomes in addition to exogenous infectious viruses.

Table 36.1 Diseases caused by retroviruses in animals

Disease/disorder	Species affected
Leukaemia	Avian, mouse, cat, primates
Lymphoma	Avian, mouse, cat, primates, fish
Carcinoma	Avian, mouse, sheep
Sarcoma	Rat, chicken, fish
Anaemia, aplasia	Cat, horse
Autoimmune disorders	Cat, primates, sheep, mouse
Immune deficiency	Cat, primates
Nervous system	Sheep, goat, mouse
Osteopetrosis	Chicken
Arthritis	Goat

Chapter 37. Only a small proportion of people infected with HTLV-1 progress to disease. The Tax protein and the bZIP factor may play a role in initiating HTLV malignancies (Yasunaga and Matsuoka, 2007).

3. Primate foamy virus (PFV) is a spumavirus originally detected in cultured nasopharyngeal carcinoma cells of a Kenyan patient. This finding marked the first report of human infection by a retrovirus (Achong *et al.*, 1971). Because the PFV genome is indistinguishable from that of the simian foamy virus type 6 of chimpanzees, it probably represents a zoonosis and several other examples of foamy virus infections transmitted to humans from various species of Old World monkeys and apes have been recorded (Heneine *et al.*, 2003). Zoonotic

PFV infection without clinical symptoms has been recorded in primate handlers who have suffered bites or puncture wounds and is frequently seen in people who worship at Hindu temples that are populated by macaques and langurs. However, there is no evidence of human-to-human transmission of PFV, not even from PFV-infected people who were blood donors (Heneine *et al.*, 2003; Murray and Linial, 2006).

4. Murine retroviruses—Since the discovery of RT in 1970 there have been numerous reports of retrovirus infections in human cells in culture, which were thought to represent human retroviruses. However, several of these infections resulted from *in vitro* contamination by murine, feline or non-human primate retroviruses, whereas others could not be firmly substantiated. Other reports recorded PCR detection of retroviral genomes in noncultured human biopsy samples, for example, of murine mammary tumour virus sequences in human breast tumour tissue. Most of these claims have been difficult to confirm or substantiate and require cautious interpretation in case they originate from the extraordinary sensitivity of detecting retroviral sequences in animal material (such as sera) contaminating human specimens in the laboratory. A good rule of thumb is that the detection of unusual, low-level retroviral sequences in DNA from human biopsies should not be taken as evidence of genuine infection unless the retroviral genomes are clearly integrated into human DNA, which can be ascertained by analysis of host sequences flanking the integration sites (Voisset *et al.*, 2008).

Table 36.2 Classification of retroviruses of vertebrates

Genus	Example	Virion morphology ^a	Genome
Alpharetroviruses	Rous sarcoma virus Avian leukosis virus	Central spherical core; C-type —	Simple
Betaretroviruses	Murine mammary tumour virus Simian retrovirus type I	Eccentric spherical core; B-type Central spherical core; D-type	Simple
Gammaretroviruses	Murine leukaemia virus	Central spherical core; C-type	Simple
Deltaretroviruses	Human T-cell leukaemia virus	Central spherical core	Complex
Epsilonretroviruses	Fish dermal sarcoma virus	Central spherical core; C-type	Simple
Lentiviruses	Human immunodeficiency virus	Cone-shaped core	Complex
Spumaviruses	Primate foamy virus	Central spherical core; long envelope spikes	Complex

^aBetaretroviruses and spumaviruses have condensed cores visible in the cytoplasm of infected cells, whereas in the other retroviruses the cores condense as crescent-shaped bodies during maturation and budding at the cell membrane.

5. XMRV—Evidence of retroviral integration into human DNA has been provided for a gammaretrovirus, xenotropic MuLV-related virus (XMRV), which is closely related to xenotropic MuLV. Endogenous strains of MuLV are denoted ecotropic if their host range is restricted to rodents, xenotropic if they cannot reinfect murine cells but can infect ‘foreign’ species such as human cells, and amphotropic if they can infect both murine and human cells. XMRV has been found in stromal cells of prostate cancer biopsies in patients homozygous for a variant of the RNaseL enzyme that gives reduced activity (Dong *et al.*, 2007). RNaseL is a downstream effector of the interferon pathway to which XMRV is sensitive. The natural history of XMRV is not yet elucidated, and it might represent a zoonotic infection from mice that only infects people with defects in the interferon response pathway. There is also preliminary evidence of human infection by a virus related to murine mammary tumour virus, a betaretrovirus, in patients with primary biliary cirrhosis (Voisset *et al.*, 2008).
6. Human endogenous retroviruses (HERVs) are mendelian loci in human chromosomes representing ‘fossil’ infections of the germline (Weiss, 2006). Approximately 8% of human DNA sequences are derived from HERV insertions and some 42% of human DNA derives from retrotransposons (de Parseval and Heidmann, 2005). The best-characterized HERV genomes are related to mammalian gamma- and betaretroviruses (HERV-K; which uses tRNA for lysine as a primer) but others exist too. No deltaretroviruses are known to have become endogenous. HERV genomes are almost all defective, that is, human endogenous retroviral genomes have not been rescued in infectious form, in contrast to baboon endogenous virus (BaEV) and porcine endogenous virus (PERV) of pigs, which threaten the safety of human xenotransplantation from these

sources (Magre *et al.*, 2003). Many HERV genomes express envelope and other proteins. For example, HERV-W (which uses tRNA for tryptophan as a primer) in the human placenta may be necessary to effect cell fusion in the syncytiotrophoblasts (de Parseval and Heidmann, 2005). HERV-K is expressed in testicular tumours and in the pancreas of type 1 diabetes while HERV-W is expressed in multiple sclerosis brain lesions, yet none of these viral genomes have been shown to play a causal role in these diseases (Voisset *et al.*, 2008).

RETROVIRAL VECTORS

Retroviruses have been turned to clinical use as vectors in gene therapy and immunotherapy (Gaspar and Thrasher, 2005). Because retroviral genomes naturally integrate into host chromosomal DNA they provide an excellent means of delivering therapeutic genes or genes encoding antigens for vaccination. MuLV, a gammaretrovirus, was pioneered for gene therapy, using three plasmids in packaging cells: expressing MuLV *gag* and *pol* to provide core proteins and viral enzymes, vesicular stomatitis G protein as an envelope, and the therapeutic gene in a vector carrying packaging signals and the LTR of MuLV. However, in a successful, curative clinical trial of gene therapy of infants with severe combined immune deficiency due to adenine deaminase deficiency, some patients developed leukaemia as a result of the integration of the vector with an active LTR adjacent to a host oncogene. Safer vectors have been developed without promoters and enhancer sequences in the LTR, or omitting the need for integration altogether (Philpott and Thrasher, 2007).

Lentiviral vectors, usually based on the HIV-1 genome, or that of equine infectious anaemia virus, have an advantage over MuLV vectors in their capacity to infect

nondividing target cells (Philpott and Thrasher, 2007). Foamy virus vectors are under development because this type of retrovirus has not been associated with disease in animals or humans (Murray and Linial, 2006; Rethwilm, 2007).

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The Human T-Lymphotropic Viruses

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INTRODUCTION

The HTLV-BLV viruses are a subfamily of the family *Retroviridae*. They comprise the more well-known human T-cell lymphotropic virus types 1 and 2 (HTLV-1, HTLV-2); bovine leukaemia virus (BLV); and an increasing number of simian T-lymphotropic viruses/primate T-lymphotropic viruses (STLVs/PTLVs), which are closely related to HTLV-1 and -2, including the recently described HTLV-3 (Calattini *et al.*, 2005; Wolfe *et al.*, 2005) and HTLV-4 (Wolfe *et al.*, 2005). HTLV-1 infection is usually asymptomatic but is associated with malignant and inflammatory diseases and a mild or selective impairment of immune function in a minority. Disease associations with HTLV-2 are less well established. STLVs have been associated with malignant disease in non-human primates, but not necessarily in the primary host species. In a small proportion of naturally infected cattle, BLV is associated with a B-cell leukaemia. However, leukaemia or lymphoma is very common in sheep experimentally infected with BLV.

HISTORY

The discovery of HTLV-1 as an important human pathogen was the result of two distinct lines of research. One, the long search for cancer-causing retroviruses in humans, was dependent on the earlier discovery and refinement of tests for reverse transcriptase (RT), together with the identification and use in cell culture of T-cell growth factor, now known as interleukin 2 (IL-2). In 1980, Gallo's team

found that one of their many transformed T-cell lines derived from leukaemia/lymphoma patients contained a retrovirus (Poiesz *et al.*, 1980). This virus was HTLV-1, also sometimes referred to as the human T-cell leukaemia/lymphoma virus type I. The second was the recognition (1974) and description (1977) of a new disease entity, adult T-cell leukaemia-lymphoma (ATLL) by Takatsuki and colleagues (Uchiyama *et al.*, 1997). The clustering of this disease particularly in south-western Japan suggested an environmental or infectious aetiology. Gallo's cell line was derived from the lymphocytes of an Afro-American patient with an aggressive form of cutaneous T-cell lymphoma subsequently recognized to be cutaneous ATLL.

In 1981 Miyoshi and colleagues produced an immortalized T-cell line by the co-culture of peripheral blood lymphocytes from a woman with ATLL with cord blood cells from a male baby (Miyoshi *et al.*, 1981). This cell line (MT-2), which has an XY karyotype, was observed to produce numerous extracellular type C retroviral particles and was positive for adult T-cell leukaemia antigen by indirect immunofluorescence (Hinuma *et al.*, 1981). Using viral antigens from MT-2 cells to develop a serological test they demonstrated that nearly all ATLL patients and a high proportion of their relatives had antibodies to this virus (Yoshida *et al.*, 1982). Known initially in Japan as adult T-cell leukaemia virus (ATLV), sequence analysis showed that ATLV was almost identical with HTLV-1. Seroprevalence studies revealed that ATLV/HTLV-1 was endemic in south-western Japan with 15% of the population seropositive, rising to 30% in some villages. In central Japan only 1% of the population were seropositive, with higher rates again in the north. The inhabitants

of central Japan are believed to have come from mainland Asia about 300 BC, displacing the 'older' population to the north and south-west. This distribution suggests that the arrival of HTLV-1 in Japan preceded this migration.

A high seroprevalence of HTLV-1 in patients in Martinique with tropical spastic paraparesis (TSP) was first described by Gessain *et al.*, (1985) and the description of an identical condition, HTLV-1-associated myelopathy (HAM) in Japan was published the following year (Osame *et al.*, 1986). This disease is commonly referred to as HAM/TSP but here the acronym HAM will be used as the data presented refer only to HAM. HTLV-1 seronegative TSP occurs.

A related virus, HTLV-2 was isolated from the cells of a patient with an atypical hairy cell leukaemia (HCL) by Kalyanaraman *et al.*, (1982). HCLs are usually of B-cell origin but the infected cell line expressed T-cell markers. Although HTLV-2 was isolated from a second patient with HCL, subsequent extensive investigation has failed to confirm any association between HCL and HTLV-2 infection. HTLV-2 shares 60–70% sequence homology with HTLV-1 and anti-HTLV-2 antibodies are detected by HTLV-1 lysate or whole virus-based assays.

STLVs have been found in macaques and other Old World monkeys, and STLV-1 appears to be more closely related to HTLV-1 than to other STLV/PTLVs. HTLV-1/STLV-1 strains cluster geographically rather than by host species. During the last few years a number of STLVs closely related to, but discrete from, HTLV-1 have been characterized. These include relatives of HTLV-2 (Giri *et al.*, 1994) and viruses that cluster neither with HTLV/STLV-1 nor -2. The first of these, found in an Eritrean baboon (*Papio hamadryas*), was designated PTLV-L, after Leuven where the baboon was residing and the virus was isolated (Goubau *et al.*, 1994). STLVs isolated from wild-caught red-capped mangabeys (*Cercocebus torquatus*) from Cameroon are distinct from, but cluster with, PTLV-L in the newly designated PTLV-3 type (Meertens *et al.*, 2002). In 2005 an HTLV isolated from natives of Cameroon was found to demonstrate seroreactivity similar to HTLV-2 but genomic sequencing independent from HTLV-1 and HTLV-2 and distinct clustering with the PTLV-3s. HTLV-III having been used, along with lymphadenopathy virus (LAV) as an early name for HIV-1, this virus has been designated HTLV-3 (Calattini *et al.*, 2005). Thus far, HTLV-3 has been found only in subjects with a connection with the bush-meat trade and there is no evidence of disease association or nonzoonotic transmission. In addition to HTLV-3, an independent group also identified multiple human infections with STLV-1 viruses and a further distinct isolate, HTLV-4 (Wolfe *et al.*, 2005).

THE VIRUS

Morphologically HTLV-1 and -2 resemble C-type retroviruses (Figure 37.1). They can be grown *in vitro* in immortalized lines obtained by culturing patients' cells with phytohaemagglutinin (PHA) and IL-2. *De novo* infection of T cells and cell lines requires co-cultivation using irradiated or mitomycin-C-treated HTLV producer cell lines. The HTLVs are not readily transmissible in cell-free form and this is reflected in the observation that only whole fresh blood transfusion and not plasma or other cell-free fractions results in transmission (Okochi *et al.*, 1984).

Susceptible cell lines can be identified by the formation of syncytia (giant multinucleated cells) upon contact with virus-producing cells. This interaction requires the presence of the HTLV-1 envelope and specific cell membrane ligands (receptors). The syncytial assay has proved useful in studying HTLV-1, particularly for the detection of neutralizing antibodies and cellular receptors (Clapham *et al.*, 1984). *In vitro* many cell types from a broad range of species, including astrocytes, can be infected. *In vivo* HTLV-1 primarily infects CD4⁺ lymphocytes whilst HTLV-2 infects CD8⁺ lymphocytes. However HTLV-1 infection of CD8⁺ lymphocytes does occur and is more common among HTLV-1-specific cytotoxic CD8⁺ lymphocytes than other CD8⁺ cells (Hanon *et al.*, 2000). Infection of B cells, monocytes (Koyanagi *et al.*, 1993) and natural killer (NK) cells (Igakura *et al.*, 2003) has been reported, but Richardson found no evidence of such infection cells *in vivo* (Richardson *et al.*, 1990) Whether HTLV-1 infection of neural cells occurs *in vivo* remains controversial.

The glucose transport protein GLUT-1 (Manel *et al.*, 2003) has been identified as a receptor for HTLV-1 but since this is widely distributed while HTLV-1 tropism is narrow, an additional receptor seems probable. The

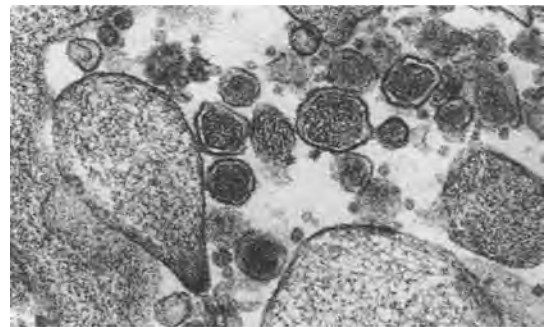


Figure 37.1 HTLV-1 electron micrograph. Electron micrograph of HTLV-1 particles produced by a T-cell line transformed *in vitro*. Note the variable size of the particles and the diffuse spherical core.

envelope proteins of HTLV-1 and HTLV-2 are the target for neutralizing antibodies. Sera from British or American patients and asymptomatic carriers of HTLV-1 neutralize equally viruses from other countries including Japan (Clapham *et al.*, 1984). This suggests that there is a single worldwide serotype for HTLV-1.

The 9 kb genome of HTLV-1 contains the three major open reading frames (ORFs), *gag*, *pol* and *env*, of all retroviruses flanked by two long terminal repeats (LTRs) with an additional regulatory region, pX (Figure 37.2). The names, product size and functions of the genes of HTLV-1 are summarized in Table 37.1. The HTLV-2 genome is very similar except that five ORFs have been identified in pX.

The LTR containing the viral promoter and other regulatory elements are divided into three regions, U3, R and U5. The U3 region contains elements that control proviral transcription, messenger RNA termination and polyadenylation.

The first major reading frame (*gag*) encodes a 429 amino acid precursor polyprotein Pr53^{Gag}. A second major reading frame within the *gag-pol* complex encodes a second precursor Pr76^{Gag-Pro} which gives rise to the viral protease. This frame slightly overlaps with *gag* and is generated by a frameshift suppression of the *gag* terminator codon. During viral assembly the *gag* precursors accumulate at the inner face of the plasma membrane and are anchored there by the matrix domain. In the absence of protease immature viral particles are released. During and early after viral budding cleavage of Pr53^{Gag} into the three major structural proteins of the core of the virus, 19 kDa (matrix protein), 24 kDa (capsid protein) and 15 kDa (nucleocapsid protein), results in the formation of the mature virion. The viral genome is transported to the cell envelope bound to the *gag* precursor. The matrix domain of the *gag* precursor is involved in targeting of the *gag* precursor-genome complex to the plasma membrane (this also requires myristylation of the polyprotein) and in

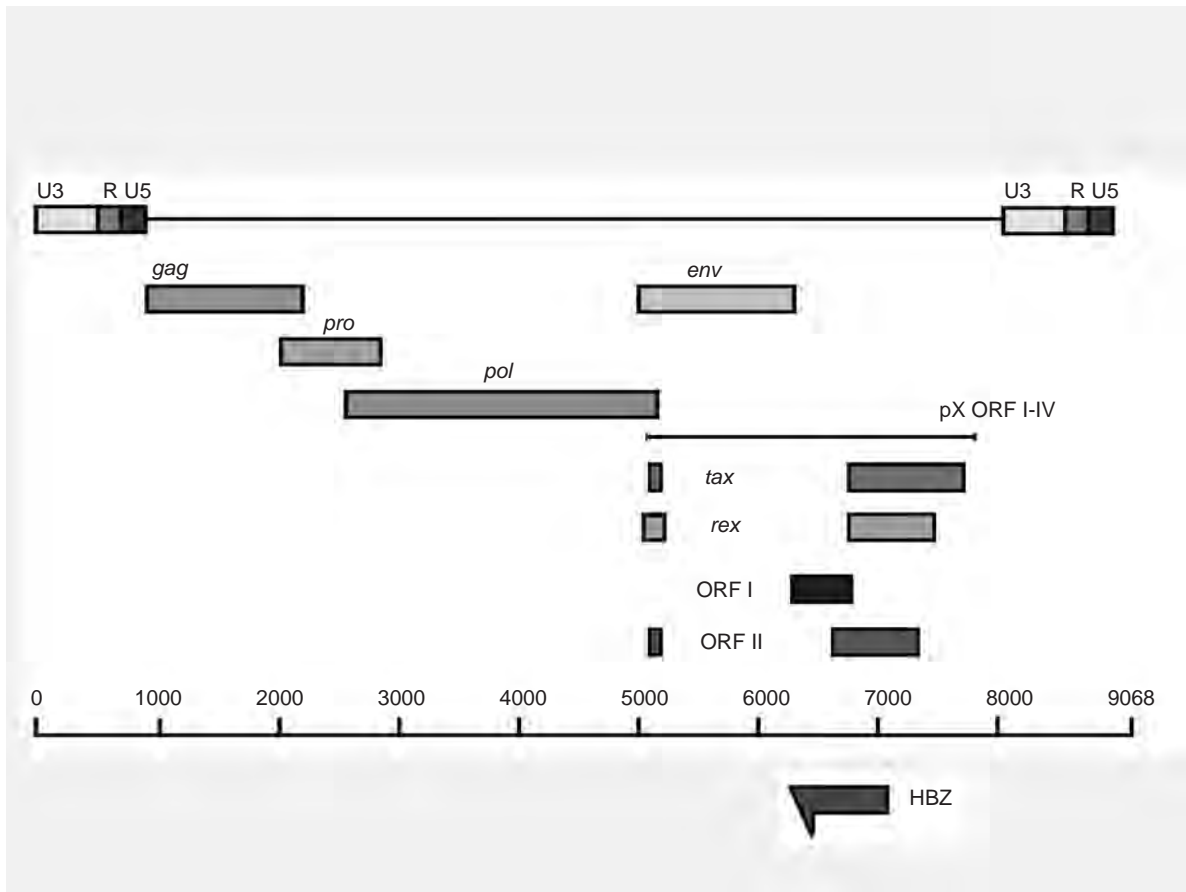


Figure 37.2 Schematic representation of the HTLV-1 genome. (Source: Modified from Franchini, G. (1995) *Blood*, **86**, 3619–39. Reproduced with permission.)

Table 37.1 Products of HTLV-1 genes

5' LTR		Contains regulatory elements essential for viral replication			
<i>gag</i>	Group <i>antigen</i>	Nucleocapsid proteins	p19 p24 p15	Matrix Capsid Nucleocapsid	
<i>pol</i>	<i>Polymerase</i>	Reverse transcriptase	RT	transcription of DNA from RNA	
		Proteinase		cleavage of protein precursors	
		RNaseH		digestion of RNA template	
		Integrase		integration of proviral DNA into host genome	
<i>Env</i>	<i>Envelope</i>	Surface glycoprotein	gp46	SU	
		Transmembrane	gp21	TM	
<i>Px</i>	ORF I		p12 ^I	(Rof) infectivity	
	ORF II		p13 ^{II} p30 ^{II}	mitochondrial protein	
				(Tof) modulation of cellular gene transcription	
	ORF III		p27	Rex	Regulatory gene of 'X' region
			p21 ^{rexIII}		Cytoplasmic protein unknown function
	ORF IV		p40	Tax	Transactivating gene of 'X' region
3' LTR		Contains regulatory elements essential for viral replication			
HBZ		Antisense strand of Px		Separate roles of RNA and protein	

efficient budding of the viral particle from the cell. The mature (cleaved) matrix protein also plays a role early in the replication cycle, in viral entry, as virions with defective matrix proteins have reduced infectivity even when properly released (Le Blanc *et al.*, 1999). The capsid proteins assemble during viral maturation to form the shell around the core of the nucleus. The N-terminal domain is essential for viral particle formation (Rayne *et al.*, 2001). In the viral particle the nucleocapsid protein is also a key component of the capsid and is in close contact with the genome. In the cell the nucleocapsid domain of the *gag* precursor polyprotein is important for recognition and packaging of the viral genome. Retroviral *gag* function is reviewed in (Wills and Craven, 1991).

The remaining products of *pol*, RT and integrase are encoded by a different ORF and are cleaved from a 99 kDa precursor protein. RT transcribes the single viral RNA strand, generating first the complementary DNA strand

and then, using that as a template, the viral DNA version of the original RNA; the latter is digested by RNase H. The double-stranded viral DNA is then integrated into a host cell chromosome. Having no proofreading mechanism, RT is error prone, and a high frequency of mutation would be expected to result in HTLV/STLV sequence diversity. However, as we will see later, this does not occur.

Env encodes a 481-amino-acid protein that after glycosylation has a molecular weight of 62 kDa and is processed into an outer surface protein of 46 kDa and a transmembrane protein of 21 kDa. The envelope precursor is translated from a 4.2 kb mRNA from which *gag* and *pol* have been spliced out.

The fifth genomic region, referred to as pX, is located between *env* and the 3' LTR. The region codes for *tax* and *rex* which are translated from a double spliced 2.1 kb mRNA as well as three additional proteins encoded by

ORFs I, II and III, whose functions are slowly being revealed. Tax, a 40 kDa protein that is expressed early, drives viral transcription from Tax responsive elements (21 bp repeats) in the LTR U3 and is an important upregulator of viral replication. In addition *tax* transactivates a range of host cellular genes through which it is thought to affect cell replication and play an important role in the pathogenesis of ATLL. The immunodominant peptides for the host cytotoxic T-lymphocyte response are also usually found in the Tax protein. This may be important both for the control of infection and in the pathogenesis of inflammatory disease associated with HTLV-1, of which the archetype is HAM.

Rex is a 27 kDa phosphoprotein which determines the export of unspliced *gag-pol* mRNA and singly spliced *env* mRNA from the nucleus, thereby controlling the production of viral proteins and infectious virus. Rex production results in increased export of *env* mRNA and unspliced viral RNA for the Gag/Pol proteins essential for the production of new virions and aids a shift from doubly spliced P_x RNA to *gag-pol* and *env* RNA. Thus the overall effect of Rex may be virion production soon after infection followed by downregulation of viral expression. This may protect the infected cell from death due to the cytolytic effect of virion production and protect both the infected cell and the virus from the host immune response (Hidaka *et al.*, 1998).

The Tax (p37) and Rex (p26) proteins of HTLV-2 are slightly smaller than those of HTLV-1. HTLV-2 Tax shares 72–74% amino acid homology with HTLV-1 Tax. However the C-termini of HTLV-2 Tax (both HTLV-2a and HTLV-2b) are different from HTLV-1 Tax. Tax IIa has a 22-amino-acid truncation at the C-terminus while the C-terminus of Tax IIb has 25 amino acids which are totally different from Tax I (Lewis *et al.*, 2000). *In vitro* HTLV-2 Tax proteins activate transcription of NF- κ B and the c-AMP response element-binding protein (CREB) pathways just as well as HTLV-1 Tax, although Tax from some HTLV-2a isolates had much less transcriptional activity possibly due to low levels of expression of Tax (Lewis *et al.*, 2002). However both Tax IIa and Tax IIb are less efficient at transforming rat fibroblast cell lines than Tax I (Endo *et al.*, 2002).

The additional proteins are p13 and p30 (ORF II) and p12 from ORF I. P30^H localizes to the nucleus and may modulate transcription of cellular genes. P13^H localizes to mitochondria and *in vitro* disrupts the mitochondrial inner membrane potential. Although not essential for viral replication these proteins are important for viral infectivity. P12^I exhibits weak oncogenic activity and is important in infectivity. (Reviewed by Ciminale *et al.*, 1996 and by Johnson *et al.*, 2001).

Encoded from the antisense strand of the P_x region is the HTLV-1 bZIP protein (HBZ) (Gaudray *et al.*, 2002). This

leucine zipper protein inhibits CREB binding to the viral promoter, thus countering the action of Tax (Lemasson *et al.*, 2007). Conversely, HBZ mRNA has been shown to contribute to T-cell proliferation (Satou *et al.*, 2006).

DIAGNOSIS

Detection of HTLV-1 and -2 infection is primarily by serology. A particle agglutination assay based on whole viral lysate is sensitive for both viruses and relatively inexpensive but limited by poor specificity. First-generation commercial enzyme immunoassays (EIAs) were more specific than the agglutination assays but less sensitive, particularly for HTLV-2. EIAs incorporating recombinant peptides are highly sensitive and specific for HTLV-1 and HTLV-2. Immunofluorescence assays using fixed infected cells with uninfected cells as controls can be used to confirm and type infections but are subjective. Peptide-based assays that discriminate between HTLV-1 and HTLV-2 have been developed but HTLV-1 infection is usually confirmed by the detection of antibodies to gag (p19 and p24) and env (gp21 and gp46) by Western blot (Figure 37.3), although radioimmune precipitation assays (RIPAs), radioimmune binding assays (RIBAs), line immunoprecipitation assays (LIAs) and competitive enzyme-linked immunosorbent assays (ELISAs) can also be used. p19 antibody is often absent in, and is not required to confirm, HTLV-2 infection. Recombinant env peptides have improved the sensitivity and specificity of Western blotting and type-specific antigens rgp46-I and rgp-46-II enable the infections to be discriminated without resort to molecular methods. Minimal criteria for the diagnosis of HTLV infection are the detection of antibodies to one gag and one env protein. Western blots that reveal some virus-specific bands but which are not sufficient to make a positive diagnosis are termed 'indeterminate'. Further investigation is then warranted, including DNA amplification using generic or type-specific primers and repeat serology after a few weeks. Viral culture may be required to confirm an infection but is costly, time consuming and must be conducted in a category 3 laboratory (The HTLV European Research Network, 1996). Although HTLV-1 viral DNA load is frequently high in asymptomatic carriers, very low levels, <1 HTLV DNA copy per 100 000 peripheral blood mononuclear cells (PBMCs), can be found and a negative polymerase chain reaction (PCR) does not exclude the diagnosis of HTLV infection. In European studies HTLV-1/2 infection was not confirmed in the majority of low-risk subjects with Western blot indeterminate sera, whereas among HIV-infected injecting drug users HTLV-2 infection might be found by DNA amplification. Indeterminate Western blot patterns are common in the tropics and may be more common than confirmed

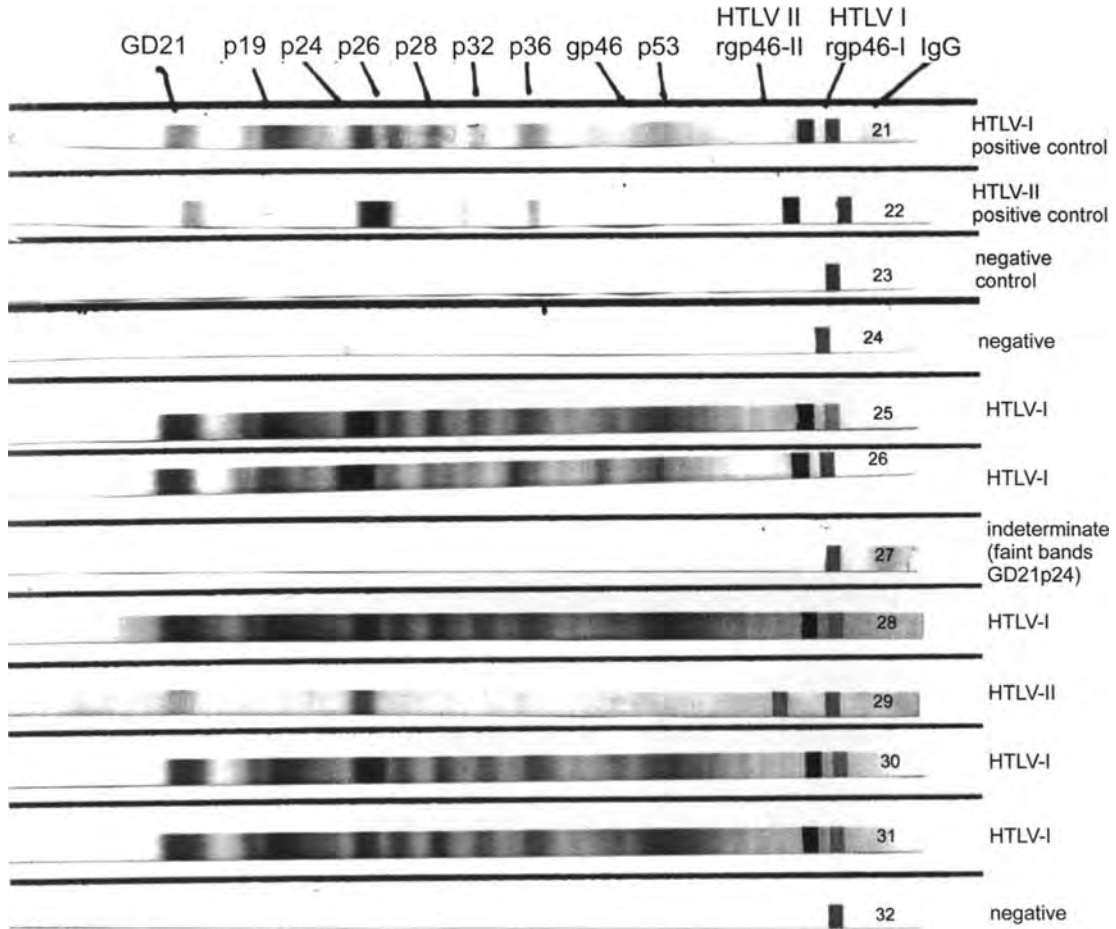


Figure 37.3 HTLV-1 and -2 Western blot with recombinant peptides which discriminate between HTLV-1 and HTLV-2 infection. (Genelabs HTLV 2.4, Singapore.)

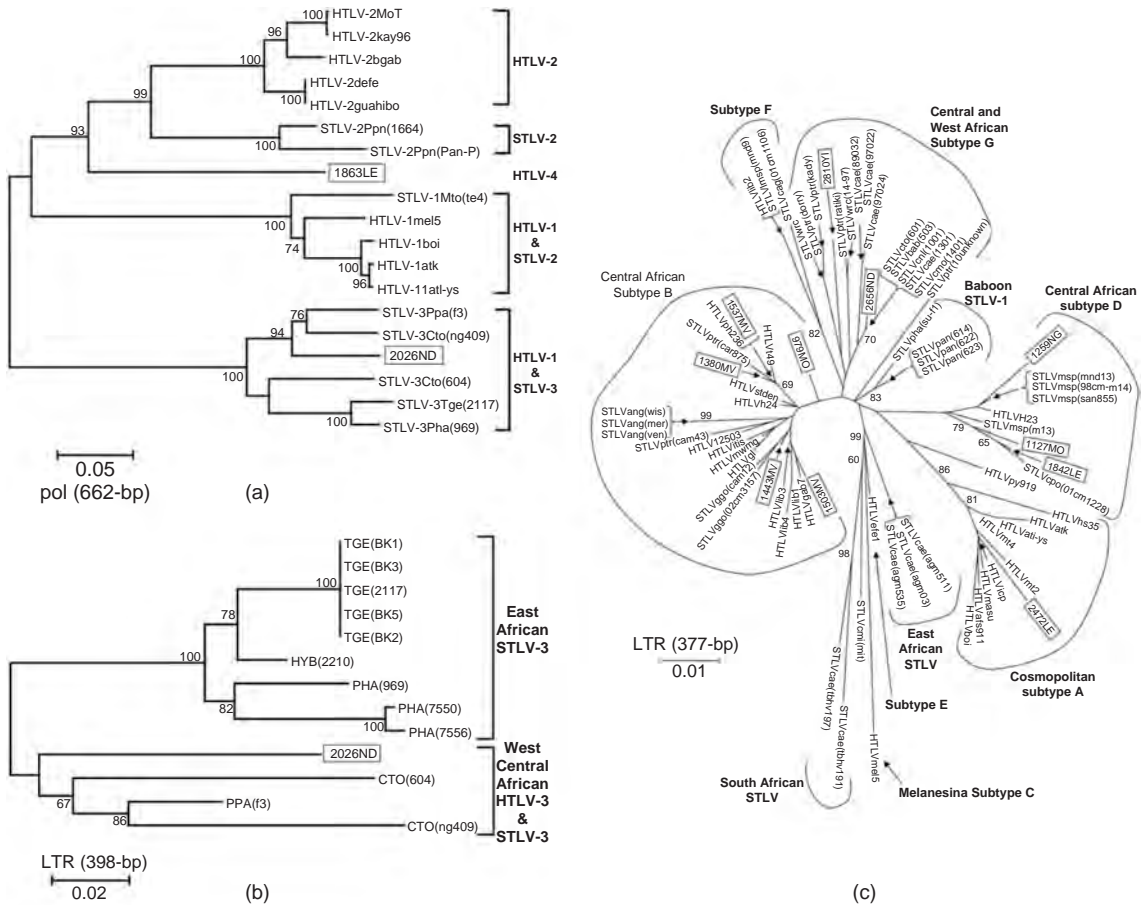
HTLV-1 infection, even in endemic areas. In this setting antibodies to Gag proteins only have been associated with a low risk of HTLV infection (Rouet *et al.*, 2001).

The presence of the recombinant surface membrane glycoprotein (RD21 in the HTLV 2.4 Western blot kit, Genelabs, Singapore) alone may reflect recent infection and a follow-up sample should be obtained. Antibodies to the Gag proteins p19 and p24 usually appear early following seroconversion whereas antibodies to the transmembrane glycoprotein gp46 appear later. Anti-Tax antibodies occur late or not at all (Manns *et al.*, 1991).

VIRAL VARIATION

Despite the high mutation rate seen with RT, HTLV-1 has a highly conserved genome with only about 4% sequence variation between isolates from around the world. The

only exceptions are isolates from Australian aborigines and Melanesians which form a distinct genotype or clade, HTLV-1_{MEL}, and which have up to 8% diversity in LTR and/or env. Most other isolates from Africa, Japan or the Caribbean belong to the Cosmopolitan clade, HTLV-1_{COS}. However, three other clades have been recognized, from Central Africa, from West Africa and from Japan (Ureta-Vidal *et al.*, 1994). In phylogenetic analyses, STLV-1, which is almost identical to HTLV-1, clusters with HTLV-1 by geography rather than by species (Figure 37.4). This suggests that there have been a number of simian-human transmissions (Koralnik *et al.*, 1994). HTLV-2 shares 60–70% sequence homology with HTLV-1. HTLV-2 isolates generally belong to genotype a or b. HTLV-2c (Eiraku *et al.*, 1996) has to date only been described in Brazil among Amazonian Indians



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Figure 37.4 Phylogenetic analysis showing the relationship between HTLV-1/STLV-I subtypes.

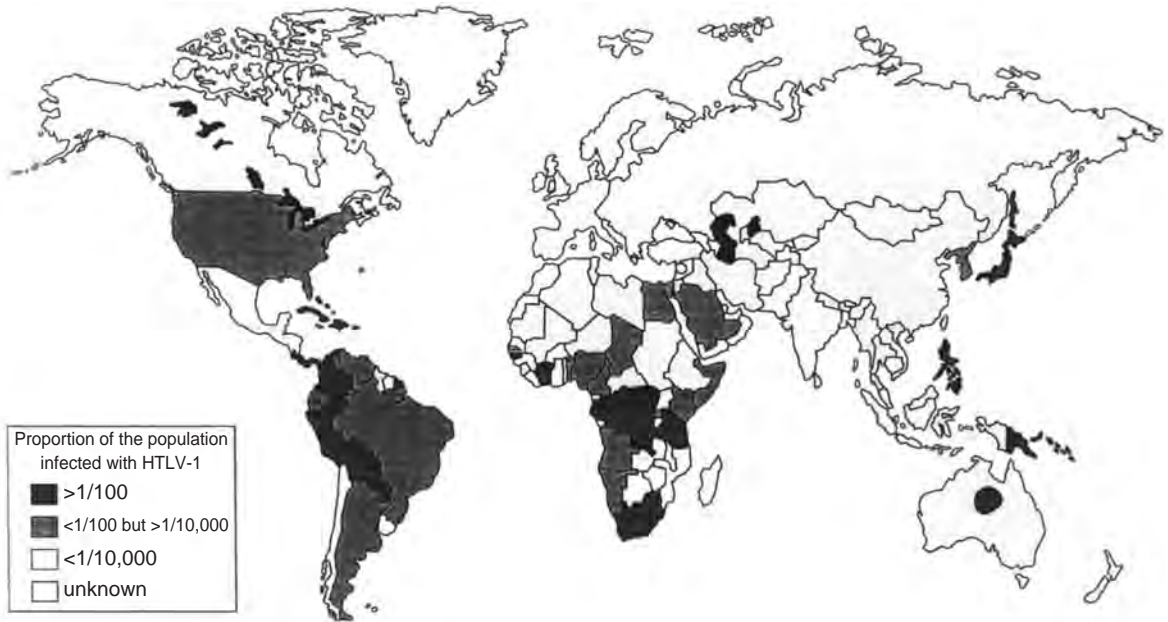
(Ishak *et al.*, 2007) while HTLV-2d was described in Congolese pygmies (Vandamme *et al.*, 1998).

The highly conserved nature of HTLV-1, HTLV-2 and their simian counterparts is unexpected for a retrovirus and quite distinct from the rapid evolution of HIV and hepatitis C virus (HCV). RNA viruses usually evolve at 1/100 to 1/10 000 nucleotide substitutions/site/year whereas HTLV-2 is mutating at a rate of 1.7–7.31/10 000 nucleotide substitutions/site/year in the LTR. From family studies involving two to three generations it appears that HTLV-1 only undergoes a few replication cycles, perhaps early following infection, in each generation (Van Dooren *et al.*, 2001). It was suggested that HTLV-2 is evolving faster in injecting drug users than in endemic populations: 2.7 nucleotide substitutions per 10 000 sites per year in injecting drug users, which would be consistent

with the virus being transmitted from one host to another after a shorter period than in the endemic populations in whom mother-to-child transmission plays an important role in maintaining the virus in the population (Salemi *et al.*, 1999).

EPIDEMIOLOGY

It has been estimated that 20 million people worldwide are infected with HTLV-1, including 1.2 million in Japan. Other endemic areas are the Caribbean, parts of the south-eastern United States, Melanesia and parts of sub-Saharan Africa, especially West and Central Africa (Figure 37.5). Recently HTLV-1 infection has been found to be relatively common in southern Africa,



(a)



(b)

Figure 37.5 Global seroprevalence of (a) HTLV-1 and (b) HTLV-2.

particularly Natal. Up to 0.5% of blood donors in Brazil are HTLV-1 seropositive, with considerable regional variation. HTLV-1 infection is recognized among many native South American indigenous peoples as well as among black and Japanese immigrants. However, population mixing has been extensive and in Brazil the proportion of neurology patients with HAM/TSP is equal among the main racial groups. Iran is another recently described area of HTLV-1 infection, with cases described in neighbouring Middle East and Central Asian countries. The prevalence of HTLV-1 among European Union blood donors is low (2–7 per 100 000) but remarkably similar across the length and breadth of the European Union. Seroprevalence rates 50–100 times higher have been found among women attending antenatal clinics and among men and women attending sexually transmitted disease (STD) clinics. In Central and Eastern Europe very few studies have been conducted, but cases of HTLV-1-related pathology have been diagnosed in patients from Bulgaria, Romania and in a Georgian family. Despite the importance of injecting drug users in the spread of HIV-1 infection in member states of the former USSR, HTLV-2 has not yet been detected in these high-risk populations (reviewed in Taylor, 1996). The most important recent developments in European epidemiology of HTLV infection have been (i) the confirmation, in a large multicentre study, that HTLV-1/2 infection in the European Union is 10-fold more common among pregnant women than among blood donors (Taylor *et al.*, 2005.) and (ii) the emergence of data showing HTLV-1 infection and ATLL to be relatively common in Romania. Romania can be considered to be the most recently described endemic area for HTLV-1 infection and the first to be described in a white population. The occurrence of ATLL indicates that HTLV-1 is unlikely to have arrived in Romania recently.

