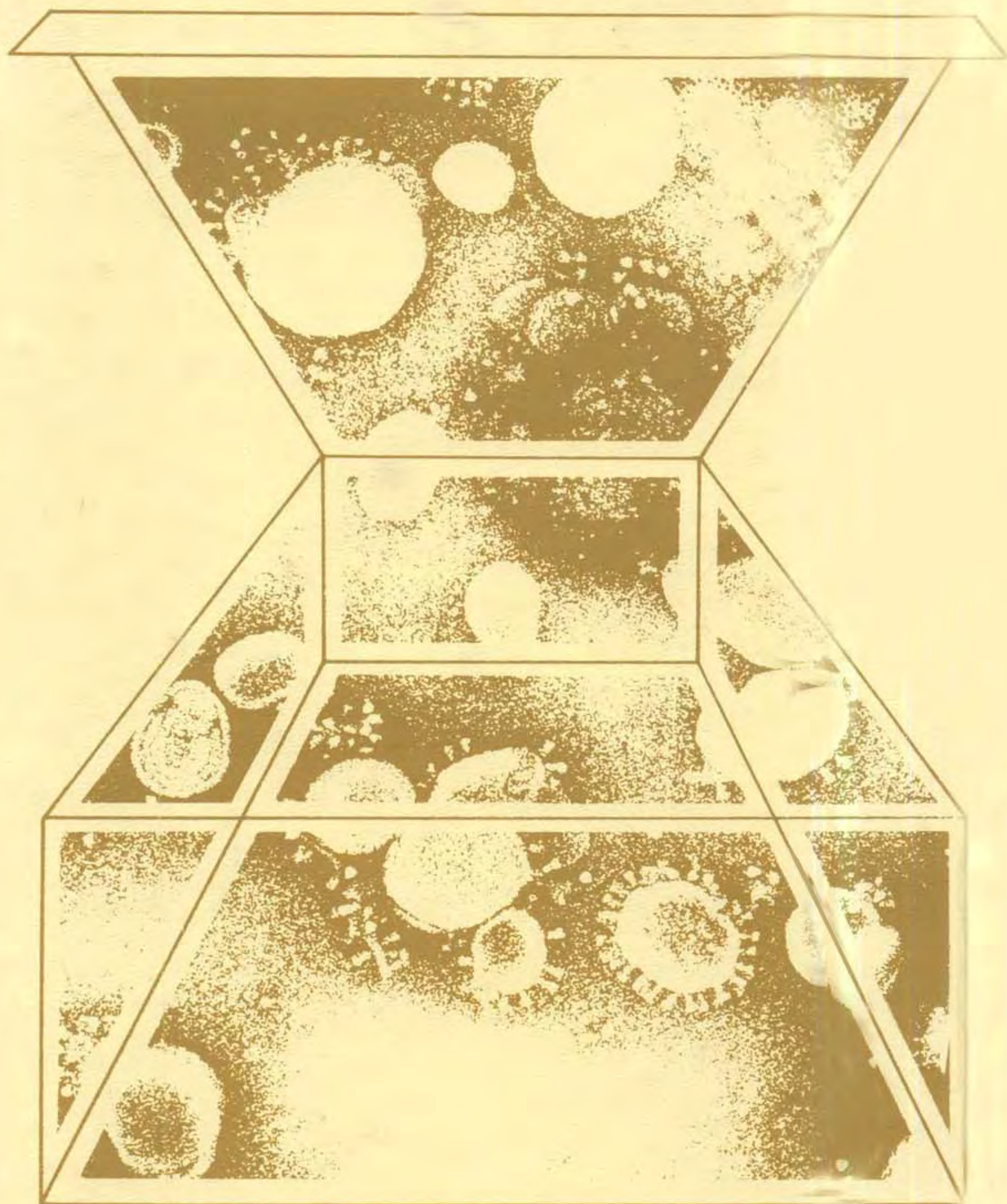
CONQUEST OF VIRAL DISEASES



A Topical Review of Drugs and Vaccines

J. S. Oxford ELSEVIER B. Öberg

Conquest of Viral Diseases

A Topical Review of Drugs and Vaccines

Perspectives in Medical Virology

Volume 1

General Editor

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Conquest of Viral Diseases

A Topical Review of Drugs and Vaccines

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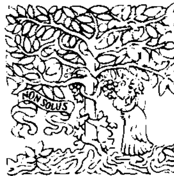

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Preface

The recent explosive progress in medical virology resulted to a large extent from rapid advances in recombinant DNA techniques, in molecular and cell biology, in protein and nucleic acid chemistry, the use of monoclonal antibodies, progress in antiviral research and the application of modern technology to fundamental biological processes. These advances in knowledge of structural and biochemical components of viruses and mode of viral replication, understanding of cell pathology and immunopathogenesis have many health implications which include rapid, precise and specific diagnosis, epidemiology of viral infections, and the development of new types of vaccines. Strengthening of the links between the laboratory, field work and the clinician is essential for implementing the strategy of the World Health Organisation's programme of health for all by the year 2000. Infectious diseases – and viral infections – are still responsible for most of the problems encountered daily in primary health care in many parts of the world.

The number of new journals introduced during the last decade and the avalanche of information have made it virtually impossible for microbiologists, epidemiologists, pathologists, scientists and physicians to keep abreast of the scientific literature. We recognise the age of rapid dissemination of information retrieval by computerised systems, the ever increasing published proceedings of meetings and symposia, abstract and rapid literature surveys, but it is secondary literature in the form of review series which has become useful as sources of information. However, their value depends intrinsically on authoritative and critical evaluation of the original data by experts in particular topics. The series on Perspectives in Medical Virology was conceived after many discussions with teachers of postgraduate students, research workers, medical virologists, students and many colleagues.

A number of distinguished practising virologists were then invited to assemble the important information available and to integrate research at the basic level with

clinical practice in selected subjects for the new series. We hope that in this way the series will promote dissemination of information, useful discussion and exchange of ideas and stimulate further interest and research in medical virology.

Finally, I am happy to acknowledge the enthusiastic support of the authors, of my friends and colleagues and of the Publishers and in particular the help of Dr Louis Ter Meer.

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Foreword

The control of viral diseases has been a long crusade for the biomedical investigator. Two approaches have evolved for the control of viral infections. First, since the successful development of a smallpox vaccine by Jenner over two centuries ago, efforts directed at the control of viral infections have focused for the most part on prophylaxis by vaccination. Significant advances in vaccination have been achieved for the prevention of rubella, measles, polio, and rabies. These accomplishments have had a major impact on worldwide public health. Even more notably, we have witnessed the eradication of smallpox through a combined effort co-ordinated by the World Health Organization. However, the physician today encounters viral diseases for which contemporary technology has not provided quick and simple mechanisms of prevention. The unique propensities of some viruses and social limitations have not allowed for the proper application of some existing vaccines. From a social organization standpoint, the simple lack of uniform refrigeration in many Third World countries and the inordinate costs of vaccine preparation, deployment and delivery in man hours have prevented successful universal immunization against measles and polio. Advances in viral genetics and the biochemistry of both viral replication and host response are leading to the development of immunogens which, hopefully, will be of clinical benefit, albeit many years from realization. These immunogens have been predicated upon utilization of contemporary viral genetic engineering, the synthetic production of immunogens and the cloning of key microbial elements.

Drs. Oxford and Öberg have taken a broad and comprehensive view of the complex problems encountered in developing prophylaxis of viral diseases. In so doing, the vaccine successes of smallpox, measles and polio are contrasted with the inherent problems encountered in the development of vaccines to prevent herpesvirus, influenza, and rhinovirus infections. Because of these inherent difficulties, alter-

native methods for the control of viral diseases have led to the second approach, namely, utilization of therapeutics as chemotherapy, interferon, or biologic response modifiers. The herpesviruses as a group have spearheaded the development of antiviral chemotherapeutics.

Antiviral therapy for human viral infections has evolved slowly over the past twenty-five years and far more so than the development of vaccines. The first series of antiviral agents tested in clinical trials arose from screening programmes for the development of anti-cancer drugs. These drugs were identified as 'first generation' antiviral agents because of their non-selective inhibition of both host cell and viral replication and include idoxuridine, cytosine arabinoside and vidarabine. In the early 1960s, idoxuridine was shown to have clinical activity for topical therapy of herpes simplex keratoconjunctivitis in animals and humans. This observation set the stage for the realization that antiviral therapy could be effective for the treatment of human disease. While idoxuridine was under development, amantadine, a cyclic amine, was established as useful for prevention of influenza A infections. As recently as five years ago, little clinical attention was paid to amantadine, in spite of clinical efficacy, because of the concern for potential toxicity following prophylactic administration. However, many of these concerns have been alleviated with its use as a therapeutic for Parkinson's disease. More recently, rimantidine has attracted clinical interest because of less toxicity.

During the 1970s, vidarabine was shown to be effective against life-threatening human viral infections, being proved useful for the treatment of herpes simplex encephalitis, varicella-zoster virus infections in the immunocompromised host (shingles and chickenpox), and neonatal herpes simplex virus infection.

During the late 1970s, the first of the 'second generation' antiviral drugs, namely selective inhibitors of viral replication, began to enter clinical investigations. Acyclovir is the prototype of these drugs, being selectively activated by an enzyme unique to herpesvirus replication, herpes simplex thymidine kinase. Subsequently, it has been shown useful for the treatment of primary genital herpes simplex virus infections and severe progressive mucocutaneous herpes simplex virus infections when administered by either parenteral or topical routes. The recognition of acyclovir's ability to inhibit herpesvirus replication was a fortuitous observation rather than one strategically planned to utilize enzymes unique to herpesvirus replication. Nevertheless, the scientific advances offered by the synthesis and development of acyclovir for treatment of human herpesvirus infections are significant. In essence, its development will allow for the more rational development of antiviral agents in the future.

Other second generation compounds are receiving attention for treatment of herpesvirus infections. These include phosphonoformate – a compound being extensively studied for herpes simplex and cytomegalovirus infections in Sweden; bromovinyl deoxyuridine – a compound currently under evaluation for shingles and chickenpox in Belgium; and ribavirin – a compound being assessed for treatment

of respiratory syncytial virus and influenza infections as well as some of the more common herpesvirus infections. In the immediate future, additional analogues of acyclovir such as dihydroxypropoxy methyl guanine will be entering phase I clinical evaluations. Thus, a great deal of interest has evolved for the development of antiviral drugs both in academia and in industry.

In spite of the advances in the treatment of herpesvirus infections, several pieces of the puzzle leading to successful therapy of human viral disease remain strikingly elusive. These pieces are applicable to the successful therapy of all viral infections and not just those of the herpesvirus family. One significant missing piece of data is our lack of understanding of the mechanisms by which all herpesviruses establish latency and subsequently recur. Even with the introduction of antiviral therapy early in the disease course for primary genital herpes simplex virus infections, it has not been possible to significantly alter the frequency of recurrences let alone completely prevent them, regardless of the mode of drug delivery. Thus, although therapy may be of clinical benefit for the acute episode of disease, it does not alter the subsequent frequency or severity of recurrences. The development of medications to achieve this endpoint will require strategies different from those currently available. For other viruses, a better understanding of the common mechanisms of rhinovirus replication is essential for the development of specific drugs as the numerous serotypes preclude utility of a vaccine.

Since most viral infections lead to peak progeny viral production prior to the onset of clinical symptomatology, it becomes encumbent upon the biomedical investigator to identify diagnostic procedures which would allow for early and specific identification of patients with infection. Utilization of contemporary principles of molecular biology for the development of specific antiviral agents and for rapid diagnostics will likely redefine the natural history of many human viral infections, allowing the selection of populations at high risk for development of infection and for whom prophylactic antivirals or improved vaccines might prove useful. Examples would include family members at risk for development of rhinovirus or coronavirus infection, sexual partners of individuals with genital herpes simplex virus infection, or immunocompromised patients at risk for development of cytomegalovirus infection. If diagnostic procedures are improved, prophylactic antivirals might prove useful.

Drs. Oxford and Öberg's book addresses these issues in detail, providing insight in the directions mandatory for further improvement of the control of viral infections. Our current understanding of viral infections and the need to develop drugs which act at specific targets of viral replication can only lead to further improvement in the treatment and management of viral infections of humans. These insights will require the careful melding of talents of both the basic scientist and the clinical investigator.