HTLV-2 infection is found among native American Indians in North, Central and South America and was until recently considered a New World virus. It seems likely that HTLV-2 was introduced into the injecting drug user population of the United States during the 1970s and into Europe slightly later. In Europe HTLV-2 is common among injecting drug users in Eire, Spain, Italy and Scandinavia, uncommon in the United Kingdom, and rare in Germany and France. The first isolations of HTLV-2 in Africa were from pygmy tribes both from Ethiopia and from West Africa (Gessain *et al.*, 1995; Goubau *et al.*, 1992). HTLV-2 infection in West African prostitutes (which may not imply a local origin), and in the Gabon in a male with no history to suggest exposure to an imported virus have been described. This virus is genetically close both to the Cameroonian pygmy isolate and to a North American isolate, emphasizing the conserved nature of these viruses (Letournier *et al.*, 1998).

These findings, and the identification of an HTLV-2-like primate virus in Central Africa, suggest that HTLV-2, like HTLV-1, may have originated in Africa. However, a number of questions remain as HTLV-2 is widely spread throughout the Americas but has been found in few populations in Africa.

To date PTLVs have not been identified in New World primates and with the exception of a report of HTLV-2 in Mongolia, which may have been a recently imported infection, there is no population-based evidence of HTLV-2 migrating from the Old to the New World.

TRANSMISSION

For both viruses transmission may be by three routes: from mother to child; through sexual intercourse; and through blood–blood contact. Family studies in Japan suggested that HTLV-1 was mainly transmitted from mother to child (Kajiyama *et al.*, 1986). HTLV-1 was identified in lymphocytes in breast milk and transmission through breastfeeding was demonstrated in marmosets and rabbits. The mother-to-child transmission rate in Japan was 25% if babies were breastfed, but only 5% if babies were bottlefed. It may be that maternal anti-HTLV antibodies protect breastfeeding infants until their titre starts to decline, as in one study in Japan short-term breastfed babies were no more likely to be infected than bottlefed babies (Takahashi *et al.*, 1991). Breastfeeding for six months is associated with higher rates of transmission that continue to increase if weaning is further delayed. After weaning there is no further risk of transmission (Ando *et al.*, 2003) until children reach adolescence and start to engage in other risk activities. Although in one study HTLV-1 was detected in cord blood none of the cord-blood positive babies became infected. HTLV-1 has been detected but not quantified in cervico-vaginal secretions (Zunt *et al.*, 2002) and the contribution of *in utero* and perinatal infections to the total infection rate is uncertain.

In the Gabon the mother-to-child transmission rate was 9.7% with transmission associated with higher maternal viral load (Ureta-Vidal *et al.*, 1999). There are conflicting data on mother-to-child transmission of HTLV-2 but among the Kayapo Indians in Brazil the risk of transmission from an HTLV-2-infected mother to her child was 30–50% (Ishak *et al.*, 1995).

Family studies also indicated that infection was likely to pass from husband to wife. In a Japanese cohort of 100 discordant couples practising unprotected sexual intercourse there were seven seroconversions during five years of observation. Uninfected females were 3.9 times more likely to become infected than uninfected males (Stuver *et al.*, 1993). Higher rates of transmission have

been reported: 60% of wives of seropositive husbands over 10 years in one study, but only 0.4% of husbands of seropositive wives; 50% of wives during one to four years of marriage in another. In a study of HTLV-1 and HTLV-2-infected blood donors in the United States, those with higher HTLV-1 proviral loads were more likely to have an infected partner. A similar association of transmission with high proviral load was seen in HTLV-2, although the viral burden is generally much less than in HTLV-1 (Kaplan *et al.*, 1996). Both HTLV-1 and HTLV-2 are more common in females. It is reasonable to suggest that condoms will efficiently protect against transmission, but no data are available.

HTLV-1 is transmitted by cell-containing blood products but not by plasma or plasma-derived products. This has been demonstrated in the rabbit model and through clinical observation. Fresh blood is more infectious than older blood due to the short life of stored lymphocytes. Infection has occurred following transfusion of 41 ml blood. Following transfusion with HTLV-1-infected blood the median time to seroconversion was 51 days (Manns *et al.*, 1994). The mean incubation period of HAM following infection by transfusion is three years, shorter than by other routes. In a post-mortem study the average time to first symptoms was eight and a half months but the duration of symptoms was not different from that in other patients with HAM (Iwasaki, 1990). The risk of HAM was 7.7-fold higher than expected among transfusion recipients in Japan and following the introduction of blood donor screening in 1986 the incidence of HAM fell by 16%. HTLV-1 is also transmitted through injecting drug use but this route is more commonly associated with HTLV-2. Indeed outside of the endemic areas of the Americas HTLV-2 is primarily transmitted through re-use of injection paraphernalia.

HTLV-ASSOCIATED DISEASE

HTLV-1 is recognized as a carcinogen by the International Agency for Research on Cancer (1996). HTLV-1 is clearly associated with ATLL and serological documentation of HTLV-1 infection is an essential part of confirming this diagnosis, whereas the demonstration of clonality is only required in unusual cases. The search for sero-epidemiological evidence of an association between HTLV-1 and other malignancies has been complicated by the frequent history of previous blood transfusion in patients with malignant disease included in such studies. However, two studies have demonstrated an increased rate of cervical carcinoma in patients with HTLV-1 and in one of these studies patients with HTLV-1 were also found to have more advanced disease. An interaction between HTLV-1 and human papillomavirus (HPV) directly

or through an effect on HTLV-1 on cell-mediated immunity is biologically plausible. However, since both HPV and HTLV-1 are sexually transmitted the association may reflect sexual activity rather than a biological interaction between two oncogenic viruses (International Agency for Research on Cancer, 1996). HTLV-1 has been implicated as a co-factor in the development of hepatocellular carcinoma due to HCV in a number of epidemiological studies in Japan (Arisawa *et al.*, 2006; Boschi-Pinto *et al.*, 2000) with evidence that HTLV-1 can upregulate HCV replication (Zhang *et al.*, 2007).

Although the initial association with atypical HCL was disproved, HTLV-2 infection was also reported in two patients with large granular lymphocytosis and one with large granular lymphocytic leukaemia, but the virus was found in the T lymphocytes and not the abnormal cells. Rare cases of CD8⁺ lymphoproliferation in patients with HIV-1/HTLV-2 co-infection have been reported, including one in which clonal expansion of HTLV-2-infected CD8⁺ lymphocytes was demonstrated (Poiesz *et al.*, 2000). In summary, there are as yet inadequate data to afford HTLV-2 carcinogenic status although it immortalizes T cells *in vitro* (reviewed in Araujo *et al.*, 2002).

ATLL may present as acute, chronic or smouldering leukaemia or as a lymphoma including a cutaneous presentation. ATLL cells are usually CD3⁺, CD4⁺, CD25 (IL-2 receptor- α)⁺ and in the leukaemic form characteristic poly-lobed 'flower' cells (Figure 37.6) can be easily identified. Although the cells are morphologically mature, the malignancy is aggressive and survival, despite therapy, measured in months. The most common presentations are acute leukaemia and lymphoma. The infrequent chronic and smouldering forms are associated with longer survival. Hypercalcaemia, due to expression of parathyroid hormone-related protein (Nadella *et al.*, 2007), is a common and characteristic finding (Kiyokawa *et al.*, 1987). Patients may present with acute renal failure and lytic bone lesions. Atypical presentation with opportunistic infections should also be considered.

HTLV-1 is associated with a number of inflammatory conditions characterized by a lymphocytic infiltration of the target organ (Figure 37.7). The lifetime risk of HAM in HTLV-1-infected people is 2–7%, except in Japan where it has been estimated at 0.25%. HAM has been diagnosed in about 200 patients in the United Kingdom, mostly of Caribbean origin, but also in the white population. This includes more than one case following transfusion with HTLV-1-infected blood in the United Kingdom. The condition is a chronic progressive spastic paraparesis without the relapsing/remitting character of multiple sclerosis (although with similarity to primary progressive multiple sclerosis). Chronic backache, hyperactive bladder, constipation and impotence are common.

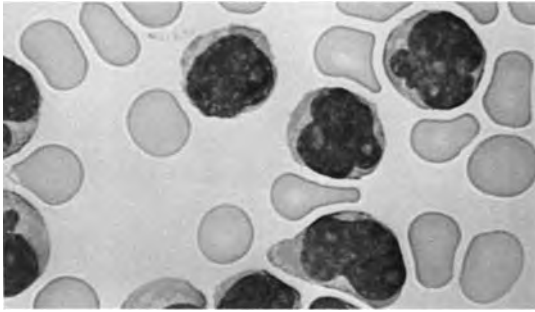


Figure 37.6 Flower cells A blood smear showing characteristic convoluted T-cells in ATLL. (Source: Courtesy of Dr E. Matutes and Professor D. Catovsky.)

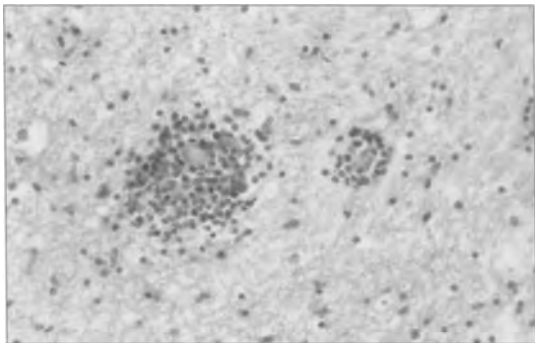


Figure 37.7 Perivascular lymphocytic infiltration in CNS. (Source: Courtesy of Dr Margaret Esiri.)

Sensory signs and upper limb disease are unusual but can be found in long-standing disease. Other causes of myelopathy including cord compression should be excluded (Table 37.2). The female-to-male ratio is 2 : 1 and the onset is most common in the third and fourth decades, resulting in decades of morbidity with more than 50% eventually becoming wheelchair dependent. The condition is occasionally rapidly progressive and fatal within two years of onset.

In endemic areas uveitis is more common in HTLV-1 patients than in the general population. HTLV-1-associated uveitis usually responds to treatment with topical steroids, less often systemic steroids are required. The condition is generally mild but recurs in 25% of cases (Mochizuki *et al.*, 1992). In a prospective study of 200 patients with HTLV-1 in Martinique 14.5% had uveitis, but 37% had keratoconjunctivitis sicca and interstitial keratitis was also common (Merle *et al.*, 2002).

Polymyositis, alveolitis, arthritis and thyroiditis have been reported in subjects with HTLV-1, often in patients

Table 37.2 Differential Diagnosis of HAM

Compressive myelopathy
Syringomyelia
Familial spastic paraparesis
Primary lateral sclerosis
Multiple sclerosis
Transverse myelitis
Devic's disease
Motor neuron disease
Vitamin B12 deficiency
Folate deficiency
Syphilis
Human immunodeficiency virus
Schistosomiasis
Sarcoidosis
Neurological lupus
Behçet's disease
Sjögren's syndrome
Carcinomatous meningitis
Paraneoplastic syndrome

with HAM. While these associations have not been confirmed epidemiologically, the histology/cytology characterized by lymphocytic inflammatory infiltration is consistent with an HTLV-1 aetiology.

Infective dermatitis is an eczematous condition of children that only responds to long-term antibiotic treatment against streptococcal and staphylococcal species that are otherwise not usually considered pathogenic. Skin biopsies reveal an inflammatory infiltrate of CD8⁺ lymphocytes. HTLV-associated infective dermatitis is rare outside the tropics. Encrusted (Norwegian) scabies has been reported in patients with HTLV-1 and suggested as a marker for the development of ATLL.

Studies in Japan have repeatedly shown reduced delayed hypersensitivity to tuberculin in HTLV-1-infected people; conversely there was no abnormality in delayed hypersensitivity skin testing to mumps virus or *Candida albicans* antigens in a cohort of HTLV-1-infected blood donors (Murphy *et al.*, 1997; Murphy *et al.*, 2001). Murphy described an increased adjusted odds risk of previous tuberculosis (TB) in HTLV-2-positive blood donors in the United States (Murphy *et al.*, 1993), while in Lima, Peru TB is significantly more common among patients with HTLV-1 infection than the general population (Verdonck *et al.*, 2007). It is not clear whether bronchiectasis, which appears to be more common in patients with HAM (Okada *et al.*, 2006), represents part of the inflammatory or immunosuppressive spectrum of HTLV-associated diseases.

Failure to clear *Strongyloides stercoralis* despite appropriate treatment is also recognized with HTLV-1 infection (Terashima *et al.*, 2002) and investigation for HTLV-1 is

considered an essential part of the management of patients with proven strongyloidiasis and the literature is littered with case reports of HTLV-1 infection and disseminated strongyloidiasis (strongyloides hyperinfection syndrome). The endemic regions for strongyloidiasis and HTLV-1 infection overlap considerably. Prolonged and repeated courses of ivermectin and albendazole may be required. Increased rates of infection and treatment failure have also been reported for *Schistosoma mansoni* in Brazil (Porto *et al.*, 2002).

A myelopathy similar to that seen with HTLV-1 has been reported in several subjects with HTLV-2 infection in the absence of HIV infection (Murphy *et al.*, 1997; Black *et al.*, 1996; Jacobson *et al.*, 1993) although some patients also have ataxia (Harrington *et al.*, 1993). Although the frequency of this condition is unknown and the causative role of HTLV-2 unproven, many specialists accept that there is a growing body of evidence to suggest that HTLV-2-associated myelopathy is a real entity.

In a cohort study of US blood donors, respiratory and urinary infections were more common among the HTLV-2-infected donors than in matched controls (Murphy *et al.*, 1999)

An association between *Mycosis fungoides* and the presence of HTLV-1 tax DNA has now been refuted. There appears to be an association between HTLV-1 and Sjögren's syndrome in endemic areas but this has not yet been confirmed in properly aged-matched studies. HTLV-1-defective provirus has been amplified from the salivary glands of seronegative patients with Sjögren's syndrome. Rare cases of seronegative but PCR-positive HAM/TSP are reported with defective provirus. *Tax* sequences are present in all these conditions. The absence of both humoral and cellular immune responses in these exceptional cases makes an understanding of the pathogenesis of these inflammatory conditions even more elusive.

PATHOGENESIS

Our understanding of the pathogenesis of ATLL is incomplete, based to a large extent on *in vitro* studies of HTLV-1 infected cells and Tax constructs and has to take into account a number of apparent discrepancies. HTLV-1 can immortalize T lymphocytes *in vitro* and these cells express HTLV-1 proteins but there appears to be little expression of HTLV-1 by ATLL cells *in vivo*. Only a small proportion (2–4%) of those infected with HTLV-1 develop ATLL and then only after many decades of infection. Indeed the observation that only a proportion of the mothers of patients with HAM but all the mothers of ATLL patients are carriers of HTLV-1 suggests that infection during infancy is important for the development

of ATLL. In common with other malignancies it is likely that transformation is a multistep phenomenon with the infection of a lymphocyte by HTLV-1 only one of several events leading to ATLL. Since HTLV-1 does not contain an oncogene and malignant transformation is not related to the integration-disturbing cellular genes important in oncogenesis, the transactivating characteristics of Tax assume importance. Although adult T-cell leukaemic cells contain integrated HTLV-1 that may be defective, *tax* is generally preserved.

In vitro, HTLV-1 induces T-cell activation and proliferation and immortalizes primary human lymphocytes that can become IL-2 independent. In these cells, as in other tumour virus models, Jak/Stat proteins are constitutively activated (Johnson *et al.*, 2001). Tax has been shown to stabilize and inactivate the tumour suppressor p53. Tax also interacts with cell cycle genes inducing phosphorylation of cyclin D1-cdk4/6 and cyclin D3 which may contribute to the shift from G₁- to S-phase in the cycle. Tax inactivates a cdk inhibitor and the human mitotic arrest deficiency 1 (MAD-1) protein.

Other examples of cellular genes transactivated by Tax are listed in Table 37.3. Tax has an inhibitory effect on β -polymerase, a DNA-repair gene. This may increase the likelihood of mutagenesis. Tax does not act directly on the cellular genes, or on the promoter sequences in the viral LTR, but binds to a number of specific transcription factors with resulting enhancement of their interaction with the target genes: nuclear factor- κ B (NF- κ B); activator protein 1 (AP1); CREBs/activating transcription factors (ATFs); serum-response factor (SRF) and nuclear factor of activated T-cells (NFATs).

The interaction between the IL-2R α gene and Tax has been of particular interest because of the high expression of IL-2R α (CD25) by T lymphocytes in ATLL. IL-2R α expression normally only occurs after antigenic stimulation by the T-cell receptor. However it appears that Tax is able to induce constitutive expression of the IL-2R α gene. There is a 12 bp sequence in the 5' IL-2R α gene promoter required for *tax* transactivation that shows homology with the NF- κ B-binding site. The NF- κ B precursor, a p105 heterodimer, is usually found in the cytoplasm because its nuclear localization signal is masked. Tax causes NF- κ B to dissociate from its inhibitor, I κ B, which results in increased transport of NF- κ B to the nucleus where the NF- κ B p50 is able to activate transcription of IL-2R α . A secondary effect of Tax is that the dissociation of p50 from the heterodimer releases the p65 protein which is then free to dimerize with the product of the c-Rel oncogene. This p65-Rel heterodimer is also able to activate the NF- κ B motif. Thus Tax may further enhance the activation of the many genes under the influence of NF- κ B.

Table 37.3 Cellular genes transactivated by Tax

Regulated through AP-1	NFAT	NF-κB	CREB
<i>AP-1 proteins</i>	IL2	IL-2Rα (CD25)	ETR101
FOS	IL13	IL-15R	
FOSL1(FRA-1)	TNFSF6 (Fas ligand)	IL-8	
JUN		TERT (telomerase)	
JUND	IRF4 (Interferon regulatory factor 4)	MYC	
TGFB1 (TGF-β)		TNF (TNF-α)	
NR4A1 (TR3/nur77)		LTA (TNF-β)	
TIMP-1		PTHR1 (PTHr-P)	
IL-8		TNFRSF4 (OX40/OX40L)	
		GM-CSF	

Based on Taylor (2007).

One theory of T-cell transformation by HTLV-1 has been that by promoting activity of both IL-2R and IL-2 genes Tax gives rise to continuous proliferation of the cells by positive feedback, the autocrine loop theory. However, stimulation of Tax-transduced T cells can occur in an IL-2-independent manner, tax-transfected cells continue to grow after anti-CD3 stimulation in the absence of IL-2 and not all tax-transformed T cells express IL-2, although they all express IL-2R.

Other mechanisms potentially important in HTLV-1-associated leukemogenesis include: the promotion of S-phase in the cell cycle; interactions with tumour suppressor genes for example, p53 and Rb; and effects on genomic stability.

While the oncogenic potential of HTLV-1 has been recognized from the time of its discovery a similar role has not been proven for HTLV-2. This could merely be a function of their different epidemiology; the HTLV-2 endemic populations are small and often remote and therefore rare malignancies might be missed while in industrialized states HTLV-2 is mostly spread among adults who may not incubate the infection for long enough. With the exception of a few isolates of HTLV-2a in which Tax protein expression is low, the transactivating function of HTLV-2 Tax via NF-κB does not differ significantly from HTLV-1 *in vitro* (Lewis *et al.*, 2002).

The pathogenesis of HAM is also incompletely understood. It is rare to obtain histopathology at the time of the initial symptoms but a perivascular lymphocytic infiltration of the spinal cord, which is at first CD4⁺ and later predominantly CD8⁺ is found, followed by demyelination and atrophy. HTLV-1 is rarely found in the lesions and when detected is probably in circulating CD4⁺ (and to a lesser extent CD8⁺) lymphocytes. The peripheral lymphocyte proviral load is approximately 10-fold higher in HAM than in asymptomatic carriers although there is considerable overlap between the ranges. There appears to be a threshold of about 1 HTLV DNA copy per 100

PBMCs above which the risk of HAM increases exponentially (Nagai *et al.*, 1998). Higher viral load in the CSF than in the peripheral blood has been associated with HAM (Lezin *et al.*, 2005) and these viral load measures may be useful to discriminate HTLV-1 infection from other causes of neurological disease in HTLV-seropositive patients (Puccioni-Sohler *et al.*, 2007), especially if the presentation is atypical. Finally, at a given viral load, *ex vivo* PBMC Tax expression (in short-course unstimulated culture) is independently associated with HAM (cf. asymptomatic carriage) (Asquith *et al.*, 2005a).

HTLV-1 Tax-specific cytotoxic T lymphocytes (CTLs) while found in the majority of patients with HAM (Jacobson *et al.*, 1990) are also detected in asymptomatic carriers (Parker *et al.*, 1992). Limiting dilution chromium release assays have shown a high frequency of such CTLs (Daenke *et al.*, 1996) and using MHC-peptide tetramers as many as 10% of circulating CD8 lymphocytes have been found to recognize a single HTLV-1 epitope (Bieganowska *et al.*, 1999). Chromium release assays are laborious and estimate much lower frequencies of virus-CTL than tetramers but the latter do not measure lytic activity. ELISpots can be used to identify the number of cells releasing specific cytokines, for example, interferon gamma (IFN-γ) or IL-2, when incubated with peptides and again high frequencies are found (Goon *et al.*, 2004). A lytic efficiency assay has been described. This is dependent on the observation that infected PBMCs spontaneously express viral proteins during unstimulated *ex vivo* culture, peaking at 12–18 hours. Removal and measured addition of autologous CD8 cells alters the absolute number of CD4 Tax-positive cells and the killing rate of each CD8 cell can be determined. In these assays it becomes clear that at a set viral load the lytic efficacy of CTL from patients with HAM is less than that from asymptomatic carriers (Asquith *et al.*, 2005b).

T-helper responses to HTLV-1 have been difficult to study but using a short-incubation ELISpot assay higher

frequencies of CD4 cells secreting IFN- γ in response to HTLV-1 Env and Tax peptides have been found in patients with HAM (median 2/1000 CD4 cells) compared with asymptomatic carriers (Goon *et al.*, 2002).

Polyclonal expansion of HTLV-1-infected lymphocytes has been reported in patients (without malignancy) with high proviral load, particularly patients with HAM. As with the oligo/monoclonal proliferation of T lymphocytes in ATLL, viral replication in these cells occurs through cell division, without the need for reverse transcription

and integration. Thus, HTLV-1 appears to be able to replicate through two quite distinct mechanisms –cell division and virion production. The relative contribution of ‘cellular’ and ‘viral’ replication to HTLV-1 proviral load in patients with HAM and in asymptomatic carriers is uncertain. The continuous presence of a strong anti-Tax CTL response suggests continuing expression of Tax by some cells. It is possible that this may drive the proliferation of the clonally expanding cell populations. Despite the low rate of detection of HTLV-1 proteins *in vivo* there

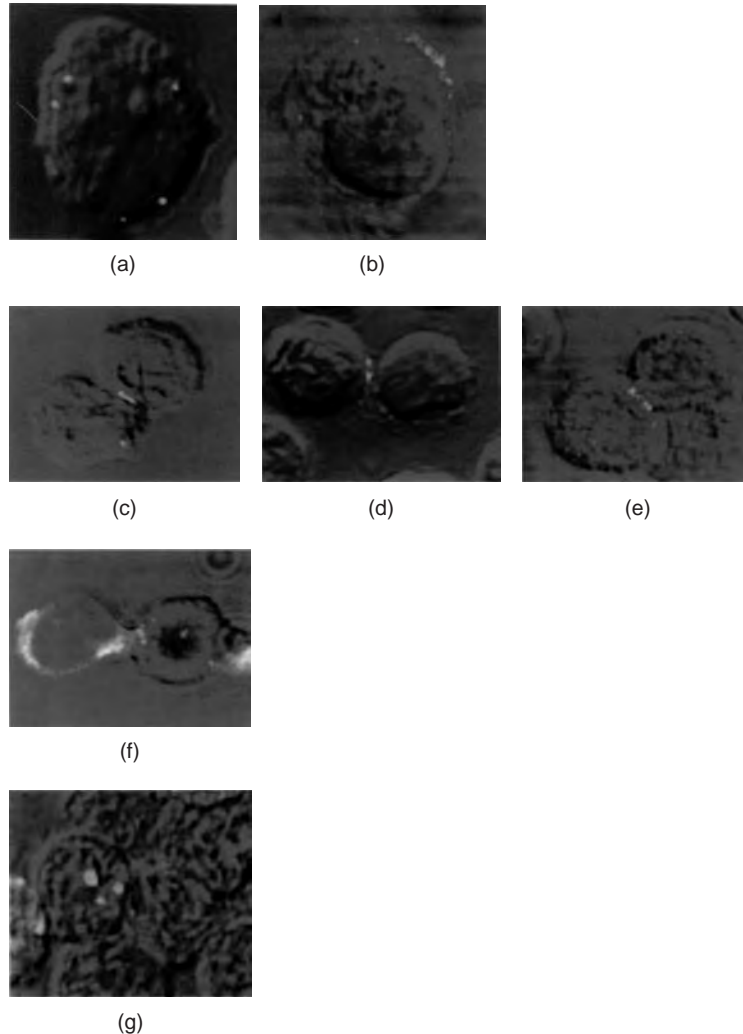


Figure 37.8 Cell-to-cell transfer of HTLV-1 gag protein. In an isolated HTLV-1-infected CD4 lymphocyte HTLV-1 gag p15 (red) is unpolarized (a), while HTLV-1 env gp46 (red) accumulates at the cell surface (b). Forty minutes after contact with an uninfected CD4 lymphocyte, HTLV-1 p15 (c), p19 (d) and gp46 (e) polarize at the cell–cell junction. (f) HTLV-1 gag p19 (red) from the infected (CFSE stained –green) CD4 lymphocyte is transferred to the uninfected CD4 lymphocyte. (g) The transfer of HTLV-1 genome (red) from the CFSE stained infected CD4 lymphocyte to the uninfected control CD4 cell can be demonstrated by peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH) after 120 minutes. (Source: Courtesy of Professor C.R.M. Bangham.)

is now good evidence that HTLV-1-infected CD4 and CD8 cells are capable of expressing viral proteins and that such cells are lysed by HTLV-1-specific CTLs. It has been proposed that the efficiency of these CTLs *in vivo* at identifying and lysing virus protein-expressing cells contributes significantly to the apparent absence of viral expression described. Recently 'direct' cell-to-cell spread of HTLV-1 following the development of a virus-induced synapse has been demonstrated in primary cells *ex vivo* (Figure 37.8) (Igakura *et al.*, 2003). It is now clear that contact with an uninfected cell realigns the cell structure, resulting in localization of viral proteins (and genome) at the site of contact (Nejmeddine *et al.*, 2005). Electron microscopy shows the expression of viral particles at the synapse with a very narrow space between the cells (Figure 37.9). This, if it occurs to any degree *in vivo*, may help explain the absence of free virions in plasma.

There are three main theories concerning HAM pathogenesis: (i) the inflammatory response is directed against virus in the CNS, (ii) the inflammatory response against HTLV-1 targets similar host CNS peptides, and (iii) CNS tissue is an innocent by-stander damaged when CTLs recognize migrating HTLV-1-expressing CD4⁺ cells. The latter has been considered most probable, although the potential role of HTLV-specific CD4 cells and NK cells might also be considered. Molecular mimicry between HTLV-1 and the human neural ribosomal nucleoprotein A1 has, however, been reported (Levin *et al.*, 2002). The effect of Tax on matrix metalloproteinases and on tissue inhibitors of metalloproteinases disturbing the neuronal

milieu which has been reported *in vitro* could be implicated in the degeneration of neurons.

Although HAM is associated with high proviral load, differences in the rates of HAM per HTLV-1 infected subjects in different populations suggest that other, possibly host factors, are important. Of the candidate factors, human leukocyte antigen (HLA) types have attracted considerable interest. HLA types are known to be associated with other inflammatory diseases but could also influence outcome by controlling viral replication. In Japanese ATLL patients HLA-A26, B61 and DR9 were found at an increased frequency while HLA0A24 and HLA-Cw1 were less frequently found than in controls. Conversely, in patients with HAM HLA-Cw7, B7 and DR1 were found more commonly than in controls and patients with ATLL. It has been suggested that in Japan the A26Cw3B61DR9DQ3 haplotype is representative of ATLL and is associated with a low immune response, while A24Cw7B7DR1DQ1 is the representative haplotype of HAM and is associated with a high immune response. Among different ethnic groups HLA class I haplotypes were variable but examination of class II suggested that some haplotypes associated with disease are panethnic while others are ethnospecific (Sonoda *et al.*, 1996). In a study of Japanese blood donors and patients with HAM, possession of HLA-A*02 and HLA-Cw*08 were found to protect against HAM through an association with lower HTLV-1 viral burden, whereas HLA-B*5401 was associated with an increased risk of disease (Jeffery *et al.*, 1999, 2000). Further studies, of single nucleotide polymorphisms in the same population, have revealed that TNF-963A predisposes to HAM (odds ratio = 9.7), SDF-1+801A 3' UTR reduced the risk of HAM by half, while the IL-15 191C allele also conferred protection against HAM by being associated with lower proviral loads (Vine *et al.*, 2002).

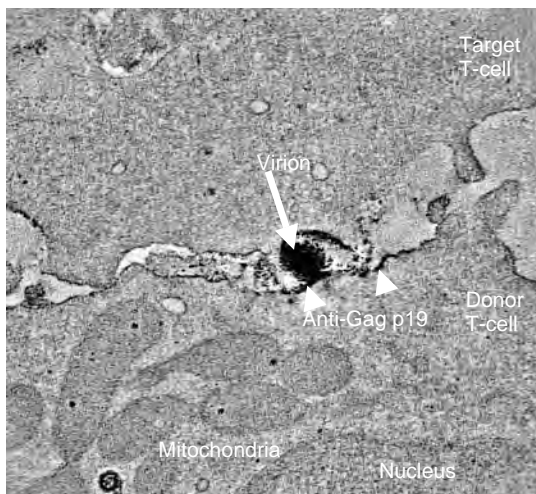


Figure 37.9 Viral budding at the virological synapse. (Source: Courtesy of Dr Endre Majorovits and Dr Mohamed Nejmeddine.)

TREATMENT

Few instances of curative treatment of ATLL have been reported and there have been no randomized controlled clinical trials. Until recently the overall management has been to treat the disease as per other malignancies and the regimens for non-Hodgkin's lymphoma such as cyclophosphamide, adriamycin, vincristine and prednisolone (CHOP) are favoured. Although successive improvements in chemotherapy have increased remission rates to 42%, ATLL is essentially a highly drug-resistant malignancy and survival remains less than 12 months for the acute leukaemia and lymphoma presentations. Enhanced transcription of the multidrug resistance gene (MDR1) with significant P-glycoprotein-mediated drug efflux in

the T cells of HTLV-1-infected individuals, with and without malignancy, has been demonstrated, as has the ability of the HTLV-1 tax protein to activate the MDR1 promoter. Other treatments such as deoxycoformycin, interferons β and γ , and topoisomerase II have been tried with limited success. Since CD25 is expressed by all ATLL cells, anti-Tac (CD25) antibodies have been tried although with limited success. Improved remission rates (56%) were obtained with $^{90}\text{yttrium}$ -labelled anti-Tac antibodies (Waldmann *et al.*, 1995). There are also minimal data on anti-CD52 (Campath-1h).

Successful eradication, not only of ATLL cells but also of HTLV-1 infection, has been reported following stem cell transplantation and this approach is increasingly used for selected patients. Reduced intensity conditioning may be appropriate for older patients but early deaths from complications remain a significant problem and at present only a minority of patients with ATLL are suitable for SCT either through lack of a matched donor or clinical considerations. Grafting of patients not in remission has not proved successful.

Improved remission rates and survival have been reported in uncontrolled studies, with the combination of IFN- α and zidovudine (Gill *et al.*, 1995; Hermine *et al.*, 1995; Matutes *et al.*, 2001). The mechanism of activity of these compounds in ATLL is not clear; they are not effective when given independently and do not appear to have a cytotoxic effect. Interest in this treatment followed anecdotal improvement in a patient with ATLL and HIV. At present, this approach carries the best outcome but the timing (whether to start early, during chemotherapy) and dosing of therapy needs further refinement. The use of molecular markers such as p53 to predict response to therapy holds promise.

Novel approaches under investigation include the combination of arsenic with interferon, proteasome inhibitors and blocking the transferrin receptor. The immune suppression seen in patients with ATLL is more severe than in other malignancies, and prophylaxis against *Pneumocystis pneumonia*, *Cryptococcus* and herpes viruses is recommended. Where appropriate, infection with *Strongyloides stercoralis* should be sought and treated.

The treatment of HAM is also difficult and there have been, to date, only two randomized controlled studies. Early reports of the use of pulsed high-dose steroids, steroid-sparing cytotoxics and plasmapheresis, all targeting the immune response, were encouraging (Osame *et al.*, 1990) but the improvements have not been long term (Matsuo *et al.*, 1989). High-dose vitamin C (Kataoka *et al.*, 1993) and oxpentifylline have also been promoted (Shirabe *et al.*, 1997). Extensive research into the treatment of HIV-1 has resulted in the licensing of five

classes of antiretroviral therapy. Unfortunately the current protease inhibitors and non-nucleoside RT inhibitors have no activity against HTLV-1. The nucleoside analogue reverse transcription inhibitors (NRTIs) zidovudine and zalcitabine have been shown to be active against HTLV-1 *in vitro* and in animal models. Unfortunately zalcitabine and two other NRTIs, didanosine and stavudine, are neurotoxic. Zidovudine was reported to improve mobility in ambulant patients in one study (Sheremata *et al.*, 1993) but not in another (Gout *et al.*, 1991). Neither reported proviral load measurements. In the management of HAM it is important to consider that in long-standing disease demyelination and atrophy may prevent improvement even if viral replication or the production of damaging cytokines has been reduced. Clinical improvement with a reduction in HTLV-1 proviral load was reported with lamivudine (Taylor *et al.*, 1999) but in a randomized placebo-controlled study zidovudine plus lamivudine was not effective (Taylor *et al.*, 2006).

The earliest studies of IFN- α were of short duration but temporary benefit was observed (Shibayama *et al.*, 1991; Kuroda *et al.*, 1992). In a randomized study of IFN- α given for four weeks with two months follow-up, a dose related effect was observed. Thus the 16 patients prescribed 0.3×10^6 IU did not benefit but a clinical response was reported in 3/17 and 6/16 patients randomized to 1×10^6 and 3×10^6 IU daily respectively (Izumo *et al.*, 1996). IFN- β 1A for six months did not alter the clinical status of 12 patients in an observational study and although a reduction in CTL activity was documented in three patients this was not specific to HTLV-1 (Oh *et al.*, 2005).

Symptomatic management remains the mainstay of therapy. Bladder spasticity with urgency, frequency, nocturia and incontinence is often distressing. Some patients respond well to oxybutinin. The anabolic steroid danazol has been reported to improve these symptoms. Intravesical instillation of capsaicin has been shown to reduce the bladder spasticity in these patients, with symptomatic improvement lasting months (Dasgupta *et al.*, 1996) but this approach is no longer favoured. Intranasal antidiuretic hormone cautiously used may reduce nocturia in refractory cases. In a minority of patients the bladder is hypotonic and catheterization, intermittent or permanent, is necessary both to relieve symptoms and to protect renal function. A titrated dose of baclofen may reduce limb spasticity –tizanidine may be tried if baclofen is not tolerated. The combination of urinary frequency with impaired mobility is particularly frustrating for patients with HAM. Lumbar pain, which has a radicular pattern, is common. Management can be complex and long-acting local anaesthesia can be useful in more severe cases. Some

patients respond to specific analgesia such as amitriptyline, carbamazepine, gabapentin or pregabalin. Although less commonly used as an analgesic sodium valproate has additional potential as a histone deacetylase inhibitor both in ATLL and HAM (Achachi *et al.*, 2005; Lezin *et al.*, 2007; Mosley *et al.*, 2006).

With the exception of HTLV-1-associated uveitis, which usually responds to topical corticosteroids, very little is known about the management of other HTLV-1-associated inflammatory diseases. Where possible, patients with HTLV infection and disease should be referred to specialized centres and offered access to clinical trials.

PREVENTION OF DISEASE

There is no vaccine for HTLV-1 or HTLV-2 infection. Although the association of HAM with high proviral load and viral expression suggests that load-reducing therapy may in the future be part of the treatment or prevention of this disease, current strategies must be directed at preventing infection. Two transmission routes can be targeted relatively easily. Many blood transfusion services now include HTLV-1/2 antibody screening of all or of new donors. This was first introduced in Japan in 1986 where blood transfusion accounted for up to 60% of seroconversions in the Kyushu region of south-western Japan (Kamihira *et al.*, 1987). Blood donor screening has been standard practice in the United States and Australia for many years and more recently introduced in South American countries. With the commercial production of suitably sensitive and specific screening assays HTLV-1/2 blood donor testing has been introduced in the majority of EU countries. In France more than 300 HTLV-1 positive blood donors have been detected since screening began in 1991. In the United Kingdom, donor screening was introduced in August 2002. Prior to that the risk of transmission was almost certainly reduced by leukodepletion introduced in 1999 to protect recipients from the transmission of prions. Screening of blood donors undoubtedly prevents infection in recipients, but cost effectiveness in terms of preventing HTLV-1-associated disease in them has been questioned (Tynell *et al.*, 1998). In such analyses more weight has been given to the 'cost' of developing ATLL following transfusion rather than HAM although the latter causes more morbidity and is a much more common consequence.

HTLV-1, and probably HTLV-2, can also be transmitted through organ donation. In endemic areas it has been suggested that donors and recipients should be matched for HTLV-1 antibody status. Elsewhere the arguments that apply to blood donor screening should apply to donated organs.

Eighty per cent of mother-to-child transmission can be prevented by avoidance of breastfeeding. In Japan carrier mothers are identified through antenatal screening programmes and during the next few years the impact of these programmes should become apparent as the first generation of screened babies are now reaching child-bearing age. In the United Kingdom the prevalence of HTLV-1/2 infection in women attending metropolitan antenatal clinics is approximately 1 : 200 (cf. HIV-1) but HTLV-1 antibody testing has never been offered as a routine part of antenatal care. Although breastfeeding is less common in the United Kingdom there is now increasing concern to prevent the transmission of other viral infections by this route and recognition that when offered there is a high uptake, by the mothers, of interventions to reduce transmission. Reduction of HTLV-1 transmission from mothers to their infants is likely to prevent most cases of HTLV-associated disease in the United Kingdom –ATLL by preventing infection of the infant and HAM by preventing both early infection and by reducing the pool of infected people who will transmit HTLV-1 sexually in adult life. Any effect on the incidence of disease would not be seen for several decades.

HIV AND HTLV CO-INFECTION

Co-infections of HTLV-1 or HTLV-2 with HIV-1 occur where risk factors are shared. Thus HTLV-1 and HIV-1 co-infections are likely to be most common in Latin America, the Caribbean and in West/Central Africa whereas HTLV-2 co-infection with HIV-1 is common among injecting drug users in Europe and North America.

HTLV-1 and HIV-1 share tropism for CD4⁺ lymphocytes but CD4⁺ lymphocyte counts and CD4/CD8 ratios are normal in HTLV-1 infection alone. Studies in Brazil and French Guiana have reported decreased survival in patients co-infected with HTLV-1 and HIV-1 and more advanced HIV disease than anticipated from the CD4 counts. Thus the initiation of prophylaxis against *Pneumocystis* pneumonia and/or antiretroviral therapy at CD4 levels higher than currently recommended should be considered. No effect of HTLV-2 on HIV-1 outcome was found in a study of North American and Italian injecting drug users whereas increased risk of sensory motor polyneuropathy has been observed with this co-infection. HAM is more common in HIV-1 co-infection. HTLV-2 viral load seems to be inversely related to HIV-1 viral load, though whether this association is causal is uncertain. HTLV-2 infection may be more difficult to detect serologically and indeterminate Western blot results warrant further, molecular, investigation.

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Human Immunodeficiency Viruses

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INTRODUCTION AND CLASSIFICATION

Acquired immune deficiency syndrome (AIDS) first came to the notice of physicians and epidemiologists in 1981 in the United States when a handful of homosexual men presented with *Pneumocystis carinii* pneumonia (PCP) and Kaposi's sarcoma (KS). These diseases were previously extraordinarily rare in young adults and indicated an underlying immune deficiency. The patients showed a selective depletion of CD4⁺ T-helper lymphocytes in the peripheral blood. It was noted that a larger proportion of gay men suffered from generalized lymphadenopathy. In 1982, investigators at the Centers for Disease Control and Prevention (CDC) in Atlanta, USA, detected similar cases of what soon became known as AIDS among injecting drug users, sex workers and recipients of blood transfusions and blood products, especially of pooled clotting factors administered for haemophilia. These epidemiological observations led to the conclusion that AIDS was not simply a consequence of gay lifestyle, but was caused by an infectious agent, probably a virus that was sexually and parenterally transmitted (Weiss, 2008).

Human immunodeficiency virus type 1 (HIV-1) was first isolated in 1983 (Barré-Sinoussi *et al.*, 1983). Several further HIV-1 isolates were reported in 1984 from the United States which, together with serological studies of prevalence, made a convincing case for HIV being the cause of AIDS. HIV-2 was first isolated in 1986 (Clavel *et al.*, 1986). Retrospective serological surveys indicated that HIV-1 had begun to spread among American gay men from 1977 onwards, showing a considerable incubation period before the manifestation of AIDS. The earliest known positive blood sample was collected in 1959 in Congo. However, molecular clock analyses of diversity suggest a common origin for HIV-1 (Group M) dating

from around 1931 (Korber *et al.*, 2000) and for HIV-2 from around 1940 (Lemey *et al.*, 2003).

HIV-1 and HIV-2 represent two separate viruses with distinct origins. Both viruses belong to the *Lentivirus* genus of retroviruses (see Chapter 36) and have a similar genome organization, but some of their accessory genes differ (Figure 38.1).

The origin of HIV-1 was almost certainly a zoonotic infection from chimpanzees, which harbour a related lentivirus, SIVcpz, whereas HIV-2 came from sooty mangabey monkeys in West Africa (Heeney *et al.*, 2006). HIV-1 is divided into three groups: the main group (M), the new group (N) and the outlier group (O). These groups represent three separate transfers from the chimpanzee or, in the case of group O, possibly from a gorilla (Van Heuverswyn *et al.*, 2006). Groups N and O remain largely confined to a part of West Central Africa (Gabon and Cameroon), although sporadic infection through contact with people from that region occurs. Only group M has radiated to cause the AIDS pandemic. The HIV-1 subtypes or clades lettered A to K all belong to group M. There also exist a number of so-called circulating recombinant forms (CRFs) of HIV-1 because genetic recombination is a feature of retrovirus replication (see Chapter 36). The most prevalent are CRF_01AE in South East Asia and CRF_02AG in West Africa. The genomic and antigenic variation manifest by HIV-1 groups and subtypes is important for diagnostic virology based on genomic and serological assays (Figure 38.2).

HIV-1 and HIV-2 strains can also be classified according to cell tropism phenotype, which does not relate directly to their major genotypic classification. Thus within each HIV-1 subtype there are virus isolates that are syncytium inducing (SI) or nonsyncytium inducing (NSI) for CD4 cells *in vitro*. Most primary, transmitting HIV-1

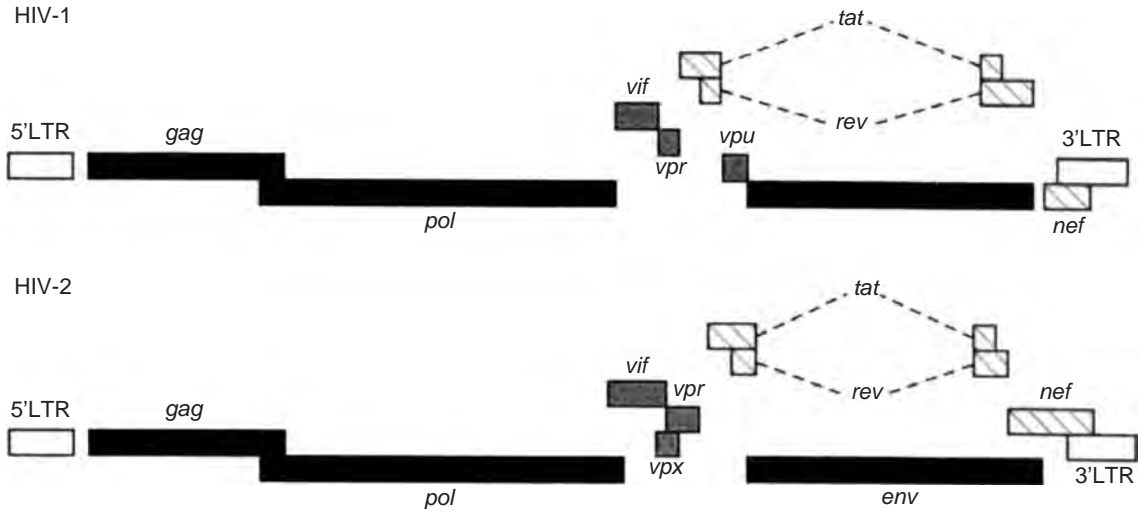


Figure 38.1 HIV-1 and HIV-2 genome maps.

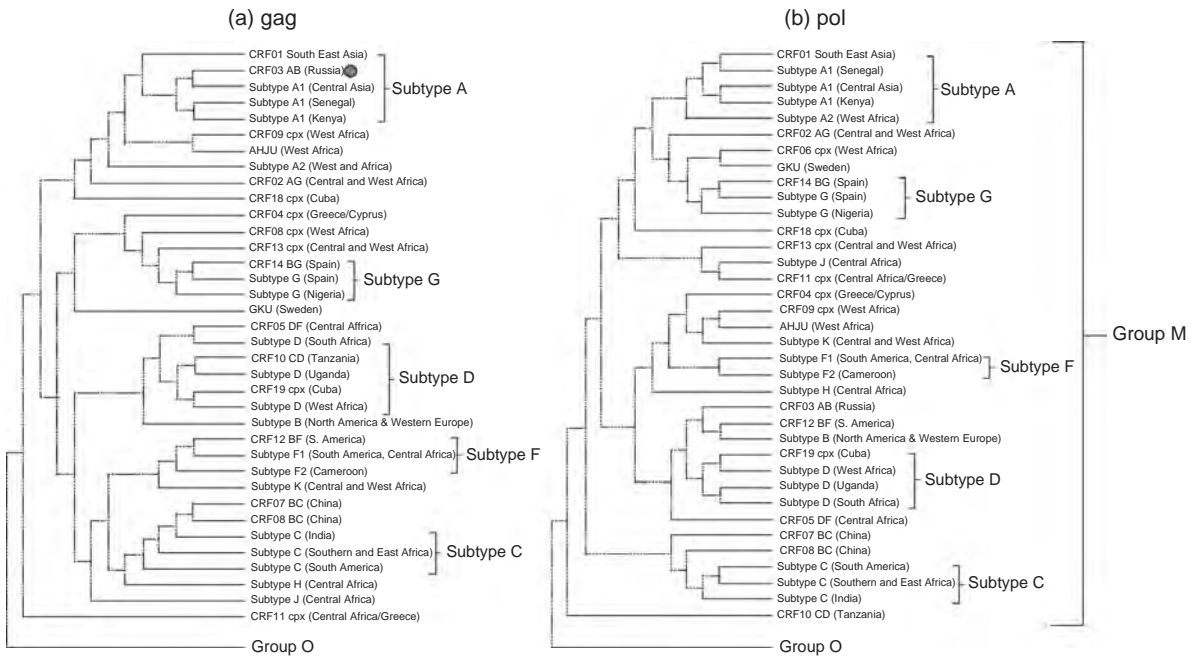


Figure 38.2 Strain level classification of HIV group M diversity, using gag or pol gene sequences. (Source: From Gifford et al., 2007.)

strains have an NSI phenotype, while SI substrains tend to appear in infected individuals later as they progress to AIDS. This phenotypic classification is related to cellular tropism for macrophages or T-cell lines reflecting which kind of chemokine co-receptor the virus uses to gain entry

into cells. Most NSI strains utilize the CCR5 co-receptor and are known as R5 viruses, whereas most SI strains utilize the CXCR4 co-receptor and are known as X4 viruses (Berger et al., 1998). Some viruses are dual-tropic, known as R5X4 viruses.

EPIDEMIOLOGY

Risk Factors

The AIDS epidemic was first identified in the United States within the men having sex with men (MSM) risk group, in men and boys with haemophilia who were exposed to contaminated clotting factors, in recipients of blood transfusion and in intravenous injecting drug users. However, in Africa (where it was originally recognized as 'slim' disease (Serwadda *et al.*, 1985)) and when it spread to Asia, HIV has from the beginning been heterosexually transmitted, affecting men and women alike. With the rapid introduction of screening following the development of commercial antibody tests in 1985, HIV transmission through blood and blood products virtually disappeared, although this remains a problem in countries where screening is not stringent. As is described below, the epidemic has grown enormously since the early 1980s, with the major risks of transmission continuing to include heterosexual and homosexual intercourse, vertical transmission and intravenous drug use. With the improved understanding of the pathogenesis of HIV disease, it is now well established that the concentration of virus in the plasma of an infected individual is a good surrogate of infectivity to others. Thus, maternal plasma viral load is a predictor of perinatal transmission (Jourdain *et al.*, 2007). Similarly, the period of primary infection, when plasma viraemia is very high, is a particular risk for transmission, with some studies suggesting that 30% of new onward infections occur during this phase (Wawer *et al.*, 2005). These observations are important, since HIV is rarely diagnosed during early infection, and this period

therefore remains refractory to many standard intervention approaches, such as education or antiviral therapy. Another specific co-factor for sexually transmitted infection is coexisting genital ulceration, which may lead to enhanced local replication of HIV, and thus transmission.

Geography and Prevalance

UNAIDS (the Joint United Nations Programme on HIV/AIDS) estimates that 32.7 million people are living with HIV infection in 2006. Half of these are women, and 2.5 million are children. Each day around 7000 individuals are infected, and over 5700 people die from AIDS. Although recent models of the epidemic have revised these estimated figures downward, it is clear that the epidemic remains one of the world's leading health crises. Sub-Saharan Africa is most affected (22 million), with South Africa hosting the largest number of infections in the world. There are large differences in HIV prevalence in different cities in Africa for reasons that are still not clear, although sexually transmitted diseases associated with genital ulceration like herpes simplex type 2 infection, and lack of male circumcision are risk factors; women become infected at younger ages than men (Avert *et al.*, 2001). India is estimated to have 2.2 million infections, and the epidemic is also growing in Eastern Europe (Figure 38.3).

Within the United Kingdom, new diagnoses continue to rise, reaching over 7000 in 2006. These comprise two epidemics: that amongst heterosexuals, who are mainly infected in sub-Saharan Africa, and that within MSM. The latter group of transmissions occurs within the United Kingdom, which suggests that we are witnessing a failure in safe sex public health messages.

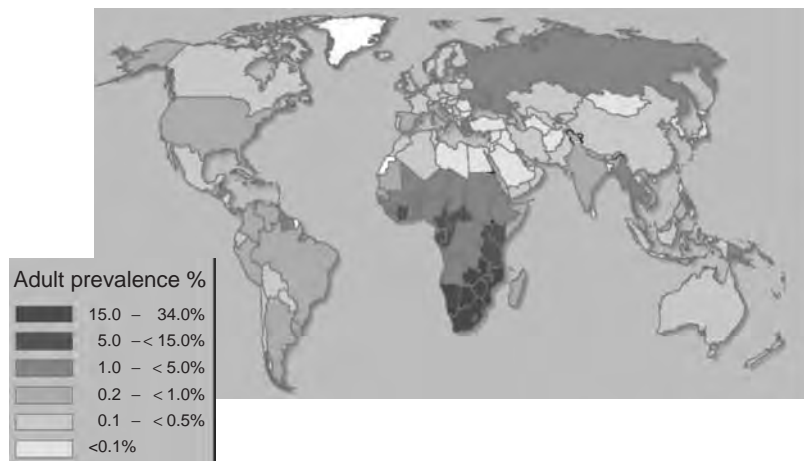


Figure 38.3 A global view of HIV infection. 38.6 million (33.4–46.0 million) living with HIV, 2005. Source: From World Health Organization http://www.who.int/topics/hiv_infections/en/

Molecular Epidemiology

The M group of HIV-1 can be separated into a number of subtypes, or clades, based on genetic differences. These subtypes are characteristically associated with geographical regions and/or risk groups of infected individuals (Figure 38.2). It is currently thought that these viruses all emerged from the epicentre of the epidemic, in what is now the Democratic Republic of Congo, a theory supported by the finding of all major viral clades circulating there (Vidal *et al.*, 2000). Thus, the presence of different subtypes around the world is likely to represent founder effects.

Recent advances in evolutionary methodologies have allowed insights into the likely progression of different lineages of HIV-1. These techniques include estimation of the timing and growth of epidemics based on sampling of contemporaneous, as well as historical, gene sequences, and include the concept of phylodynamics (Grenfell *et al.*, 2004). This refers to the detailed study of how pathogens evolve in the context of selective pressures and transmission bottlenecks, allowing the identification of the key determinants of epidemic history. Such approaches are most appropriately applied to highly variable pathogens such as RNA viruses. One example is the elucidation of the history of subtype B HIV-1. Using viral sequences of viruses isolated from the early Haitian epidemic in the 1980s, Gilbert *et al.* (2007) confirmed that spread of subtype B virus from Africa occurred during the 1960s, and that this virus subsequently was transferred to the United States, leading, at least in part, to the widespread subtype B epidemic. It is suggested that this represented one of the earlier transfers of the virus from Africa, and thus explains the broad diversity of subtype B viruses. Although only representing around 5–10% of the world's HIV, subtype B is the most intensely studied since it reflects the MSM and intravenous drug user epidemics in North America and western Europe, which is where most clinical HIV research has been undertaken.

At least three lineages of HIV-1 subtype C have now been identified, circulating in southern Africa, India and Brazil (together representing around 50% of worldwide infections). Similarly, subtype A can be differentiated between Kenyan, West African, Central Asian and South-West Asian epidemics (Gifford *et al.*, 2007)

In view of the large proportion of imported infections within the United Kingdom, the epidemic here is one of the most genetically diverse worldwide. Although the MSM epidemic remains overwhelmingly subtype B, recent data suggest that an increasing number of new MSM infections are with non-subtype B viruses, illustrating how the future molecular epidemiology may evolve. Indeed, the large number of sequences now generated through HIV drug resistance testing (although representing the

pol gene alone, only 10% of the HIV genome) provides a unique opportunity within infectious diseases to incorporate real-time phylogeny into existing surveillance schemes to monitor the epidemic.

There is increasing interest in biological and clinical differences between subtypes, particularly related to potential vaccine efficacy and antiviral efficacy. For instance, of the two coexisting subtypes in Uganda, subtype A infections appear to progress more quickly than subtype D (Kiwanuka *et al.*, 2008). *In vitro* studies on subtype C virus suggest that it remains less pathogenic than subtype B virus but the significance of these findings remains unclear (Arien *et al.*, 2005).

REPLICATION

HIV Culture and Isolation

HIV can be propagated in short-term cultures of CD4⁺ peripheral blood mononuclear cells (PBMCs) stimulated by phytohaemagglutinin and interleukin 2 (IL-2). The SI strains can adapt to growth in immortal CD4⁺ T-cell lines, which become chronic virus producers. R5 strains, however, need to be propagated in PBMCs, where they may be cytopathic, and in noncytopathic macrophage culture. Virus isolation is useful for determining the phenotype of HIV strains, and whether they have developed drug resistance, though genotyping is more rapid and is now more generally used. Figure 38.4 shows HIV particles produced by the CEM T-cell line in culture.

Isolation of HIV in cell culture can be detected by several means: (i) a cytopathic effect (CPE), including syncytia for X4 strains; (ii) detection of viral antigens in infected cells by antibodies, for example immunofluorescence, enzyme-linked immunocytology of infected cells, enzyme-linked immunosorbent assay (ELISA) for p24 antigen in cells or in supernatant medium from infected cultures; (iii) reverse transcriptase (RT) assay, either by enzyme activity or by ELISA, and (iv) genome detection, using polymerase chain reaction (PCR).

Genes and Proteins

Figure 38.1 shows the proviral genomes of HIV-1 and HIV-2. The gene maps are similar except that HIV-2 lacks *vpu* but carries *vpx*. The core and matrix proteins are encoded by *gag*. The Gag proteins of the mature virus are p17, p24, p7 and p6, and are processed by cleavage of the p55 precursor protein by the viral protease. The matrix antigen p17 is localized to the inner layer of the viral envelope and requires myristylation, which allows it to be tightly bonded to the inner envelope; matrix antigen is indispensable for budding. The core shell is made of

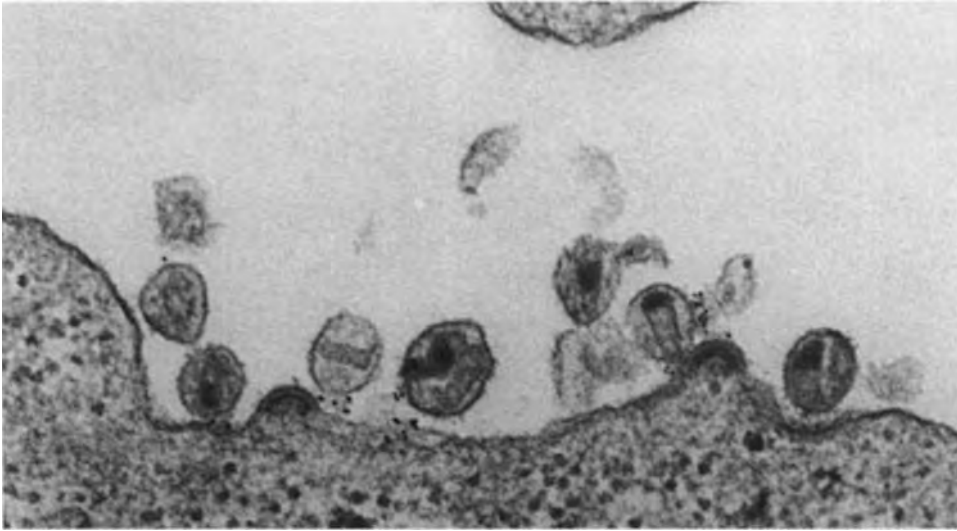


Figure 38.4 HIV-1 particles produced by CEM cells. Note the crescent-shaped core in budding particles and the condensed cone-like core in mature particles of approximately 100 nm diameter. The black spots are indirect immunogold labelling of antibody from an AIDS patient absorbed to the virus-producing cells.

p24 capsid antigen. This is the viral protein most usually detected clinically as a measure of antigenaemia. The *pol* gene encodes the enzymes protease, RT and integrase. The *env* gene encodes the gp41 and gp120 envelope glycoproteins, cleaved by cellular enzymes (furins) from the gp160 precursor.

In addition to *gag*, *pol* and *env*, HIV-1 and HIV-2 carry seven regulatory and accessory genes (Emerman and Malim, 1998). The *tat* gene encodes a protein that binds to TAR RNA sequences in the 5' long terminal repeat (LTR) to upregulate viral RNA transcription through complexes of cellular transcription factors and cyclin T. Rev serves to aid export of long HIV transcripts from nucleus to cytoplasm through recognition of Rev-response elements (RREs) in unspliced or singly spliced viral mRNA. Nef has multiple functions in signal transduction and downmodulation of CD4 cell surface expression. Vpr allows transport of newly infected pre-integration complexes (PICs) of the HIV proviral DNA into the nucleus for integration (by integrase) into host chromosomal DNA. Vpr also arrests cells in the G₂-phase of the mitotic cycle, enhancing virus production. Vpu has two known functions: degradation of cellular CD4 and enhancement of viral particle release, the latter by antagonizing tetherin function (Neil *et al.*, 2008). Tetherin is a host cell protein thought to represent an innate antiviral mechanism by inhibiting the release of enveloped viral particles from the cell. Vif is incorporated into virus particles and helps infection in new target cells by abrogating restriction to replication exerted by ABOBEC 3C (Sheehy *et al.*, 2002).

Although SI strains of HIV-1 can be propagated in T-cell lines with deletions or nonfunctional mutations in *nef*, *vpu* or *vif*, such mutations adversely affect HIV replication in PBMCs and macrophages so that attenuated infection or no replication occurs. Thus all the viral genes are required for efficient infection and pathogenesis *in vivo* (Stevenson, 2003).

HIV Replication and Antiviral Targets

HIV infects mainly CD4⁺ cells by binding to CD4 as a receptor (Dalgleish *et al.*, 1984). However, many of these cells are not lymphocytes. For example, monocytes, macrophages, dendritic cells (DCs), Langerhans cells and some brain cells, such as the microglia, express the CD4 receptor, and many are susceptible to infection by HIV. It is likely that infection of these cell types plays a major role in the pathogenesis of disease (Stevenson, 2003; Weiss, 2003). Importantly, HIV binds to the DC-SIGN receptor on DCs without infecting them, and hence can be delivered to CD4 lymphocytes in the lymph node (Geijtenbeek *et al.*, 2000; Pope and Haase, 2003).

The CD4 cell surface antigen is necessary but not sufficient for HIV-1 infection (Dalgleish *et al.*, 1984; Maddon *et al.*, 1986). While CD4 is required for high-affinity binding of gp120, co-receptors are required for subsequent steps leading to fusion between the viral envelope and cell membrane. The co-receptors were identified in 1996 to be chemokine receptors and help to explain the differential tropism for lymphocytes and macrophages (Berger *et al.*,

1999). Primary NSI strains of HIV mainly utilize CCR5 (R5 strains), which is the receptor for the chemokines MIP-1 α , MIP-1 β and RANTES, whereas SI strains utilize CXCR4 (X4 strains), the receptor for stromal derived factor 1 (SDF-1). Following gp120 binding to CD4, the co-receptor-binding site becomes exposed, allowing further attachment to CCR5 or CXCR4 receptor. The discovery that chemokine receptors act as co-receptors to CD4 (Feng *et al.*, 1996) helped to explain why these chemokines can inhibit HIV-1 infection *in vitro*. The tolerance of heterozygous and homozygous gene deletions of the CCR5 gene ($\Delta 32$) in the human population (see section on Host genetic determinants for HIV/AIDS below) with no apparent ill-effects has stimulated the development of CCR5 antagonists, which compete with HIV for binding to the co-receptor, and therefore inhibit viral replication.

The binding of gp120 to the cellular receptor CD4 and a chemokine co-receptor triggers a series of complex conformational changes in gp41 that lead to the fusion of viral and cellular membranes. There are three functional regions in the ectodomain of gp41: the fusion peptide, heptad repeat 1 (HR1) and HR2, and the transmembrane region. Crystallographic studies have demonstrated that the fusion-active (fusogenic) conformation of gp41 is a six-helix bundle structure in which three N-terminal helices (HR1) form a central trimeric coiled coil and three C-terminal helices (HR2) pack in an anti-parallel manner into hydrophobic grooves of the coiled core. The hairpin interactions between the HR1 and HR2 domains of gp41 bring the viral and host cell membranes together for fusion. One fusion inhibitor, enfuvirtide (ENF), is approved for clinical use. This is a synthetic 36-amino-acid molecule that corresponds to the HR2 peptide, and thus inhibits the normal fusogenic bundle formation.

Recent work has started to unravel the early stages of replication after cell entry and uncoating. The TRIM5 family of proteins is thought to confer species specificity on HIV-1 infection. Thus, monkey TRIM5 α restricts infection with HIV-1, through binding to the viral gag, and this restriction is conferred on human cells by expression of the same monkey TRIM species. Human TRIM5 α binds HIV-1 capsid poorly, explaining the lack of protection in humans.

Reverse transcription is initiated by the viral RNA-dependent DNA polymerase within the incoming virion. The viral genome comprises two identical strands of RNA, and the enzyme jumps strands during transcription. This provides the basis for recombination in a cell infected with more than one virus, since strands from each virus can be utilized within the jump. The other major mechanism of HIV variation lies in the error-prone nature of the RT process, since the

enzyme lacks proofreading mechanisms. The error rate is estimated at 10^{-4} , approximating to one mutation for every daughter virus produced. One further potential cause of viral genetic variation, particularly G-to-A hypermutation (a characteristic of HIV-1), is the activity of the host cell enzyme APOBEC3, which is a cytidine deaminase. It is thought that this family of proteins represents a long-standing innate antiviral defence mechanism. In response, the HIV vif protein acts to increase APOBEC destruction by cell proteasomes, thus maintaining the viability of proviral DNA species. Despite this, the detrimental impact of G-to-A mutations can be observed within proviral DNA, compared to plasma RNA (representing productive infections) (Gifford *et al.*, 2008). The viral RT remains the major target of antiviral drugs.

The synthesis of full-length proviral DNA within the reverse transcription complex produces the PIC. This comprises a number of viral proteins, including integrase, and host proteins such as the cellular transcription co-activator lens-epithelium-derived growth factor (LEDGF/p75). This PIC crosses the nuclear envelope into the nucleus. A key feature of HIV-1 is its ability to infect nondividing as well as dividing cells, such as macrophages and microglia cells, and thus nuclear entry is independent of mitosis.

Following nuclear entry, tethering of the viral integrase to host chromatin is thought to require LEDGF/p75 (Ciuffi *et al.*, 2005; Llano *et al.*, 2006). Subsequently, integrase catalyses integration via a number of linked steps. In the first step, the enzyme binds to the reverse transcribed double-stranded viral DNA in the cytoplasm and cleaves a GT dinucleotide from each 3' end of the viral DNA, in a reaction termed 3'-processing, to produce 3'-hydroxyl ends (CAOH-3'). In the second step, the PIC formed by the proviral DNA, the integrase and cellular and viral proteins, enters the nucleus, where the proviral DNA is inserted into the host genome in a process known as strand transfer. This involves cleavage of 4–6 bp in the host DNA and the joining of the CAOH-3' viral DNA ends to the 5'-phosphate ends of the host DNA. It is this stage which is inhibited by currently available integrase inhibitors. It is thought that integration is targeted at transcriptionally active areas of the host cell chromosome. Proviral DNA can also exist in an episomal, non-integrated form, as one or two LTR circles. The implications of this viral state for pathogenesis remains unclear.

Over 40 species of mRNA are produced by alternate splicing, but around half are estimated to be full-length RNA, which function as mRNA and genomic RNA for subsequent packaging into secreted virions.

The production of new HIV-1 virions is driven by the production of the gag precursor, incorporating the matrix

(MA), capsid (CA), spacer peptide 1 (SP1), nucleocapsid (NC), SP2 and p6 proteins. A frameshift also leads to expression of gag-pol polyproteins, incorporating the RT, protease and integrase enzymes. Production of viable virions occurs through a complex assembly process at the plasma membrane. The MA targets the gag to the assembly site, and the CA domain is involved in multimerization of gag polyproteins. The NC is responsible for packaging the genomic RNA for export. Simultaneously with budding, the protease acts to cleave the gag polyprotein into its constituent proteins. Cleavage of gag is a highly ordered process, initially at the SP2/NC site, followed by MA/CA and then p1/p6 sites. A new maturation inhibitor, named Berivimat, acts to inhibit the SP1-CA cleavage. By contrast, the protease inhibitor (PI) class of drugs act to inhibit all cleavage steps, leading to the production of immature virions which are non-infectious. The late stage of budding involves the binding of the PTAP motif in the viral p6 to the Tsg101 component of the host endosomal sorting complex ESCRT-1. A further host protein, the apoptosis-linked gene 2 interacting protein (Alix), appears to play an important role in this p6-Tsg101 interaction.

HOST GENETIC DETERMINANTS FOR HIV/AIDS

A number of host genetic factors affect either the risk of acquiring infection when exposed to HIV, or the rate of progression to AIDS following infection (O'Brien and Nelson, 2004). Different major histocompatibility complex (MHC) class I, class II and natural killer (NK) killer inhibitory receptor (Kir) alleles can be categorized as 'good' or 'bad' genes for AIDS progression. Since the frequency of MHC alleles varies in different populations, these may influence disease progression and also responses to immunotherapy.

Another set of polymorphic genes that impacts HIV/AIDS are those encoding chemokines that bind to CCR5, the co-receptor for R5 strains of HIV, and the CCR5 gene itself. The major ligands for CCR5 are CCL5 (RANTES) and CCL3L1 (MIP-1 α P). The higher the chemokine concentration in the plasma, the lower the risk of infection, and the slower the progression to AIDS when untreated, because CCL5 and CCL3L1 compete with HIV gp120 for binding to CCR5. CCL3L1 genes vary in copy number, and individuals with relatively high copy numbers express higher plasma concentrations of the chemokine (Gonzalez *et al.*, 2005).

The CCR5 gene is polymorphic (O'Brien and Nelson, 2004). A common mutation in the noncoding promoter region of CCR5 influences the degree of gene expression,

and consequently the density of CCR5 on the surface of target cells for HIV infection. In fact, it is the combination of high CCL3L1 expression coupled with lower CCR5 expression that is protective for HIV infection and for progression to AIDS. In Europeans, but not Africans, there is a relatively frequent mutation in the CCR5 gene in which 32 base pairs are deleted from the coding region, known as CCR5 Δ 32, so this allele does not express a functional CCR5 molecule. Indeed, CCR5 Δ 32 homozygotes (approximately 1% of the white population) are resistant to infection by R5 strains of HIV, although they remain susceptible to the generally less transmissible X4 strains. The CCR5 Δ 32 mutation first came to light through the study of exposed uninfected individuals. CCR5 Δ 32 heterozygotes express lower levels of CCR5 receptor than normals and hence exhibit a slower progression to AIDS.

The reason for the high frequency of CCR5 Δ 32 alleles in the white population remains unresolved. Its frequency increases with latitude, being highest in Scandinavian countries. Some have speculated that CCR5 Δ 32 may have given protection against a highly pathogenic agent in the past, such as smallpox or the plague. Another polymorphism is a mutation in CCR2, but it is not clear whether this mutation acts through CCR2 itself, or by affecting CCR5 expression, because CCR5 is an adjacent gene on the chromosome.

There are also human polymorphisms in the intracellular HIV restriction factors TRIM5 α and APOBEC3 (Holmes *et al.*, 2007; Towers, 2007). Whether different alleles affect susceptibility to HIV/AIDS remains to be definitively shown. However, interspecies polymorphisms in TRIM5 α (differences between humans and simians) does affect HIV and SIV susceptibility and may help to restrict cross-species lentivirus infection in situations of human exposure to infected non-human primates.

With the advent of whole-genome scanning for single nucleotide polymorphisms, new genetic loci are coming to light that affect susceptibility to HIV/AIDS, including those involved in interferon responses (Fellay *et al.*, 2007; Loeuillet *et al.*, 2008). This is a rapidly developing area of AIDS research and it is likely that polymorphisms in human genes that affect the metabolism of antiretroviral drugs will also be increasingly frequently defined (Telenti and Zanger, 2008).

VIRAL DYNAMICS AND PATHOGENESIS

Viral Dynamics

In untreated individuals, HIV infection is characterized by high levels of virus replication, generation of genetically diverging virus variants through the emergence of

mutations and recombination events, and reciprocal interactions with the immune system. Approximately 10^{10} new virus particles are produced daily and rapidly cleared, with a free plasma virus clearance rate of only a few hours. The half-life of virus-producing cells is less than one day, leading to an infected cell loss rate of 10^8 – 10^9 per day (Ho *et al.*, 1995; Ramratnam *et al.*, 1999; Markowitz *et al.*, 2003; Wei *et al.*, 1995). The high virus turnover ensures rapid adaptation to changing selective pressures and provides an opportunity for drug resistance, immune escape and cell tropism to evolve, while the resulting chronic immune activation leads to the dysfunction and gradual collapse of the immune system. Within the diverse virus variants circulating in plasma ('the quasispecies'), dominant populations represent the expression of a dynamic equilibrium between escape from selective pressures and preserved ability to replicate and infect ('viral fitness').

HIV replicates preferentially in activated CD4 T cells. Although most infected cells have a short lifespan, a fraction of cells survive long enough to revert to a resting memory state. The exclusion of host transcription factors (e.g. NF- κ B) from the nucleus virtually silences HIV gene expression in resting cells, and HIV persists as a stable, integrated and transcriptionally silent provirus, which is unaffected by antiretroviral therapy (Han *et al.*, 2007; Wong *et al.*, 1997). A proportion of latently infected cells can resume full virus production following activation. Thus, the latently infected CD4 T cells act as a reservoir, which is established early during primary infection and shows a slow subsequent rate of decay. Although the frequency of latently infected cells is only 1 in 10^6 resting CD4 T cells, the reservoir can rapidly repopulate the plasma quasispecies when antiviral therapy is interrupted, and represents the main barrier to eradicating the infection (Chun *et al.*, 1997, 1998; Siliciano *et al.*, 2003; Strain *et al.*, 2003). In viraemic individuals, the reservoir is continuously replenished, creating an archive for all quasispecies that have replicated at a high level, including drug-resistant mutants, with long-term implications for treatment (Monie *et al.*, 2005).

Cell Tropism and HIV Receptors

The expression of CCR5 or CXCR4 on different CD4 cells defines their susceptibility to infection by the corresponding R5 or X4 strains, thus determining virus tropism. The sequence of the V3 loop is the major determinant of co-receptor use, as a result of the structural homology between the crown of the V3 loop and the β 2– β 3 loop in the natural chemokine ligands. Additional determinants are found in V1 and V2.

R5 viruses appear to be more efficiently transmitted than X4 viruses and predominate in the initial and asymptomatic phases of the infection regardless of the route

of exposure (Pope and Haase, 2003). The mechanisms of the preferential selection are unclear. X4 viruses tend to emerge as the CD4 count declines, and are eventually detected in 40–60% of patients, coinciding with expanded cell tropism, increased virus replication rate, faster disease progression, and the onset of AIDS (Connor *et al.*, 1997). It is still debated whether the emergence of X4 viruses is the cause or consequence of disease progression. One proposed mechanism of enhanced pathogenicity is the ability of X4 viruses to infect immature thymocytes, the precursors of mature CD4 T cells (Correa and Muñoz-Fernández, 2001). R5 viruses detected in advanced disease can also differ from those detected in early infection by showing reduced dependence on high CD4/CCR5 levels for entry, decreased susceptibility to RANTES, and enhanced pathogenicity (Kwa *et al.*, 2003).

Dendritic Cells

Dendritic cells are professional antigen-presenting cells and important mediators of the adaptive immune response against HIV, but are also responsible for uptake, trafficking and dissemination of the virus (Pope and Haase, 2003). DCs express CD4, chemokine co-receptors, Fc receptors, DC-SIGN and other C-type lectin receptors that can mediate HIV uptake. DCs can capture HIV without becoming productively infected, transmit the captured virions by forming synapses with T cells and transport the virus to the lymphoid organs. They are also susceptible to infection. Although virus replication is generally less efficient than in T cells, progeny virus can be released to propagate the infection (Hladik *et al.*, 2007; Wu and KewalRamani, 2006). It remains to be determined whether DCs can provide a cellular reservoir for latent infection. Langerhans cells are a subset of DCs found in tissues, including the genital mucosal epithelium. Alongside intraepithelial CD4 T cells and subepithelial DCs, CD4 T cells and macrophages, Langerhans cells encounter HIV early after sexual exposure. It has been proposed that the virus ability to establish a systemic infection depends on its early interaction with Langerhans cells (Boggiano and Littman, 2007). Follicular DCs are found in the B-cell follicles and germinal centres of peripheral lymphoid tissues. They do not become productively infected with HIV, but in lymphoid tissues trap and retain large quantities of infectious virus particles on their surface, proximal to highly susceptible CD4 T cells.

CD4 T-cell Depletion

Approximately 3×10^7 CD4 T cells are lost every day, but the mechanisms of HIV-induced CD4 depletion

are not entirely known. The highest rate of CD4 T-cell destruction occurs during primary infection before the emergence of the adaptive immune response. HIV transmission is followed rapidly by dissemination to the lymphoid organs, particularly gut-associated lymphoid tissues (GALT), where around 60% of total CD4 T cells reside (Li *et al.*, 2005). Within days of the infection there is massive depletion of memory CD4 T cells in the GALT, independently of the route of transmission (Brenchley *et al.*, 2004; Li *et al.*, 2005). CD4 depletion in GALT persists after the acute phase of the infection and is only partially restored by antiretroviral therapy (Guadalupe *et al.*, 2006). Conversely, the depletion is not observed in HIV-infected long-term nonprogressors who remain clinically and immunologically well over many years of infection. These findings suggest that GALT plays a central role in HIV pathogenesis (Centlivre *et al.*, 2007).

Several explanations for HIV-induced CD4 T-cell loss have been proposed: impaired T-cell production by the thymus, decreased proliferative capacity of CD4 T cells, and increased death due to direct and indirect mechanisms. HIV can cause death of CD4 T cells in several ways (Letvin and Walker 2003; Pope and Haase, 2003): (i) by a direct CPE, including cell fusion by SI strains; (ii) by cytotoxic T lymphocytes and other immune responses that target infected cells and (iii) by apoptosis of infected and uninfected cells, due to lymphocyte activation and a changed cytokine and chemokine milieu. For patients with a CD4 count ≥ 500 cells μl^{-1} , the median time to a CD4 cell decline < 350 cells μl^{-1} is 2.5 years, ranging from 0.7 years in those with a plasma HIV RNA load ≥ 500 000 copies ml^{-1} to 4.7 years in those with a viral load < 1000 copies ml^{-1} (UK Collaborative HIV Cohort (CHIC) Study Steering Committee, 2007). These data clearly indicate that viral replication is a major driver of CD4 T-cell loss. However, the chronic immune activation characteristic of HIV infection seems to drive CD4 T-cell loss more than direct virus-mediated destruction. There exists a strong association between activation of the immune system, T-cell apoptosis, immunological exhaustion, uncontrolled HIV replication, plasma viral load and disease progression (Choudhary *et al.*, 2007; Day *et al.*, 2006; Deeks *et al.*, 2004; D'Souza *et al.*, 2007; Hazenberg *et al.*, 2003; Kaufmann *et al.*, 2007; Oxenius *et al.*, 2004). The mechanisms by which immune activation induces CD4 T-cell depletion are still incompletely understood. In HIV-infected people both CD4 and CD8 T cells show a significant upregulation of the inhibitory receptor programmed death 1 (PD-1). The observation that *in vitro* exposure of CD4 T cells to high concentrations of HIV gp120 induces Fas ligand expression at the cell surface provides one pathway by which Fas–Fas ligand-mediated T-cell apoptosis may occur. There are

also clear but not absolute correlates between the rate of disease progression and the immunogenetic background of the host (Carrington and O'Brien 2003). Several human leukocyte antigen (HLA) markers have been found to be associated with either faster (e.g. HLA-B*35 and C ω 4) or slower (e.g. HLA-B*27 and HLA-B*57) HIV disease progression among untreated individuals.

IMMUNE RESPONSES

As with other virus infections, systemic infection by HIV typically elicits both cellular and humoral immune responses. These help to control virus replication, but do not clear the infection entirely from the body. Hence there is no recovered, immune state for HIV, or for most other lentivirus infections of animals.

After primary infection, seroconversion occurs when antibodies first become detectable, and specific CD4⁺ and CD8⁺ T cells appear around the same time. Because virus-neutralizing antibodies do not appear until several months later, it was assumed that cell-mediated immunity was chiefly responsible for clearance of peak primary viraemia before the establishment of the viral load 'set point'. Indeed strong cellular immune responses aid survival, particularly a powerful T-helper cell response in so-called 'elite' controllers (Walker, 2007). However, recent studies indicate that non-neutralizing antibodies may play a role in virus clearance, mediated through complement (C'). Antibody plus C' can inactivate virus infection through lysis of the HIV envelope and other inhibitory mechanisms; C' may also reduce the number of virus-producing cells through antibody-dependent cellular cytotoxicity. But the action of C' may be a two-edged sword, because it can also enhance HIV infection of cells bearing C' receptors, such as macrophages and DCs. Thus innate immune components may exert contradictory protective and virulent effects during the course of HIV infection (Willey and Aasa-Chapman, 2008).

The immune response undoubtedly shapes HIV, just as HIV eventually takes its toll on the immune system. Early in the course of infection, HIV genetic variation is minimal, but a quasispecies of genomes representing a high degree of genetic variation follows soon after seroconversion. HIV is adept at throwing up immune escape mutants out of this quasispecies. This immune evolution is evident both for escape from recognition of Gag and Tat peptides recognized by cytotoxic T cells, and for escape from neutralization by antibodies. By the time that neutralizing antibodies appear, new variants of HIV also appear so that HIV keeps ahead of this host—virus interplay.

HIV particles also cover the most vulnerable neutralization epitopes on gp120 and gp41 under a carapace of

carbohydrate groups. The CD4-binding site on gp120 remains available as a relatively constant feature (though in a cleft) but other epitopes such as the co-receptor site on gp120 and the fusion site on gp41 are only revealed during induced-fit interactions with the receptors, so that antibodies have little time to act. X4-tropic viruses, however, have more exposed sites, and may be more readily controlled by humoral immunity. This may explain why X4 viruses tend to appear late in infection because they are opportunist HIV variants that only thrive in an already immunocompromised host (Weiss *et al.*, 2008; Willey and Aasa-Chapman, 2008).

THE LABORATORY DIAGNOSIS OF HIV INFECTION

Screening and Confirmatory Tests

The laboratory diagnosis of HIV infection is based on methods that detect antibodies and methods that detect the virus or virus components in blood. The chronological order of detection after primary infection is on average 9–11 days for viral RNA and 21–25 days for anti-HIV antibodies. Serology remains the cornerstone of the diagnosis of HIV infection in adults. Although early studies suggested that a small percentage of people may require up to six months for antibody to appear, the vast majority of infected individuals develop detectable antibodies within three to eight weeks of the infection when tested with current assays. Serological assays can be divided into screening tests and confirmatory (supplemental) tests. Screening tests are generally designed to detect infection with high sensitivity (low false-negative rate), whereas confirmatory tests detect the infection with high specificity (low false-positive rate). In practice, a combination of tests is used to allow diagnosis with high sensitivity and specificity, and to differentiate between HIV-1 and HIV-2.

Enzyme-linked immunoassay (EIA) and EIA-like formats for HIV antibody detection have undergone refinement through the years to increase sensitivity (>98%), specificity (>99%), and automation. Third-generation assays employ a mixture of recombinant viral proteins and synthetic peptides to detect antibodies against both HIV-1 and HIV-2, including all subtypes of HIV-1 group M and the outlier group O. The antigen-sandwich format is considered the most sensitive screening method, given its ability to detect all isotypes of antibody (including IgM). The test detects antibodies within three to four weeks, but does not usually differentiate between HIV types. Fourth-generation assays allow the combined detection of anti-HIV antibodies and p24 antigen, thereby increasing diagnostic sensitivity in recent infection by approximately one week.

The interpretation of screening assay results is as follows:

- *Negative screening test.* Unless a recent infection is suspected, a negative screening test result is considered sufficient to exclude infection.
- *Reactive screening test.* A reactive result requires confirmation and differentiation between HIV-1 and HIV-2. A positive report is released if consensus results are obtained from all assays, and a second blood is requested to confirm patient identity. Non-consensus in the confirmatory tests may be due to early infection, non-specific reactivity, or, rarely, a new non-reactive HIV subtype.
- *Recent infection.* In people with recent infection, anti-HIV antibodies may be initially low or undetectable. Follow-up testing demonstrates rising antibody responses confirming the diagnosis. The Western Blot will demonstrate the evolution of the serological response to specific HIV antigens. If a clinical diagnosis of primary infection is suspected, the diagnosis can be achieved by detection of viral RNA or proviral DNA. Viral load assays are widely used for this purpose. Approximately 50% of cases will also show detectable p24, but p24 antigen detection has been largely replaced by the more sensitive molecular methods.
- *Non-specific reactivity.* For patients with non-specific reactivity, the clinical follow-up involves establishing that they are viral RNA and proviral DNA negative and that they have non-evolving serology. They are followed over time for reassurance, and in the majority the reactivity will eventually wane.

Confirmatory tests traditionally include Western blot, line immunoassay (LIA), indirect fluorescent assay (IFA) and radioimmunoprecipitation assay (RIPA). The tests are highly specific, but labour-intensive and expensive. Western blot is considered the gold standard for validation of HIV test results. The assay uses antigens prepared from whole cultured virions, separated by electrophoresis and blotted onto a nitrocellulose membrane which is then cut into strips. The HIV-1 viral antigens are separated as follows (from top to bottom): gp160, gp120, p66, p55, p51, gp41, p31, p24, p17 and p15. Antibodies to the Gag proteins (p17, p24 and p55) appear first in the course of infection, decreasing in titre during disease progression. Antibodies to the Env glycoproteins (gp160, gp120 and gp41) appear later and persist in advanced disease. Although the interpretation criteria vary, reactivity to at least two of gp160/120, gp41 or p24 is required for a positive result. It is generally accepted that a negative result is the absence of all bands, although it has been proposed that results can be reported as negative if there is only a very

weak p17 band. Indeterminate classifications occur when there is reactivity to one or more antigens, but not fulfilling the criteria for positivity. LIA uses a similar principle to Western blot, but employs recombinant antigens and synthetic peptides applied on nitrocellulose strips, improving standardization and reducing background reactivity. A positive result for HIV-1 is usually defined by reactivity to p24 and gp41.