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Introduction

We have attempted to write a book totally orientated towards *prevention and therapy* of viral disease, either by antivirals or vaccines. There are many excellent text books and reviews of basic and molecular virology, viral diagnosis of infectious disease and immunological effects of virus disease but, more often than not, in these texts, vaccines and antivirals are mentioned *en passant* at the end of the book and certainly only briefly. 'Vaccinology' encompassing new developments in immunogenic short peptides, genetically cloned viral antigens and site-specific mutagenesis to produce live attenuated viruses is a fast moving field; so is antivirals, with exciting developments with new and effective compounds inhibiting herpes viruses. It is an opportune moment to look in a broad way at viral diseases, to see how much progress has been made, how far there is to go and in what direction. Viewed in this way, the achievements have already been many and varied. But there have been some serious setbacks as well and these should be thoroughly comprehended and are discussed in this book.

Effective vaccines have existed for two decades or more against polio, measles, rubella, rabies and flaviviruses and we have all witnessed the successful eradication of smallpox. So we seem at first sight to be left with the more 'difficult' viruses such as herpes (with latency and disease in spite of immunity) or influenza (with antigenic drift and shift) or rhinoviruses and arboviruses (with many antigenically different serotypes). But life (and particularly microbial life) is not as simple as this: although polio and measles vaccines are successful in industrialized countries there are major problems in underdeveloped countries of the world. Live polio virus vaccines have had some setbacks in these regions, whilst some newly developed vaccines such as rabies are too expensive for widespread use in some poorer countries. Also, some European countries have not made any serious attempt to control measles and rubella, using vaccines. 'No man is an island' and so many of these infective viral dis-

eases must be confronted as 'international diseases', with no frontiers. Influenza moves rapidly between China, USSR and the USA, knowing no political barrier! If in a short time measles is eradicated in the USA then continual vaccination will be essential because of new introductions by travellers from Europe.

We have tried to give a perspective to some of these viral diseases and their problems and characteristics related to a strategy of control or prevention, and give the reader an up-to-date analysis of a wide range of viruses, a recent history of preventative and therapeutic measures and, more difficult, an estimate of prospects for the future. However, this is not a textbook in bedside medicine and we have not included other aspects than those concerning specific prevention and therapy. The scope of this book has made it necessary to discuss subjects where we lack personal experience and we apologize to the specialists for the mistakes we have made, hoping that they are not distorting to the main theme. We suggest that the reader, particularly the non-specialist, should read the more general chapters on vaccines and antivirals and drug resistance before plunging into the deep end of herpes or myxovirus virology. The reference lists may look frightening – may look indeed as if *everything* is known about human viruses. This is certainly not the case – quite the opposite in fact. Like history, medical and molecular virology has to be continually rewritten and re-interpreted. We are not dealing with a static subject. This book is not intended as a bible or article of faith but an introduction to a fascinating and expanding area of study. A wide range of people will contribute (as they did with smallpox) to the demise of certain of these viruses, not simply scientists and clinicians. We therefore encourage and urge non-specialists particularly to persevere with the text and are confident that you will find it worthwhile!

John S. Oxford
Bo Öberg

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Abbreviations and Synonyms

2′,5′-A	(2′,5′)-oligoisoadenylate triphosphate
AAV	adeno associated virus
ACG	9-(2-hydroxyethoxymethyl)guanine, acycloguanosine, acyclovir, ACV
ACGMP	ACG monophosphate
ACV	ACG
AdThd	5′-amino-5′-deoxythymidine
AHC	acute haemorrhagic conjunctivitis
AIDS	acquired immune deficiency syndrome
AIU	5′-amino-2′,5′-dideoxy-5-iodouridine
AMV	avian myeloblastosis virus
ara-A	9-β-D-arabinofuranosyladenine
ara-AMP	ara-A 5′-monophosphate
ara-C	1-β-D-arabinofuranosylcytosine
ara-CTP	ara-C 5′-triphosphate
ara-T	1-β-D-arabinofuranosylthymidine
ATG	antithymocyte globulin
Biolf 62	DHPG
BK virus	human polyoma virus
bp	base pairs
BTH	butylhydroxytoluene, 2,6-di-tert-butyl-4-methylphenol
BVara-U	E-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil
BVDU	E-5-(2-bromovinyl)-2′-deoxyuridine
BVDUTP	BVDU 5′-triphosphate
ca	cold adapted
CCID$_{50}$	cell culture infectious dose (50%)
CEF	chick embryo fibroblast cells
CHO	carbohydrate
CMI	cell mediated immunity
CMV	cytomegalovirus
CNS	central nervous system
cRNA	complementary RNA
CSF	cerebrospinal fluid
17D	vaccine strain of yellow fever virus
3-DG	3-deazaguanine
dGTP	2′-deoxyguanosine 5′-triphosphate
DHBG	9-(3,4-dihydroxybutyl)guanine
DHF	Dengue haemorrhagic fever
DHPG	(9-[(2-hydroxy-1-hydroxymethyl)-ethoxy)methyl]guanine, 2′NDG, Biolf 62
DI	defective interfering particles
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DP	diphosphate
ds	double-stranded
EBV	Epstein-Barr virus
ED$_{50}$	effective dose (50%)
EDU	5-ethyl-2′-deoxyuridine
EEV	Eastern equine encephalitis virus
EHNA	erythro-9-(2-hydroxy-3-nonyl)-adenine
eIF-2	eukaryotic peptide initiation factor 2
ELISA	enzyme linked immunosorbent assay

EM electron microscopy
F fusion protein
FANA 2-deoxy-2,3-dehydro-N-trifluoro-acetyl-neuraminic acid
FIAC 1-(2-fluoro-2-deoxy-β-D-arabino-furanosyl)-5-iodocytosine
FMAU 2′-fluoro-5-methyl-1-β-D-arabin-ofuranosyluracil
FMDV foot and mouth disease virus

Gal galactose

H1, H2, H3 influenza haemagglutinin (sub-types)
HA haemagglutinin
HAV hepatitis A virus
HBB 2-(α-hydroxybenzyl)benzimidaz-ole
HBcAg hepatitis B virus core antigen
HBeAg hepatitis B virus e antigen
HBG 9-(4-hydroxybutyl)guanine
HBIG hepatitis B virus immune globulin
HBsAg hepatitis B virus surface antigen
HBV hepatitis B virus
HCMV human cytomegalovirus
HDC human diploid cell
HI haemagglutination inhibition
HNANB hepatitis non-A non-B virus
HNIG human immunoglobulin
HPUara 6-(4-hydroxyphenyl-hydraz-ino)uracil
HPV human papilloma virus
HSV-1,2 herpes simplex virus types 1 and 2
HSV-TK herpes simplex virus thymidine kinase
HTLV human T-cell leukaemia/lympho-ma virus
HuIFN-α human leukocyte interferon
HuIFN-β human fibroblast interferon
HuIFN-γ human immune interferon