Simple and rapid confirmatory assays have been developed and alternative confirmatory strategies have been proposed. One example is the combination of two screening tests that use either antigens derived from different sources or different assay configurations, or a combination of screening test and virus detection test, to be followed by a confirmatory test only in case of discrepancies. Traditional confirmatory tests still have a place for sera yielding indeterminate results.

In general, plasma HIV RNA quantitative detection for primary diagnostic purposes is not recommended because the assay has been explicitly developed for use in known infected individuals, and may give a low false positive result in an uninfected individual.

Rapid Anti-HIV Antibody Assays for Point-of-Care Testing

The availability of antiretroviral therapy makes the early diagnosis of HIV infection imperative. In western countries, despite the success of voluntary testing services and the adoption of routine, opt-out testing in antenatal and genito-urinary clinics, a significant proportion of infected people remain unaware of their infection. These patients are known to repeatedly seek medical care for a series of minor and major ailments, but are not identified as HIV-infected until late in the disease course. A call has been made internationally to increase uptake of HIV testing and improve recognition of undiagnosed infection.

Two strategies have been proposed: wider adoption of routine opt-out HIV testing (Table 38.1) in a variety of health-care setting where HIV-infected patients may present (e.g. accident and emergency, general medical wards, TB clinics), and use of rapid point-of-care HIV antibody tests.

Rapid HIV tests use capillary blood collected by finger-prick or oral fluids, require no sophisticated equipment, can be performed by trained staff with limited technical expertise, provide a result in less than 30 minutes, and can be read visually. These features make them suitable for point-of-care (near-patient) testing. 'Salivary' antibody tests (e.g. the OraQuick Advance) use the crevicular fluid from capillaries beneath the tooth-gum margin, which is a transudate of blood. The concentration of antibodies in oral fluids is about 1/400 of that in plasma, requiring a high sensitivity of detection for acceptable assay performance.

The common assay formats are flow-through, lateral-flow and agglutination assays. They usually incorporate an internal control based on the detection of human IgG. Approved tests perform well when compared with laboratory screening assays (Branson 2007) and can detect group M and group O HIV-1 and HIV-2. In a voluntary testing service with a HIV-1 seroprevalence of 3.3%, the specificity of the Abbott Determine test with capillary blood is 99.6% (99.3–99.8%) and the positive predictive value 90.5% (82.8–95.6%). This translates into a false-positive rate of 1 : 11. In addition, relative to third-generation serological assays, the Determine and other rapid tests show an average delay of five to seven days in the detection of anti-HIV antibodies. Thus, point-of-care tests are best suited for specific settings where the advantages clearly outweigh the risk of false positivity and possible delays in recognizing early infection. They are most useful in situations when immediate results are required to guide management, such as testing pregnant women who present during labour, or the assessment of source people in exposure incidents to inform the use of post-exposure

Table 38.1 Centers for Disease Control and Prevention (CDC) definitions of HIV testing

Diagnostic testing: Performing an HIV test for people with clinical signs or symptoms consistent with HIV infection.

Screening: Performing an HIV test for all people in a defined population.

Targeted testing: Performing an HIV test for subpopulations of people at higher risk, typically defined on the basis of behaviour, clinical or demographic characteristics.

Informed consent: A process of communication between patient and provider through which an informed patient can choose whether to undergo HIV testing or decline to do so. Elements of informed consent typically include providing oral or written information regarding HIV, the risks and benefits of testing, the implications of HIV test results, how test results will be communicated, and the opportunity to ask questions.

Opt-out screening: Performing HIV screening after notifying the patient that (i) the test will be performed and (ii) the patient may elect to decline or defer testing. Assent is inferred unless the patient declines testing.

HIV-prevention counselling: An interactive process of assessing risk, recognizing specific behaviours that increase the risk for acquiring or transmitting HIV, and developing a plan to take specific steps to reduce risks.

prophylaxis (PEP). The tests are also valuable in clinical settings where a significant proportion of people tested by conventional methods fail to return for their results. The availability of rapid results allows appropriate counselling and management of the infected people, while avoiding the need for follow-up visits for those who test negative. Furthermore, the avoidance of venupuncture and the rapid availability of results can be an incentive for people to present for testing. Because the assay format is simple and most tests are stable within a wide temperature range, rapid HIV tests can also be used outside of traditional clinical settings to improve uptake among at-risk populations that may be otherwise difficult to reach.

The main advantages and disadvantages of rapid HIV tests are summarized in Table 38.2. The tests should be performed under conditions that satisfy accreditation requirements, including the establishment of rigorous training, quality assurance and audit programmes. Arrangements should also be in place for rapid confirmation of reactive results, to avoid unnecessary anxiety in patients who test falsely reactive. The World Health Organization recommends that prior to their implementation, point-of-care HIV tests first be evaluated at a reference laboratory and then at points-of-service, and that measures to ensure quality of testing should be in place.

Methods to Identify Recently Acquired HIV-1 Infection

Following seroconversion, several laboratory methods have been developed to allow differentiation of early versus established (greater than four to six months) HIV infection. The assays are used to study the dynamics of the epidemic and transmission networks, inform public health intervention programmes, and identify candidates for clinical trials and vaccine strategies targeting early infection (Table 38.3) (Gupta *et al.*, 2007).

The detuned assay has been widely applied but currently has limited availability. The test is based on the principle that in early infection a sample reactive in the

Table 38.2 Advantages and disadvantages of rapid point-of-care HIV tests

Advantages	Disadvantages
Easy to perform and interpret	Expensive
Require simple equipment	Reduced sensitivity in early infection
Avoid venupuncture	Prone to operator errors
Include a positive control	Subjective interpretation of results

Table 38.3 Assays for differentiating early (less than four to six months) from established infection

Assay	Principle
Detuned assay with Vironostika-LS EIA	High-sensitivity/low-sensitivity dual EIA
Barin 'Peptide' assay	High-sensitivity/low-sensitivity dual EIA
Calypte [®] BED Incidence HIV EIA	Proportion of HIV-1-specific IgG in a serum or plasma relative to the total IgG
AxSYM and Vitros avidity assays	Employ guanidine to elute low-avidity and low-affinity antibodies

sensitive EIA becomes nonreactive in a less-sensitive assay. The 'peptide' assay follows the same principles but offers the advantage of being independent of commercial platforms (Barin *et al.*, 2005). The Calypte[®] BED Incidence HIV EIA is a quantitative assay that detects gradually increasing levels of anti-HIV IgG after seroconversion. Avidity assays employ guanidine to disrupt low-avidity binding between IgG antibodies and corresponding antigen, using as platform the Abbott AxSYM HIV 1/2gO MEIA assay or the Ortho Anti-HIV 1 and 2 VITROS ECi assay (Chawla *et al.*, 2007; Suligo *et al.*, 2002). Although there is individual variability in avidity maturation, validated breakpoints can identify seroconversion within the previous four to six months. The choice of the breakpoint ultimately depends on the intended application of the assay. Adopting a lower cut-off value increases specificity, but may result in some loss of sensitivity.

HIV Diagnosis in Children

The diagnosis of HIV infection in infants is confounded by the transfer of maternal antibodies from approximately 32 weeks of gestation. It takes up to 18 months for the passive antibody to disappear. Early diagnosis may be obtained by virus detection and recommended methods include proviral DNA detection by PCR (gold standard). With DNA PCR, which predominantly uses in-house methodologies, strict quality control is required to minimize the risk of contamination. In view of the genomic diversity of HIV, a maternal sample should always be amplified with the first infant sample to confirm that the primers detect the maternal virus. If a maternal virus cannot be detected, a different primer set or a different test (e.g. HIV RNA detection or virus culture) should be used. A positive DNA PCR at any time requires a repeat test on a second blood sample for confirmation. Infected infants are usually DNA-positive by week 4 and HIV can

be reasonably excluded with two negative tests, one at six weeks of age and the other at age three months or older. Testing is usually done at zero to two days, six weeks and three months of age. If all these tests are negative and the baby is not being breastfed, then parents can be informed that the child is not HIV infected. An HIV antibody test should be done at 18 months of age to confirm loss of maternal antibodies. Detection of viral RNA by viral load tests is practical, but data on assay performance in this setting are limited. For an untreated infant, RNA tests may be more sensitive than either DNA PCR or culture

(Lambert *et al.*, 2003). A low copy number result, however, should not be assumed to indicate infection as it may represent false positivity.

THE NATURAL HISTORY OF HIV INFECTION AND ITS CLINICAL MANIFESTATIONS

The natural course of HIV infection can be divided into three phases (Figure 38.5). In the acute phase, the plasma viral load increases exponentially and then

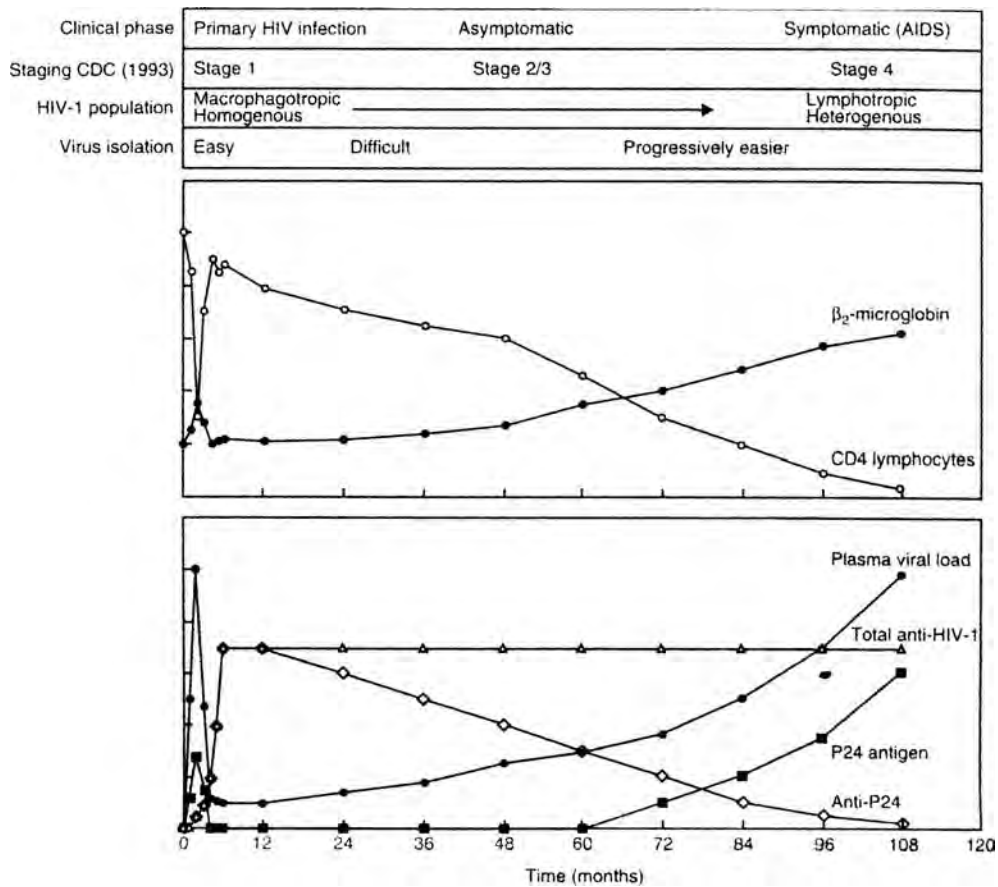


Figure 38.5 The clinical, virological and immunological phases of HIV infection. The natural course of HIV infection can be divided into three phases: primary infection, asymptomatic stage and symptomatic stage. In the top panel, CD4 T-cell counts (O) show a rapid decline immediately after infection coinciding with the burst of virus replication, followed by recovery to near-normal levels, subsequent gradual loss over several years of asymptomatic infection, and accelerated loss in late disease. The reciprocal of these changes is shown by the β_2 -microglobulin level (●), a plasma measure of immune activation. In the bottom panel, the plasma viral load (●) achieves a peak during primary infection, which is mirrored by the p24 antigen (■), declines to a relatively stable set-point in the early asymptomatic infection and raises gradually over time, with an accelerate increase in late disease. Anti-HIV antibodies (Δ) become detectable within three to eight weeks of the infection and persist throughout the infection. However certain antibody subsets (i.e. anti-p24 antibodies, \diamond) may decline in advanced disease.

declines sharply in line with the emerging immune response and/or exhaustion of cellular targets (Perelson, 2002). After approximately six months, the viral load reaches a relatively constant level known as the set-point. The asymptomatic phase, lasting for 6–15 years, is characterized by gradual CD4 T-cell depletion which precedes and predicts the clinical manifestations of advanced disease. The viral load set-point ranges generally between 10^3 and 10^5 copies ml^{-1} and is highly correlated with the rate of CD4 T-cell loss and disease progression (Mellors *et al.*, 1996). The viral load increases over time by 0.1–0.2 \log_{10} copies ml^{-1} per year, and ultimately the immune destruction and the direct pathogenic effects of the virus result in the range of opportunistic infections, malignancies and neurological and gastrointestinal disorders characteristic of AIDS. The depletion of CD4 T cells accounts for the profound immune deficiency. Other manifestations of disease (e.g. CNS infection) probably reflect infection of other cell types, including macrophages, microglial cells, astrocytes and neural progenitor populations (Schwartz and Major, 2006). The rates of progression vary among infected individuals. A small proportion (5–8%) remain clinically free of disease with high CD4 cell numbers (CD4 counts) for more than 15 years following infection. The majority (70–80%) show a progressive deterioration in the course of seven to eight years, and a further subset (10–15%) experience rapid progression within five years. Table 38.4 outlines the classification most widely used to stage HIV infection. AIDS-defining conditions are summarized in Table 38.5.

Primary Infection

In 50–90% of cases, primary HIV infection is accompanied by the acute onset of a constellation of symptoms referred to as the acute retroviral syndrome or acute seroconversion illness (Figure 38.6). Symptoms develop rapidly one to six weeks after infection and in over 50% of cases include fever $>38^\circ\text{C}$, fatigue, pharyngitis, body aches and cervical lymphadenopathy (Vanhems and Beaulieu, 1997). The rash is erythematous, maculopapular and nonpruritic, and affects the face, trunk

Table 38.5 AIDS-defining conditions

Candidiasis of bronchi, trachea or lungs
Candidiasis, oesophageal
Cervical cancer, invasive
Coccidioidomycosis, disseminated or extrapulmonary
Cryptococcosis, extrapulmonary
Cryptosporidiosis, chronic intestinal (>1 mo duration)
Cytomegalovirus disease (other than liver, spleen or nodes)
Cytomegalovirus retinitis (with loss of vision)
Encephalopathy, HIV related
Herpes simplex: chronic ulcer(s) (>1 mo duration) or bronchitis, pneumonitis, or oesophagitis
Histoplasmosis, disseminated or extrapulmonary
Isosporiasis, chronic intestinal (>1 mo duration)
Kaposi's sarcoma
Lymphoma, Burkitt's (or equivalent term)
Lymphoma, immunoblastic (or equivalent term)
Lymphoma, primary, of brain
<i>Mycobacterium avium</i> complex or <i>M. kansasii</i> , disseminated or extrapulmonary
<i>Mycobacterium tuberculosis</i> , any site (pulmonary or extrapulmonary)
<i>Mycobacterium</i> , other species or unidentified species, disseminated or extrapulmonary
<i>Pneumocystis carinii</i> pneumonia
Pneumonia, recurrent
Progressive multifocal leukoencephalopathy
Salmonella septicaemia, recurrent
Toxoplasmosis of brain
Wasting syndrome due to HIV

and occasionally the extremities including the palms and soles. Neurological manifestations are observed in 12% of cases, most commonly in the form of meningitis or meningo-encephalitis. Acute opportunistic infections may occur, including PCP and oral and oesophageal candidiasis.

The GALT, rich in activated memory CD4 T cells, is the main site of early HIV replication. HIV has also been detected in cerebrospinal fluid (CSF) soon after infection.

Table 38.4 Centers for Disease Control and Prevention (CDC) staging of HIV infection

CD4 counts (cells mm^{-3})	Clinical group		
	A: asymptomatic, PHI or PGL ^a	B: symptomatic, non-C ^b	C: AIDS-defining conditions
>500	A1	B1	C1
200–500	A2	B2	C2
<200	A3	B3	C3

^aPHI, primary HIV infection; PGL, persistent generalized lymphadenopathy.

^bSymptomatic but not included in C are those conditions associated with defective immunity.

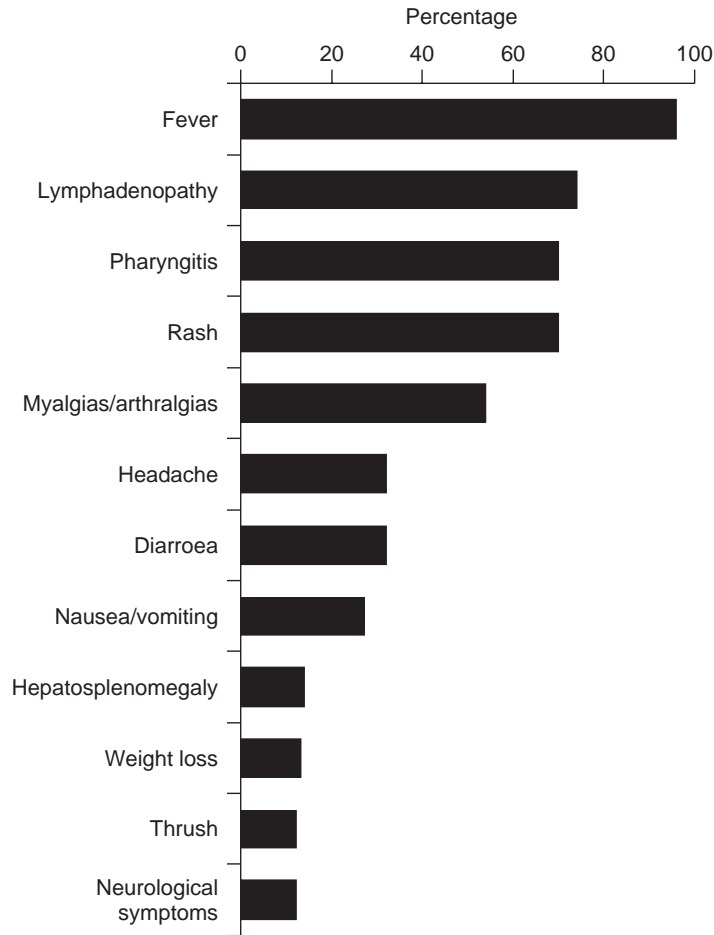


Figure 38.6 Symptoms that accompany primary HIV infection (acute retroviral syndrome or acute seroconversion illness).

Marked changes in the peripheral lymphocyte count are seen; the CD4 : CD8 ratio is reversed, CD8 cells increase and CD4 cells decrease. CD4 lymphopenia occurs early and is generally short lived, beginning to improve after day 9. The symptomatic phase of primary HIV infection lasts less than 14 days on average and is self-limiting. Complete clinical recovery is the norm. Multiple, severe and protracted symptoms, candidiasis, neurological involvement, and persistently high plasma viral load predict a rapid subsequent rate of disease progression. Whether antiretroviral treatment should be started in patients with primary HIV infection remains controversial. Early therapy may preserve immune responses but the long-term clinical benefit has not as yet been clearly demonstrated. In clinical practice, therapy is often used to limit symptoms in a severe seroconversion illness.

Asymptomatic Phase

Primary HIV infection is followed by an asymptomatic phase lasting 6–15 years. Clinically, patients are relatively free of symptoms, although lymphadenopathy may be a complaint in some cases. Virologically this does not represent a period of latency but rather the lymphoid tissue is actively infected by HIV (Stevenson, 2003) and there is a high turnover of HIV production and of infected lymphocytes with equally active replenishment (Ho *et al.*, 1995; Markowitz *et al.*, 2003; Ramratnam *et al.*, 1999; Wei *et al.*, 1995). The duration of the asymptomatic phase is variable and related to the severity of primary HIV infection, the phenotypic characteristics of the infecting viruses, the status of the host immunity, the lifestyle of the host and the use of antiretroviral therapies. This phase of clinical care is generally supportive, involving

surveillance for features of early progression and is the optimum time to commence antiretroviral therapy.

Symptomatic Phase

In untreated patients, early features of symptomatic disease are often nonspecific and associated with dermatological manifestations, such as eczema and human papillomavirus (HPV) lesions, as well as oral and vaginal candidiasis, recurrent chest infections, night sweats, weight loss and lymphadenopathy. Oral lesions become common, with gingivitis, candidiasis, herpes simplex lesions and aphthous ulcers. Oral hairy leukoplakia presents as white, ribbed lesions on the lateral margins of the tongue; it may be asymptomatic or produce soreness within the mouth. It is associated with Epstein—Barr virus (EBV) infection and responds to high-dose acyclovir or valaciclovir. As disease progresses and the CD4 count declines to <200 cells μl^{-1} , the risk of AIDS-defining conditions increases. HIV-induced disease affects multiple systems and organs through viral-mediated pathogenicity and immunodeficiency, leading to opportunistic infections and malignancies (Table 38.6).

Opportunistic Infections

Opportunistic infections seen in symptomatic HIV-1 disease may be categorized into: (i) those that do not cause disease in the immunocompetent host (e.g. *P. carinii*); (ii) those that cause mild disease in the normal host (e.g. herpes simplex virus (HSV), *Toxoplasma gondii*) and (iii) those that are conventional pathogens (e.g. *Mycobacterium tuberculosis*) but can produce widespread debilitating disease in the immunocompromised host.

Pneumocystis carinii

PCP is nearly always seen at CD4 counts <200 cells μl^{-1} . It is characterized by the subacute onset of fever, fatigue, dry cough and progressive shortness of breath. Diffuse, bilateral infiltrations with a batwing appearance are characteristic, but occasionally the chest radiograph may be normal at presentation. Diagnosis is made by detection of the organism in induced sputum or bronchoalveolar lavage. Treatment is usually with co-trimoxazole. Routine prophylaxis with co-trimoxazole in patients with CD4 cell counts <200 cells μl^{-1} markedly reduces the incidence of the infection.

Mycobacteria

The risk of active tuberculosis (TB) is high among HIV-infected people, and TB is the leading cause of death among HIV-infected people worldwide. *M. tuberculosis* causes disease in all stages of HIV infection. In

patients with CD4 counts <300 cells μl^{-1} the features of TB are similar to those of TB in immunocompetent hosts, with pulmonary disease the most common manifestation. At CD4 counts <200 cells μl^{-1} presentation can be atypical and nonpulmonary and disseminated disease may occur. Almost every tissue and organ can be affected. *M. avium* and *M. intracellulare* (together known as the *M. avium-intracellulare* complex (MAI)) are two closely related species of ubiquitous environmental organisms which can cause disseminated disease in patients with advanced disease, almost exclusively when the CD4 count is <50 cells μl^{-1} . Symptoms are nonspecific and include fatigue, fever, night sweats, weight loss, anaemia, abdominal pain and diarrhoea.

Cytomegalovirus

Cytomegalovirus (CMV) disease is common in untreated patients with advanced disease, especially at CD4 counts <50 cells μl^{-1} . Its manifestations primarily include progressive chorioretinitis, oesophagitis, colitis and neurological disease (e.g. encephalitis, polyradiculopathy, myelitis and mononeuritis multiplex). CMV retinitis occurred in 30–40% of AIDS patients prior to the introduction of highly active antiretroviral therapy (HAART). The lesions are initially asymptomatic but, as the perivascular exudates and haemorrhages involve the macula, vision becomes impaired. Replacement of retinal cells with fibroblasts may facilitate retinal detachment. Bilateral involvement is common. CMV pneumonitis is rare in AIDS patients because it requires active recruitment of the immune system to cause disease. Progression of CMV infection can be restricted by antiviral therapy with ganciclovir (or valganciclovir), foscarnet and cidofovir. The incidence of CMV retinitis is reduced by 80% in the era of HAART. Detection of CMV viraemia by PCR correlates with increased mortality (Deayton, 2004) and one non-randomized clinical trial reports reduced mortality when CMV retinitis was treated with systemic, rather than local, ganciclovir (Kempen, 2003).

Herpes Simplex Virus

In the early 1980s, chronic persistent ano-genital ulceration due to HSV was one of the first recognized manifestations of AIDS. Over the subsequent decades, a strong epidemiological association emerged between HSV-2 and HIV, reflecting common risk factors for infection as well as reciprocal promoting effects. Bidirectional interactions have been demonstrated between the two infections. Both HSV disease and subclinical HSV shedding recur frequently in HIV-infected people. Disease may be prolonged and respond poorly to therapy in patients with low CD4 counts. Frequent HSV reactivations enhance

Table 38.6 Major systems and organs affected by HIV infection

System or organ	Examples
Chest	Bacteria (e.g. <i>Streptococcus pneumoniae</i> , <i>Haemophilus influenzae</i> , <i>Staphylococcus aureus</i> , <i>Pseudomonas aeruginosa</i>) <i>Pneumocystis carinii</i> pneumonia Mycobacterial infections Fungi (e.g. <i>Histoplasma</i> , <i>Cryptococcus</i> , <i>Aspergillus</i>) KS, lymphoma, bronchogenic carcinoma Lymphocytic and nonspecific interstitial pneumonitis
Mouth	Candidiasis Gingivitis and periodontitis Viral infections causing ulcerations (HSV, VZV, CMV) Oral hairy leukoplakia (EBV) Warts (HPV) KS, lymphoma, carcinoma Idiopathic parotid enlargement
Eye	Keratoconjunctivitis sicca Infectious keratitis (e.g. VZV) Retinal microvasculopathy Infectious retinitis (e.g. CMV, VZV, HSV, toxoplasma, syphilis)
Skin	Staphylococcal folliculitis Eosinophilic and other pruriginous folliculitis Bacillary angiomatosis Viral eruptions (e.g. HSV, VZV, molluscum contagiosum, HPV) Insect bite reactions, photosensitivity, drug reactions Seborrheic dermatitis, psoriasis, Reiter's syndrome KS
Gastrointestinal and hepatic	Oesophagitis (e.g. HSV, CMV, <i>Candida</i> , idiopathic) Diarrhoea Colonic disease (e.g. CMV) Proctitis (e.g. HSV) Enhanced progression of viral hepatitis B and C Biliary tract disease (e.g. CMV, <i>Cryptosporidium</i>) Pancreatitis
Nervous system	Infections (e.g. <i>Toxoplasma</i> , <i>Cryptococcus</i> , PML, CMV) Malignancies (e.g. primary central nervous system lymphoma) HIV-encephalopathy Distal sensory polyneuropathy Functional disorders (e.g. anxiety, depression, psychosis)
Haematological	Anaemia, granulocytopenia, thrombocytopenia Coagulopathy
Endocrine and metabolic	Dislipidaemia, lipodistrophy Hypogonadism
Cardiovascular	Pericarditis, pulmonary hypertension, atherosclerosis
Renal	HIVAN and immune-mediated renal disease

KS, Kaposi's sarcoma; PML, progressive multifocal leukoencephalopathy; HIVAN, HIV-associated nephropathy.

HIV replication, increasing HIV viral load in both plasma and genital tract, with implications for HIV transmission and disease progression. Conversely, HSV suppression with acyclovir or valaciclovir reproducibly reduces HIV RNA load in plasma and the genital tract by 0.3–0.4 log₁₀ copies ml⁻¹. Consistent with these findings, HSV-2 infection has been shown to increase the risk of HIV

transmission and acquisition by approximately two- to threefold (Ramaswamy and Geretti, 2007; Wald and Link, 2002), although HSV interventions have not been shown to reduce incidence to date. There is also evidence for a small but significant positive impact of anti-HSV therapy on HIV disease progression. Whether widespread use of suppressive anti-HSV therapy can lower HIV incidence

in at-risk populations is currently being evaluated in clinical trials. HIV infection is associated with a significant impairment of HSV-specific T-cell immunity, which is restored gradually by HAART. However, immunological recovery may remain partial (Ramaswamy *et al.*, 2007) and treated patients continue to experience more frequent HSV reactivations and shedding than HIV-negative people.

Varicella-zoster Virus

Patients with HIV infection can develop severe illness with either varicella or zoster. Progressive primary varicella, a syndrome with persistent new lesion formation and visceral dissemination may be life-threatening. In the pre-HAART era, approximately 25% of patients hospitalized with varicella developed severe complications, including haemorrhagic rash, pneumonitis and fulminant infection with disseminated intravascular coagulation. HIV-infected people have a higher frequency of zoster than the general population and zoster continues to be common in the HAART era. Varicella-zoster virus (VZV) reactivation is often seen at CD4 counts >200 cells μL^{-1} as one of the early manifestations of declining immune function. Although most have an uncomplicated clinical course, multi-dermatomal, disseminated and chronic atypical skin rashes can occur. Acute retinal necrosis and neurological syndromes including encephalitis, myelitis and meningitis can develop in the absence of rash.

Progressive Multifocal Leukoencephalopathy

Progressive multifocal leukoencephalopathy (PML) is a subacute, slowly progressing condition associated with JC virus producing oligodendritic lysis and selective white matter destruction. JC virus may be detected by PCR in the CSF (70–85% sensitivity) or brain biopsy. HAART has proven efficacy in prolonging the survival of patients with PML, but the combined use of HAART and cidofovir has failed to demonstrate a benefit compared with HAART alone.

HIV-associated Encephalopathy

HIV-associated encephalopathy (HIVE) is a syndrome complex of progressive cognitive, motor and behavioural dysfunction also known as AIDS dementia complex (ADC), HIV/AIDS dementia or HIV-associated cognitive motor complex. The syndrome occurs commonly in patients with advanced disease (CD4 count <200 cells μL^{-1}). Its hallmark is encephalitis affecting the white and subcortical grey matter. HIV RNA is frequently detected in CSF. Multiple pathogenic mechanisms are thought to be involved, including HIV replication in

brain macrophages and microglial cells, neurotoxic effects of HIV proteins, and inflammatory responses (Ellis *et al.*, 2007; Schwartz and Major, 2006). With the advent of HAART, a syndrome of minor cognitive motor disorder has become more common than ADC. HAART also leads to improvements of cognitive function in patients who already have ADC. Nonetheless, HIVE continues to occur in treated patients despite suppressed viraemia and CD4 count recovery, perhaps reflecting ongoing viral replication in the CNS as a 'sanctuary site', or immunopathogenic mechanisms independent of viral replication. Some antiretroviral drugs (e.g. zidovudine, lamivudine, nevirapine) show better penetration in the CSF than others, but is not known if they are more effective at treating HIVE.

Hepatitis B and Hepatitis C Co-infection

In the last two decades co-infection with hepatitis C virus (HCV) has emerged as a leading cause of liver morbidity and a frequent cause of death in people with HIV. It is estimated that approximately 30% of HIV-infected individuals worldwide are also infected with HCV, and HCV should be regarded currently as one of the most important opportunistic infections in HIV-infected patients (Vallet-Pichard and Pol, 2006). HIV/HCV co-infected patients show more aggressive liver disease than people infected with HCV alone, with accelerated progression of fibrosis. Development of cirrhosis and hepatocellular carcinoma occur within an average of 7 and 18 years of HCV infection respectively, compared with 23 and 28 years respectively in patients with HCV alone. Furthermore, proportionately fewer co-infected patients achieve a sustained virological response with pegylated interferon α and ribavirin compared with those infected with HCV alone. Co-infected patients also have an increased risk of HAART-related hepatotoxicity, although HAART is beneficial in these patients because it slows the progression of liver fibrosis.

Around 10% of HIV-infected individuals have chronic hepatitis B virus (HBV) infection and co-infection promotes fast progression to cirrhosis and increases the risk of liver-related morbidity and mortality (Thio *et al.*, 2002). Several nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) have activity against both HIV and HBV, including lamivudine, emtricitabine (FTC), adefovir (lower doses required to treat HBV than HIV), tenofovir (TDF) and entecavir (Bottecchia *et al.*, 2007). The effectiveness of lamivudine in the treatment of chronic hepatitis B is well documented, but the benefit is limited by the emergence of drug resistance, leading to increases in serum HBV DNA load and liver enzymes, and a risk of fulminant liver failure. HBV resistance mutations can be recognized in 94% of viraemic patients who have received

lamivudine for over four years. Currently, the combination of TDF with FTC (or lamivudine) is recommended for treating HBV in HIV co-infected patients, and this should be given in the context of a triple drug regimen against HIV. Options for treating HBV in patients who do not require HIV treatment due to high CD4 counts are limited. They may include pegylated interferon α , adefovir (at low dose, but poor efficacy as monotherapy) and/or telbivudine. Earlier initiation of HAART should be considered.

Malignancies

Historically, three types of malignancies have been regarded as AIDS-defining cancers: Kaposi's sarcoma, non-Hodgkin's lymphoma (NHL), and invasive cervical cancer. However, non-AIDS-defining cancers represent an increasing proportion of the malignancies observed in HIV-infected people. Compared with the general population, HIV-infected people have an increased risk of anal and other HPV-related genital cancers, lung cancer, liver cancer, cancers of the head and neck, and Hodgkin's lymphoma (HL), and show poor overall survival (Boshoff and Weiss, 2002; Grulich *et al.*, 2007; Long *et al.*, 2008). In addition, cancers of oesophagus, stomach, kidney, and brain, melanoma and non-melanoma skin cancers, myeloma, leukaemia, and possibly testicular seminoma have been observed at rates greater than expected in the general population. In contrast, rates of prostate, breast, ovarian and colorectal cancer appear to be comparable to those in the general population. Many of the cancers that occur at increased rates in HIV infection are those with a known or suspected infectious cause, including EBV, HBV, HCV, human herpes virus 8 (HHV-8) and HPV. The increased incidence of specific non-AIDS-defining cancers in HIV-infected populations has also been attributed to high rates of smoking and alcohol use. The incidence of KS, HL, NHL, liver cancer and anal cancer is also greater in HIV-infected people than in immunocompromised transplant recipients (Grulich *et al.*, 2007).

Kaposi's Sarcoma

Together with PCP, KS was one of the sentinel diseases that in 1981 alerted epidemiologists and physicians to the incipient AIDS epidemic. The incidence of KS in HIV-infected people started to decrease before 1996 and declined sharply after the introduction of HAART. However KS remains common in HIV-infected people. The incidence is related to human HHV-8 prevalence in a population and historically KS was seen more frequently in homosexual males than injecting drug users or haemophiliacs. Histopathologically KS is a multicentric angioproliferative disorder characterized by spindle cell proliferation,

neo-angiogenesis, inflammation and oedema, with the proposed involvement of a multipotential mesenchymal cell expressing markers of lymphatic endothelium (Dupin *et al.*, 1999). The pathogenesis is complex. It involves interaction between HHV-8 and HIV, leading to increased cell proliferation and survival, dysregulated angiogenesis, evasion of immunity and malignant progression. Infection with HHV-8 precedes the development of KS and is found in every case of the disease (Greene *et al.*, 2007; Hansen, Boshoff and Lagos, 2007). The genome of HHV-8 contains a number of potential oncogenes and the virus seems able to transform human endothelial cells. In addition the HIV Tat protein may also contribute to the disease process. It has been proposed that HHV-8 DNA load in plasma is associated with disease progression, but the findings have not been consistent.

KS often runs an aggressive course in patients with AIDS, with cutaneous as well as visceral involvement. Typically single or multiple purple lesions appear on the skin; these may range from benign and indolent to aggressive and invasive forms. Lesions may occlude the lymphatics, producing lymphoedema. Gastrointestinal KS can produce dysphagia or gastrointestinal bleeding. Pulmonary KS causes space-occupying bronchial lesions, producing wheeze, cough, dyspnoea and life-threatening haemoptysis.

The prognosis of KS is dependent upon the disease stage, the severity of HIV-associated immunosuppression and other systemic illnesses (Boshoff and Weiss, 2002). Usually, immune reconstitution with HAART leads to resolution of KS, but chemotherapy may be necessary. Pegylated liposomal doxorubicin has been established as the treatment of choice for patients with AIDS-associated KS in western countries.

Lymphomas

The incidence of lymphoma in patients with HIV infection greatly exceeds that of the general population. Histologically three groups are recognized (Grogg *et al.*, 2007): (i) Hodgkin's and non-Hodgkin's lymphomas also occurring in immunocompetent hosts (e.g. Burkitt's and Burkitt-like lymphomas, diffuse large-cell lymphomas); (ii) lymphomas occurring more specifically in HIV-infected patients (e.g. primary effusion lymphoma (PEL)) and (iii) lymphomas and lymphoproliferative disorders also seen in patients with other forms of immunosuppression. Aggressive B-cell NHL that also occurs in immunocompetent patients accounts for the vast majority of cases, but the relative risk of NHL is increased 60- to 200-fold in HIV-infected patients when compared with the general population. The lymphomas are usually widespread at presentation and frequently involve extranodal sites, as in primary CNS lymphoma.

The pathogenesis is thought to be multifactorial, including chronic immune activation and dysfunction and the oncogenic roles of EBV and HHV-8. EBV is detected in approximately 50% of lymphomas arising in the setting of HIV, ranging from nearly 100% of primary CNS lymphomas and HL, to 60–80% of immunoblastic diffuse large B-cell lymphomas and PEL, and 30–50% of Burkitt's lymphomas. HHV-8 is also linked to PEL.

Changes in the relative frequency of specific lymphoma subtypes have been seen following the introduction of HAART. Burkitt's lymphoma and centroblastic diffuse large B-cell lymphomas continue to occur in patients with normal or slightly diminished CD4 counts; PEL and immunoblastic diffuse large B-cell lymphomas (including primary CNS lymphoma) are generally seen in people with marked immunodeficiency and have become less common in the HAART era. Primary CNS lymphoma occurs most frequently as multiple lesions in the cerebrum. A few studies have proposed that detection of EBV DNA by PCR in the CSF of HIV-infected patients with a CNS lesion infers a diagnosis of lymphoma. However, EBV detection in the CSF *per se* has a low positive predictive value for primary CNS lymphoma (Ivers *et al.*, 2004), making interpretation problematic in the absence of other diagnostic signs.

Ano-genital Squamous Carcinoma

Rapidly progressive, squamous intraepithelial carcinomas can develop in the cervical canals of women and anorectal junction of men infected with HIV. Each site has a squamous-columnar border and there is evidence of viral causation (Palefsky, 2007). These regions are commonly infected with HPV in these patient groups and, especially as immunosuppression increases, the HPV-encoded oncoproteins (E6 and E7) known to promote genetic instability in cells may lead to tumour formation. Studies confirm an increased risk particularly of *in situ* cervical cancer but also of invasive disease in HIV-infected women and the disease course can be aggressive (Boshoff and Weiss, 2002). The incidence of cervical cancer has not changed since 1996, and rigorous gynaecological screening among HIV-infected populations is paramount. The role of routine anal cytology in men has not been clearly established.

The Effects of Antiretroviral Therapy on Morbidity and Mortality

The introduction of HAART has changed the natural history of HIV infection, with a decline in overall mortality and morbidity, and a change in the pattern of HIV-related illnesses. The incidence and mortality of AIDS-defining infectious diseases such as PCP, MAI and CMV and

for the AIDS-defining cancers KS and NHL have decreased (Hooshyar *et al.*, 2007). At the same time, there is increased incidence and mortality for non-infectious diseases such as liver disease, hypertension and other cardiovascular disease, non-AIDS malignancies, pulmonary disease and alcohol abuse. Although the incidence of most AIDS events declines after initiation of HAART, the decline is more rapid for some conditions than others, and greatest for events with a viral aetiology (d'Arminio Monforte *et al.*, 2005).

There is evidence to indicate that a CD4 cell nadir <200 cells μl^{-1} prior to restoration with HAART is not predictive of increased risk of opportunistic infections, leading to safe discontinuation of both primary and secondary prophylaxis. At the same time, it has now become apparent that unique presentations of many HIV-related conditions can occur upon the introduction of antiretroviral therapy, especially in people with low CD4 counts. The immune reconstitution inflammatory syndrome (IRIS) is characterized by worsening clinical, laboratory or radiological findings after initiation of HAART, coinciding with viral load suppression and CD4 count increase, and reflecting restoration of immune responses. IRIS may manifest as the apparent worsening of a recognized illness, but may also unmask previously unrecognized disease. For instance, cystoid macula oedema and vitritis due to CMV may be observed. In addition to infectious diseases, IRIS can also occur with autoimmune or malignancy-related conditions such as KS. The prognosis is generally favourable (Lipman and Breen, 2006).

ANTIRETROVIRAL THERAPY—A HISTORICAL PERSPECTIVE

Antiretroviral therapy, where available, has transformed HIV-1 infection into a treatable chronic condition rather than a death sentence (Pomerantz and Horn, 2003). With hindsight, many now recognize the suboptimal policies made during the early period of antiretroviral therapy. Zidovudine (ZDV) was the first licensed drug against HIV-1 in the late 1980s following demonstration of clinical benefit in a placebo-controlled trial, and there was widespread demand for monotherapy at this time. Controversy was fuelled by results of the French—UK Concorde study, which showed little benefit of initiating zidovudine therapy early, compared to later in disease. In the early 1990s dual therapy with ZDV and didanosine (ddI) or zalcitabine (ddC) (or other nucleoside analogues) proved beneficial over single therapy, which provided the underpinning for later combination treatments using three or more drugs. Monitoring of therapy response was undertaken by CD4

cell count changes during this period, as well as the time to clinical endpoints, such as AIDS and death.

The mid 1990s marked a paradigm shift in HIV therapy, driven by two independent advances. First, assays for accurate and sensitive quantitation of plasma viraemia became commercialized and therefore widely available. Second, results of the first clinical studies of protease inhibitor (PIs) demonstrated the profound potency of this class of drugs when combined with two nucleoside analogues, defined by reductions in viral load. Since the application of plasma virus quantitation to natural history cohorts showed viral load to be predictive of disease progression (Mellors *et al.*, 1996), a leap of logic was taken to postulate that a therapy-induced fall in viral load would lead to improved prognosis, and this was demonstrated subsequently. Since viral replication (as measured by plasma viral load) became central to theories of pathogenesis, the pendulum swung back to early initiation of therapy. This approach was exemplified by the statement at the time ‘it’s the virus, stupid’, and various estimates were made of the duration of therapy required to eradicate the virus from infected individuals.

Unfortunately, this optimism was tempered by two practical manifestations of therapy—adherence problems and toxicity, as well as the recognition of long-lasting reservoirs of infection within the body. Long-term PI therapy generated severe lipid abnormalities leading to body shape changes and cardiac problems (although some of these are now also known to be associated with long-term nucleoside analogue treatment) (Carr, 2003). The large pill burden of early triple therapy regimens also caused difficulty in drug compliance, and rates of therapy failure were significant. By the late 1990s, non-nucleoside reverse transcriptase inhibitors (NNRTIs), such as nevirapine (NVP) and efavirenz (EFV), had started to replace PIs within first-line regimens following encouraging clinical trial data.

In the early 2000s, the emerging toxicities associated with PIs and, with hindsight, nucleoside analogues, led to a reassessment of the ‘start early’ principle, and risk—benefit arguments were used to suggest initiation of therapy at a CD4 cell count of 200–350 cells μl^{-1} . With improvements in drug formulations, and management of these toxicities, the timing of starting therapy is currently being readdressed.

MONITORING OF ANTIRETROVIRAL THERAPY AND RESISTANCE

CD4 Count

The CD4 count is the major clinical indicator of immunocompromise and guides the initiation of antiretroviral

therapy and the use of chemoprophylaxis for opportunistic infections. Guidelines generally recommend starting HAART at a CD4 count <350 and well above 200 cells μl^{-1} , a threshold associated with a significant risk of AIDS-defining events. Earlier initiation of therapy at CD4 counts >350 cells μl^{-1} is currently being evaluated with the aim of further reducing morbidity and mortality, improving tolerability, and reducing the risk of onward HIV transmission. The potential benefits of starting therapy at higher CD4 cells counts must be balanced against the long-term risk of drug toxicity and declining levels of compliance over time, leading to treatment failure and emergence of drug resistance. Most patients commencing HAART with CD4 counts <350 cells μl^{-1} experience good virological responses and significant immunologic recovery. The immunological success of therapy is defined as a CD4 count increase of 100–150 cells μl^{-1} per year during the first few years of therapy, with most patients showing a plateau after seven years.

Plasma Viral Load

Molecular techniques for quantifying the viral load have become a pivotal component of clinical management. In untreated people, the viral load is a strong predictor of CD4 count decline, rate of disease progression and risk of mortality (Mellors *et al.*, 1996). In the HAART era, a viral load $>100\,000$ copies ml^{-1} serves as an indicator that therapy should be considered at a CD4 count closer to 350 than 200 cells μl^{-1} . Viral load reduction with HAART translates into immunological and clinical benefit, establishing viral load as a key surrogate marker of therapeutic success. Treatment guidelines recommend that the main goal of antiretroviral therapy is to achieve and maintain an ‘undetectable’ viral load, initially <400 copies ml^{-1} and more recently <50 copies ml^{-1} . Although it seems intuitive that viral load reduction to below the lowest measurable threshold is preferable, the target level of suppression is mainly defined by the technical properties of the viral load assay. While evidence indicates that viral replication continues in people with undetectable viral load, achieving and maintaining a viral load <50 copies ml^{-1} is clearly associated with long-term virological suppression, lack of evidence of viral escape, and sustained immunological and clinical benefit.

Viral load assays have evolved over the years to improve the sensitivity and range of quantification, ensure optimal performance with prevalent HIV-1 group M subtypes, and increase automation. First-generation viral load assays were based either on amplification of the signal in the branched chain DNA assay (Bayer VERSANT HIV-1 RNA assay) or on amplification of the target, including reverse transcriptase PCR (Roche RT-PCR), ligase chain reaction (Abbott LCX

assay) and nucleic acid sequence-based amplification or NASBA (BioMérieux Nuclisens HIV-1 QT assay). Second-generation assays are based on real-time PCR methods (Roche COBAS® TaqMan® HIV-1 Test and Abbott RealTime HIV-1 assay) or combined NASBA and real-time molecular beacon detection (BioMérieux NucliSens EasyQ assay). Commercial assays show good overall correlation and are reported to reliably detect and quantify all major HIV-1 group M subtypes (Schutten *et al.*, 2007). It is nonetheless important to remain vigilant about performance. The Abbott assay also detects group O viruses. A range of in-house real-time PCR-based assays for HIV-2 have been published.

The expanded access to antiretroviral therapy in developing countries requires the availability of simple, accurate and inexpensive methodologies for viral load monitoring. Methods evaluated include the PerkinElmerUltra p24 antigen assay, the CaviDi ExaVir Load RT enzyme activity test and home-brew real-time PCR techniques. In the CaviDi assay, the RT enzyme is extracted from the virus particle, followed by RT-mediated synthesis of BrdU-DNA from a poly(A) template bound to a 96-well plate. Synthesized DNA is then quantified using anti-BrdU conjugated to alkaline phosphatase. The assay is easy to perform and requires simple and inexpensive equipment, although throughput is low.

Drug Resistance

A key moment in the HIV replication cycle is the production of double-stranded DNA from the viral RNA template by RT. The enzyme has low fidelity, with no proofreading activity and an estimated error rate of 10^{-4} nucleotides. Misincorporations become fixed in the newly produced genome, mostly as point mutations, although duplications and insertions also occur. The high rate of virus replication further contributes to generating a high degree of viral diversity, and all mutations, including those responsible for drug resistance, can occur daily in an infected patient. Some of the mutants will not be functional, whereas others will persist within the quasispecies and will be archived in proviral DNA. It has been estimated that the prevalence of single drug-resistant mutants is relatively high within the quasispecies, whereas double and especially triple mutants are considerably less frequent. Resistant mutants generally have a reduced fitness relative to drug-susceptible wild-type virus, which will therefore dominate in the absence of drug pressure. Once therapy is introduced, if viral replication continues, the pre-existing mutants acquire a selective advantage and outgrow the wild-type virus. With ongoing virus replication, single mutants evolve to acquire further mutations, some increasing the level of drug resistance and others compensating for reduced fitness. Recombination events involving different virus strains

Table 38.7 Main principles of antiretroviral drug resistance

Drug-resistant mutants:

- pre-exist the introduction of therapy and are selected rather than created by drug pressure under incomplete virological suppression
 - carry mutations within proteins that play an important role in virus replication and as a result are generally less fit than wild-type virus and in the absence of drug pressure exist as minority, low-frequency species
 - continue to evolve under drug pressure to increase the level of resistance and over time acquire compensatory changes that restore viral fitness
 - lose the replication advantage if drug pressure is discontinued, with outgrowth of the wild-type virus which becomes again dominant
 - persist as low-frequency species and archived resistance once therapy is discontinued, ready to re-emerge upon reintroduction of drug pressure
-

co-infecting the same cell are a further source of genetic evolution. Thus, ongoing replication leads towards the emergence of virus which is simultaneously highly resistant and fit to replicate and infect (Table 38.7).

Antiretroviral drug resistance testing provides a valuable guide to the management of HIV-infected patients. Sequencing the viral genomic region of interest—also known as genotyping—is typically performed by automated population sequencing, which produces a consensus sequence of the dominant plasma quasispecies, allowing the identification of mutations that are known to be associated with drug resistance. The results are described by the codon position preceded by the wild-type amino acid and followed by the mutated amino acid. For example, the mutation associated with lamivudine (3TC) resistance M184V involves the substitution of a methionine for valine at codon 184 in RT. The International AIDS Society-USA (IAS-USA) regularly updates a list of significant resistance-associated mutations (Johnson *et al.*, 2007). Established assays target RT and protease, and new assays are becoming available to sequence other genomic regions of interest, including gp41 (fusion inhibitors), gp120 (CCR5 inhibitors), gag (PIs) and integrase (integrase inhibitors).

Phenotypic assays use the viral genes of interest derived from plasma RNA and transfected into a laboratory vector to generate a recombinant virus that can be grown *in vitro* in the presence of escalating concentrations of individual antiretroviral agents. Replication is measured as the expression of a reporter gene. Results are reported as fold changes in the drug concentration required to inhibit virus growth by 50 or 90% relative to a control

wild-type virus (inhibitory concentration, IC_{50} and IC_{90}). The virtual phenotype is a genotype interpretation method that matches the patient's derived viral sequence to genotype—phenotype pairs within a large dataset to predict the phenotype. The relative advantages and disadvantages of these approaches are summarized in Table 38.8.

A meta-analysis (Torre and Tambini, 2002) concluded there was benefit for using genotyping but not phenotyping to support clinical care, and that expert opinion added significantly to the effect. Both resistance testing systems can provide useful information however (Table 38.9), and both require appropriate interpretation of results. The interpretation of genotypic resistance test result is done through software-based algorithms that assign scores to individual mutations or apply specific rules to certain mutation patterns. The effects of mutations are not simply

additive and analysis of all but the simplest mutation patterns must take into account reciprocal synergistic and antagonistic interactions. Furthermore, the phenotypic and clinical effects of rarer mutations are often unknown. Over 20 rules-based genotypic interpretation systems have been proposed and several noncommercial software programmes are publically available (e.g. ANRS, DMC, REGA and Stanford). All systems require updating as new drugs enter clinical use and evidence for both new and old drugs grows. Differences between interpretation systems exist and the levels of agreement are highest for common mutation patterns. There are ongoing efforts to refine the systems and improve discrimination and concordance. However, although each of the major interpretation systems may consider slightly different mutations to be relevant for each antiretroviral drug, they can predict virological response to an overall similar extent (Fox *et al.*, 2007).

Table 38.8 Genotypic and phenotypic antiretroviral drug resistance assays

	Genotypic	Phenotypic
Availability	Wide	Limited
Turn-around time	1–2 wk	3–4 wk
Cost	++	++++
Commercial assays	Yes	Yes
Quality assurance	Available through national and local schemes	Not available
Sensitivity for low-frequency species	~20%	~20%
Output	Provides a consensus sequence of the viral population in the individual patient	Provides an average of susceptibility of the viral population in the individual patient
Recommended viral load (copies ml^{-1})	> 1000	> 1000
Interpretation	Specialist advice recommended	Specialist advice recommended

Table 38.9 Use of resistance testing in clinical practice

Clinical scenario	Resistance test	Comment
Primary infection	Recommended	Genotypic testing offers the highest sensitivity for the detection of transmitted resistance
New diagnosis of established infection	Recommended	Testing demonstrated to be cost-effective if prevalence of transmitted resistance is 4% or higher. Genotypic testing offers the highest sensitivity for the detection of transmitted resistance
Treatment failure	Recommended	Genotypic testing is generally cost-effective. Phenotypic testing should be considered in the presence of complex resistance patterns in multidrug experienced patients. Testing should be performed while on therapy as results are unreliable if therapy was discontinued more than 2–4 wk prior to testing
Pregnancy	Recommended	Recommended before starting therapy in drug-naive women Recommended in all women with a detectable viral load while on therapy

In phenotyping, cut-offs are employed to interpret the measured fold-changes. The cut-offs are either biologically defined as the spread of phenotypic susceptibilities measured in drug-naïve people, or clinically defined as the level of resistance that translates into reduced (lower cut-off) or abolished (upper cut-off) responses to treatment. Although a more intuitive indicator of drug resistance, clinical cut-offs represent an oversimplification. With most available antiretrovirals, increasing resistance and loss of activity should be seen as a continuum. Furthermore, clinical cut-offs are defined for individual drugs within a combination regimen, thus requiring complex statistical analyses to extrapolate the contribution of the drug of interest. Validation in treated populations other than those from which the cut-off was originally derived may not be readily available. The cut-offs must be regarded as drug-specific. For example, the 3TC resistance mutation M184V confers >100-fold phenotypic resistance to the drug and this is highly significant. In contrast, the TDF resistance mutation K65R confers only small shifts (less than twofold) in phenotypic susceptibility to the drug. Yet, TDF activity is virtually abolished in the presence of K65R. As with genotypic testing, information on biological and clinical cut-offs may not be available for newer drugs. With all systems, guidelines recommend that clinicians should consult a specialist to assist with the interpretation of results and selection of an optimized combination regimen.

One important limitation of both genotypic and phenotypic tests is their inability to detect resistance present at low frequency within the quasispecies (Flys *et al.*, 2005; Johnson *et al.*, 2005; Kassaye *et al.*, 2007; Palmer *et al.*, 2006). Emerging evidence indicates that low-frequency resistant mutants can have a negative impact on responses to antiretroviral therapy, at least in the context of regimens based on two NRTIs with NNRTIs. Methodologies have been developed to detect mutants with a frequency as low as 0.1%, including mutation-specific real-time PCR assays, single genome sequencing, parallel allele-specific sequencing (PASS), pyrosequencing and ultra-deep sequencing. The methodologies are currently either too laborious or too expensive for routine clinical practice.

Table 38.10 Methods for determining HIV tropism

Assay	Method
MT2	Culture of virus isolate on MT2 cells to assess ability to form syncytia, allowing detection of X4 virus
Culture on indicator cells line	Culture of virus isolate on cell lines engineered to express CD4 with CCR5 or CXCR4 (e.g. glioma human malignant glioma cell line U87)
Recombinant phenotypic assay	Recombinant virus containing the envelope gene sequence from the patient's plasma is used to infect engineered cell lines expressing CD4 with CCR5 or CXCR4
Genotypic	Prediction of tropism based on the sequence of the V3 loop and other parameters

Tropism

The introduction of small molecule (sm) CCR5 inhibitors in clinical practice requires the determination of the viral tropism prior to their use. In addition, assays may be needed in the near future for assessing patients who experience treatment failure on these new compounds (Table 38.10). Tropism assays commonly involve generation of recombinant viruses containing the *env* gene from plasma virus, and infectivity is tested within cells expressing relevant co-receptors (Coakley *et al.*, 2005). HIV tropism may also be predicted from the sequence of the V3 loop (Low *et al.*, 2007; Soulié *et al.*, 2008). Key genotypic determinants include V3 amino acids 11 and 25, but some of the models also use clinical determinants such as the CD4 count to improve prediction. Currently, the correlation of genotypic results with the virus phenotype remains imperfect, but several interpretation algorithms are undergoing optimization.

Fitness

Viral fitness can be described as the capacity of a virus to infect, replicate and produce mature infectious progeny in a defined host environment (Geretti, 2005; Nijhuis *et al.*, 2001). To escape from drug pressure, resistant mutants require changes in viral proteins that play a key role in virus replication and infectivity. Drug resistance carries therefore a fitness cost to the virus and the virus replication capacity is inversely correlated to the number and type of resistance mutations. It should be noted, however, that both drug-resistant and drug-susceptible viruses exhibit a wide range of replication capacities, reflecting variations in the many determinants of viral fitness.

Growth competition experiments assess viral fitness *in vitro* by mixing different strains in cell culture and determining the 'prevailing' virus. For instance, virus variants containing the protease mutations D30N or L90M are consistently out-competed by wild-type virus *in vitro*, while the D30N mutant is overtaken by the L90M mutant. Thus, both D30N and L90M have reduced fitness relative to wild-type virus, but the fitness cost is higher for the D30N mutant.

The fitness cost associated with a resistance mutation can to some extent be extrapolated from the kinetics of its disappearance from the dominant quasispecies once drug pressure is removed. The 3TC mutation M184V, for instance, disappears as early as two weeks after discontinuation of therapy, suggesting a significant impact on viral fitness. Conversely, when patients with drug resistance discontinue antiretroviral therapy, outgrowth of wild-type dominant quasispecies can be accompanied by an increase in plasma viral load and a drop in CD4 counts. The magnitude of the effect provides an indirect estimate of the fitness cost of the mutations, but can also reflect residual antiviral activity despite detectable resistance. The effect is not consistently seen. It is most often demonstrated with the NRTIs and variably with the PIs, but generally not with the NNRTIs (Deeks *et al.*, 2005).

Although the fitness cost of antiretroviral resistance can translate into a virological and immunological benefit, the optimal strategies for exploiting the effect remain to be determined. Evidence to date suggests that there is little rationale for continuing failing NNRTI therapy, whereas there is evidence for a beneficial effect associated with continuing NRTI or PI-based therapy. However, whether it is preferable to continue the NRTIs, the PIs or both remains controversial.

ANTIRETROVIRAL DRUG CLASSES

Five classes of antiretrovirals are currently available in clinical practice (Table 38.11), targeting virus entry, reverse transcription, integration and protein cleavage during maturation (Figure 38.7). Since the introduction of

HAART in the mid 1990s there has been a remarkable evolution towards effective, simple and convenient regimens. In the early days of triple therapy a patient on stavudine (d4T), 3TC and indinavir (IDV) received 10 pills and the dosing schedule was three times daily. Just over 10 years later, the first triple co-formulation has become available, containing TDF, FTC and EFV in one pill to be taken once daily. Despite increased convenience of dosing and improved efficacy and tolerability, successful antiretroviral therapy continues to demand high levels (>95%) of adherence to maintain virological suppression. The most common drug resistance detected in treated patients worldwide is directed against NRTIs and NNRTIs. This reflects the extensive use of these drugs in current therapy, but also the use of mono and dual NRTI therapy in the pre-HAART era and of suboptimal triple therapy regimens in early HAART era. In addition, first-generation NNRTIs such as NVP and EFV offer a low barrier against the emergence of resistance, due to the fact that a single mutation is sufficient to confer high-level resistance to the drugs.

Cross-resistance between drugs of the same class is extensive to complete for the NRTIs, complete for first-generation NNRTIs, and generally partial for the PIs, although accumulation of multiple protease mutations can lead to substantial to complete cross-resistance. New drugs within existing drug classes have been designed to be effective against drug-resistant strains. At the same time new drug classes offer additional treatment options for patients with drug resistance. Nonetheless, new drugs and drug classes remain susceptible to loss of activity. There is no cross-resistance between the fusion inhibitor ENF and smCCR5 inhibitors, but

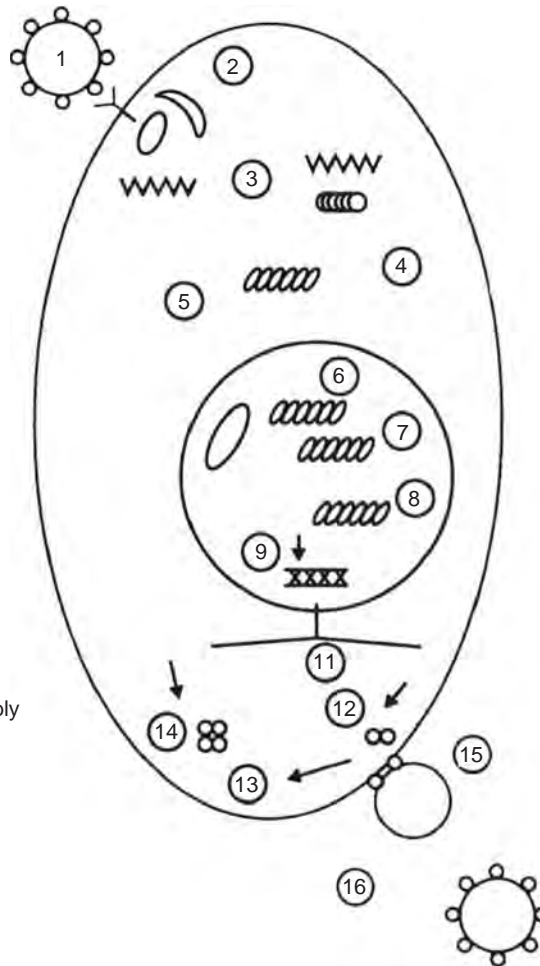
Table 38.11 Current classes of antiretrovirals

Class	Examples
Nucleoside/nucleotide RT inhibitors (NRTIs)	Zidovudine, didanosine, stavudine, lamivudine, abacavir, tenofovir, emtricitabine Co-formulations: zidovudine/lamivudine, zidovudine/lamivudine/abacavir, abacavir/lamivudine, tenofovir/emtricitabine, tenofovir/emtricitabine/efavirenz
Non-nucleoside RT inhibitors (NNRTIs)	Nevirapine, efavirenz
Protease inhibitors (PIs) ^a	Etravirine, rilpivirine Saquinavir, indinavir, nelfinavir, fosamprenavir, atazanavir, tipranavir, darunavir Co-formulations: lopinavir/ritonavir
Entry inhibitors	Fusion inhibitors: enfuvirtide Small-molecule CCR5 inhibitors: maraviroc, <u>vicriviroc</u>
Integrase inhibitors	Raltegravir, <u>elvitegravir</u>

Underlining indicates unlicensed drugs in advanced development.

^aRitonavir is only used at low doses for boosting of other PIs.

1. Attachment and fusion
2. Uncoating
3. Reverse transcription
4. RNase H degradation
5. DNA synthesis
6. Migration to the nucleus
7. Integration
8. Latency
9. Viral transcription
10. RNA nuclear transport
11. RNA stability
12. Protein synthesis
13. Protein glycosylation
14. Packaging and assembly
15. Release
16. Maturation



Potential therapeutics

1. Enfuvirtide,
sCD4-Ig proteins
Modified chemokines
DMD 3200
MAbs
2. Hypericin
3. NRTIs, Tenofovir
- 4-6. None
7. Integrase inhibitors
8. None
9. TAR decoy
10. RRE decoys
Rev trans
dominant
11. Ribozymes
12. Antisense
molecules
GLQ 223
13. *N*-butyl
DNJ
14. U-81749, etc.
15. Interferon
16. Protease inhibitors

Figure 38.7 The life cycle of HIV, indicating the stages at which potential therapeutics might act.

cross-resistance can occur between maraviroc (MCV) and candidate smCCR5 inhibitors, and there seems to be complete cross-resistance between the integrase inhibitors raltegravir (RAL) and elvitegravir (ELV). Furthermore, clinical trials in treatment-experienced patients have clearly demonstrated that the use of any new drug requires support from other active drugs in the combination regimen, to prevent functional monotherapy and loss of activity. Thus, preventing resistance and cross-resistance remains an important goal of antiretroviral therapy.

Nucleoside/Nucleotide RT Inhibitors

Nucleoside and nucleotide analogues act to inhibit the viral RT. They are analogues of one of the four natural nucleosides (thymidine, cytidine, adenosine and guanosine) and, following triphosphorylation within the cell by

host cell enzymes, they compete with natural nucleotides as substrates for the viral RT. The nucleotide analogue TDF is a phosphonate, structurally similar to a nucleoside monophosphate, and requires two phosphorylations for activation. Incorporation of the NRTI triphosphate prevents the addition of further nucleotides to the DNA chain and terminates viral replication.

The NRTIs share the ability to inhibit the cellular DNA polymerase γ responsible for synthesis of mitochondrial DNA. The inhibition, alongside other proposed mechanisms, leads to a risk of mitochondrial toxicity. A wide range of adverse events have been attributed to mitochondrial toxicity, including lactic acidosis, hepatic steatosis, neuropathy, myopathy, pancreatitis, probably lipodystrophy and possibly polyneuropathy, pancytopenia, proximal

tubular dysfunction and osteopaenia. As a result of unfavourable toxicity profiles, d4T and the combination of d4T and ddI are no longer recommended for initial therapy, and TDF or abacavir (ABC) are preferred to ZDV for first-line therapy to reduce the risk of bone marrow suppression and lipoatrophy over prolonged ZDV use. Many studies implicate d4T and ZDV in the development of lipoatrophy, and switch studies with both ABC and TDF demonstrated a significant improvement in loss of fat. Zalcitabine (ddC) has modest potency and causes peripheral neuropathy and is now rarely used in clinical practice.

ZDV was the first licensed drug against HIV-1 (Pomerantz and Horn, 2003). Together with d4T, it is a thymidine analogue, and the two drugs are antagonistic, presumably due to competition for the same intracellular phosphorylation pathways. The acquisition of high-level ZDV resistance requires the sequential accumulation of several changes in the RT, involving amino acid positions 41, 67, 70, 215 and 219. Accumulation of three or more mutations confers significant resistance to ZDV and cross-resistance to d4T, ABC, ddI and TDF (Geretti, 2006). The mutations, known as thymidine analogue mutations (TAMs), tend to cluster according to two groups, namely that including M41L, L210W and T215Y (TAM I) and that including D67N, K70R, T215F and L219Q/E (TAM II). The determinants of one or other route remain unclear, but the first pathway is associated with greater levels of ZDV resistance and NRTI cross-resistance than the second pathway. For many years, d4T resistance was thought to be associated only with changes at RT position 75, which are rarely observed *in vivo*. Subsequently, it became evident that d4T can select TAMs and shares a significant degree of cross-resistance with ZDV. There is also evidence that the RT mutation K65R confers a degree of resistance to d4T and emergence of K65R has been observed in a small proportion of patients receiving first-line therapy with d4T/3TC/EFV.

Didanosine is an adenosine analogue which requires deamination prior to phosphorylation. Resistance is associated with mutations at codons 65, 69 and 74 in RT. In addition, the presence of multiple TAMs reduces ddI activity. ABC is a guanosine analogue, which is converted to carbovir triphosphate as the active component. A severe hypersensitivity reaction (HSR) occurs in approximately 5% of patients as a result of an immune-mediated reaction typically seen within the first six weeks of therapy. Rechallenge with ABC is contraindicated due to the risk of precipitating a life-threatening reaction. Identification of patients at risk of developing HSR can be achieved through genetic screening for the human leukocyte antigen HLA-B*5701. A large cohort study has demonstrated that ddI and ABC use is associated with a higher risk of

cardiovascular events, although this observation requires confirmation (D:A:D Study Group, 2008).

Resistance to ABC is mediated by the emergence of mutations at RT codons 65, 74, 115 and 184. In addition, the presence of multiple TAMs reduces susceptibility to the drug, and the effect is augmented by the presence of M184V. Clinical data, however, indicate that M184V in isolation does not significantly compromise responses to ABC.

TDF is a nucleotide analogue structurally related to adefovir. The drug undergoes renal excretion by glomerular filtration and active tubular secretion. Renal toxicity halted the development of adefovir for the treatment of HIV infection. With TDF, clinical cohorts have detected a low risk of generally mild renal functional abnormalities, although cases of acute proximal toxicity have been reported. The RT mutations selected by TDF are K65R and less commonly K70E/G. The presence of multiple TAMs is also associated with reduced drug activity. TDF is also a potent HBV inhibitor.

3TC and FTC are both cytidine analogues and structurally similar. Both also have activity against HBV. High-level resistance is generated by the M184V mutation in RT. The mutation emerges within weeks of 3TC monotherapy and is also the first mutation to emerge with triple therapy regimens containing 3TC or FTC. One interesting observation from the NUCA 3001 study was that people receiving monotherapy with 3TC experienced an initial suppression of viral load followed by rebound after two weeks, presumably coinciding with the emergence of M184V. However, the viral load remained suppressed by approximately $0.5 \log_{10}$ copies ml^{-1} relative to the level measured pre-therapy (Eron *et al.*, 1995). More recently, a randomized clinical trial demonstrated that patients with M184V continuing 3TC monotherapy maintained a lower viral load (by $0.5 \log_{10}$ copies ml^{-1}) and showed a slower decline in CD4 counts over 48 weeks than did patients who discontinued all therapy, thus confirming the earlier observations (Castagna *et al.*, 2006). A number of explanations for these findings have been put forward. First, the fitness of the M184V mutant may be reduced. Second, residual antiviral activity may occur despite high-level resistance.

The main resistance mutations that confer resistance to the NRTIs are summarized in Figure 38.8. TAMs and T69 insertions (which usually occur with TAMs) reduce susceptibility by promoting excision of the incorporated NRTI, using pyrophosphate or ATP as acceptor and allowing the elongation of the DNA chain to resume. The mechanism is known as primer unblocking or pyrophosphorolysis. Other mutations such as M184V, K65R and L74V are discriminatory, as they prevent NRTI triphosphate incorporation. The M184V mutation shows several

	M	D	K	L	T	T	K	K	T	K	L	V	V	Y	M	T	Q	A	V	F	F	F
	41	67	70	210	215	215	219	65	69	70	74	75	75	115	184	69	151	62	75	77	77	116
	L	N	R	W	Y	F	Q/E	R	D	E/G	V/I	T	M	F	V/I	Ins	M	V	I	L	I	Y
ZDV	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black
d4T	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black
ABC	Black	Grey	White	Grey	Black	Grey	White	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black
ddI	Black	Grey	White	Grey	Black	Grey	White	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black
TDF	Black	Grey	Grey	Grey	Black	Grey	White	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black
3TC	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black
FTC	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black

Figure 38.8 Major resistance mutations for the NRTIs. Black shadowing indicates high impact on resistance; grey shadowing indicates contribution to resistance; white indicates no significant resistance effect.

interesting effects in *in vitro* systems, including enhanced RT fidelity, which in turn would reduce the rate at which new mutants (including drug-resistant mutants) are generated. In addition, the presence of M184V antagonizes pyrophosphorolysis, thus enhancing the chain termination effect of the nucleoside analogues. In clinical practice, the combination of ZDV with 3TC has been shown to delay the emergence of ZDV resistance whereas emergence of TAMs occurs more rapidly with combinations such as ZDV and ddI. In addition, M184V partially restores susceptibility to ZDV, d4T and TDF in the presence of TAMs and clinical evidence is available of improved virological responses to TDF in the presence of the mutation. Other mutations associated with hypersusceptibility effects include K65R, which increases susceptibility to ZDV, and L74V/I and the NNRTI mutations L100I and Y181C which increase susceptibility to ZDV and TDF.

The classic multi-nucleoside resistance pathways include a diverse cluster of amino acid insertions and deletions between positions 67 and 70, commonly 69S-(S-S) or 69S-(S-G), which usually occur with TAMs, and the Q151M complex. The first pattern confers resistance to all available NRTI. The Q151M mutation confers low-level resistance to TDF, 3TC, and FTC, and high-level resistance the other NRTIs. In combination with mutations at positions 75, 77, and 116, Q151M confers intermediate resistance to 3TC, FTC and TDF and high-level resistance to other NRTIs. Both patterns have become rather uncommon in treated patients. More commonly, multi-nucleoside resistance is associated with the presence of multiple TAMs or K65R with M184V, the latter

seen in patients failing therapy with TDF plus 3TC or FTC.

Non-nucleoside RT Inhibitors

The NNRTIs are a structurally diverse group of compounds, of which NVP and EFV are approved for use; etravirine (ETV) is a second-generation NNRTI. Delaviridine is licensed in the United States but rarely used. The drugs bind within a hydrophobic pocket near the active site of RT causing its allosteric inhibition. The pocket has a different structure in HIV-2 and group O HIV-1, so the NNRTIs are ineffective against these viruses.

NVP and EFV have proven efficacy in first-line therapy in combination with two NRTIs. With EFV, the major side effects are insomnia and vivid dreams, especially in the first weeks after starting treatment. With NVP, up to 13% of patients develop a mild to moderate rash, usually within the first six weeks of therapy. However, there is a risk of life-threatening reactions, including severe hypersensitivity, Stevens—Johnson syndrome, toxic epidermal necrolysis, and fulminant hepatitis. The risk appears to be gender- and CD4 count-dependent at least in some populations. As a result, NVP is currently contraindicated in women with CD4 counts >250 cells μl^{-1} and in men with CD4 counts >400 cells μl^{-1} . Whereas use of the drug has declined in recent years in western countries, NVP remains an important component of triple therapy in developing countries. Both drugs are metabolized primarily by cytochrome P450 (CYP) 2B6, leading to a potential for significant drug—drug interactions. There is also considerable inter-individual variability in the metabolism of

NVP and EFV, and this is at least in part related to host genetics. For example, a CYP2B6 G to T polymorphism at position 516 is associated with reduced clearance and higher drug levels, with implications for drug toxicity and prolonged drug persistence after discontinuation of therapy (Rotger *et al.*, 2007).

Failure of EFV- or NVP-containing regimens is usually accompanied by the presence of detectable mutations that cause high-level resistance to the drugs. Figure 38.9 summarizes the main RT mutations involved in resistance to the NNRTIs. NVP may select K103N or Y181C in RT. The genetic pathway to resistance is dependent on co-therapies. Thus, resistance to NVP monotherapy is usually caused by the Y181C mutation. By contrast, in the presence of ZDV, other mutations, such as the K103N are the preferred route, possibly because Y181C suppresses the emergence of ZDV resistance. The K103N mutation confers high-level phenotypic cross-resistance to EFV. Although the Y181C mutation confers only modest phenotypic resistance to EFV, complete cross-resistance is observed *in vivo*. This is attributed to the concomitant selection of low-frequency K103N in people with dominant Y181C. Failure of EFV-containing regimens is associated with the K103N mutation in up to 90% of cases, producing phenotypic cross-resistance to NVP.

Structural studies have contributed to the design of second-generation NNRTIs. Two new compounds, ETV (or TMC125) and TMC278 have improved activity against resistant viruses, both *in vitro* and *in vivo*. The introduction of ETV in routine clinical care demands a shift in our current understanding of antiretroviral treatment strategies. The raised genetic barrier relative to first-generation compounds indicates that it is now possible to sequence drugs within the NNRTI class, with an approach similar to that used for the NRTIs and PIs. Although cross-resistance does occur, multiple mutations are required to confer resistance to the drug, probably reflecting a high degree of molecular flexibility (Vingerhoets *et al.*, 2005). However, progressive loss of

susceptibility is seen as resistance mutations accumulate and activity is rapidly lost in patients with one or more NNRTI resistance mutations in the absence of other fully active drugs in the regimen. Within better structured regimens, a gradual loss of response to ETV occurs with increasing numbers of NNRTI resistance mutations (Lazzarin *et al.*, 2007; Madruga *et al.*, 2007; Nadler *et al.*, 2007). More precise predictors of ETV response will emerge though ongoing analyses.

Protease Inhibitors

In the mid 1990s, the combination of the first PIs with two NRTIs established the efficacy of triple therapy and marked a major step forward in HIV management. Over time, new drugs and drug formulations have become available that improve efficacy and dosing schedule and PIs are currently a key component of the treatment of drug-experienced patients. In addition, clinical trials have clearly established long-term efficacy in first-line therapy. During HIV replication, *gag* and *gag-pol* gene products are produced as precursor polyproteins which are subsequently cleaved by the protease enzyme at nine distinct sites to yield the structural proteins p17, p24 p9 and p7 and the viral enzymes protease, RT and integrase. PIs are competitive inhibitors of the protease enzyme thereby preventing maturation into a fully functional and infectious viral particle. All PIs are metabolized, at least partially, through the CYP3A4 iso-enzyme of the CYP system. This leads to significant interactions with other drugs, and also inter-individual differences in plasma drug levels. CYP3A4 is found primarily in the intestine and liver and is important in first-pass drug metabolism. A further major step forward in HIV treatment was the recognition that inhibition of CYP3A4 with a low dose of ritonavir (100–200mg) reduces hepatic and intestinal metabolism of concurrently administered PIs, thereby boosting drug levels, improving pharmacokinetic profiles, and improving treatment responses in both

	A	L	K	K	K	V	V	V	E	V	V	Y	Y	Y	G	G	P	F	F	M	K
	98	100	101	101	103	106	106	108	138	179	179	181	188	188	190	190	225	227	227	230	238
	G	I	E	P	N/S	A	M	I	K	D/E	F	C/I/V	L	H/C	A	S/E	H	L	C	L	N/T
NVP																					
EFV																					
ETV																					

Figure 38.9 Major resistance mutations for the NNRTIs. Black shadowing indicates high impact on resistance; grey shadowing indicates contribution to resistance; white indicates no significant resistance effect.

treatment-naïve and treatment-experienced patients. The boosting effect of ritonavir may be mediated by additional mechanisms, including interference with efflux channels that actively transport PIs out of cells, and reducing protein binding of the PI.

Ritonavir boosting is required for PIs such as saquinavir (SQV), lopinavir (LPV), tipranavir (TPV) and darunavir (DRV) to achieve adequate plasma concentrations. Although optional for fosamprenavir (FPV) and atazanavir (ATV), co-administration of ritonavir improves the efficacy of these PIs relative to their unboosted counterparts. Increased drug levels also improve the barrier to the emergence of drug resistance in treatment-naïve patients and can overcome low to intermediate levels of PI resistance in treatment-experienced patients. The downside is that ritonavir boosting may lead to an increased frequency of side effects such as diarrhoea and raised blood lipids, and promote other metabolic abnormalities such as reduce glucose tolerance and abnormal fat distribution. The enhanced efficacy of ritonavir-boosted PIs (PI/r) has led to recent clinical trials of monotherapy with LPV/r, ATV/r and DRV/r and initial data indicate that a substantial number of patients may be able to maintain virological suppression. The strategy is subject to ongoing clinical trials.

The protease enzyme is a homodimer, each monomer comprising 99 amino acids. Three main domains are recognized: the active site, the dimerization domain and the flaps. The protease is highly polymorphic in PI-naïve patients. Although it has been proposed that certain polymorphisms may facilitate the emergence of resistance,

there is no evidence for a significant effect on drug susceptibility. The main PI resistance mutations are shown in Figure 38.10. Major mutations are generally selected first and have clear effects on drug susceptibility (e.g. D30N, G48V, V82A/F/T, I84V and L90M). Minor mutations accumulate in viral genomes already containing one or more primary mutations, have a less discernible effect on resistance, but may be selected because they improve viral fitness. In general terms, the total number of major PI mutations predicts response to the PIs. Mutations at codons 82, 84 and 90 in particular have wide resistance effects. First-generation PIs such as SQV, IDV and nelfinavir (NFV) are structurally poorly adaptable to mutations in the protease enzyme and the presence of only a few resistance mutations can cause significant loss of affinity and activity (Martinez-Cajas and Wainberg, 2007). Newer PIs including FPV, ATV, LPV, TPV and DRV have high to very high binding affinities and can tolerate higher numbers of mutations than can older PIs. Protease mutations however have a different impact on individual PIs and resistance testing should guide the selection of the most appropriate PI in patients with resistance (Figure 38.10).

Clinical trials in drug-naïve patients have repeatedly shown that the combination of a PI/r with two NRTIs as first-line therapy rarely results in the emergence of protease resistance at the time of virological failure. The determinants of viral load rebound in the absence of detectable resistance remain unclear. Overall high drug levels and rapid pharmacokinetics suggest that there may be limited opportunity for the optimal selection of resistance

	D 30 N	V 32 I	V 33 F	M 46 I/L	I 47 A	I 47 V	G 48 V	I 50 L	I 50 V	I 54 V	I 54 A	I 54 L/M	L 76 V	V 82 A	V 82 F	V 82 T	V 82 S	V 82 L	I 84 V	N 88 S	N 88 D	L 90 M
ATV																						
DRV																						
FPV																						
IDV																						
LPV																						
NFV																						
SQV																						
TPV																						

Figure 38.10 Major resistance mutations for the PIs. Positions 30, 32, 47, 48, 50, 82 and 84 are in the protease substrate cleft. Positions 46, 47, 48, 50 and 54 are in the protease flap. Black shadowing indicates high impact on resistance; grey shadowing indicates contribution to resistance; white indicates no significant resistance effect.

in patients with incomplete adherence. The proposed hypothesis is that drug levels are either too high to allow significant viral replication to occur or, in the case of missed doses, become rapidly too low to exert significant selective pressure. Other important determinants of the high barrier to resistance are related to the structural interactions of the PI with the protease and the number of mutations required to confer significant phenotypic resistance. Protease resistance mutations can be associated with a significant fitness cost for the virus. However, compensatory mutations can emerge rapidly that restore virus fitness. In addition, changes may occur in the gag cleavage sites that serve as the natural substrate for the enzyme, which improve processing and increase resistance and fitness (Nijhuis *et al.*, 2007).

Indinavir (IDV)

The use of IDV/r in clinical practice has declined in recent years as a result of the significant risk of nephrolithiasis. Mutations selected by IDV occur at codons 46, 47 (V), 48, 54, 76, 82, 84, 88 and 90.

Saquinavir

The first PI to be developed and approved, SQV has undergone several re-formulations to improve bioavailability and tolerability. The hard gel capsule dosed with ritonavir remains an important option for PI-naïve patients. SQV/r may also retain activity against certain viruses with limited PI resistance. Key resistance mutations include G48V and L90M; the latter may be accompanied by mutations at codon 84.

Nelfinavir

NFV levels are relatively unaffected by co-administration of ritonavir, although improved drug exposure is achieved by administration with high fat meals. The drug is rarely used currently due to the frequent occurrence of diarrhoea and the availability of more potent PIs. Predominant use has been in PEP and pregnancy. Possible contamination of NFV stock with ethyl methanesulfonate has recently led to the withdrawal of NFV pending further toxicology studies. NFV resistance develops along one of three pathways, involving the mutations D30N (with or without N88D), L90M, or N88S, all with or without M46I/L.

Fosamprenavir

FPV is the pro-drug of amprenavir. It can be used unboosted in drug-naïve patients, but it is usually used in combination with ritonavir. FPV/r can be used once daily in treatment-naïve patients, but twice daily administration

is recommended in treatment-experienced patients. Mutations selected by FPV involve codons 32, 46, 47 (V), 50 (V), 54, 76 and 84. Due to structural similarities with DRV, there is a degree of cross-resistance between the two drugs.

Lopinavir

LPV is the only available PI co-formulated with ritonavir, initially in a capsule formulation and more recently as a tablet. Mutations selected by LPV follow one of two pathways: an IDV-like pathway associated with mutations at positions 46, 54 and 82, and a FPV-like pathway associated with mutations at positions 32, 47, 50 (V) and 76. In treatment-naïve patients the combination of LPV/r with two NRTIs has only rarely resulted in the emergence of PI resistance at the time of virological failure. Pre-existing protease resistance mutations reduce the genetic barrier and accelerate the selection of further resistance, although LPV/r generally retains good activity in PI-experienced patients with low to intermediate levels of PI resistance. The major toxicities of LPV/r are diarrhoea and raised lipids, especially triglycerides. LPV/r can be used once daily in treatment-naïve patients, but twice daily administration is generally preferred in patients with resistance.

Atazanavir

ATV is approved for use once daily either with or without ritonavir, although current guidelines recommend ritonavir boosting due to improved virological efficacy. Mutations selected by ATV include I50L and N88S. The signature mutation I50L does not confer cross-resistance to other PIs. Rather, I50L has been linked to hypersusceptibility effects for several PIs, although the clinical significance of the finding is currently unclear. A key benefit of ATV is the limited effect on lipid levels. ATV/r maintains activity in PI-experienced patients with a limited number of PI mutations, but is generally less effective than LPV/r in this setting.