ID_{50} inhibitory dose (50%)
IDC 5-iodo-2′-deoxycytidine
IDU 5-iodo-2′-deoxyuridine
IEM immune electron microscopy
IF interferon
IMP inosine 5′-monophosphate
IND investigational new drug
IPV inactivated polio virus (vaccine)

ISG immune serum globulin

JBE Japanese B encephalitis, JE, JEV, JV
JC virus human polyoma virus
JE JBE
JEV JBE
JV JBE

-K M.W. molecular weight $\times 10^{-3}$

LCM lymphocytic choriomeningitis virus
LD_{50} lethal dose (50%)

MDBK Madin Darby bovine kidney cells
MK monkey kidney cells
MP monophosphate
mRNA messenger RNA

N1, N2 influenza neuraminidase (sub-types)
NA neuraminidase
NDA new drug application
2′NDG DHPG
NeuAc N-acetylneuraminic acid
NK natural killer (cells)
NS non structural (protein)
NT neutralizing activity

OPV oral polio virus (vaccine)

PAA phosphonoacetic acid
PEG polyethylene glycol
PFA phosphonoformic acid, foscarnet
PML progressive multifocal leukoence-phalopathy
pol polymerase
Poly I:C polyriboinosinic polyribocytidylic acid
PrdUrd 5-propyl-2′-deoxyuridine

RF replicative form (RNA synthesis)
RI replicative intermediate (RNA synthesis)
RNA ribonucleic acid
RNase ribonuclease
rRNA ribosomal RNA
RSV respiratory syncytial virus
RV rhinovirus

S-DHPA	(S)-9-(2,3-dihydroxypropyl)adenine
SFV	Semliki forest virus
SLE	St Louis encephalitis virus
SRD	single radial immunodiffusion
SV	sindbis virus
SV40	simian virus number 40
TBE	tick borne encephalitis virus
$TCID_{50}$	tissue culture infective dose (50%)
TFT	trifluorothymidine
TK	thymidine kinase
TP	triphosphate
tRNA	transfer RNA
ts	temperature sensitive
VCA	viral capsid antigen
VEE	Venezuelan equine encephalitis virus
Vero	continuous line of monkey kidney cells
VP	virus protein
vRNA	virion RNA
VSV	vesicular stomatitis virus
VZIG	varicella-zoster immune globulin
VZV	varicella zoster virus
WEE	Western equine encephalitis virus
ZIG	zoster immune globulin
ZIP	zoster immune plasma

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CHAPTER 1

The need for chemotherapy and prophylaxis against viral diseases

1.1. Introduction

1984 marks the centenary of the first use of one of the most effective viral vaccines (rabies, Pasteur, 1884) as well as being the famous milestone of fictional political forecasting (Orwell, 1949). This 100 year period has witnessed the industrialization of countries throughout the world and, along with this social and economic development, marked changes in patterns of infectious disease both accompanying and in some cases caused by the population and social changes. Excellent examples of these medical changes are shown in Figs. 1.1–1.3, where mortality in different age groups is compared for the two years, 1910 and 1967 in Germany for infectious diseases, heart and circulation disease and neoplastic disease, respectively. A dramatic drop in mortality at all ages, but particularly in children, from infectious disease is noted between 1910 and 1967, whereas, conversely, an increase in mortality in older persons (but not children) is noted which is caused by heart and circulation diseases and neoplasms. Disease patterns are still changing today and new viral diseases are discovered regularly (witness the fevers of Marburg and Lassa in the last decade, and, more recently, the different viruses associated with AIDS). Indeed there is every reason to assume that continual change in infectious diseases is to be expected in the future. Even the arrival of potent antiviral compounds may not eradicate diseases such as herpes, but rather may alter the pathogenic process of the virus itself as strong selective pressures are brought to bear. A similar phenomenon has been happening with bacteria such as *Staphylococci* or *Streptococci* as, over the years, strong selective pressures exerted by antibiotics have led to the emergence of organisms with reduced or altered pathogenicity. In the case of malaria, wide-

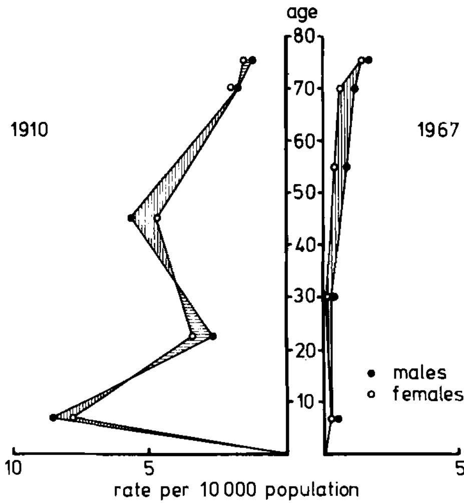

Fig. 1.1. Age distribution of mortality in 1910 and 1967 for the Federal Republic of Germany: infectious diseases. ●, male; ○, female.

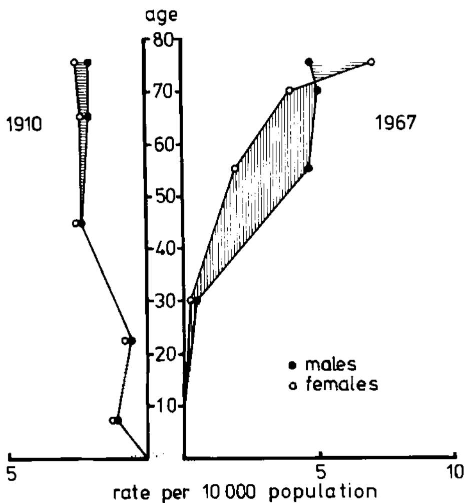

Fig. 1.2. Age distribution of mortality in 1910 and 1967 for the Federal Republic of Germany: heart and circulation.

spread use of antimalarials on the one hand has led to a gradual and threatening build up of drug resistant strains of parasite and also the use of DDT to destroy the mosquito has led to development of resistance in the vector. Therefore both eukaryotes and prokaryotes possess a dramatic ability to adapt to a changed environment, and viruses are no exception.

As bacteriologists before us have done, we should ask ourselves which viral dis-

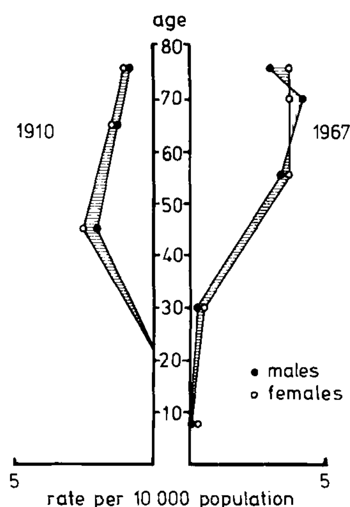

Fig. 1.3. Age distribution of mortality in 1910 and 1967 for the Federal Republic of Germany: neoplasm.

eases are important causes of mortality and morbidity, and, amongst these which would be the most suitable for ultimate control or even eradication. In fact, the question is more complex than this because in different parts of the world different viral diseases are important and even the same virus (such as measles) may cause very different disease syndromes. Whilst in most major European countries respiratory viruses are an important cause of morbidity and mortality, in third world countries measles, polio and diarrhoeal diseases predominate in this respect. A potential for distorted programmes of control of viral disease may easily occur, with most research centred on developed countries and therefore oriented towards viral disease in those countries. An excellent example is in the area of antiviral chemotherapy. At the present time most research effort is undoubtedly in the area of herpes infections, particularly HSV-2 causing genital infections, and this is closely followed by the search for new compounds against influenza A virus and rhinoviruses. Also, although influenza A is a pandemic virus causing mortality and morbidity throughout the world the same cannot be said for herpes viruses. Although infections with herpes viruses are universal, the impact of these nevertheless pale into insignificance beside medical problems with measles, arboviruses and hepatitis A and B in third world countries. So the direction of research effort needs continuous reassessment but, more importantly, some international perspective and direction. The World Health Organisation has provided this perspective for the eradication of smallpox from the whole world and continues to provide direction with its integrated 'Health for All by the year 2000' programme (Fig. 1.4) which calls on member states to pursue a programme aimed at attainment by all people of the world, of a level of health that will permit them to live a socially and economically produc-

Fig. 1.4. A, collage of aspects of the WHO programme 'Health for all by the year 2000.' B, piped water reaches a small community in the Soloman Islands; satisfying needs for water calls for a complex interlocking planning and action at many levels. C, inequality in the great cities of the world. (From 'World Health', WHO publication.)

tive life. The programme includes sections on the control of certain viral diseases causing respiratory infection and diarrhoeal diseases. The WHO conference at Alma-Ata in the USSR in 1978 stressed the importance of primary health care: "essential health care must be based on practical scientifically sound and socially acceptable methods and technology made universally accessible to individuals and families in the community through their full participation and at a cost that the community and the country can afford to maintain at every stage of their develop-

ment in the spirit of self reliance and self determination".

This programme does not, at present, envisage eradication of any particular viral disease, as smallpox was eradicated, because this may not gain the necessary full support of all countries. Individual countries might, and are, considering 'national' eradication of certain virus infections such as measles in the USA, Cuba, Bahamas, Canada and Barbados where mortality rates of less than 1 measles death per 100 000 population have now been achieved. Polio is another example of a viral disease which can be eradicated nationally, but of course a 'national' eradication always means that re-introduction from outside can constantly occur.

1.2. Important viral diseases in third world countries

Table 1.1 illustrates illness and death caused by infectious diseases (viruses, bacteria and parasites) in Africa, Asia and Latin America. Viruses are the single chief cause of mortality, amounting to many millions of persons per year. Among these virus infections the worst culprits are respiratory viruses such as influenza, and measles and rotaviruses. Although an estimated 5–10 million persons die of enteric infections in these three continents each year, viruses will cause only a proportion of these cases and a similar situation will be true for respiratory illness. We shall examine the methods developed for the prevention of measles, influenza, polio and rotaviruses later on (Chapters 8, 7, 4 and 9, respectively) but it may be mentioned here that live polio vaccines used so successfully in industrialized countries are much more difficult to apply successfully in third world countries where problems of vaccine administration, heat lability control and viral interference become very important. Measle's programmes are being initiated now in certain third world countries (reviewed by McKenzie, 1983), but vaccines against diarrhoeal diseases are in their infancy. Research has been orientated towards the development of successful inactivated or live vaccines or chemoprophylactic agents against influenza for the last 40 years with only limited success and even less success against other respiratory viruses such as respiratory syncytial virus and parainfluenza viruses.

Little emphasis is given in Table 1.1 to mortality from arboviruses other than dengue, and from hepatitis A and B viruses. A successful yellow fever vaccine was developed in the 1940s but is not used for mass immunization but rather for containment, whereas the development of vaccines or chemoprophylactic agents against hepatitis is only beginning and so far has only resulted in vaccines and drugs with a very limited use on a large scale because of the expense involved.

Rabies vaccine, on the other hand, and the topic of our introductory sentence has been a success story in developed *and* underdeveloped countries. Cheap and effective rabies vaccines have been produced locally, with the help of the network of Pasteur Institutes throughout the world, to contain this terrifying disease (Chapter 6).

TABLE 1.1.
Estimates of illnesses and deaths associated with major infectious diseases of Africa, Asia, and Latin America, 1977–1978 (after Kapikian et al., 1980)

Disease	No. of cases per year	No. of deaths per year
Diarrhoeas	3–5 billion	5–10 million
Respiratory illnesses	Not estimated	4–5 million
Malaria	150 million	1.2 million
Measles	80 million	900 000
Schistosomiasis	20 million	0.5–1 million
Whooping cough	20 million	250 000–450 000
Tuberculosis	7 million	400 000
Neonatal tetanus	120 000–180 000	100 000–150 000
Diphtheria	700 000–900 000	50 000–60 000
Hookworm	1.5 million	50 000–60 000
South American trypanosomiasis	1.2 million	60 000
Onchocerciasis skin disease	2–5 million	low
River blindness	200 000–500 000	20 000–50 000
Meningitis	150 000	30 000
Amebiasis	1.5 million	30 000
Ascariasis	1 million	20 000
Poliomyelitis	2 million	10 000–20 000
Typhoid	500 000	25 000
Leishmaniasis	12 million	5000
African trypanosomiasis	10 000	5000
Leprosy	12 million	very low
Trichuriasis	100 000	low
Filariasis	2–3 million	low
Giardiasis	500 000	very low
Dengue	1–2 million	100
Malnutrition	not estimated	2 million