Tipranavir

TPV is a nonpeptidic PI used with 200 mg of ritonavir twice daily, a higher dose of ritonavir than used to boost other PIs. TPV/r is effective in patients with extensive PI resistance, but its use is limited by problematic drug—drug interactions, poor gastrointestinal tolerability and concerns over hepatic and lipid-related adverse events and a possible risk of intracranial haemorrhage. Nonetheless, given the limited cross-resistance with DRV/r, TPV/r provides a valuable treatment option for patients with certain protease resistance patterns. Key mutations selected by TPV include V32I, I47V, I54V/A/M, V82L/T

and I84V. Resistance mutations predictive of virological responses in highly treatment-experienced patients have been identified and assigned a 'weight'. These include T74P and I47V (each +6), Q58E and V82L/T (+5), N83D (+4), I54A/M/V (+3), M36I, K43T and I84V (+2), and L10V and M46L (+1). Some mutations have hypersusceptibility effects and have been assigned a negative score: L24I and L76V (-2), I50L/V (-4) and I54L (-7). Responses decline with a score between -3 and 10 and are essentially lost with a score >10.

Darunavir

DRV is a nonpeptidic analogue of amprenavir with enhanced activity against resistant virus (McCoy, 2007). It is dosed twice daily with 100 mg of ritonavir in treatment-experienced patients and has shown excellent activity in the presence of extensive drug resistance. A once daily dose is currently under evaluation for PI-naïve people. Gastrointestinal symptoms and headache are the most commonly reported events. A genotypic score has been derived from studies in highly treatment-experienced patients, including the mutations V11I, V32I, L33F, I47V, I50V, I54L/M, G73S, L76V, I84V and L89V. Responses have been shown to decline in these highly experienced populations in the presence of three or more mutations. In phenotypic studies, I50V, I54M, L76V and I84V reduce susceptibility to the greatest extent. V32I, L33F, I47V, I54ML, G73CS and I89V have been the most common mutations to emerge with DRV/r treatment failure.

Entry Inhibitors

Current entry inhibitors target either gp41-mediated fusion of the viral envelope with the cell membrane (ENF, T20 or ENF) or the interaction between the viral gp120 and the CCR5 co-receptor (Esté, 2007). MCV is the first smCCR5 inhibitor to enter clinical practice and vicriviroc is in advanced development. In addition, the monoclonal antibody PRO140 is a new CCR5 inhibitor undergoing fast track development.

Enfuvirtide

ENF, the first fusion inhibitor to enter clinic practice (Lalezari *et al.*, 2003), is a synthetic peptide that mimics amino acids 127–162 of HIV-1 gp41. By binding the first HR1 region of gp41, ENF prevents the hairpin formation necessary for fusion. ENF must be administered twice daily by subcutaneous injection, and injection site reactions are frequent. Due to the complexity of the molecule, production costs are high. Use in clinical practice is limited to patients with extensive drug resistance, although the best activity of ENF is seen in the presence of other active drugs in the combination regimen.

Unless well supported, ENF is vulnerable to loss of activity through the rapid emergence of resistance. The best characterized resistance mutations occur in the highly conserved region of HR1 spanning amino acids 36–45 (e.g. G36D/E, V38A/E, Q40H, and N43D), and to a lesser extent, in other *env* regions. Continued therapy in the presence of resistance provides little or no virological benefit and is not generally recommended. Some interesting data however suggest that patients with resistance mutations at codon 38 may experience improvements in CD4 counts despite ongoing viraemia, possibly reflecting fitness or immunological effects of the mutant.

Maraviroc

MCV is a functional antagonist of the CCR5 receptor, which inhibits CCR5-mediated signalling without triggering internalization of the co-receptor. After binding, MCV shows prolonged co-receptor occupancy which is thought to explain the delayed viral load rebound observed after treatment discontinuation. Approximately 20% of drug-naïve and 60% of drug-experienced people harbour X4 or dual tropic strains as dominant quasiespecies and MCV has no significant antiviral activity in these patients. In addition, HIV-infected people may carry low frequencies of X4 and dual tropic viruses and these may be selected by MCV if virological suppression is incomplete. Two main mechanisms of HIV resistance to smCCR5 inhibitors have been described. A first mechanism is characterized by a switch in viral tropism from R5 to X4 or dual use. In most cases the switch is thought to occur within the viral population when selective suppression of R5 virus allows outgrowth of the pre-existing X4 and dual tropic strains. In the second mechanism, changes in the envelope allow the virus to bind to the co-receptor despite the alteration in shape caused by the presence of the inhibitor (Kuhmann *et al.*, 2004). The effect is mediated by mutations in the stems of the V3 loop and allows resistant virus to bind with higher affinity to the drug-bound co-receptor than wild-type virus. To date the mutation pattern remains to be clearly defined, making the genotypic identification of resistance problematic. The phenotypic effect is shown by a plateau in the maximal percentage inhibition (MPI) observed with increasing drug concentrations.

MCV has shown excellent tolerability in short-term clinical trials. There remain concerns about the long-term safety of CCR5 inhibition and on the possible pathogenic effects of causing an expansion of X4 viruses as a result of CCR5 inhibition. The chemokines that bind to CCR5 orchestrate leukocyte trafficking in tissues and play an important role in regulation of immunological processes. Although individuals homozygous for the CCR5 Δ 32 have normal life expectancy, it is not known whether they carry additional genetic changes that compensate for the

lack of CCR5 function. Furthermore, there is evidence that CCR5 Δ 32 is associated with an increased risk of symptomatic West Nile virus infection, and with improved recovery from HBV infection, suggesting significant immunological effects. Limited data indicate that after MCV discontinuation resistant virus reverts to minority species and in cases of tropism switch, R5 virus again becomes the dominant quasispecies. These observations suggest that the viral population selected during MCV therapy has no inherent advantage over the pre-treatment population in the absence of drug pressure. The long-term impact of the possible enrichment of resistant variants or X4 and dual tropic virus remains unknown at present.

Integrase Inhibitors

Several candidate integrase inhibitors have been developed. Current integrase inhibitors inhibit strand transfer (Nair and Chi, 2007). RAL is a pyrimidinone carboxamide and the first integrase inhibitor to enter clinical practice. In clinical trials, RAL showed high potency, good tolerability and no significant drug–drug interactions. A randomized clinical trial in treatment-naïve people compared the activity of RAL to that of EFV in combination with two NRTIs. Although the percentage of patients who achieved virological suppression was similar overall in the two treatment arms, patients receiving RAL showed a more rapid viral load response. The significance of this finding is currently under investigation (Murray *et al.*, 2007). RAL however has a low barrier to the emergence of resistance and patients with virological failure show mutations in the integrase gene. Several pathways of resistance have been identified, the most common involving mutations at codons 148, 155 or less commonly 143. Additional key resistance mutations occur at codons 74, 92, 138, 140, 157 and 163. Virtually complete cross-resistance has been observed with ELV, another integrase inhibitor undergoing clinical trials and work has started on the development of second-generation integrase inhibitors with enhanced barrier to the emergence of resistance.

TRANSMISSION OF DRUG RESISTANCE

Transmission of drug-resistant species of HIV-1 is well recognized, and in some populations has reached up to 10–15% of new infections. Following transmission, resistance can persist over months and years, unlike the situation of stopping therapy after emergence of resistance which leads to rapid outgrowth of wild-type virus that is archived from prior to treatment (Pao *et al.*, 2004 Little *et al.* 2008). One explanation for this difference is that transmission events involve a single or small number

of infectious virions. These are likely to be homogeneous. Therefore, in the face of transmission of resistant viruses, nonresistant virus is not also present to allow subsequent outgrowth. Nevertheless, some transmitted mutants can revert back to wild type, and minority species assays demonstrate that this is likely the case for M184V (Metzner *et al.*, 2005).

Transmitted resistance can compromise the response to first-line therapy. This is particularly the case for transmitted NNRTI resistance, which leads to more complete class resistance, than viruses carrying one or two nucleoside analogue or PI resistance mutations (Kuritzkes *et al.*, 2008). Many national and international guidelines on antiretroviral therapy now recommend routine resistance testing at time of diagnosis, in order to guide appropriate first-line therapy.

Despite case reports to the contrary, there is no evidence that disease progression following transmission of resistance *per se* is any different to cases of transmission of nonresistant viruses.

PREVENTION

The vast majority of HIV transmissions worldwide are due to sexual transmission. Therefore, safe sex messages remain the cornerstone of any prevention campaign. Nevertheless, the continuing epidemic, more than 20 years after the modes of transmissions were understood, suggests that such simple messages are far more complicated to implement. Indeed, there is some evidence that the impact of antiretroviral therapy in shifting HIV infection from a life-threatening condition to a chronic disease state has led to a relaxation in concern amongst some risk groups.

Short-term antiretroviral therapy has been utilized to prevent vertical transmission, and also as post-exposure prophylaxis for many years. More recently, this concept has been extended to pre-exposure settings, and this is discussed below. However, amongst infected individuals, antiretroviral use remains focused on providing benefit for that individual. There is increasing discussion about starting treatment at earlier stages of disease than current guidelines specify, particularly with improvements in the management of drug-related toxicities, and easier to take drug formulations. This will also have public health benefits, since earlier treatment will decrease the duration of high, infectious viraemia.

Vertical Transmission

Mother-to-Child Transmission

Historically, the rates of vertical mother-to-child transmission of HIV varied between African (30–50%), North

American (20–30%) and European (14%) studies, probably due to the inclusion of populations at different stages of disease. HIV may be transmitted throughout pregnancy, during delivery and as a consequence of breastfeeding (Lehman and Farquhar, 2007). Evidence indicates that the majority of transmission events occur perinatally and during breastfeeding. There is a linear correlation between maternal viral load and risk of transmission, but there is no evidence for a safe threshold and transmission has occurred in women with a viral load <400 copies ml^{-1} . HIV suppression in plasma is generally associated with undetectable HIV RNA in the genital tract, but dissociation can occur, leading to HIV shedding in cervico-vaginal secretions. Mode of delivery, duration of membrane rupture and delivery before 32 weeks of gestation are important predictors of transmission.

Strategies for interruption of vertical transmission have had a dramatic impact on the numbers of children infected with HIV in the developed world. In the pivotal study, treatment of the mother with zidovudine during the last trimester of pregnancy and the infant for the first six weeks of life produced a 60–70% reduction in vertical transmission (Connor *et al.*, 1994). In developed countries, some authorities still regard ZDV monotherapy as an option for selected women with a low viral load willing to deliver by planned caesarean section (PLCS). The risk of developing ZDV resistance during monotherapy appears to be low in this selected group. HAART is recommended in pregnant women who require treatment for their own health, have a high viral load ($>10\ 000$ copies ml^{-1}) or have evidence of drug resistance. In addition, PLCS is recommended for all women taking ZDV monotherapy and for those on HAART with detectable viraemia, whereas elective vaginal delivery is an option for women with no detectable viraemia on HAART. For PEP, most infants can be treated with ZDV monotherapy for four weeks, but HAART should be given to infants born to untreated mothers or mothers with detectable viraemia despite HAART. Exclusive formula-feeding is also recommended to all HIV-positive mothers. With these combined strategies, vertical transmission of HIV can be reduced to negligible levels (Fowler *et al.*, 2007). Approximately 30% of vertically infected children develop severe disease within the first year of life, but most develop disease over several years. Infections and failure to thrive are common features of paediatric HIV infection. Prognosis has been improved substantially with the use of HAART, but untreated children progress rapidly to disease, especially in resource-poor settings where mortality is greater than 50% by two years of age (Prendergast *et al.*, 2007).

The availability of HAART is limited in many developing countries. Trials of a single dose of NVP given to women in labour and to the infant have shown good

results, reducing transmission by almost 50%. However, one concern is related to the emergence of NVP resistance (and EFV cross-resistance) in a large proportion of treated women and their infants, which may compromise the activity of first-line NNRTI-based regimens (Flys *et al.*, 2005; Johnson *et al.*, 2005; Kassaye *et al.*, 2007; Palmer *et al.*, 2006). Avoiding breastfeeding in the developing world is also problematic. Lack of conferred immunity through breast milk and poor hygiene in preparation of bottle feeds lead to significant infant morbidity and mortality from gastrointestinal infections. In addition, formula-feeding is often stigmatized as indicating that the mother is HIV-positive.

Sexually Transmitted Infections

Sexually transmitted infections are a risk factor for acquisition of HIV. In addition local genital tract inflammation increases HIV shedding. It follows that control of sexually transmitted infections may be an important, and cost-effective, method for reducing transmission of HIV. The most compelling evidence relates to herpes simplex 2 infections. Thus, a randomized study of valaciclovir in HSV-seropositive, antiretroviral-naive HIV-infected women demonstrated a significant reduction in plasma and genital tract HIV RNA (Nagot *et al.*, 2007), although the evidence to date is for a lack of impact of HSV treatment on HIV acquisition (Watson-Jones *et al.*, 2008).

Circumcision

Observational studies have demonstrated that circumcision has a protective effect on acquisition of HIV. A number of mechanisms have been proposed; foreskin contains high concentrations of cells permissive for HIV infection, such as DCs, macrophages and CD4 lymphocytes; reduced genital ulceration; foreskin may contain more disrupted epithelium compared with the more keratinized shaft of the penis.

More recently, three randomized clinical trials, all based in sub-Saharan Africa, have shown benefit of circumcision in reducing incidence of infection in men by 50–70%, and there is growing opinion that this may provide an important arm of HIV-prevention programmes worldwide (Weiss *et al.*, 2008).

However, there are a number of limitations of this strategy. First, benefit is conferred on the circumcised man, rather than women. This is of concern, since the risk of infection per coital act is higher for women than men, although any reduction of infections at the population level would be expected to lead indirectly to reductions in female infections. Second, the protection against transmission through anal sex (whether heterosexual or homosexual) remains unknown. Third, the degree to which the

benefit observed in the resource-poor world can be extrapolated to Northern countries is unclear. Finally, there will be a number of cultural and educational hurdles to overcome prior to widespread implementation of circumcision.

Microbicides

Topical administration of microbicides to vagina and/or rectum has been proposed as appropriate for women in particular, who otherwise may not be able to convince sexual partners to adopt safer sex practices. Compounds in development include polyanions, such as cellulose sulfate and PRO-2000, and non-ionic surfactants such as nonoxynol-9. These are thought to act by disrupting the virus—cell interaction. Progress in the field was slowed by the unexpected finding from two randomized trials of cellulose sulfate of enhanced rates of infection in recipients of the active compound. Possible reasons for these findings include an inflammatory effect of the compounds on genital epithelium, alterations to epithelial permeability and/or alterations to natural genital microbial flora. Nevertheless, further work is being undertaken, including the topical use of antiretroviral drugs, within the microbicides programme (van der Wigjert and Shattock, 2007).

Post-exposure Prophylaxis

Post-exposure prophylaxis (PEP) was initially addressed in the context of occupational exposure to HIV-1, such as needlestick injuries. Epidemiological studies have indicated that the average risk for HIV transmission after percutaneous exposure to HIV-infected blood in health-care settings is about 3 per 1000 injuries. After a mucocutaneous exposure, the average risk is estimated at less than 1 in 1000. It has been considered that there is no risk of HIV transmission where intact skin is exposed to HIV-infected blood.

More recently, attention has turned to post-exposure prophylaxis following sexual exposure (PEPSE), where the risk of acquisition is increased in the passive partner in anal intercourse.

A case—control study conducted by the CDC concluded that the administration of ZDV prophylaxis to healthcare workers occupationally exposed to HIV was associated with an 81% reduction in the risk for occupationally acquired HIV infection (Cardo *et al.*, 1997). Four factors were associated with increased risk of occupationally acquired HIV infection: (i) deep injury, (ii) visible blood on the needle, (iii) injury with a needle which had been placed in a source patient's artery or vein and (iv) terminal HIV-related illness in the source patient (now explained through a high viral load).

In the absence of randomized studies addressing the interval between a risk incident and initiation of PEP,

three lines of evidence provide guidance: (i) animal studies; (ii) human perinatal transmission studies and (iii) consideration of virological/immunological studies on the natural history of primary infection.

First, SIV/maaque animal models demonstrated a protective effect of TDF when administered within 72 hours of inoculation, and such protection was enhanced by treatment for more than 10 days. By contrast, in one study, PEP with intravenous ZDV, 3TC and IDV as early as 4 hours post infection in a SIV/HIV chimera (SHIV) infection of macaques did not prevent infection. One human perinatal transmission intervention study is also informative. In a subset of participants in the ACTG 076 protocol, where antenatal treatment of the pregnant woman with ZDV was omitted (through choice or limited clinical care), neonatal ZDV started within 48 hours of birth was associated with an HIV transmission rate of 9.3%, compared with a rate of 18.4% when ZDV was started at a later time (Connor *et al.*, 1994). Recent studies of the SIV—maaque model, as well as natural history studies following HIV-1 transmission in humans, demonstrate extensive infection of gut-associated CD4 lymphocytes, and their preferential depletion is evident at the time of primary infection. This suggests there is only a brief window of opportunity to prevent or abort infection (through administering PEP) before irreversible systemic infection and HIV seroconversion occur (Centlivre *et al.*, 2007).

Together, these studies provide some evidence that PEP is most likely to be effective when initiated as soon as possible (within hours, and certainly within 48–72 hours of infection), and continued for at least 28 days. It should be noted that the evidence base on which these conclusions are based is limited.

In HIV-infected patients, triple therapy has proved more effective than mono or dual therapy in suppressing HIV replication and avoiding the emergence of viral resistance. The US guidelines recommend two-drug PEP regimens following lower risk incidents and three-drug regimens only for higher risks (www.cdc.gov/mmwr/preview/mmwrhtml/rr5409a1.htm3). This two-tier approach adds to the complexity of the risk assessment process, at the expense of greater potency and protection for the exposed worker. The main arguments in favour of two-drug PEP (fewer side effects, better adherence and course completion rates) are being addressed through switching to better-tolerated agents with lower pill burdens. At the same time, a potent three-drug PEP regimen is preferred because resistance to antiretroviral drugs is found at significant levels in both treated and untreated infected individuals in the United Kingdom. Current guidance suggests truvada (TDF and FTC) and kaletra (LPV/r) as the PEP of choice, to be taken over four weeks. Data on the optimal duration of follow-up

are limited. However, based on expert opinion, UK guidelines now recommend, as a minimum standard, that follow-up should be for at least 12 weeks after the HIV-exposure event or, if PEP was taken, for at least 12 weeks from when PEP was stopped.

Pre-exposure Prophylaxis (PREP)

A number of animal model SIV experiments demonstrate the efficacy of pre-exposure prophylaxis (PREP) (García—Lerma *et al.*, 2008). There is increasing interest in the use of antiretrovirals with low toxicity prior to possible exposure. Such an approach is relevant for, for example, sex workers. Trials of TDF, with or without FTC, are ongoing. Concerns with this approach include the risk of resistance in cases where the recipient is already HIV infected, or indeed, rapid development of resistance in cases of transmission, and also the potential increase in risky behaviour consequent on the reassurance given by use of this approach.

VACCINES

A safe, efficacious vaccine that prevented HIV infection would provide the single most important means of curtailing the AIDS pandemic. Sadly, there is no imminent prospect of developing a prophylactic vaccine. Large phase III trials of vaccines based on envelope glycoproteins designed to elicit neutralizing antibodies did not show efficacy (Graham and Mascola, 2005). Similarly, recent vaccine trials of non-enveloped components designed to elicit cell-mediated immunity were curtailed early, owing to more cases of HIV infection in the vaccine arm than in the placebo arm (Sekaly, 2008).

The lack of a vaccine is not for want of effort. Part of the explanation is the sheer degree of variability of HIV. It is some 10 times greater even than influenza A virus, for which the WHO must annually decide what substrain to use. Moreover, unlike influenza virus, chronic infection by HIV means that there is no 'prevalent strain' in circulation, but rather thousands of strains distributed among a dozen major antigen subtypes and recombinant forms.

Many approaches to developing vaccines have been attempted: whole inactivated virus particles, subunit recombinant proteins, viral proteins expressed through DNA-based vaccines and delivery of genes in expression vectors such as vaccinia and adenoviruses. The one approach that has led to cross-protection against different SIV strains in macaques is a live-attenuated vaccine in which *nef* and other accessory genes are attenuated. However, it is doubtful that a live vaccine, however

'disabled', could be licensed for human use given the propensity for HIV to repair its fitness, and to recombine with incoming virus. Besides, the mechanism of protection by live-attenuated SIV strains remains a mystery, because it does not appear to be related to either humoral or cell-mediated immune responses. Therefore we do not even have a scientific principle to aid us in developing a safer vaccine.

In the absence of a prophylactic vaccine, effort has been turned towards a therapeutic vaccine or immunotherapy. It was hoped that HAART might sufficiently reduce virus load so that an immunogenic boost would lead to long-term suppression of virus by immunological means. However, there is little promise that this approach will be useful.

Nonetheless, experimental protection of macaques against challenge by the same virus strain as the immunogen has been achieved. For example, passive transfer of neutralizing antibody in sufficient concentration can block infection if administered at the time of challenge. SHIV may be useful in testing future envelope-based vaccine candidates. Much effort will continue to be placed on vaccine discovery and development. However, for the time-being HIV has humbled the vaccinologists.

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Human Prion Diseases

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INTRODUCTION TO PRIONS AND HISTORICAL PERSPECTIVE

The prion diseases, or transmissible spongiform encephalopathies, are a closely related group of neurodegenerative conditions that affect both humans and animals. They have been called previously the subacute spongiform encephalopathies, slow virus diseases and transmissible dementias. The prototypic disease is scrapie, a naturally occurring disease of sheep and goats, which has been recognized in Europe for over 200 years and is present in many countries worldwide. Other animal prion diseases described over the last few decades include transmissible mink encephalopathy (TME) and chronic wasting disease of mule deer and elk, both principally in the United States, and since the 1980s bovine spongiform encephalopathy (BSE)—first described in the United Kingdom and now seen in most European Union (EU) and some other countries. The more recently described feline spongiform encephalopathy of domestic cats and spongiform encephalopathies of an increasing number of species of zoo animals (Kirkwood *et al.*, 1990) are also now also recognized as animal prion diseases.

The human prion diseases have been classified traditionally into Creutzfeldt—Jakob disease (CJD), Gerstmann—Sträussler syndrome (GSS) (also known as Gerstmann—Sträussler—Scheinker disease) and kuru. Although these are rare neurodegenerative disorders, affecting about 1–2 per million worldwide per annum, remarkable attention has been focused on these diseases in recent years. This is because of the unique biology of the transmissible agent or prion, and also because of the fears that the epizootic of BSE could pose a threat to public health through dietary exposure to infected tissues.

Scrapie was demonstrated to be transmissible by inoculation between sheep (and goats) following prolonged incubation periods in 1936. It was assumed that some type of virus must be the causative agent and Sigurdsson coined the term ‘slow virus infection’ in 1954. There was considerable interest in the 1950s in an epidemic of a neurodegenerative disease, kuru, characterized principally by a progressive ataxia, amongst the Fore linguistic group of the Eastern Highlands of Papua New Guinea. Subsequent fieldwork, by a number of investigators, suggested that kuru was transmitted during cannibalistic feasts. Hadlow (1959) drew attention to the similarities between kuru and scrapie at the neuropathological, clinical and epidemiological levels, leading to the suggestion that these diseases may also be transmissible. A landmark in the field was the transmission by intracerebral inoculation with brain homogenates into chimpanzees of first kuru and then CJD by Gajdusek and colleagues in 1966 and 1968, respectively (Gajdusek *et al.*, 1966; Gibbs *et al.*, 1968). Transmission of GSS followed in 1981. This work led to the concept of the ‘transmissible dementias’. The term ‘Creutzfeldt—Jakob disease (CJD)’ was introduced by Spielmeyer in 1922, drawing from the case reports of Creutzfeldt (1920) and Jakob (1921) and was used in subsequent years to describe a range of neurodegenerative conditions, many of which would not meet modern diagnostic criteria for CJD. The criterion of transmissibility allowed diagnostic criteria for CJD to be assessed and refined; atypical cases could be classified as CJD on the basis of their transmissibility. All the animal and human conditions share common histopathological features. The classical diagnostic triad of spongiform vacuolation (affecting any part of the cerebral grey matter), neuronal loss and astrocytic proliferation may be accompanied by amyloid plaques.

The nature of the transmissible agent in these diseases has been a subject of intense and often heated debate for many years. The understandable initial assumption that the agent must be some form of virus was challenged, however, both by the failure to directly demonstrate such a virus (or any immunological response to it) and by evidence indicating that the transmissible agent showed remarkable resistance to treatment expected to inactivate nucleic acids (such as ultraviolet radiation or treatment with nucleases). Such findings had led to suggestions as early as 1966 by Alper and others that the transmissible agent may be devoid of nucleic acid (Alper *et al.*, 1966, 1967). Such findings led Griffith to suggest in 1967 that the transmissible agent might be a protein, and to propose several hypothetical mechanisms for replication (Griffith, 1967). Progressive enrichment of brain homogenates for infectivity resulted in the isolation of a protease-resistant sialoglycoprotein, designated the prion protein (PrP), by Prusiner and co-workers in 1982. This protein was the major constituent of infective fractions and was found to accumulate in affected brains and sometimes to form amyloid deposits. The term prion (from proteinaceous infectious particle) was proposed by Prusiner (1982) to distinguish the infectious pathogen from viruses or viroids. Prions were defined as 'small proteinaceous infectious particles that resist inactivation by procedures which modify nucleic acids'.

The protease-resistant PrP extracted from affected brains was of 27–30 kDa and became known as PrP^{27–30}. N-terminal sequencing of PrP^{27–30} enabled production of isocoding mixtures of oligonucleotides that were used to screen cDNA libraries prepared from scrapie-infected hamsters. These studies led to recovery of cognate cDNA clones by Weissmann and colleagues in 1985. Remarkably, PrP^{27–30} was encoded by a single-copy host chromosomal gene rather than by a putative nucleic acid in fractions enriched for scrapie infectivity. PrP^{27–30} was found to be derived from a larger molecule of 33–35 kDa designated PrP^{Sc} (denoting the scrapie isoform of the protein) (Oesch *et al.*, 1985). The normal product of the PrP gene, however, is protease sensitive and was designated PrP^C (denoting the cellular isoform of the protein). No differences in amino acid sequence between PrP^{Sc} and PrP^C have been identified. PrP^{Sc} is known to be derived from PrP^C by a post-translational process (Borchelt *et al.*, 1990; Caughey and Raymond, 1991).

Many of the key advances in understanding the pathogenesis of the prion diseases have come from study of the various forms of human prion disease. In particular, the recognition that the familial forms of the human diseases are autosomal dominant inherited conditions, associated with *PRNP* coding mutations (Hsiao *et al.*, 1989; Owen *et al.*, 1989), as well as being transmissible to laboratory

animals by inoculation, strongly supported the contention that the transmissible agent, or prion, was composed principally of an abnormal isoform of prion protein.

STRUCTURAL BIOLOGY OF PRIONS

A wide body of data now supports the idea that prions consist principally or entirely of an abnormal isoform of a host-encoded protein, the PrP, designated PrP^{Sc} (for review see Prusiner, 1991). PrP^{Sc} is derived from PrP^C by a post-translational mechanism (Borchelt *et al.*, 1990) and no covalent differences (Caughey and Raymond, 1991) between PrP^C and PrP^{Sc} have been demonstrated. It is proposed that PrP^{Sc} acts as a template which promotes the conversion of PrP^C to PrP^{Sc} and that this conversion involves only conformational change.

The conformation of the cellular isoform was first established by nuclear magnetic resonance (NMR) measurements of recombinant mouse PrP (Riek *et al.*, 1996). Since then NMR measurements on recombinant hamster, human and other mammalian PrPs show that they have essentially the same conformation.

Following cleavage of an N-terminal signal peptide, and removal of a C-terminal peptide on addition of a glycosylphosphatidylinositol (GPI) anchor, the mature PrP^C species consists of an N-terminal region of about 100 amino acids, which is unstructured in the isolated molecule in solution, and a C-terminal segment, also around 100 amino acids in length. The C-terminal domain is folded into a largely α -helical conformation (three α -helices and a short anti-parallel β -sheet) and is stabilized by a single disulfide bond linking helices 2 and 3. There are two asparagine-linked glycosylation sites (see Figure 39.1).

The N-terminal region contains a segment of five repeats of an eight-amino-acid sequence (the octapeptide repeat region), expansion of which by insertional mutation leads to inherited prion disease. While unstructured in the isolated molecule, this highly conserved region contains a tight binding site for a single Cu²⁺ ion with a dissociation constant (K_d) of 10⁻¹⁴ M. A second tight copper site ($K_d = 10^{-13}$ M) is present upstream of the octa-repeat region but before the structured C-domain (Hornshaw *et al.*, 1995; Jackson *et al.*, 2001). Clearly, it is possible that the unstructured N-terminal region may acquire structure following copper binding. A role for PrP in copper metabolism or transport is possible and disturbance of this function by the conformational transitions between isoforms of PrP could be involved in prion-related neurotoxicity.

The structured C-domain folds and unfolds reversibly in response to chaotropic denaturants and recent work

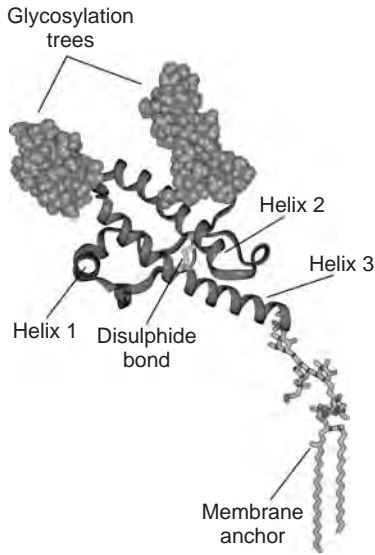


Figure 39.1 Model of glycosylated human prion protein indicating positions of *N*-linked glycans (in blue), the single disulfide bond-joined helices 2 and 3 and GPI anchor to outer surface of cell membrane.

on the folding kinetics of mouse PrP^C (Wildegger *et al.*, 1999) demonstrates that there are no populated intermediates in the folding reaction and that the protein displays unusually rapid rates of folding and unfolding. These findings have been reinforced by hydrogen/deuterium exchange measurements on the human protein which show that the overall equilibrium constant describing the distribution of folded and unfolded states is the same as the protection factor (Hosszu *et al.*, 1999). This shows that there are no partially unfolded forms or intermediates that have a population greater than the unfolded state. The data suggest that PrP^{Sc} is unlikely to be formed from a kinetic folding intermediate, as has been hypothesized in the case of amyloid formation in other systems. In fact, on the basis of population it would be more likely that PrP^{Sc} were formed from the unfolded state of the molecule.

Inherited prion diseases may produce disease by destabilizing PrP^C, which would predispose the molecule to aggregate. Alternatively a mutation could facilitate the interaction between PrP^C and PrP^{Sc} or affect the binding of a ligand or co-protein. In order to relate the folding stability of PrP^C to its propensity for forming PrP^{Sc}, several of the human mutations have been copied into the recombinant mouse protein (Liemann and Glockshuber, 1999). Although this work concluded broadly that there is no absolute correlation between stability and disease, all of the fully penetrant pathogenic mutations show

significant destabilization, while non-pathogenic polymorphisms have little effect.

PrP^{Sc} is extracted from affected brains as highly aggregated, detergent-insoluble material that is not amenable to high-resolution structural techniques. However, Fourier transform infrared (FTIR) spectroscopic methods show that PrP^{Sc}, in sharp contrast to PrP^C, has a high β -sheet content (Pan *et al.*, 1993). PrP^{Sc} is covalently indistinguishable from PrP^C (Pan *et al.*, 1993; Stahl *et al.*, 1993).

The underlying molecular events during infection which lead to the conversion of PrP^C to the scrapie agent remain ill-defined. The most coherent and general model thus far proposed is that the protein, PrP, fluctuates between a dominant native state, PrP^C, and a series of minor conformations, one or a set of which can self-associate in an ordered manner to produce a stable supramolecular structure, PrP^{Sc}, composed of misfolded PrP monomers. Once a stable 'seed' structure is formed, PrP can then be recruited leading to an explosive, autocatalytic formation of PrP^{Sc}. Such a system would be extremely sensitive to three factors: (i) overall PrP^C concentration; (ii) the equilibrium distribution between the native conformation and the self-associating conformation and (iii) complementarity between surfaces which come together in the aggregation step. All three of these predictions from this minimal model are manifest in the aetiology of prion disease: an inversely proportional relationship between PrP^C expression and prion incubation period in transgenic mice (Bueler *et al.*, 1993; Collinge *et al.*, 1995b; Prusiner *et al.*, 1990; Telling *et al.*, 1995); predisposition by relatively subtle mutations in the protein sequence (Collinge, 1997); and a requirement for molecular homogeneity for efficient prion propagation (Palmer *et al.*, 1991; Prusiner *et al.*, 1990).

Little is known for certain about the molecular state of the protein that constitutes the self-propagating, infectious particle itself. It is hypothesized that prions are self-propagating fibrillar or amyloid forms of PrP in which the ends of the propagating fibrils constitute the infectious entity and the exponential rise in prion titre is a consequence of fibre fragmentation. While this concept of prion propagation as nucleation-dependent polymerization has been around for a long time (Come *et al.*, 1993; Gajdusek, 1988; Jarrett and Lansbury, 1993), there is now direct experimental evidence that PrP fibrillized *in vitro* may be infectious (Legname *et al.*, 2004).

Direct *in vitro* mixing experiments (Bessen *et al.*, 1995; Kocisko *et al.*, 1994, 1995) have been performed in an attempt to produce PrP^{Sc}. In such experiments an excess of PrP^{Sc} is used as a seed to convert recombinant PrP^C to a protease-resistant form (designated PrP^{RES}). However, the relative inefficiency of these reactions has precluded determining whether new infectivity has been generated. An

artificial species barrier has, however, been exploited to address this issue, and such conversion products, expected to have a different host specificity (and so which can be bioassayed in the presence of an excess of starting material), have not shown any detectable infectivity (Hill *et al.*, 1999a). These results argue that acquisition of protease resistance by PrP^C is not sufficient for the propagation of infectivity. However, more recently methods that have demonstrated *in vitro* amplification of prions have been reported which involve sequential cycles of sonication and incubation with brain homogenate as a source of PrP^C and presumably important co-factors (Castilla *et al.*, 2005).

The difficulty in performing structural studies on native PrP^{Sc} has led to attempts to produce soluble β -sheet-rich forms of PrP, which may be amenable to NMR or crystallographic structure determination. It is now recognized that the adage 'one sequence, one conformation' is not strictly true. Depending on solvent conditions, probably any protein chain can adopt a variety of conformations in which there is a degree of periodic order (that is extensive regions of secondary structure). However such alternative states do not have precisely and tightly packed side chains which are the hallmark of the native state of orthodox globular proteins.

Studies on a large fragment of the human PrP⁹¹⁻²³¹ have shown that at acidic pH PrP can fold to a soluble monomer comprised almost entirely of β -sheet in the absence of denaturants (Jackson *et al.*, 1999). Reduction of the native disulfide bond was a prerequisite for β -sheet formation and these observations of alternative folding pathways dependent upon solvent pH and redox potential could have important implications for the mechanism of conversion to PrP^{Sc}. Indeed this monomeric β -sheet state was prone to aggregation into fibrils with partial resistance to proteinase K digestion, characteristic markers of PrP^{Sc}. Unusually for a protein with a predominantly helical fold, the majority of residues in PrP⁹¹⁻²³¹ have a preference for β -conformation (55% of non-glycine/proline residues). In view of this property, it is possible that the PrP molecule is delicately balanced between radically different folds with a high-energy barrier between them; one dictated by local structural propensity (the β -conformation) and one requiring the precise docking of side chains (the native α -conformation). Such a balance would be influenced by mutations causing inherited human prion diseases. It is also worthy of note that individuals homozygous for valine at polymorphic residue 129 of human PrP (where either methionine or valine can be encoded) are more susceptible to iatrogenic CJD (Collinge *et al.*, 1991a), and valine has a much higher β -propensity than does methionine.

The precise subcellular localization of PrP^{Sc} propagation remains controversial. However, there is considerable

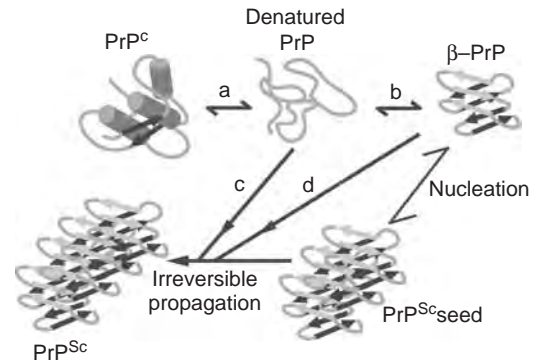


Figure 39.2 Possible mechanism for prion propagation. Largely α -helical PrP^C proceeds via an unfolded state (a) to re-fold into a largely β -sheet form, β -PrP (b). β -PrP is prone to aggregation in physiological salt concentrations. Prion replication may require a critical 'seed' size. Further recruitment of β -PrP monomers (c) or unfolded PrP (d) then occurs as an essentially irreversible process.

evidence implicating either late endosome-like organelles or lysosomes (Arnold *et al.*, 1995; Laszlo *et al.*, 1992; Mayer *et al.*, 1992; Taraboulos *et al.*, 1992). The environments of these organelles are evolved to facilitate protein unfolding at low pH prior to degradation by acid-activated proteases. It is possible that the α -PrP to β -PrP conversion, caused by reduction and mild acidification, is relevant to the conditions that PrP^C would encounter within the cell, following its internalization during recycling. Such a mechanism could underlie prion propagation, and account for the transmitted, sporadic and inherited aetiologies of prion disease (see Figure 39.2). Initiation of a pathogenic self-propagating conversion reaction, with accumulation of aggregated β -PrP, may be induced by exposure to a 'seed' of aggregated β -PrP following prion inoculation, or as a rare stochastic conformational change, or as an inevitable consequence of expression of a pathogenic PrP^C mutant that is predisposed to form β -PrP. It remains to be demonstrated whether such alternative conformational states of the protein are sufficient to cause prion disease in an experimental host or whether other cellular co-factors are also required.

A critical test of the protein-only hypothesis, both with respect to infectivity and 'strain-ness' would be to produce discrete prion strains synthetically from defined components.

Important steps in this endeavour have been reported. Purified, bacterially expressed recombinant N-terminally truncated mouse PrP has been aggregated into fibrillar material and bioassayed in transgenic mice (designated Tg9949) expressing very high levels of the same truncated mouse PrP (Legname *et al.*, 2004). Following prolonged

incubation periods, these Tg9949 mice developed a prion disease transmissible to wild-type as well as to further Tg9949 mice. The transmission characteristics at both primary and second passage of infectivity were consistent with production of a novel prion strain type, distinct from commonly used mouse-adapted laboratory strains. However, there remain major problems in interpretation of these data and the synthetic production of high-titre infectivity directly transmissible to wild-type animals has not yet been confirmed (Collinge and Clarke, 2007).

NORMAL CELLULAR FUNCTION OF PrP

While PrP plays a central role in pathogenesis of prion diseases, and mice devoid of PrP^C are resistant to scrapie and do not accumulate PrP^{Sc} or propagate infectivity (Bueler *et al.*, 1992), its normal biological function remains unclear. PrP is highly conserved among mammals, has been identified in marsupials, amphibians and birds, and may be present in all vertebrates. It is expressed during early embryogenesis and is found in most tissues in the adult (Manson *et al.*, 1992). However, highest levels of expression are seen in the central nervous system (CNS). The protein is found predominantly in neurons, particularly at synapses in cholesterol-rich microdomains or caveolae, known to play a central role in neuronal signalling events (for review see Anderson, 1993). PrP is also expressed widely in cells of the immune system (Dodelet and Cashman, 1998). Mice lacking PrP as a result of gene knockout (*Prnp*^{0/0}) showed no gross phenotype (Bueler *et al.*, 1992), although they were completely resistant to prion disease following inoculation and did not replicate prions (Bueler *et al.*, 1993). However, these mice were then shown to have abnormalities in synaptic physiology (Collinge *et al.*, 1994) and in circadian rhythms and sleep (Tobler *et al.*, 1996). While none of these observations define a molecular role for PrP^C, it has been argued that PrP may act as a receptor for an, as yet, unidentified extracellular ligand. Newly synthesized PrP^C is transported to the cell surface and then cycles rapidly via a clathrin-mediated mechanism, with a transit time of around an hour, between the surface and early endosomes (Shyng *et al.*, 1994).

It is possible that *Prnp*^{0/0} mice may be viable and healthy due to secondary compensatory mechanisms during neurodevelopment as has been documented in other models of targeted gene knockout. The most direct approach to answering this question was by knocking out neuronal PrP expression in a developed nervous system, avoiding potential compensatory mechanisms activated during neurodevelopment, and thus to observe directly the effects of acute depletion of PrP, revealing any critical function. This has now been achieved and excludes

PrP loss of function as a sufficient cause of prion neurodegeneration (Mallucci *et al.*, 2002).

PRION STRAINS

A major problem for the 'protein-only' hypothesis of prion propagation has been how to explain the existence of multiple isolates, or strains, of prions. Dickinson, Fraser and colleagues isolated multiple distinct strains of naturally occurring sheep scrapie in mice. Such strains are distinguished by their biological properties: they produce distinct incubation periods and patterns of neuropathological targeting (so-called lesion profiles) in defined inbred mouse lines (for review see Bruce *et al.*, 1992). As they can be propagated serially in inbred mice with the same *Prnp* genotype they cannot be encoded by differences in PrP primary structure. Furthermore, strains can be re-isolated in mice after passage in intermediate species with different PrP primary structures (Bruce *et al.*, 1994). Conventionally, distinct strains of conventional pathogen are explained by differences in their nucleic acid genome. However, in the absence of such a scrapie genome, alternative possibilities must be considered. Weissmann (1991) proposed an 'unified hypothesis' where, although the protein alone was argued to be sufficient to account for infectivity, it was proposed that strain characteristics could be encoded by a small cellular nucleic acid, or 'co-prion'. Although this hypothesis leads to the testable prediction that strain characteristics, unlike infectivity, would be sensitive to UV irradiation, no such test has been reported. At the other extreme, the protein-only hypothesis (Griffith, 1967) would have to explain how a single polypeptide chain could encode multiple disease phenotypes. Clearly, understanding how a protein-only infectious agent could encode such phenotypic information is of considerable biological interest.

Support for the idea that strain specificity may be encoded by PrP itself was provided by study of two distinct strains of TME prions which can be serially propagated in hamsters, designated hyper (HY) and drowsy (DY). These strains can be distinguished by differing physicochemical properties of the accumulated PrP^{Sc} in the brains of affected hamsters (Bessen and Marsh, 1992). Following limited proteolysis, strain-specific migration patterns of PrP^{Sc} on polyacrylamide gels were seen which related to different N-terminal ends of HY and DY PrP^{Sc} following protease treatment and implying differing conformations of HY and DY PrP^{Sc} (Bessen and Marsh, 1994).

Distinct human PrP^{Sc} types have been identified which are associated with different phenotypes of CJD (Collinge *et al.*, 1996b; Parchi *et al.*, 1996). The different fragment sizes seen on Western blots following treatment

with proteinase K suggests that there are several different human PrP^{Sc} conformations. However, while such biochemical modifications of PrP are clearly candidates for the molecular substrate of prion strain diversity, it is necessary to be able to demonstrate that these properties fulfil the biological properties of strains. In particular, that they are transmissible to the PrP in a host of both the same and different species. This has been demonstrated in studies with CJD isolates, with both PrP^{Sc} fragment sizes and the ratios of the three PrP glycoforms (diglycosylated, monoglycosylated and unglycosylated PrP) maintained on passage in transgenic mice expressing human PrP (Collinge *et al.*, 1996b). Furthermore, transmission of human prions and bovine prions to wild-type mice results in murine PrP^{Sc} with fragment sizes and glycoform ratios which correspond to the original inoculum (Collinge *et al.*, 1996b). Variant Creutzfeldt—Jakob disease (vCJD) is associated with PrP^{Sc} glycoform ratios which are distinct from those seen in classical CJD. Similar ratios are seen in BSE and BSE when transmitted to several other species (Collinge *et al.*, 1996b). These data strongly support the ‘protein-only’ hypothesis of infectivity and suggest that strain variation could be encoded by a combination of PrP conformation and glycosylation. Furthermore, polymorphism in PrP sequence can influence the generation of particular PrP^{Sc} conformers (Collinge *et al.*, 1996b). Transmission of PrP^{Sc} fragment sizes from two different sub-types of inherited prion disease to transgenic mice expressing a chimaeric human mouse PrP has also been reported (Telling *et al.*, 1996). As PrP glycosylation occurs before conversion to PrP^{Sc}, the different glycoform ratios may represent selection of particular PrP^C glycoforms by PrP^{Sc} of different conformations. According to such a hypothesis, PrP conformation would be the primary determinant of strain type with glycosylation being involved as a secondary process. However, since it is known that different cell types may glycosylate proteins differently, PrP^{Sc} glycosylation patterns may provide a substrate for the neuropathological targeting that distinguishes different prion strains (Collinge *et al.*, 1996b). Particular PrP^{Sc} glycoforms may replicate most favourably in neuronal populations with a similar PrP glycoform expressed on the cell surface. Such targeting could also explain the different incubation periods which also discriminate strains, targeting of more critical brain regions, or regions with higher levels of PrP expression, producing shorter incubation periods.

Recent work has shown strain-specific protein conformation to be influenced by metal binding to PrP^{Sc} (Wadsworth *et al.*, 1999). Two different human PrP^{Sc} types, seen in clinically distinct subtypes of classical CJD, can be interconverted *in vitro* by altering the metal-ion occupancy. The dependence of PrP^{Sc} conformation on the

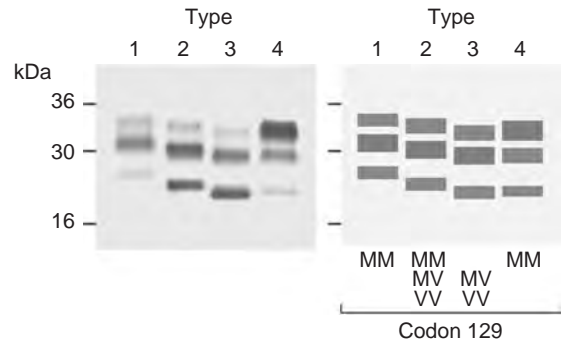


Figure 39.3 Molecular strain typing of human prions.

binding of copper and zinc represents a novel mechanism for post-translational modification of PrP, and for the generation of multiple prion strains.

Molecular strain typing of prion isolates can now be applied to molecular diagnosis of vCJD (Collinge *et al.*, 1996b; Hill *et al.*, 1997a) and to produce a new classification of human prion diseases with implications for epidemiological studies investigating the aetiology of sporadic CJD (Figure 39.3) (Hill *et al.*, 2003; Parchi *et al.*, 1996, 1999). Such methods allow strain typing to be performed in days rather than the one to two years required for classical biological strain typing. However, an internationally agreed classification of human prion strains has yet to emerge.

This technique may also be applicable to determining whether BSE has transmitted to other species (Collinge *et al.*, 1996b), and thereby pose a threat to human health, for instance to sheep (Hill *et al.*, 1998; Hope *et al.*, 1999; Kuczius *et al.*, 1998).

Such ability of a single polypeptide chain to encode information specifying distinct phenotypes of disease raises intriguing evolutionary questions. Do other proteins behave in this way? The novel pathogenic mechanisms involved in prion propagation may be of far wider significance and relevant to other neurological and non-neurological illnesses. Other prion-like mechanisms have now been described and the recognition that certain heritable traits in yeast could be explained by conformational switching and aggregation in two yeast proteins, Ure2p and Sup35p, led to the emergence of the field of ‘yeast prions’ (Wickner *et al.*, 2004). These proteins have no sequence similarity to PrP. While there are differences to mammalian prions, which are naturally infectious lethal pathogens, the study of these analogous phenomena has led to rapid advances in investigating the processes of seeded fibril formation and the molecular basis of strain diversity and transmission barriers. Indeed a series of elegant studies have clearly established that yeast prions are

composed of protein fibrils, propagating by seeding, and that strain diversity is explained by distinct conformers (Brachmann *et al.*, 2005; Diaz-Avalos *et al.*, 2005; King and Diaz-Avalos, 2004; Krishnan and Lindquist, 2005; Tanaka *et al.*, 2004, 2005).

NEURONAL CELL DEATH IN PRION DISEASE

The precise molecular nature of the infectious agent and the cause of neuronal cell death remains unclear. The current working hypothesis is that an abnormal isoform of PrP is the infectious agent and, to date, the most highly enriched preparations contain one infectious unit per 10^5 PrP monomers (Bolton *et al.*, 1982). Various hypotheses have been proposed to explain the mechanism of spongiform change and neuronal cell loss. These have included direct neurotoxic effects from a region of the PrP encompassing residues 106–126 (Brown *et al.*, 1994; Forloni *et al.*, 1993; Tagliavini *et al.*, 1993) to increased oxidative stress in neurons as a result of PrP^C depletion which has been proposed to function as an antioxidant molecule (Brown *et al.*, 1997). Neurotoxicity of PrP 106–126 is, however, controversial (Kunz *et al.*, 1999). It has also been suggested that PrP^C plays a role in regulating apoptosis with disturbance of normal cellular levels of PrP during infection leading to cell death (Kurschner and Morgan, 1995, 1996). Certainly there have been numerous recent reports of apoptotic cells being identified in the neuronal tissue of prion disease brains (Williams *et al.*, 1997). Although PrP^C expression is required for susceptibility to the disease, a number of observations argue that PrP^{Sc} and indeed prions (whether or not they are identical) may not themselves be highly neurotoxic. Prion diseases in which PrP^{Sc} is barely or not detectable have been described (Collinge *et al.*, 1995a; Hsiao and Prusiner, 1990; Lasmézas *et al.*, 1997; Medori *et al.*, 1992c). Mice with reduced levels of PrP^C expression have extremely high levels of PrP^{Sc} and prions in the brain and yet remain well for several months after their wild-type counterparts succumb (Bueler *et al.*, 1993). Conversely, Tg20 mice, with high levels of PrP^C, have short incubation periods and yet produce low levels of PrP^{Sc} after inoculation with mouse prions (Fischer *et al.*, 1996). In addition, brain grafts producing high levels of PrP^{Sc} do not damage adjacent tissue in PrP knockout (*Prnp*^{0/0}) mice (Brandner *et al.*, 1996). The cause of neurodegeneration in prion diseases remains unclear. It remains possible that prion neurodegeneration is related, at least in part, to loss of function of PrP^C. That *Prnp*^{0/0} mice (other than those associated with overexpression of the *Prnp*-like gene *Dpl* (Moore *et al.*, 1999) do not develop neurodegeneration could be due to compensatory adaptations during

neurodevelopment. Complete or near complete ablation of PrP expression in an adult mouse using conditional gene expression methods has not yet been achieved. A recent study has demonstrated that mice inoculated with Sc237 hamster prions replicate prions to high levels in their brains, but do not develop clinical signs of prion disease during their normal lifespan, arguing that PrP^{Sc} and indeed prions (whether or not they are identical) may not themselves be highly neurotoxic (Hill *et al.*, 2000). An alternative hypothesis for prion-related neurodegeneration is that a toxic intermediate (PrP^L) is produced in the process of conversion of PrP^C to PrP^{Sc}, with PrP^{Sc}, present as highly aggregated material, being a relatively inert end-product (Hill *et al.*, 2000). The steady state level of such a toxic monomeric or oligomeric PrP intermediate could then determine rate of neurodegeneration. One possibility is that Sc237-inoculated mice propagate prions very slowly and that such a toxic intermediate is generated at extremely low levels that are tolerated by the mouse. A general protein-only model of prion propagation, encompassing both infectious and toxic species has been proposed (Collinge and Clarke, 2007).

THE 'SPECIES BARRIER'

Transmission of prion diseases between different mammalian species is restricted by a 'species barrier' (Pattison, 1965). On primary passage of prions from species A to species B usually not all inoculated animals of species B develop disease and those that do have much longer and more variable incubation periods than those that are seen with transmission of prions within the same species, where typically all inoculated animals would succumb with a relatively short, and remarkably consistent, incubation period. On second passage of infectivity to further animals of the species B, transmission parameters resemble within-species transmissions with most if not all animals developing the disease with short and consistent incubation periods. Species barriers can therefore be quantitated by measuring the fall in mean incubation period on primary and second passage, or, perhaps more rigorously, by a comparative titration study. The latter involves inoculating serial dilutions of an inoculum in both the donor and host species and comparing the LD₅₀s obtained. The effect of a very substantial species barrier (for instance that between hamsters and mice) is that few, if any, animals succumb to disease at all on primary passage, and then at incubation periods approaching the natural lifespan of the species concerned.

Early studies of the molecular basis of the species barrier argued that it principally resided in differences in PrP primary structure between the species from which the

inoculum was derived and the inoculated host. Transgenic mice expressing hamster PrP were, unlike wild-type mice, highly susceptible to infection with Sc237 hamster prions (Prusiner *et al.*, 1990). That most sporadic and acquired CJD occurred in individuals homozygous at *PRNP* polymorphic codon 129 supported the view that prion propagation proceeded most efficiently when the interacting PrP^{Sc} and PrP^C were of identical primary structure (Collinge, 1991). However, it has been long recognized that prion strain type affects ease of transmission to another species. Interestingly, with BSE prions the strain component to the barrier seems to predominate, with BSE not only transmitting efficiently to a range of species, but maintaining its transmission characteristics even when passaged through an intermediate species with a distinct PrP gene (Bruce *et al.*, 1994). For instance, transmission of CJD prions to conventional mice is difficult, with few if any inoculated mice succumbing after prolonged incubation periods, consistent with a substantial species barrier (Collinge *et al.*, 1995b; Hill *et al.*, 1997a). In sharp contrast, transgenic mice expressing only human PrP are highly susceptible to CJD prions, with 100% attack rate and consistent short incubation periods which are unaltered by second passage, consistent with a complete lack of species barrier (Collinge *et al.*, 1995b). However, vCJD prions (again comprising human PrP of identical primary structure) transmit much more readily to wild-type mice than do classical CJD prions, while transmission to transgenic mice is relatively less efficient than with classical CJD (Hill *et al.*, 1997a). The term 'species barrier' does not seem appropriate to describe such effects and 'species-strain barrier' or simply 'transmission barrier' may be preferable (Collinge, 1999). Both PrP amino acid sequence and strain type affect the three-dimensional structure of glycosylated PrP which will presumably, in turn, affect the efficiency of the protein—protein interactions thought to determine prion propagation.

Mammalian PrP genes are highly conserved. Presumably only a restricted number of different PrP^{Sc} conformations (that are highly stable and can therefore be serially propagated) will be permissible thermodynamically and will constitute the range of prion strains seen. PrP glycosylation may be important in stabilizing particular PrP^{Sc} conformations. While a significant number of different such PrP^{Sc} conformations may be possible amongst the range of mammalian PrPs, only a subset of these would be allowable for a given single mammalian PrP. Substantial overlap between the favoured conformations for PrP^{Sc} derived from species A and species B might therefore result in relatively easy transmission of prion diseases between these two species, while two species with no preferred PrP^{Sc} conformations in common would have a large barrier to transmission (and indeed transmission would

necessitate a change of strain type). According to such a conformational selection model (Collinge, 1999) of a prion transmission barrier, BSE may represent a thermodynamically highly favoured PrP^{Sc} conformation that is permissive for PrP expressed in a wide range of different species, accounting for the remarkable promiscuity of this strain in mammals. The contribution of other components to the species barrier is possible and may involve interacting co-factors which mediate the efficiency of prion propagation, although no such factors have yet been identified.

Recent data have further challenged our understanding of transmission barriers (Hill *et al.*, 2000). The assessment of species barriers has relied on the development of a clinical disease in inoculated animals. On this basis there is a highly efficient barrier limiting transmission of hamster Sc237 prions to mice. Indeed, the hamster scrapie strain Sc237, which is similar to the strain classified as 263K (Kimberlin and Walker, 1978), is regarded as non-pathogenic for mice (with no clinical disease in mice observed for up to 735 days post inoculation; Kimberlin and Walker, 1978) and was used in studies of species barriers in transgenic mice (Kimberlin and Walker, 1979; Prusiner *et al.*, 1990; Scott *et al.*, 1989). It was demonstrated that transgenic mice expressing hamster PrP (in addition to endogenous mouse PrP), in sharp contrast to conventional mice, were highly susceptible to Sc237 hamster prions with consistent short incubation periods which were inversely correlated to hamster PrP expression levels (Prusiner *et al.*, 1990; Scott and Fraser, 1989). Importantly, however, these studies defined transmission using clinical criteria and did not report PrP^{Sc} levels and types, or prion titres in the brains of clinically unaffected animals. However, while not developing a clinical disease, and indeed living as long as mock-inoculated mice, Sc237-inoculated mice may accumulate high levels of prions in their brains (Hill *et al.*, 2000). Previous studies on the species barrier between hamsters and mice (using the Sc237 or 263K strain) did not report if PrP^{Sc} and/or infectivity were present in clinically unaffected animals (Prusiner *et al.*, 1990; Scott *et al.*, 1989) or have attempted passage from mice only up to 280 days post inoculation (Kimberlin and Walker 1978). The barrier to primary passage appears in this case to be to the development of rapid neurodegeneration and the resulting clinical syndrome rather than a barrier to prion propagation itself.

PATHOGENESIS

In some experimental rodent scrapie models when prions are inoculated outside the CNS, and in natural sheep scrapie, infectivity is first detectable in the spleen and other lymphoreticular tissues (for review see Fraser *et al.*,

1992). Spleen titres rise to a plateau early in the incubation period, a considerable period before neuro-invasion is detectable. CNS prion replication then rises to high levels and the clinical phase occurs.

The route of entry of prions following oral exposure may follow invasion of Peyer's patches and other gut lymphoid tissues, the relative protease resistance of prions presumably allows a significant proportion of infectivity to survive the digestive tract. It is unclear how prions transit the intestinal mucosa although M cells may be involved (Heppner *et al.*, 2001). It has been suggested that myeloid dendritic cells mediate transport within the lymphoreticular system (LRS) (Aucouturier *et al.*, 2001). While mature B cells are required for peripheral prion propagation, this appears to be because they are required for maturation of follicular dendritic cells (FDCs). PrP^{Sc} accumulates in FDCs which are a long-lived cell type and it is thought that they are the site of prion propagation in the spleen (Mabbott *et al.*, 2000, 2003; Montasio *et al.*, 2000). However, neuro-invasion is possible without FDCs, indicating that other peripheral cell types can replicate prions (Oldstone *et al.*, 2002; Prinz *et al.*, 2002). Neuro-invasion involves the autonomic nervous system innervating lymphoid tissue with retrograde spread to the spinal cord or via the vagus to the brain stem (Beekes *et al.*, 1998; Bencsik *et al.*, 2001). Prions have been detected in the blood at low levels in some rodent models and experimental BSE-infected primates (Bons *et al.*, 2002; Brown *et al.*, 1999; Holada *et al.*, 2002) and transmission of BSE prions between sheep by transfusion has been reported (Hunter *et al.*, 2002). It is now clear that vCJD is transmissible by blood transfusion (see secondary vCJD section) (Wroe *et al.*, 2006).

While prominent lymphoreticular involvement is seen in some experimental models or natural prion diseases, it is undetectable in others (see Fraser *et al.*, 1992, for review). Both host and prion strain effects are relevant. For example infection of sheep with BSE prions results in a wide tissue distribution of infectivity but infection of cattle with this strain does not, infectivity being largely confined to the CNS. In humans infected with sporadic CJD prions, infectivity is largely confined to the CNS, while in vCJD there is prominent involvement of lymphoreticular tissues (Hill *et al.*, 1997b, 1999b; Wadsworth *et al.*, 2001). It is possible that species barrier effects are also relevant, and it has been suggested that on passage of prions in a new species, there is an obligate lymphoreticular phase.

ANIMAL PRION DISEASES

An increasing number of animal prion diseases are recognized. Scrapie, a naturally occurring disease of sheep

and goats, has been recognized in Europe for over 200 years and is present endemically in many countries. Accurate epidemiology is lacking, although scrapie appears to be relatively common in some countries. Remarkably little is known about its natural routes of transmission. TME and chronic wasting disease of mule deer and elk were described from the 1940s onwards, principally in the United States. It has more recently become apparent that chronic wasting disease is a common condition in wild deer and elk in Colorado. Again the routes of transmission are unclear. TME has occurred as infrequent epidemics amongst ranched mink and may result from food-borne prion exposure.

The appearance of BSE in UK cattle from 1986 onwards, which rapidly evolved to a major epidemic (Anderson *et al.*, 1996; Wilesmith *et al.*, 1988), was widely attributed to transmission of sheep scrapie, endemic in the United Kingdom and many other countries, to cattle via contaminated feed prepared from rendered carcasses (Wilesmith *et al.*, 1988). However, an alternative hypothesis is that epidemic BSE resulted from recycling of rare sporadic BSE cases, as cattle were also rendered to produce cattle feed. Whether or not BSE originated from sheep scrapie, it was however clear from 1990 onwards, with the occurrence of novel spongiform encephalopathies amongst domestic and captive wild cats, that its host range was different to scrapie. Many new species have developed spongiform encephalopathies coincident with or following the arrival of BSE, including greater kudu, nyala, Arabian oryx, scimitar horned oryx, eland, gemsbok, bison, ankole, tiger, cheetah, ocelot, puma and domestic cats. Several of these have been confirmed to be caused by a BSE-like prion strain (Bruce *et al.*, 1994; Collinge *et al.*, 1996b), and it is likely that most or all of these are BSE-related. More than 180 000 BSE cases have been confirmed in cattle in the United Kingdom, although the total number of infected animals has been estimated to be around 2 million. BSE has since been reported in many European countries, with significant epidemics reported in Switzerland and Portugal, and a number of other countries including Canada and Japan.

More recently, additional types of bovine prion disease have been reported in individual animals which appear to be caused by prion strains distinct from that causing epizootic BSE (Baron *et al.*, 2006). The pathogenicity of such 'atypical BSE' to humans is unknown. Also a type of sheep scrapie with a distinctive PrP^{Sc} type, known as 'atypical scrapie' has been increasingly recognized in several EU countries (Buschmann *et al.*, 2004). While this may be caused by a pre-existing sheep scrapie strain, perhaps selected by selective breeding programmes that have been used to try to eliminate endemic sheep scrapie, it will be important to investigate its potential to infect humans.

AETIOLOGY AND EPIDEMIOLOGY OF HUMAN PRION DISEASE

The human prion diseases have been traditionally classified into CJD, Gerstmann—Sträussler—Scheinker disease and kuru, and they can be further divided into three aetiological categories: sporadic, acquired and inherited.

Sporadic CJD makes up around 85% of all recognized human prion disease. It occurs in all countries with a random case distribution and an annual incidence of 1–2 per million. Hypothesized causes of sporadic CJD include spontaneous production of PrP^{Sc} via rare stochastic events, somatic mutation of *PRNP* or unidentified environmental prion exposure. An association with sheep scrapie is not supported by epidemiological studies which have found a fairly uniform worldwide incidence of sporadic CJD irrespective of scrapie prevalence (Brown *et al.*, 1987). Although spatiotemporal groupings of sporadic CJD have been previously reported (Adikari and Farmer, 2001; Farmer *et al.*, 1978), no direct evidence for exposure to a common source of infectious prions has been provided. Indeed, such apparent clustering of cases, while appearing to reach levels of significance when viewed in isolation, can be deemed to be expected by chance alone when analysed within the population as a whole (Collins *et al.*, 2002). However, the lack of such evidence does not exclude the possibility that a fraction of sporadic CJD is caused by environmental exposure to animal or human prions. There is marked genetic susceptibility in sporadic CJD in that most cases occur in homozygotes at codon 129 of *PRNP*, where either methionine or valine may be encoded. Heterozygotes appear significantly protected against developing sporadic CJD (Collinge *et al.*, 1991a; Palmer *et al.*, 1991; Windl *et al.*, 1996). Additionally, a *PRNP* susceptibility haplotype has been identified indicating additional genetic susceptibility to sporadic CJD at or near to the *PRNP* locus (Mead *et al.*, 2001).

The acquired prion diseases include iatrogenic CJD, kuru and vCJD and arise from accidental exposure to human prions through medical or surgical procedures, participation in cannibalistic feasts or dietary or other exposure to BSE prions. The two most frequent causes of iatrogenic CJD occurring through medical procedures have arisen as a result of implantation of dura mater grafts and treatment with human growth hormone derived from the pituitary glands of human cadavers (Brown *et al.*, 1992a, 2000). Less frequent incidences of human prion disease have resulted from iatrogenic transmission of CJD during corneal transplantation and surgical operations using contaminated instruments or apparatus (Brown *et al.*, 1992a, 2000). Primary and secondary vCJD are discussed

below. *PRNP* codon 129 genotype is also relevant to susceptibility and incubation period (see below).

Around 15% of human prion disease is inherited and all cases to date have been associated with coding mutations in the PrP gene (*PRNP*) of which over 30 distinct types are recognized (Figure 39.3). The inherited prion diseases can be diagnosed by *PRNP* analysis and the use of these definitive genetic diagnostic markers has allowed the recognition of a wider phenotypic spectrum of human prion disease to include a range of atypical dementias and fatal familial insomnia (FFI) (Collinge *et al.*, 1990, 1992; Medori *et al.*, 1992b). The protective effect of *PRNP* codon 129 heterozygosity is also seen in some of the inherited prion diseases, with a later age at disease onset in heterozygotes (Hsiao *et al.*, 1992).

The occurrence of cases of apparently sporadic CJD in unusually young people in 1995 (Bateman *et al.*, 1995; Britton *et al.*, 1995; Tabrizi *et al.*, 1996) led to concerns that BSE transmission to humans may have occurred. Arrival of further cases in 1996 led to the recognition of a novel clinicopathological type of human prion disease, new vCJD (Will *et al.*, 1996), indicating the arrival of a new risk factor for CJD in the United Kingdom (Collinge and Rossor, 1996). A link with BSE seemed highly likely on epidemiological grounds and this was strongly supported by experimental data, first from molecular strain typing studies (Collinge *et al.*, 1996b) and later by transmission studies into both transgenic and conventional mice (Hill *et al.*, 1997a; Bruce *et al.*, 1997). *PRNP* mutations are absent in vCJD, and all cases studied to date have been methionine homozygotes at codon 129.

The observation that vCJD is caused by the same prion strain as that causing BSE in cattle raised the possibility that a major epidemic of vCJD will occur in the United Kingdom and other countries as a result of dietary or other exposure to BSE prions and also (Collinge, 1999) concerns of potential iatrogenic transmission of pre-clinical vCJD via medical and surgical procedures. Transmission of vCJD by blood transfusion has now been documented. That only *PRNP* 129MM individuals are susceptible to BSE infection is questionable, since the other acquired human prion diseases, iatrogenic CJD and kuru, occur in all codon 129 genotypes as the epidemic evolves, with codon 129 heterozygotes having the longest mean incubation periods (Collinge, 1999; Lloyd *et al.*, 2002; Poulter *et al.*, 1992). Human BSE infection of other *PRNP* genotypes may simply have a longer latency (Collinge, 1999) and may also have a different phenotype due to propagation of different prion strains (Hill *et al.*, 1997a; Wadsworth *et al.*, 2004).

Estimates of the mean incubation period of human-to-human prion transmission come from study of growth hormone-related iatrogenic CJD and kuru.

The mean incubation period has been estimated in both examples to be around 12 years; in kuru, incubation periods can exceed 50 years (Collinge *et al.*, 2006). The effect of a species barrier is to considerably increase mean incubation periods and the range of incubation periods, which may approach the usual lifespan of the species concerned. The cattle-to-mouse barrier for the BSE strain results typically in a three- to fourfold increase in mean incubation period. Mean incubation periods of human BSE infection of 30 years or more should be considered (Collinge, 1999). Furthermore, prion disease in mice follows a well-defined course with a highly distinctive and repeatable incubation time for a given prion strain in a defined inbred mouse line. In addition to the PrP gene, a small number of additional genetic loci with a major effect on incubation period have been mapped. It can be anticipated that the human homologues of such loci may play a key role in human susceptibility to prion disease, both following accidental human prion exposure and exposure to the BSE agent. By definition, the patients identified to date with vCJD are those with the shortest incubation periods for BSE. These in turn, given that no unusual history of dietary, occupational or other exposure to BSE has been identified, would be expected to be predominantly those individuals with short incubation time alleles at these multiple genetic loci in addition to having the codon 129 methionine homozygous *PRNP* genotype. The vCJD cases reported to date may therefore represent a distinct genetic subpopulation with unusually short incubation periods to BSE prions. It is possible therefore that a human BSE epidemic will be multiphasic, and that recent estimates of the size of the vCJD epidemic based on uniform genetic susceptibility may substantially underestimate the eventual size (D'Aignaux *et al.*, 2001; Ghani *et al.*, 2000). Genes involved in species barrier effects, which would further increase both the mean and range of human BSE incubation periods, are also likely to be relevant. In this context, it will be very difficult to accurately predict a human epidemic until such loci are identified and their gene frequencies in the population can be determined (Lloyd *et al.*, 2001).

CLINICAL FEATURES AND DIAGNOSIS

With the advances in our understanding of their aetiology it now seems more appropriate to divide the human prion diseases into inherited, sporadic and acquired forms with CJD, GSS and kuru clinicopathological syndromes within a wider spectrum of disease. Kindreds with inherited prion disease have been described with phenotypes of classical CJD, GSS, and also with other

neurodegenerative syndromes including FFI. Some kindreds show remarkable phenotypic variability which can encompass both CJD- and GSS-like cases as well as other cases which do not conform to either CJD or GSS phenotypes (Collinge *et al.*, 1992). Cases diagnosed by PrP gene analysis have been reported which are not only clinically atypical but which lack the classical histological features entirely (Collinge *et al.*, 1990). Significant clinical overlap exists with familial Alzheimer's disease, frontotemporal dementias and amyotrophic lateral sclerosis with dementia. Inherited prion diseases are a frequent cause of pre-senile dementia and a family history is not always apparent: *PRNP* should be analysed in all suspected cases of CJD, and considered in all early onset dementia and ataxias. Although classical GSS is described below the inherited prion diseases should now be subclassified according to mutation. Acquired prion diseases include iatrogenic CJD, kuru and primary and secondary vCJD. Sporadic prion diseases at present consist of CJD and atypical variants of CJD. Cases lacking the characteristic histological features of CJD have been transmitted. As there are at present no equivalent aetiological diagnostic markers for sporadic prion diseases to those for the inherited diseases, it cannot yet be excluded that more diverse phenotypic variants of sporadic prion disease exist and novel phenotypes of human infection with BSE prions are likely to emerge. The key clinical features and investigations for the diagnosis of prion disease are given in Table 39.1.

Sporadic Prion Disease

Creutzfeldt-Jakob Disease

The core clinical syndrome of classic CJD is of a rapidly progressive multifocal dementia usually with myoclonus. The onset is usually in the 45–75 year age group with peak onset between 60 and 65. The clinical progression is typically over weeks, progressing to akinetic mutism and death often in two to three months. Around 70% of cases die in under six months. Prodromal features, present in around a third of cases, include fatigue, insomnia, depression, weight loss, headaches, general malaise and ill-defined pain sensations. In addition to mental deterioration and myoclonus, frequent additional neurological features include extrapyramidal signs, cerebellar ataxia, pyramidal signs and cortical blindness. About 10% of cases present initially with cerebellar ataxia.

Routine haematological and biochemical investigations are normal although occasional cases have been noted to have raised serum transaminases or alkaline phosphatase. There are no immunological markers and acute phase proteins are not elevated. Examination of the cerebrospinal fluid (CSF) is normal; 14-3-3 protein is usually elevated

Table 39.1 Diagnosis of prion disease

Disease	Characteristics
Sporadic CJD	Rapidly progressive ^a dementia with two or more of: myoclonus, cortical blindness, pyramidal signs, cerebellar signs, extrapyramidal signs, akinetic mutism Most aged 45–75 Serial EEG usually shows pseudoperiodic complexes CSF 14-3-3 protein usually positive CT normal or atrophy, MRI may show high signal in the striatum and/or cerebral cortex in FLAIR or diffusion-weighted images <i>PRNP</i> analysis: no pathogenic mutations, most are 129MM (VV and MV may be longer duration, clinically atypical and EEG less often positive) Brain biopsy should be considered in highly selected cases (to exclude treatable alternative diagnoses): PrP immunocytochemistry or Western blot for PrP ^{Sc} types 1–3 ^b
Iatrogenic CJD	Progressive cerebellar syndrome and behavioural disturbance or classical CJD-like syndrome with history of iatrogenic exposure to human prions (pituitary-derived hormones, tissue grafting or neurosurgery) May be young EEG, CSF and MRI generally less helpful than in sporadic cases <i>PRNP</i> analysis: no pathogenic mutations, most are 129 homozygotes Brain biopsy should be considered in highly selected cases (to exclude treatable alternative diagnoses): PrP immunocytochemistry or Western blot for PrP ^{Sc} types 1–3 ^b
Variant CJD	Early features: depression, anxiety, social withdrawal, peripheral sensory symptoms Cerebellar ataxia, chorea or athetosis often precedes dementia Advanced disease resembles sporadic CJD Most in young adults; however, age at onset 12–74 yr seen EEG nonspecific slow waves, CSF 14-3-3 may be elevated or normal MRI: pulvinar sign usually present (particularly using FLAIR sequence) but may be late feature <i>PRNP</i> analysis: no mutations, all 129MM to date Tonsil biopsy: characteristic PrP immunostaining and PrP ^{Sc} on Western blot (type 4t)
Iatrogenic vCJD	Has occurred in recipients of blood transfusion from a donor who subsequently developed clinical vCJD Known recipients of implicated blood or blood products in the UK have been notified of their risk status Clinical features and investigations as for primary vCJD
Inherited prion disease	Varied clinical syndromes between and within kindreds: should consider in all pre-senile dementias and ataxias irrespective of family history <i>PRNP</i> analysis: diagnostic, codon 129 genotype may predict age at onset in pre-symptomatic testing

CSF, cerebrospinal fluid; CT, computed tomography; MRI, magnetic resonance imaging; CJD, Creutzfeldt–Jakob disease.

^aClinical duration typically 6 mo or less but high variability: type 1 PrP^{Sc} associated with short duration (~8 wk); ~10% have duration >2 yr.

^bClassification of Hill *et al.* (2003).

in CJD and is a useful adjunct to diagnosis in the appropriate clinical context (Collinge, 1996). It is also positive in recent cerebral infarction or haemorrhage and in viral encephalitis, although these conditions do not usually present diagnostic confusion with CJD. It may also be elevated in rapidly progressive Alzheimer's disease which may be difficult to clinically distinguish from CJD. Neuronal specific enolase (NSE) and S-100b may be also elevated although also are not specific for CJD

and represent markers of neuronal injury (Otto *et al.*, 1997; Zerr *et al.*, 1995). Neuroimaging with computed tomography (CT) or magnetic resonance imaging (MRI) is crucial to exclude other causes of subacute neurological illness, but MRI has become increasingly useful in diagnosis of sporadic CJD, showing high signal in the striatum and/or cerebral cortex in FLAIR or diffusion-weighted images (Macfarlane *et al.*, 2007). Cerebral and cerebellar atrophy may be present in longer duration cases.

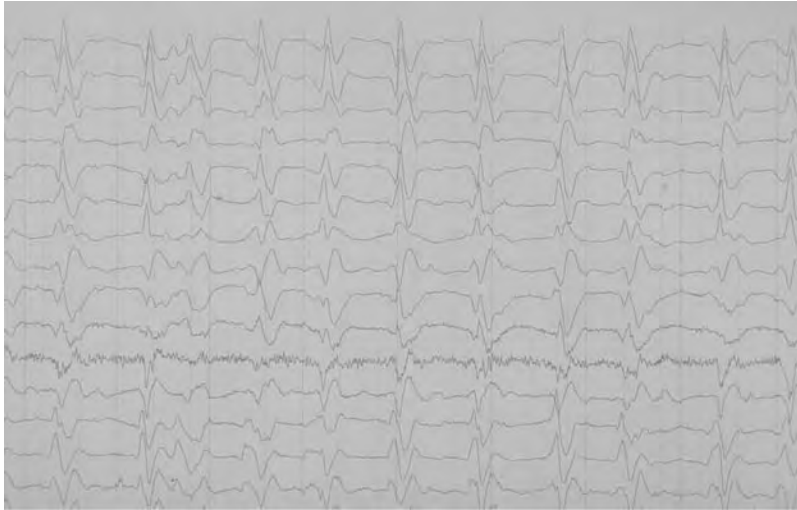


Figure 39.4 Electroencephalogram in sporadic CJD.

The electroencephalogram (EEG) may show characteristic pseudoperiodic sharp wave activity which is very helpful in diagnosis but present in only around 70% of cases (Figure 39.4). To some extent demonstration of a typical EEG is dependent on the number of EEGs performed and serial EEG is indicated to try to demonstrate this appearance.

Prospective epidemiological studies have demonstrated that cases with a progressive dementia, and two or more of the following: myoclonus; cortical blindness; pyramidal, cerebellar or extrapyramidal signs; or akinetic mutism in the setting of a typical EEG nearly always turn out to be confirmed as histologically definite CJD if neuropathological examination is performed.

Neuropathological confirmation of CJD is by demonstration of spongiform change, neuronal loss and astrogliosis (Figure 39.5). PrP amyloid plaques are usually not present in CJD although PrP immunohistochemistry, using appropriate pre-treatments, will nearly always be positive. Protease-resistant PrP, seen in all the currently recognized prion diseases, can be demonstrated by immunoblotting of brain homogenates. *PRNP* analysis is important to exclude pathogenic mutations. Genetic susceptibility to CJD has been demonstrated in that most cases of classical CJD are homozygous with respect to the common 129 polymorphism of PrP (see section on Aetiology above).

Atypical forms of Creutzfeldt—Jakob disease Atypical forms of CJD are well recognized. Around 10% of cases of CJD have a much more prolonged clinical course with a disease duration of over two years. These cases

may represent the occasional occurrence of CJD in individuals heterozygous for PrP polymorphisms. Around 10% of CJD cases present with cerebellar ataxia rather than cognitive impairment, so-called ataxic CJD. Heidenhain's variant of CJD refers to cases in which cortical blindness predominates with severe involvement of the occipital lobes. The panencephalopathic type of CJD refers to cases with extensive degeneration of the cerebral white matter in addition to spongiform vacuolation of the grey matter and has been predominately reported from Japan.

Amyotrophic variants of CJD have been described with prominent early muscle wasting. However, most cases of dementia with amyotrophy are not experimentally transmissible and their relationship with CJD is unclear. Most cases are probably variants of motor neuron disease with associated dementia. Amyotrophic features in CJD are usually seen in late disease when other features are well established.

Molecular classification of sporadic CJD The marked clinical heterogeneity observed in human prion diseases has yet to be explained. However, it has been clear for many years that distinct isolates, or strains, of prions can be propagated in the same host and these are biologically recognized by distinctive clinical and pathological features (Collinge, 2001). It is therefore likely that a proportion of clinicopathological heterogeneity seen in sporadic CJD and other human prion diseases relates to the propagation of distinct human prion strains. The identification of strain-specific PrP^{Sc} structural properties would thus allow an aetiology-based classification of CJD by typing of the infectious agent itself.

Four types of human PrP^{Sc} have now been reliably identified using molecular strain typing (Collinge *et al.*, 1996b; Hill *et al.*, 1997a, 2003; Wadsworth *et al.*, 1999) (Figure 39.3). Sporadic and iatrogenic CJD are associated with PrP^{Sc} types 1–3, while type 4 human PrP^{Sc} is uniquely associated with vCJD and is characterized by a fragment size and glycoform ratio that is distinct from PrP^{Sc} types 1–3 observed in classical CJD (Collinge *et al.*, 1996b; Hill *et al.*, 1997a, 2003; Wadsworth *et al.*, 1999). The methionine/valine polymorphism at codon 129 of *PRNP* is associated with different PrP^{Sc} types. PrP^{Sc} types 1 and 4 have so far only been detected in methionine homozygotes, type 3 cases are predominantly associated with at least one valine allele, while type 2 is seen in any *PRNP* codon 129 genotype (Collinge *et al.*, 1996a, 1996b; Hill *et al.*, 1999b, 2003; Wadsworth *et al.*, 1999). PrP^{Sc} types 1 and 2 are associated with two clinically distinct subtypes of sporadic CJD and have N-terminal structures determined by the coordination of metal ions (Hill *et al.*, 2003; Wadsworth *et al.*, 1999). Importantly, the identification of strain-specific PrP^{Sc} structural properties has enabled investigation of the influence of human PrP primary structure, in particular polymorphic residue 129, in determining PrP^{Sc} structure. Transgenic mice expressing human PrP with either valine or methionine at residue 129 have revealed that this polymorphism constrains both the propagation of distinct human PrP^{Sc} conformers and the occurrence of associated patterns of neuropathology (Asante *et al.*, 2002; Collinge *et al.*, 1996b; Hill *et al.*, 1997a) (and unpublished data). These data strongly support the biological relevance of molecular strain typing which can now be applied to rapid molecular diagnosis of classical CJD or vCJD and to produce a new classification of human prion diseases. Two such classifications are in use: no internationally agreed classification has yet emerged and it is likely that additional PrP^{Sc} types or strains will be identified (Hill *et al.*, 2003; Parchi *et al.*, 1999).

Molecular strain typing has major implications for epidemiological surveillance of sporadic CJD, whose aetiology remains obscure. While spontaneous conversion of PrP^C to PrP^{Sc} as a rare stochastic event, or somatic mutation of the PrP gene, resulting in expression of a pathogenic PrP mutant, are plausible explanations for sporadic CJD (Collinge, 1997), other causes for at least some cases, including environmental exposure to human or animal prions has not been ruled out by existing epidemiological studies (Collins *et al.*, 1999). Subclassification of sporadic CJD based upon PrP^{Sc} type immediately allows a more precise molecular classification of human prion disease and re-analysis of epidemiological data using these molecular subtypes may reveal important risk factors obscured when sporadic CJD is analysed as a single entity.

For example, it will be important to review the incidence of sporadic CJD associated with PrP^{Sc} type 2 and other molecular subtypes in both BSE-affected and unaffected countries in the light of recent findings, suggesting that human BSE prion infection may result in propagation of either type 4 PrP^{Sc} or type 2 PrP^{Sc} (Asante *et al.*, 2002). Individuals that propagate type 2 PrP^{Sc} as a result of BSE exposure may present with prion disease that would be indistinguishable on clinical, pathological and molecular criteria from that found in classical CJD.

Acquired Prion Diseases

While human prion diseases can be transmitted to experimental animals by inoculation, they are not contagious in humans. Documented case-to-case spread has only occurred during ritual cannibalistic practices (kuru) or following accidental inoculation with prions during medical or surgical procedures (iatrogenic CJD and secondary vCJD).

Kuru

Kuru reached epidemic proportions amongst a defined population living in the Eastern Highlands of Papua New Guinea. The earliest cases are thought to date back to the early part of the twentieth century. Kuru affected the people of the Fore linguistic group and their neighbours with whom they intermarried. Kuru predominantly affected women and children (of both sexes), with only 2% of cases in adult males (Alpers, 1987) and was the commonest cause of death amongst women in affected villages. It was the practice in these communities to engage in consumption of dead relatives as a mark of respect and mourning. Males after the age of 6–8 participated little if at all in mortuary feasting, which is thought to explain the differential age and sex incidence. It is thought that the epidemic related to a single sporadic CJD case occurring in the region some decades earlier. Epidemiological studies provided no evidence for vertical transmission, since most of the children born after 1956 (when cannibalism had effectively ceased) and all of those born after 1959 of mothers affected with or incubating kuru were unaffected (Alpers, 1987). From the age of the youngest affected patient, the shortest incubation period is estimated as 4.5 years, although may have been shorter, since time of infection was usually unknown. The disease has gradually declined in incidence although a small number of cases have been documented in recent years with incubation periods which may exceed 50 years (Collinge *et al.*, 2006). *PRNP* codon 129 genotype has a significant effect on kuru susceptibility and most elderly survivors of the kuru epidemic are heterozygotes (Mead *et al.*, 2003). The marked survival advantage for codon 129 heterozygotes provides

a powerful basis for selection pressure in the Fore. Remarkably, an analysis of worldwide haplotype diversity and allele frequency of *PRNP* coding and noncoding polymorphisms suggests that balancing selection at this locus is much older and more geographically widespread. Evidence for balancing selection (where there is more variation than expected in a gene due to heterozygote advantage) has been demonstrated in only a few human genes. Given recent biochemical and physical evidence of cannibalism on five continents, one explanation is that ancient and worldwide cannibalism resulted in a series of prion disease epidemics in human prehistory, thus imposing balancing selection on *PRNP* (Mead *et al.*, 2003).