In general therefore we can conclude that certain viral diseases are 'shared' between developed and underdeveloped countries (such as influenza, measles, polio and rabies) and research and technical developments in industrialized nations will hopefully be applied worldwide. An important qualification here is that new vaccines and antivirals should not be too expensive and, unfortunately, recent rabies vaccines (Chapter 6) and hepatitis vaccines (Chapter 16) are extremely costly. Live attenuated polio and measles vaccines are produced very cheaply and with more attention to their delivery (Chapters 4 and 8) can and should result in a dramatic decrease of polio and measles in these countries in the next decade.

1.3. Important viral diseases in industrialized nations of the world

As we have emphasized above, deaths from infectious viral disease in industrialized nations and also in those third world countries with well organized social infrastructure (such as China in the last 30 years) have been steadily dropping over the last 100 years, although there is still a considerable scope for further reductions in many countries. Table 1.2 shows death rates from infectious diseases in countries with 'fully developed health services' compared to those without these facilities. Table 1.3 further emphasizes marked differences in mortality caused by acute respiratory infection (bacterial and viral) in children between a third world continent (Africa), the USA and Europe. Mortality is overall 10 times higher in Africa than in the USA but nearly 60 times higher in the 1–4 year olds. Also interesting is the two fold higher mortality in children in Europe compared to the USA, at least at the beginning of the 1970s.

To put mortality from infectious diseases into some perspective Table 1.4 shows mortality in the USA in June 1982 from all causes. Deaths from pneumonia and influenza, for example, list seventh in such an analysis. The incidence of viral dis-

TABLE 1.2.
Death rates from infectious diseases (per 100 000 per annum, 1974–5) (after Lambert, 1983)

	Group A 14 countries with fully developed health service	Group B 4 countries in Central and South America
Pneumonia, influenza, bronchitis	57.5	102.0
Tuberculosis	4.1	13.7
Measles	0.1	3.6
Whooping cough	0.009	4.1
Tetanus	0.04	2.5
Poliomyelitis	0.003	0.3

TABLE 1.3.
Mortality from acute respiratory infection in children 1970–73, rate/100 000 population[a]

	Infants	1–4 yrs	5–14 yrs
Africa	1454	467	22
N. America	146	8.0	1.5
Europe	390	15	2.1
Total	762	101	8.4

[a] Pan American Health Organisation (1982)

TABLE 1.4.
Relationship between cause of death and death rate in the USA in 1982 (from MMI)

Cause of morbidity or mortality	Estimated mortality June 1982		Estimated number of physician contacts June 1982
	Number	Annual death rate/100 000	
All causes (total)	157 164	826.7	101 397 000
Accidents and adverse effects	8251	43.4	5 936 000
Malignant neoplasms	35 512	186.8	1 544 000
Diseases of heart	58 630	308.4	5 909 000
Suicides, homicides	4011	21.1	—
Chronic liver disease and cirrhosis	2110	11.1	158 000
Cerebrovascular diseases	12 091	63.6	725 000
Pneumonia and influenza	3555	18.7	716 000
Diabetes mellitus	2643	13.9	2 503 000
Chronic obstructive pulmonary diseases and allied conditions	5114	26.9	1 014 000
Prenatal care			3 310 000
Infant mortality	3200	10.6/1000 live births	

TABLE 1.5.
Incidence of virus diseases in USA (1976) (after Lambert, 1983)

	Incidence of acute conditions $\times 10^6$ (%)	
Upper respiratory disease	128	(27.7)
Influenza	110	(23.9)
Common cold	97	(21.0)
All acute conditions	461	(100)

eases in the USA is summarized in Table 1.5 and finally, visits to physicians for viral diseases are summarized in Table 1.6.

In summary, therefore, in the industrialized and more wealthy nations of the world infectious diseases including viral diseases play a much reduced role in causing mortality but amongst the important viruses in this respect are particularly respiratory viruses such as influenza A and B, and respiratory syncytial virus. Pandemic influenza A virus, in addition, causes considerable morbidity and economic disruption.

TABLE 1.6.
Visits to physicians with diagnosis of viral infection in USA (1977) (after Lambert, 1983)

Diagnosis	No. of visits $\times 10^6$
Acute urinary tract infection	23.9
Influenza	10.2
Common cold	4.2
Herpes febrilis	1.3
Herpes zoster	1.3
Varicella	0.77
Infectious hepatitis	0.64
Other viral diseases	2.04
Total office visits	1114

1.4. Disease syndromes caused by viruses

We have briefly mentioned above clinical diseases and syndromes such as influenza, respiratory tract infections, hepatitis, and arbovirus infections resulting from virus infections. But many of these disease syndromes may be caused by a multitude of viruses, making specific diagnosis without the aid of a laboratory impossible. Most antiviral agents discovered to date have a very restricted range of antiviral activity and so to use these in the field it will be necessary to identify particular viruses causing a clinical syndrome rapidly and precisely. Partly for this reason, methods of rapid virus diagnosis have been investigated rather intensively during the last few years and the advent of monoclonal antibodies, for example, should hasten this process. Meanwhile it will be useful here to briefly summarize the range of viruses responsible for these differing clinical syndromes, and finally to list the major viruses of man in a more logical 'scientific' manner, so that the reader will not end up completely confused!

1.5. Viruses causing respiratory diseases in humans

A very wide range of viruses ranging from the RNA-containing pleomorphic influenza viruses to the DNA icosahedral adenoviruses cause respiratory infection which may be completely indistinguishable by a clinician. Nevertheless some general observations are a useful guide, including the facts that rhinoviruses, ECHO viruses, reoviruses and coronaviruses rarely, if ever, cause lower respiratory tract infection but confine their attention to the upper respiratory tract and hence may often produce only mild respiratory illness (Table 1.7). Cytomegalovirus and varicella zoster, on the other hand, tend to produce only lower respiratory tract infections whereas influenza, measles, parainfluenza, RSV and adenovirus produce both. Finally, most

TABLE 1.7.
Viruses that cause respiratory disease in man

Virus	Serotypes		Syndrome
	No.	No. that cause resp. disease	
Adenovirus	38	8	Pharyngitis, lower respiratory tract disease – infants, children, adults
Coronavirus	3	3	Upper respiratory tract disease – all ages
Herpesvirus			
Simplex	2	1	Stomatitis and pharyngitis – children, young adults
CMV	1	1	Pneumonia – young infants
Influenza virus	3	3	Influenza, pneumonia – adults; croup – children (Types A and B). Mild upper respiratory tract disease (Type C)
Paramyxovirus	5	5	Croup, bronchitis, pneumonia – infants, children
Respiratory syncytial virus	1	1	Bronchiolitis, pneumonia – infants, children
Enterovirus	67	12	Pharyngitis – children, adults
Rhinovirus	150	150	Upper respiratory tract disease – all ages

(but not all) clinical cases of influenza are caused by influenza A and B viruses, whilst most cases of croup in infants are caused by RSV (Fig. 7.10 in Chapter 7).

1.6. Neurotropic and dermatological viral agents

Similarly, a wide range of viruses have a predilection for nervous tissue (Table 1.8). Some viruses such as rabies, polio and certain arboviruses target upon nerve cells, but in the case of most viruses central nervous system (CNS) effects result as an aftermath and complication of viral replication (e.g. mumps, herpes, rubella).

Certain of these disease syndromes in the CNS such as rabies can be identified by clinical examination of the patient, but even polio-like paralysis can be caused by other enteroviruses (Chapter 4). Certainly encephalitis following rash diseases such as rubella, mumps and measles presents few problems of diagnosis unless the person has been infected without a rash.

TABLE 1.8.
Neurotropic and dermatropic viral agents

Virus	Species	Number of types associated with:			Main clinical syndrome
		Total	CNS disease	Rashes	
Paramyxo	Measles virus	1	1	1	Measles
	Mumps virus	1	1	1	Mumps
	Respiratory syncytial virus	1		1	Acute resp. tract disease
	Parainfluenza virus	5	3[a]		Acute resp. tract disease
Picorna	Poliovirus	3	3[b]		Poliomyelitis
	Coxsackie virus	30	16[c]	17[d]	CNS disease
	Echovirus	33	33[e]	20[f]	CNS disease
Herpes	Herpes simplex virus	2[g]	2	2	Herpes
	Cytomegalovirus	1	1	1	Cytomegalic incl. disease
	Varicella-zoster virus	1	1	1	Varicella-zoster
	Epstein-Barr virus	1	1	1	Infectious mononucleosis
Arbo	Alpha and Flavivirus	200	18[h]	8[i]	CNS disease
Pox	Poxviruses	6	1[j]	5[k]	Poxes
Other	Rubellavirus	1	1	1	Rubella
	Lymph. choriomeningitis virus	1	1		CNS disease
	Adenovirus	33	5[l]	4[m]	Acute resp. tract disease
	SV40-like virus	3	2[n]		Progressive multifocal leukoencephalopathy
	Rabies	1	1	–	Rabies

[a] Types 1–3.
[b] Types 1–3.
[c] Types A1, 2, 4–7, 9, 10, 14, 16 and B1–6.
[d] Types A1–10, 16, 22 and B1–5.
[e] All types.
[f] Types 1–7, 9, 11, 13, 14, 16–19, 22, 25, 30, 32 and 33.
[g] Types 1 and 2.
[h] EEE, WEE, VEE, JBE, SLE, Ilheus, Looping-ill, Powassan, West Nile, etc., viruses.
[i] Chikungunya, Dengue, West Nile, Sindbis, O'Nyong-Nyong, Colorado Tick Fever, etc., viruses.
[j] Vaccinia virus.
[k] Variola (now extinct), vaccinia, paravaccinia, orf, molluscum contagiosum viruses.
[l] Types 1–3, 5 and 7.
[m] Types 1–3, and 7.
[n] JC and SV40-PML viruses.

1.7. Viruses causing rashes

Certain rashes in their typical form can be easily diagnosed as caused by the viruses of chickenpox (varicella zoster), measles or rubella (Table 1.9). However, diagnosis

TABLE 1.9.
Principal rashes in infectious virus disease in man

Virus	Disease	Features
Measles virus	Measles	Very characteristic maculopapular rash
Rubella virus	German measles	Maculopapular rashes not distinguishable clinically
Echo viruses 4, 6, 9, 16	Not distinguishable	
Coxsackie viruses A9, 16, 23		
Varicella-zoster virus	Chickenpox/zoster	Vesicular rashes
Variola virus	Smallpox	Vesicular rashes (Now extinct)
Coxsackie A/16 virus	Hand, foot and mouth disease	

of rashes caused by certain of the enteroviruses such as Echo 16 or Coxsackie A9 may be easily misdiagnosed as rubella. This would not be particularly important were it not for the propensity of rubella to cause foetal infections and abnormalities (congenital rubella syndrome).

1.8. Viruses infecting the foetus

Table 1.10 lists viruses which have been implicated in causing foetal abnormalities, abortion or postnatal infections. Undoubtedly the most significant and dramatic effects on the foetus are caused by rubella virus and the many and varied deleterious effects on the developing embryo have been encompassed as the 'expanded rubella syndrome' (Table 1.11). Infection of the mother in the first week of pregnancy may result in infection and sequelae in 90–100% of the foetuses (Chapter 9).

Herpes viruses (via infection of the cervix of the mother) may infect the foetus during delivery, whereas cytomegalovirus and vaccinia can infect the foetus in utero in the final trimester of pregnancy. Increased foetal deaths or abnormalities have been noted following infection of the mother with other viruses including polio, arboviruses, measles and influenza but these effects on the foetus are more likely to be caused via constitutional upset in the mother rather than by actual infection of the foetus or foetal tissue by the virus itself.