Kuru affects both sexes and onset of disease has ranged from age 5 to over 60. The mean clinical duration of illness is 12 months with a range of 3 months to 3 years; the course tends to be shorter in children. The central clinical feature is progressive cerebellar ataxia. In contrast to classical CJD, dementia is much less prominent, although in the later stages many patients have their faculties obtunded (Alpers, 1987). The occasional case in which gross dementia occurs is in contrast to the clinical norm. Detailed clinical descriptions have been given by a number of observers and the disease does not appear to have changed in features at different stages of the epidemic. A prodrome and three clinical stages are recognized:

Prodromal stage Kuru typically begins with prodromal symptoms consisting of headache, aching of limbs and joint pains which can last for several months.

Ambulatory stage Kuru was frequently self-diagnosed by patients at the earliest onset of unsteadiness in standing or walking, or of dysarthria or diplopia. At this stage there may be no objective signs of disease. Gait ataxia however worsens and patients develop a broad based gait, truncal instability and titubation. A coarse postural tremor is usually present and accentuated by movement; patients characteristically hold their hands together in the midline to suppress this. Standing with feet together reveals clawing of toes to maintain posture. This marked clawing response is regarded as pathognomonic of kuru. Patients often become withdrawn at this stage and occasionally develop a severe reactive depression. Prodromal symptoms tend to disappear. Astasia and gait ataxia worsen and the patient requires a stick for walking. Intention tremor, dysmetria, hypotonia and dysdiadochokinesis develop. Although eye movements are ataxic and jerky, nystagmus is rarely seen. Strabismus, usually convergent, may occur, particularly in children. This strabismus does not appear to be concomitant or paralytic and may fluctuate in both extent and type, sometimes disappearing later in the clinical course. Photophobia is common and there may be an

abnormal cold sensitivity with shivering and piloerection even in a warm environment. Tendon reflexes are reduced or normal and plantar responses are flexor. Dysarthria usually occurs. As ataxia progresses the patient passes from the first (ambulatory) stage to the second (sedentary) stage. The mean clinical duration of the first stage is around eight months and correlates closely with total duration (Alpers, 1964).

Sedentary stage At this stage patients are able to sit unsupported but cannot walk. Attempted walking with support leads to a high steppage, wide-based gait with reeling instability and flinging arm movements in an attempt to maintain posture. Hyperreflexia is seen although plantar responses usually remain flexor with intact abdominal reflexes. Clonus is characteristically short lived. Athetoid and choreiform movements and parkinsonian tremors may occur. There is no paralysis, although muscle power is reduced. Obesity is common at this stage but may be present in early disease associated with bulimia. Characteristically, there is emotional lability and bizarre uncontrollable laughter, which has led to the disease being referred to as 'laughing death'. There is no sensory impairment. In sharp contrast to CJD, myoclonic jerking is rarely seen. EEG is usually normal or may show nonspecific changes (Cobb *et al.*, 1973). This stage lasts around two to three months. When truncal ataxia reaches the point where the patient is unable to sit unsupported, the third or tertiary stage is reached.

Tertiary stage Hypotonia and hypo-reflexia develop and the terminal state is marked by flaccid muscle weakness. Plantar responses remain flexor and abdominal reflexes intact. Progressive dysphagia occurs and patients become incontinent of urine and faeces. Inanition and emaciation develop. Transient conjugate eye signs and dementia may occur. Primitive reflexes develop in occasional cases. Brainstem involvement and both bulbar and pseudobulbar signs occur. Respiratory failure and bronchopneumonia eventually lead to death. The tertiary stage lasts one to two months.

Iatrogenic Creutzfeldt—Jakob Disease

Iatrogenic transmission of CJD has occurred by accidental inoculation with human prions as a result of medical procedures. Such iatrogenic routes include the use of inadequately sterilized neurosurgical instruments, dura mater and corneal grafting, and use of human cadaveric pituitary-derived growth hormone or gonadotrophin. It is of considerable interest that cases arising from intracerebral or optic inoculation manifest clinically as classical CJD, with a rapidly progressive dementia, while those resulting from peripheral inoculation, most notably

following pituitary-derived growth hormone exposure, typically present with a progressive cerebellar syndrome, and are in that respect somewhat reminiscent of kuru. Unsurprisingly the incubation period in intracerebral cases is short (19–46 months for dura mater grafts) as compared to peripheral cases (typically 15 years or more). There is evidence for genetic susceptibility to iatrogenic CJD with an excess of codon 129 homozygotes (Collinge *et al.*, 1991a).

Epidemiological studies have not shown increased risks of particular occupations that may be exposed to human or animal prions, although individual CJD cases in two histopathology technicians, a neuropathologist and a neurosurgeon have been documented. While there have been concerns that CJD may be transmissible by blood transfusion, extensive epidemiological analysis in the United Kingdom has found that the frequency of blood transfusion and donation was no different in over 200 cases of CJD and a matched control population (Esmonde *et al.*, 1993). Recipients of blood transfusions who developed CJD had clinical presentations similar to those of sporadic CJD patients and not to the more kuru-like iatrogenic cases arising from peripheral exposure to human prions. Furthermore, experimental transmission studies have shown only weak evidence for infectivity in blood, even when inoculated via the most efficient (intracerebral) route. Iatrogenic (secondary) vCJD related to blood transfusion has, however, been recognized (see below).

Variant CJD

In late 1995, two cases of sporadic CJD were reported in the United Kingdom in teenagers (Britton *et al.*, 1995). Only four cases of sporadic CJD had previously been recorded in teenagers, and none of these cases occurred in the United Kingdom. In addition, both cases were unusual in having kuru-type plaques, a finding seen in only around 5% of CJD cases. Soon afterwards a third very young sporadic CJD case occurred. These cases caused considerable concern and the possibility was raised that they might suggest a link with BSE. By March 1996, further extremely young onset cases were apparent and review of the histology of these cases showed a remarkably consistent and unique pattern. These cases were named 'new variant' CJD although it was clear that they were also rather atypical in their clinical presentation; in fact most cases did not meet the accepted clinical diagnostic criteria for probable CJD. Extensive studies of archival cases of CJD or other prion diseases failed to show this picture and it seemed that it did represent the arrival of a new form of prion disease in the United Kingdom. The statistical probability of such cases occurring by chance was vanishingly small and ascertainment bias seemed most unlikely as an explanation. It was clear that a new risk

factor for CJD had emerged and appeared to be specific to the United Kingdom. The UK Government Advisory Committee on Spongiform Encephalopathy (SEAC) concluded that, while there was no direct evidence for a link with BSE, exposure to specified bovine offal (SBO) prior to the ban on its inclusion in human foodstuffs in 1989, was the most likely explanation. A case of vCJD was soon after reported in France. Direct experimental evidence that vCJD is caused by BSE was provided by molecular analysis of human prion strains and transmission studies in transgenic and wild-type mice (see section on Aetiology above). While it is now clear that vCJD is caused by infection with BSE prions, it is unclear why this particular age group should be affected and why none of these cases had a pattern of unusual occupational or dietary exposure to BSE. However, very little is known of which foodstuffs contained high-titre bovine offal. It is possible that certain foods containing particularly high titres were eaten predominantly by younger people. An alternative is that young people are more susceptible to BSE following dietary exposure or that they have shorter incubation periods. A possible age-related co-factor could be coexistent infection involving lymphoid tissue, for example tonsillar infection. It is important to appreciate that BSE-contaminated feed was fed to sheep, pigs and poultry and that although there is no evidence of natural transmission to these species, it would be prudent to remain open-minded about other dietary exposure to novel animal prions.

vCJD has an insidious clinical onset and its early features are highly non-specific. The clinical presentation is often with behavioural and psychiatric disturbances and in some cases with sensory disturbance. Initial referral has frequently been to a psychiatrist and the most prominent feature is depression, but anxiety, social withdrawal and behavioural change is frequent. Suicidal ideation is infrequent and response to antidepressants poor. Delusions, which are complex and unsustained, are common. Other features include emotional lability, aggression, insomnia and auditory and visual hallucinations. A prominent early feature in some is dysaesthesiae or pain in the limbs or face or pain which is persistent rather than intermittent and unrelated to anxiety levels. A minority of cases have been noted to have forgetfulness or mild gait ataxia from an early stage but in most cases overt neurological features are not apparent until some months into the clinical course. In most patients a progressive cerebellar syndrome develops with gait and limb ataxia. Overt dementia then occurs with inevitable progression to akinetic mutism. Myoclonus is seen in most patients, and chorea is often present which may be severe in some patients. Cortical blindness develops in a minority of patients in the late stages of disease. Uppage paresis, an uncommon feature

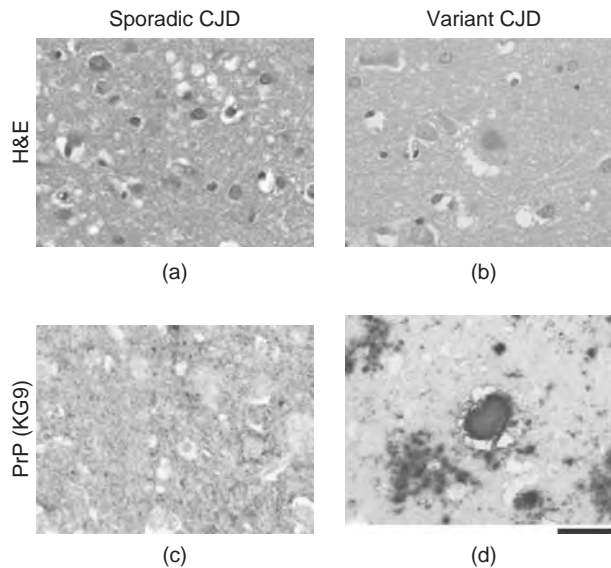


Figure 39.5 Neuropathology of sporadic and variant CJD. Mild spongiform vacuolation of the cortical grey matter is a hallmark of Creutzfeldt–Jakob disease. (a) In this case there was no deposition of kuru-like plaques but widespread deposition of abnormal prion protein in a synaptic pattern. (b) This hallmark of variant CJD can be found in several cortical areas and in the cerebellum, and may be associated with vacuolar degeneration. Immunostaining for the pathological prion protein (using monoclonal antibody KG9) highlights the florid plaques but also reveals synaptic staining. (c) In contrast, variant CJD is characterized by frequent so-called florid plaque consisting of a round amyloid core, surrounded by a daisy-like vacuolation. (d) Scale bar 50 μm .

of classical CJD, has been noted in some patients. The age at onset in the initial 14 cases reported ranged from 16 to 48 years (mean 29 years) and the clinical course was unusually prolonged (9–35 months, median 14 months). The age range of cases has since broadened, with ages at onset ranging from 12 to 74 years, although the mean remains around 28 years. The EEG is abnormal, most frequently showing generalized slow wave activity, but without the pseudoperiodic pattern seen in most sporadic CJD cases. Neuro-imaging by CT is either normal or shows only mild atrophy. The most useful non-invasive investigation in advanced cases is magnetic resonance neuro-imaging, particularly the FLAIR sequence (Collie *et al.*, 2003). Early case reports noted bilateral increased signal in the posterior thalamus (pulvinar) on T2 weighted images (Chazot *et al.*, 1996). A retrospective review of 36 histologically confirmed cases of vCJD suggested that the ‘pulvinar sign’ occurred frequently in advanced cases of vCJD (Zeidler *et al.*, 2000) with a sensitivity and specificity of up to 86% and 96% respectively (Figure 39.6). However this sign appears a late feature of the disease process. Histologically confirmed cases of vCJD with minimal or absent pulvinar changes at a mean 10.5 months during an illness of mean 15 months duration were identified in this series. Figures of 81% sensitivity and 94% specificity

have also been reported in a series including 27 cases of vCJD diagnosed by tonsil biopsy (Siddique *et al.*, 2005). As these studies suggest, the pulvinar sign is not specific for vCJD. These MRI appearances are described in sporadic CJD and paraneoplastic limbic encephalitis, both of which are important considerations in the differential diagnosis of patients with suspected vCJD. Pulvinar signal change on MRI is also reported in a number of rare conditions which might otherwise be distinguished from vCJD on clinical grounds, such as benign intracranial hypertension, status epilepticus associated with cat scratch disease, Alpers’ disease and post-infectious encephalitis. The absence of pulvinar sign does not exclude a diagnosis of vCJD.

Tonsillar biopsy remains the most sensitive and specific diagnostic procedure for vCJD (Hill *et al.*, 1997b, 1999b; Hilton *et al.*, 2004b; Wadsworth *et al.*, 2001) (Figure 39.7). Tonsillar PrP^{Sc} is uniformly present in clinically affected cases of vCJD but not in other forms of human prion disease, including iatrogenic CJD associated with use of human cadaveric-derived pituitary hormones, arguing that this distinctive pathogenesis relates to effect of prion strain rather than to a peripheral route of infection (Hill *et al.*, 1999b; Hilton *et al.*, 2004b; Wadsworth *et al.*, 2001). As infection of lymphoreticular tissues is

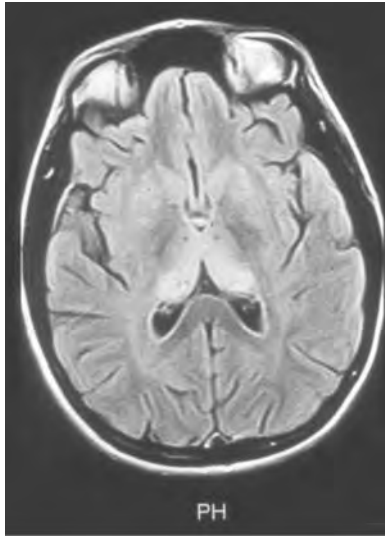


Figure 39.6 MRI signs in human prion disease: pulvinar sign in vCJD.

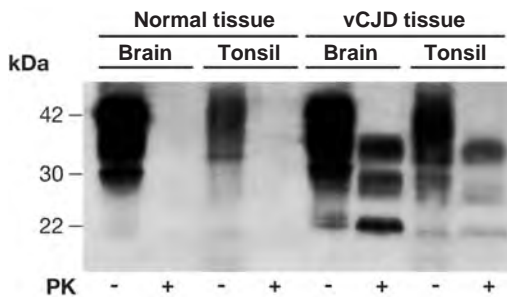


Figure 39.7 Tonsil biopsy in vCJD.

thought to precede neuro-invasion, and indeed has been detected in archived surgical samples removed prior to development of vCJD (Hilton *et al.*, 1998, 2004a), it is likely to allow firm diagnosis at the early clinical stage or indeed preclinically (Collinge, 2005b). The PrP^{Sc} type detected on Western blot in vCJD tonsil has a characteristic pattern designated type 4t. A positive tonsil biopsy obviates the need for brain biopsy which may otherwise be considered in such a clinical context to exclude alternative, potentially treatable diagnoses. CSF 14-3-3 protein may be elevated or normal. *PRNP* analysis is essential to rule out pathogenic mutations, as the inherited prion diseases present in younger patients and may clinically mimic vCJD. It is particularly important to exclude mutations prior to tonsil biopsy. Remarkably, to date all clinical cases of vCJD have been of the *PRNP* codon 129MM genotype (see section on Aetiology above).

The neuropathological appearances of vCJD are striking and relatively consistent, generally allowing differentiation from other forms of prion disease. While there is widespread spongiform change, gliosis and neuronal loss, most severe in the basal ganglia and thalamus, the most remarkable feature is abundant PrP amyloid plaques in cerebral and cerebellar cortex. These consist of kuru-like, 'florid' (surrounded by spongiform vacuoles) and multi-centric plaque types. The 'florid' plaques, seen previously only in scrapie, are a consistent feature. There is also abundant pericellular PrP deposition in the cerebral and cerebellar cortex. A further unusual feature is extensive PrP deposition in the molecular layer of the cerebellum. Western blot analysis (molecular strain typing, see section on Aetiology above) of brain tissue demonstrates PrP^{Sc} type 4 which is pathognomonic of vCJD.

Some of the features of vCJD are reminiscent of kuru, in which behavioural changes and progressive ataxia predominate. In addition, peripheral sensory disturbances are well recognized in the kuru prodrome. Kuru plaques are seen in around 70% of cases and are especially abundant in younger kuru cases. The observation that iatrogenic prion disease related to peripheral exposure to human prions has a more kuru-like than CJD-like clinical picture may well be relevant and would be consistent with a peripheral prion exposure.

The relatively stereotyped clinical presentation and neuropathology of vCJD contrasts sharply with sporadic CJD. This may be because vCJD is caused by a single prion strain and may also suggest that a relatively homogeneous genetically susceptible subgroup of the population with short incubation periods to BSE has been selected to date.

Secondary (Iatrogenic) vCJD

The prominent lymphoreticular involvement raised early concerns that vCJD may be transmissible by blood transfusion. Indeed the tissue distribution is similar to that of ovine scrapie, where prionaemia has been demonstrated experimentally. In 2004, two transfusion-associated cases of vCJD prion infection were reported amongst a small cohort of patients identified as having received blood from a donor who subsequently developed vCJD (Llewelyn *et al.*, 2004; Peden *et al.*, 2004). One patient had had a typical clinical course of vCJD although the diagnosis was not made until autopsy, and had the *PRNP* codon 129MM genotype. The second, who died of an unrelated condition, was found to have prion infection at autopsy. This patient had the *PRNP* codon 129MV genotype, which is associated with relative resistance to prion disease. Subsequently two further patients have been diagnosed with vCJD during life from this group of 23 known surviving

recipients of implicated blood. That four out of 23 patients have been infected, three dying of vCJD, in each case following transfusion with a single unit of implicated red cells, suggests the risk to recipients of blood from a silently infected donor is very substantial (Wroe *et al.*, 2006). The incubation period in the clinical cases was six to seven years. Since 2003, all known recipients of implicated blood have been notified of their status. Over 6000 individuals in the United Kingdom have been exposed to blood products prepared from large donor pools containing blood from a donor who went on to develop vCJD. None of these individuals, predominantly haemophiliacs, have yet developed vCJD.

Other Phenotypes of BSE Infection in Humans

The relatively stereotyped clinical presentation and neuropathology of vCJD contrasts sharply with sporadic CJD. This may be because vCJD is caused by a single prion strain and may also suggest that a relatively homogeneous genetically susceptible subgroup of the population with short incubation periods to BSE has been selected to date (Collinge, 1999). A widening of the recognized phenotypic range of vCJD from that based on the earliest patients can be anticipated and indeed is already emerging. Other phenotypic presentations of BSE prion infection in humans, particularly involving other *PRNP* genotypes, are to be anticipated (Hill *et al.*, 1997a; Wadsworth *et al.*, 2004). It will be important to remain open-minded about such phenotypes. Recent studies in transgenic mice expressing human PrP have shown that BSE infection can result in two distinct phenotypes, one with the neuropathological and molecular phenotype of vCJD and a second with the molecular phenotype of the commonest subtype of sporadic CJD (associated with PrP^{Sc} type 2) (Asante *et al.*, 2002). This raises the possibility that BSE infection of humans could also cause some cases of apparently sporadic CJD.

Inherited Prion Diseases

Gerstmann—Sträussler—Scheinker Disease

The first case was described by Gerstmann in 1928 and was followed by a more detailed report on seven other affected members of the same family in 1936 (Gerstmann *et al.*, 1936). The classical presentation of GSS is with a chronic cerebellar ataxia accompanied by pyramidal features, with dementia occurring later in a much more prolonged clinical course than that seen in CJD. The mean duration is around five years, with onset usually in either the third or fourth decades. Histologically, the hallmark is the presence of multicentric amyloid plaques. Spongiform change, neuronal loss, astrocytosis and white matter

loss are also usually present. Numerous GSS kindreds from several countries (including the original Austrian family described by Gerstmann *et al.*, 1936) have now been demonstrated to have mutations in the PrP gene. GSS is an autosomal dominant disorder, which can now be classified within the spectrum of inherited prion disease.

Inherited Prion Diseases

The identification of one of the pathogenic PrP gene mutations in a case with neurodegenerative disease allows not only molecular diagnosis of an inherited prion disease but also its subclassification according to mutation (Figure 39.8). Over 30 pathogenic mutations are reported in the human PrP gene and consist of two groups: (i) point mutations within the coding sequence resulting in amino acid substitutions in PrP or production of a stop codon resulting in expression of a truncated PrP and (ii) insertions encoding additional integral copies of an octapeptide repeat present in a tandem array of five copies in the normal protein (octapeptide repeat insertion (OPRI)). A suggested notation for these diseases is 'inherited prion disease (PrP mutation)', for instance: inherited prion disease (PrP 6 OPRI) or inherited prion disease (PrP P102L). Brief details of the more commonly seen types are given below (for a more comprehensive review see Collinge, 2005a). *PRNP* analysis should be considered in all early-onset dementing or ataxic disorders and is available from the UK National Prion Clinic (see <http://www.nationalprionclinic.org>).

PrP P102L This mutation was first reported in 1989 in a UK and US family and has now been demonstrated in many other kindreds worldwide. Progressive ataxia is the dominant clinical feature, with dementia and pyramidal features. However marked variability both at the clinical and neuropathological level is apparent in some families. A family with marked amyotrophic features has also been reported and cases with severe dementia in the absence of prominent ataxia are also recognized.

PrP A117V This mutation has been described in families from France, the United States and the United Kingdom. The clinical features are pre-senile dementia associated with pyramidal signs, parkinsonism, pseudobulbar features and cerebellar signs. Parkinsonian features may predominate in the early stages and mimic Parkinson's disease.

PrP D178N This mutation was originally described in two Finnish families with a CJD-like phenotype and has since been demonstrated in families in Hungary, the

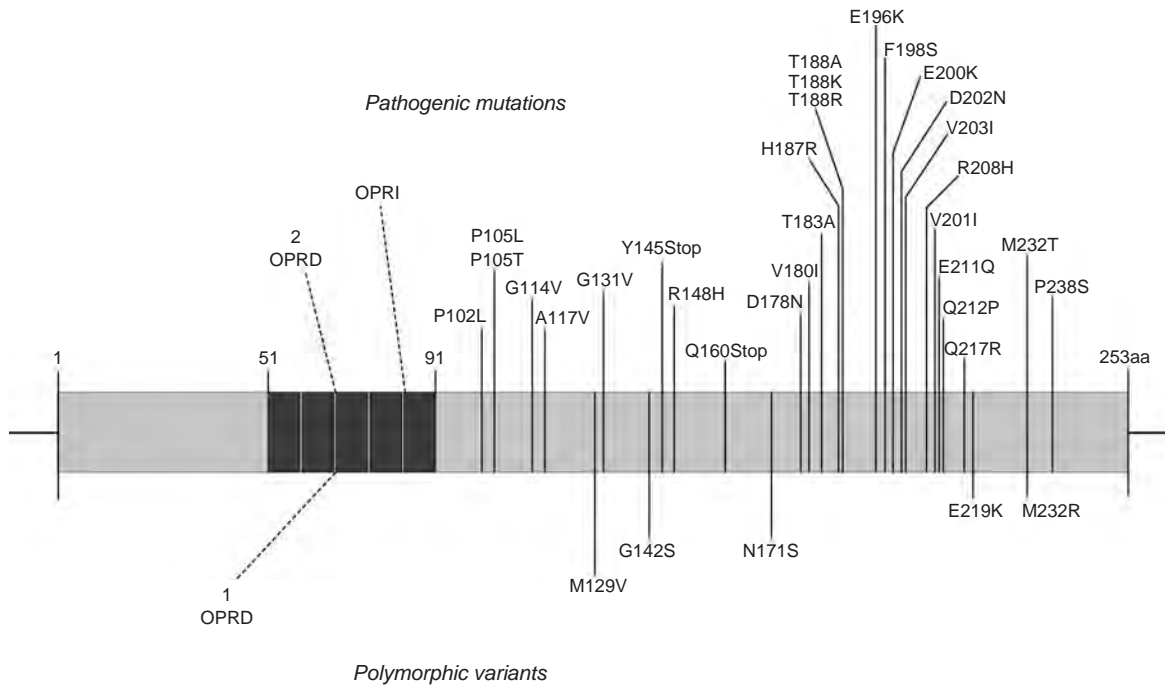


Figure 39.8 Mutations and polymorphisms in the human prion protein gene (*PRNP*). OPRI, octapeptide repeat insertion; OPRD, octapeptide repeat deletion.

Netherlands, Canada, Finland, France and the United Kingdom. This mutation was also reported in two unrelated families with FFI (Medori *et al.*, 1992a). The first cases described had a rapidly progressive disease characterized clinically by untreatable insomnia, dysautonomia and motor signs, and neuropathologically by selective atrophy of the anterior-ventral and medio-dorsal thalamic nuclei. Proteinase K treatment of extracted PrP^{Sc} from FFI cases has shown a different-sized PrP band on Western blots than PrP^{Sc} from CJD cases, suggesting that FFI may be caused by a distinct prion strain type. In a recent study, Goldfarb *et al.* (1992) reported that in all the codon 178 families they studied with a CJD-like disease the codon 178 mutation was encoded on a valine 129 allele while all FFI kindreds encode the same codon 178 mutation on a methionine 129 allele. They suggested that the genotype at codon 129 determines phenotype. Insomnia is not uncommon in CJD patients and FFI and CJD may represent extremes of a spectrum of related disease phenotypes. Recently an inherited case with the E200K mutation, which is normally associated with a CJD-like phenotype, has been reported with an FFI phenotype. An Australian family has also been reported with the FFI genotype but in which affected family members have a range of phenotypes encompassing typical

CJD, FFI and an autosomal dominant cerebellar ataxia-like illness.

PrPE200K This mutation was first described in families with CJD. Affected individuals develop a rapidly progressive dementia with myoclonus and pyramidal, cerebellar or extrapyramidal signs and a duration of illness usually less than 12 months. In marked contrast to other variants of inherited prion disease, the EEG usually shows the characteristic pseudoperiodic sharp wave activity seen in sporadic CJD. Interestingly, this mutation accounts for the three reported ethnogeographic clusters of CJD where the local incidence of CJD is around 100-fold higher than elsewhere (amongst Libyan Jews and in regions of Slovakia and Chile) (Brown *et al.*, 1992b; Goldfarb *et al.*, 1990). Now that cases can be diagnosed by PrP gene analysis, atypical forms of this condition are being detected with phenotypes other than that of classical CJD. Of interest also are reports that peripheral neuropathy can occur in this disease. Elderly unaffected carriers of the mutation have been reported. Patients with this condition have now been reported in several other countries outside the well-recognized clusters, including the United Kingdom. At least one of the UK cases does not appear to relate to the ethnogeographic clusters mentioned above,

suggesting a separate UK focus for this type of inherited prion disease.

PrP 6 OPRI This was the first PrP mutation to be reported and was found in a small UK family with familial CJD (Owen *et al.*, 1989) now known to form part of the largest known kindred with an inherited prion disease caused by an OPRI mutation. The diagnosis in the family had been based on an individual who died in the 1940s with a rapidly progressive illness characteristic of CJD. The reported duration of illness was six months. Pathologically there was gross status spongiosis and astrocytosis affecting the entire cerebral cortex, and this case is used to illustrate classic CJD histology in Greenfield's *Neuropathology*. However, other family members had a much longer duration GSS-like illness. Histological features were also extremely variable. This observation led to screening of various case of neurodegenerative disease and to the identification of a case classified on clinical grounds as familial Alzheimer's disease (Collinge *et al.*, 1989). More extensive screening work identified further families with the same mutation which were then demonstrated by genealogical studies to form part of an extremely large kindred (Collinge *et al.*, 1992; Mead *et al.*, 2006; Poulter *et al.*, 1992). Clinical information has been collected on over 80 affected individuals over seven generations. Affected individuals develop in the third to fourth decade onset of a progressive dementia associated with a varying combination of cerebellar ataxia and dysarthria, pyramidal signs, myoclonus and occasionally extrapyramidal signs, chorea and seizures. The dementia is often preceded by depression and aggressive behaviour. A number of cases have a long-standing personality disorder, characterized by aggression, irritability, antisocial and criminal activity and hypersexuality which may be present from early childhood, long before overt neurodegenerative disease develops. The histological features vary from those of classical spongiform encephalopathy (with or without PrP amyloid plaques) to cases lacking any specific features of these conditions (Collinge *et al.*, 1990). Age at onset in this condition can be predicted according to genotype at polymorphic codon 129. Since this pathogenic insertional mutation occurs on a methionine 129 PrP allele, there are two possible codon 129 genotypes for affected individuals, methionine 129 homozygotes or methionine 129/valine 129 heterozygotes. Heterozygotes have an age at onset which is about a decade later than homozygotes (Poulter *et al.*, 1992).

MOLECULAR DIAGNOSIS OF PRION DISEASE

While sporadic CJD can often, following the exclusion of other causes, be diagnosed with a high degree of

confidence on the basis of clinical criteria, atypical forms, which present much greater diagnostic difficulty, are not uncommon (Collinge, 1998). A widening of the recognized phenotypic range of vCJD from that based on the earliest patients can be anticipated and indeed is already emerging. Other phenotypic presentations of BSE prion infection in humans, particularly involving other *PRNP* genotypes, are to be anticipated. Furthermore, current clinically based diagnostic criteria for vCJD used for surveillance require the evolution of disease over at least six months and the development of several signs indicative of extensive cerebral damage. However, early diagnosis, before extensive irreversible brain damage has occurred, is crucial in such patients as they be suffering from an alternative treatable disorder. Brain biopsy may well be considered, particularly in younger patients, to exclude such conditions as cerebral vasculitis. Early tonsil biopsy, if positive, obviates the need for further investigation. The need for early, specific diagnosis is now further emphasized by the arrival of potential therapies and clinical trials for CJD. The early clinical features of vCJD—depression, anxiety, behavioural change and sensory disturbances—are highly nonspecific. Differentiation from much commoner psychiatric causes requires the arrival of overtly neurological features such as ataxia, chorea and cognitive decline, although pre-existing use of neuroleptics and other psychotropic drugs may initially delay their diagnostic recognition. While the diagnostic accuracy provided by a tonsil biopsy has to be balanced against the fact that it is an invasive procedure, early referral for investigation should allow much earlier diagnosis and access to clinical trials before extensive functional loss has occurred.

While neurologists have until recent years had to rely largely on clinical features to differentiate neurodegenerative disorders, the major advances in molecular genetics and in understanding molecular pathogenesis increasingly enable diagnosis using criteria higher in the diagnostic hierarchy of pathology. Around 15% of recognized prion disease is an inherited mendelian disorder associated with one of the more than 30 recognized coding mutations in *PRNP* (Collinge, 2001). For a single gene inherited disorder of high penetrance, such as inherited prion disease, the diagnostic supremacy of direct demonstration of causative mutation by DNA analysis is clear. Indeed, the availability of such definitive diagnostic markers has long allowed diagnosis of inherited prion disease in patients not only atypical on clinical grounds, but in whom classical neuropathological features are absent (Collinge *et al.*, 1989, 1990). Kindreds are documented in which some individuals have the classical syndromes of 'CJD' and 'Gerstmann—Sträussler—Sheinker disease' while others do not fit these rubrics at all (Collinge *et al.*, 1992).

Neuropathology in such patients is no longer the 'gold standard': rather the recognized clinicopathological manifestation of a particular inherited condition simply widens.

The acquired prion diseases, such as vCJD, although not contagious in humans, are infectious diseases. In infectious disease, while again clinical and histopathological features may be key, confirmation of diagnosis, not least in life-threatening conditions, is by identification of the infectious pathogen itself or a specific immune response to it. Isolation and strain typing of the pathogen is at the apex of the diagnostic hierarchy. Strain typing in particular may allow the source of an outbreak to be identified and the best available prognostic and therapeutic advice to be provided. While it is essential to balance the potential risks and discomfort involved in an invasive diagnostic test against the improved diagnostic accuracy, it will only be by progressing steadily to greater use of molecular analysis of neurological disease that we will be able to deliver the diagnostic and ultimately therapeutic advances to patients with neurodegenerative diseases that are so desperately needed.

PRE-SYMPTOMATIC AND ANTENATAL TESTING

Direct gene testing allows unequivocal diagnosis in patients with inherited forms of the disease and pre-symptomatic testing of unaffected but at-risk family members, as well as antenatal testing (Collinge *et al.*, 1991b). Because of the effect of *PRNP* codon 129 genotype on the age of onset of disease associated with some mutations it is possible to determine within a family whether a carrier of a mutation will have an early or late onset of disease. Most of the mutations appear to be fully penetrant, however experience with some is extremely limited. In some families, for example with E200K or D178N (FFI), there are examples of elderly unaffected gene carriers who appear to have escaped the disease. Genetic counselling is essential prior to pre-symptomatic testing and follows a protocol similar to that established for Huntington's disease. A positive PrP gene analysis has important consequences for other family members, and it is preferable to have discussed these issues with others in the immediate family before testing. Following the identification of a mutation the wider family should be referred for genetic counselling. It is vital to counsel both those testing positive for mutations and those untested but at-risk that they should not be blood or organ donors and should inform surgeons, including dentists, of their risk status prior to significant procedures as precautions may be necessary to minimize risk of iatrogenic transmission.

PREVENTION AND PUBLIC HEALTH MANAGEMENT

While prion diseases can be transmitted to experimental animals by inoculation, it is important to appreciate that they are not contagious in humans. Documented case-to-case spread has only occurred by cannibalism (kuru) or following accidental inoculation with prions. Such iatrogenic routes include the use of inadequately sterilized intracerebral electrodes, dura mater and corneal grafting, and from the use of human cadaveric pituitary-derived growth hormone or gonadotrophin. As discussed above, there is now evidence that vCJD prion infection is transmissible by blood transfusion. UK policy for some time has been to leuco-deplete all whole blood and to source plasma for plasma products from outside the United Kingdom.

A further possible route of transmission of vCJD is via contaminated surgical instruments. Prions resist conventional sterilization methods and neurosurgical instruments are known to be able to act as a vector for prion transmission: several cases of iatrogenic transmission of sporadic CJD prions via neurosurgical instruments are documented (Bernoulli *et al.*, 1977; Blattler, 2002). Recent evidence suggests that classical CJD may also be transmitted by other surgical procedures (Collins *et al.*, 1999). In the United Kingdom, all surgical instruments used on patients with suspected CJD are quarantined and not re-used unless an alternate non-prion diagnosis is unequivocally confirmed.

The pathogenesis of vCJD differs sharply from that of sporadic and other forms of 'classical' CJD. In particular, in vCJD there is extensive involvement of the LRS (lymph nodes, tonsil and spleen) with the highest levels of PrP^{Sc} outside the CNS being found in tonsil, where levels are typically 5–10% of brain levels (Wadsworth *et al.*, 2001). PrP^{Sc} is also detectable, at lower levels, in the thymus, rectum, adrenal and retina in vCJD (Wadsworth *et al.*, 2001). This wider tissue distribution raises further concerns about iatrogenic transmission of vCJD via surgical instruments previously used on patients with pre-clinical vCJD prion infection. The number of individuals incubating vCJD, but asymptomatic currently, is unknown but may be substantial. Prions adhere avidly to stainless steel and transmit the disease readily in experimental models (Flechsigs *et al.*, 2001). Tonsillar PrP^{Sc} is readily detectable in all cases of vCJD studied at autopsy and lymphoreticular involvement is a very early feature of natural prion infection in sheep and in experimental scrapie models, where replication in the LRS is detectable early in the incubation period and rises to a plateau, which considerably precedes, and is maintained in, the clinical phase (Fraser *et al.*, 1992). This suggests that tonsillar

PrP^{Sc} has probably been present for a considerable period, perhaps years, before clinical presentation of vCJD in humans and therefore tonsil biopsy should enable diagnosis at the earliest stages of clinical suspicion. In addition, this pathogenesis forms the basis of prevalence screening of the general population for infection. A retrospective study of archived surgical lymphoreticular specimens estimated a much higher prevalence of infection in the UK population of 237 per million (95% confidence interval 49–692 per million) (Hilton *et al.*, 2004b) than the number of clinical cases would so far suggest. This worrying finding led to the Chief Medical Officer commissioning a much larger, prospective, national-scale anonymous screen of discarded tonsillectomy tissue. However, it must be appreciated that the sensitivity of such tests at different points in the prolonged incubation period and in different genotypes is unknown so that such studies may underestimate significantly the prevalence of vCJD prion infection in the community.

Certain occupational groups are at risk of exposure to human prions, for instance neurosurgeons and other operating theatre staff, pathologists and morticians, histology technicians, as well as an increasing number of laboratory workers. Because of the prolonged incubation periods to prions following administration to sites other than the CNS, which is associated with clinically silent prion replication in the lymphoreticular tissue, treatments inhibiting prion replication in lymphoid organs may represent a viable strategy for rational secondary prophylaxis after accidental exposure. A preliminary suggested regimen is a short course of immunosuppression with oral corticosteroids in individuals with significant accidental exposure to human prions (Aguzzi and Collinge, 1997).

PROGNOSIS AND TREATMENT

All recognized prion diseases are invariably fatal following a progressive course. The duration of illness in sporadic patients is very short, with a mean duration of three to four months, although in some of the inherited cases the duration can be 20 years or more. There have been significant recent advances in understanding prion propagation and neurotoxicity and clear proof of principle studies of several therapeutic or secondary prophylactic approaches in animal models, suggesting effective therapeutics for human disease is realistic (Mallucci and Collinge, 2005).

A variety of drugs have been tried in individual or small numbers of patients over many years. There is no clear evidence of efficacy of any agent, and controlled clinical trials are needed. Such trials are highly challenging. Prion diseases are rare, often rapidly progressive and always fatal, which may make randomization to placebo

unacceptable. Patterns of disease overall are extremely variable with clinical durations varying from weeks to more than two years in sporadic CJD, and >20 years in some inherited prion diseases. As ‘first generation’ treatments proposed for prion disease are likely, at best, to have only a modest effect on disease progression, even using survival duration as an outcome measure requires study of large numbers to reliably assess efficacy. There is a lack of systematic natural history studies of disease progression and an absence of biological markers of disease activity. In the United Kingdom, at the request of the Government’s Chief Medical Officer, a clinical trial protocol (www.nationalprionclinic.org) and infrastructure has been developed to rigorously assess the drug quinacrine (Korth *et al.*, 2001) and to provide a framework for assessment of novel therapeutics as these become available: the Medical Research Council (MRC) PRION-1 trial. Importantly under these circumstances, a formal consultation with patient’s representatives was organized to refine the protocol so that it would be acceptable to the majority of potential participants (www.nationalprionclinic.org). Pentosan polyphosphate is another candidate anti-prion drug and has shown some efficacy in animal models. Unlike quinacrine, it does not enter the CNS readily and has been administered by intraventricular infusion in several patients. Major toxicity has been reported by this route in animal studies and such treatment was not supported by the UK’s Committee of Safety on Medicines or CJD Therapy Advisory Group. A report summarizing clinical experience to date with this treatment has been produced (Medical Research Council, 2006).

While the precise molecular events in prion propagation are not clear, it is clear that PrP^C is the essential substrate. Interference with PrP^C expression in adult brain is without serious effect and blocks onset of neurological disease in animal models (Mallucci and Collinge, 2005). It should be possible to identify small molecules which penetrate the CNS to bind to PrP^C and to prevent its recruitment into prions, or to use one of a number of emerging technologies to reduce PrP^C expression in brain. If such methods are able to reduce prion propagation rates to below those of natural clearance mechanisms it ought to be possible to cure prion infection. New methods for early diagnosis—and their timely use—will be crucial, as such methods will not reverse neuronal cell loss which is appreciable or severe by the time clinical diagnosis is typically reached. Proof of principle studies in animal models suggest that humanized anti-PrP monoclonal antibodies could be used for passive immunization in the early pathogenesis to block neuro-invasion. This treatment could be considered for known iatrogenically infected individuals (Wroe *et al.*, 2006).

CONCLUDING REMARKS

Prion diseases appear to be diseases of protein conformation and elucidating their precise molecular mechanisms may, in addition to allowing us to progress with tackling key public health issues posed by vCJD, be of wider significance in pathobiology. Common themes are emerging in neurodegenerative diseases, many of which are also associated with aggregates of misfolded protein. Also, the apparent ability of a single polypeptide chain to encode information and specify distinctive phenotypes is unprecedented and evolution may have used this mechanism in many other ways. While the protein-only hypothesis of prion propagation is supported by compelling experimental data, and now appears also able to encompass the phenomenon of prion strain diversity, the goal of the production of high-titre prions *in vitro* and atomic-level structures of infectious prion strains remains.

USEFUL WEBSITES

UK National Prion Clinic, National Hospital for Neurology and Neurosurgery, London <http://www.nationalprionclinic.org>

Medical Research Council Prion Unit, Institute of Neurology, London <http://www.prion.ucl.ac.uk/>

UK CJD Surveillance Unit, Western General Hospital, Edinburgh <http://www.cjd.ed.ac.uk/>

UK Department of Health <http://www.dh.gov.uk/PolicyAndGuidance/HealthAndSocialCareTopics/CJD/fs/en>

CJD Support Network <http://www.cjdsupport.net/>

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Subject Index

Notes:

Entries for the major viruses and the resultant infections have been kept separate for convenience. In such cases, antibodies, diagnostic techniques and vaccines have been listed under the entry relating to the infection, not the virus.

Page numbers in *italics* refer to figures and tables.

This index is in letter-by-letter order, whereby spaces and hyphens between words are excluded from alphabetization (e.g. B lymphocytes comes after blue river virus, and not at the start of the 'B' section)

Abbreviations:

3TC *see* lamivudine (3TC)

CCHF - Crimean-Congo haemorrhagic fever

CMV - cytomegalovirus

EEEV - Eastern equine encephalitis virus

HAM/TSP - HTLV-associated

myelopathy/tropical spastic paraparesis

HBV - hepatitis B virus

HCV - hepatitis C virus

HCWs - health-care workers

HHV - human herpesvirus (e.g. HHV-6)

HIV - human immunodeficiency virus

HPIV - human parainfluenza virus

HPV - human papillomavirus

HSCT - haematopoietic stem cell transplant

HSV - herpes simplex virus

KSHV - Kaposi's sarcoma-associated
herpesvirus (HHV-8)

MCD - multicentric Castleman's disease

PCR - polymerase chain reaction

PIV - parainfluenza virus

PML - progressive multifocal
leukoencephalopathy

PTLD - post-transplant lymphoproliferative
disease

SSPE - subacute sclerosing panencephalitis

TK - thymidine kinase

VEEV - Venezuelan equine encephalitis virus

WEEV - Western equine encephalitis virus
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