1.9. Viruses causing persistent infections

Most of the above mentioned viruses cause acute infection in humans following transmission from another person or from a vector such as a mosquito or other insects. But certain viruses can afterwards establish a latent or persistent infection

TABLE 1.10.
Viral infections during pregnancy implicated in foetal or neonatal disease

Virus	Potential effect on mother	Potential effect on foetus or newborn
Cytomegalovirus	Usually asymptomatic, but sometimes moderate to high fever in primary infection	Chronic infection, ?congenital malformation, mental retardation
Echoviruses	Rubella-like illness, fever, aseptic meningitis	Fatal disseminated viral infection (hepatic necrosis)
Hepatitis A and hepatitis B	Flu-like illness; chills and high fever, constitutional symptoms and jaundice, increased severity during pregnancy	Prematurity, foetal death, neonatal hepatitis vertical transmission of HBsAg
Herpes virus types 1 and 2	Oral or genital infection probably more severe in pregnancy	?Abortion ?prematurity, fatal disseminated infection
Influenza	Increased mortality in pandemics	?Increased foetal mortality
Measles (rubeola)	No special effect	Probably increased foetal mortality
Poliomyelitis	Increased susceptibility, severity and mortality during pregnancy	Foetal death, neonatal poliomyelitis
Rubella	Often asymptomatic, or very mild illness	Foetal death, chronic persisting infection, congenital malformations
Varicella-zoster	Often more severe; maternal death	Neonatal varicella, probably specific defects
Vaccinia and variola	Increased severity and mortality	Foetal death, intrauterine neonatal smallpox or vaccinia
Venezuelan and western equine encephalomyelitides	Meningoencephalitis	Neonatal encephalitis

in the patient, becoming dormant. This would by itself be of no particular consequence except for the fact that, unpredictably, certain of these viruses later become reactivated, causing a new clinical syndrome. An excellent example is shown by the herpes viruses (Table 1.12) which infect the ganglia and re-emerge, as in the case of herpes zoster or shingles a lifetime later. On re-emergence the virus can be transmitted to other persons and so the life cycle of a herpes virus can become very complex (Chapter 11). In the case of hepatitis B viruses the persistent shedding of virus into the blood stream also makes the 'carrier' a potential infector of others, via medical or dental equipment or blood transfusion.

Viruses which are able to persist form a troublesome group for prevention. Vaccines against herpesviruses are under evaluation at present but since reactivation

TABLE 1.11.
Abnormalities in congenital rubella virus infections ('expanded rubella syndrome')

	Evident in neonatal period		May not be evident until months or years later
	Common	Rare	
CNS	Encephalitis, enlarged anterior fontanelle	Microcephaly	Mental retardation, language abnormalities, motor deficits, autism
Eye	Pigmentary retinopathy, cataract, microphthalmia	Glaucoma, cloudy cornea, iris hypoplasia	Pigmentary retinopathy
Ear	Sensorineural deafness		Sensorineural hearing deficits
Skeleto-muscular	Low birth weight, postnatal growth retardation, bone radiolucencies, micrognathia	Dermal erythropoiesis	High palate, pes cavus, talipes equinovarus, finger abnormalities, dental abnormalities
Haematological	Hepatosplenomegaly, thrombocytopenia, leukopenia, adenopathy	Hepatitis, immunological dyscrasias, hemolytic anemia, hypoplastic anemia	
Cardiovascular-pulmonary	Pulmonary arterial hypoplasia, patent ductus arteriosus, coarctation of aortic isthmus	Septal defects, interstitial pneumonitis, myocardial necrosis	

occurs in the presence of high levels of neutralizing antibody such vaccines may have little or no effect, unless they stimulate cell mediated immunity (Chapter 2). Similarly, antivirals are unlikely to eliminate latent herpes viruses from the ganglia and would merely hasten the healing of a particular reactivation episode.

1.10. Sexually transmitted diseases caused by viruses

Herpes simplex venereal infections are of considerable interest to the general public, virologists and antiviral chemotherapists and data from the USA appear to suggest infections of epidemic proportions (see Chapter 12). Data from the UK show a steady increase in HSV-2 genital infections but certainly not in epidemic proportions. Indeed it is most useful to place this virus in a context of other venereal infections. Recently published data from the UK would suggest that non-specific genital infections are increasing in incidence at least as rapidly as HSV infections. Although the aetiology of non-specific genital infection is still not fully elucidated, *Chlamydia*

TABLE 1.12.
Examples of persistent viral infections

Virus	Site of persistence	Infectiousness of persistent virus	Consequence	Shedding of virus to exterior
Herpes simplex	Dorsal root ganglia Trigeminal ganglia	– –	Activation, vesicles	+
Varicella zoster	Dorsal root ganglia	–	Activation, zoster	+
EB virus	Lymphoid tissue	–	Lymphoid tumour? (Burkitt's lymphoma)	–
Cytomegalovirus	Salivary glands	+	None known	+
Hepatitis B	Liver (virus shed into blood)	+	Blood remains infectious	+
Adenoviruses	Lymphoid tissue	– +	None known	±
Measles	Brain	±	Subacute sclerosing pan-encephalitis	

trachomatis is recognized to be the commonest cause in Britain, and isolation rates from the cervix of unselected women attending sexually transmitted disease clinics in Britain may reach up to 31%.

Total new attendances at special (venereal disease) clinics in the United Kingdom rose by 4.6% in 1981 compared with 1980, continuing the increase noted each year since the early 1950s. This is less than the previous annual increase of 9% (which, however, was unusually large). The overall picture of sexually transmitted disease in British clinics in the past 30 years is one of the increasing importance of new cases requiring treatment in categories other than syphilis or gonorrhoea, which now account for only 16% of total cases requiring treatment. The largest absolute increase in new attendances by diagnostic category in 1981, apart from 'other conditions requiring treatment', was in non-specific genital infection: there were 132 391 new attendances, an increase of 6915 (5.5%) over those in 1980, but this was about half the previous annual rise of 11%. There were rises in most other diagnostic categories. The number of new attendances due to herpes simplex infection increased by 1300 (12.1%), those due to warts by 1700 (5.3%) and those due to candidiasis by 2894 (6%).

Other viruses including cytomegalovirus and hepatitis B virus are transmitted in semen from infected persons, but the general significance of this is not clear. HTLV-III has been implicated as a possible cause of acquired immunodeficiency syndrome (AIDS) in promiscuous homosexuals (see Chapter 14) and presumably transmission via person to person in semen might be a major factor.

1.11. Attempts at prevention of human viral diseases

Table 1.13 and Fig. 1.5 list, rather exhaustively (but not completely), a classification of viruses causing human disease, and Table 1.14 briefly summarizes information on currently used vaccines and chemoprophylactic agents against viral diseases. The epidemiology, strategy of replication and physical and antigenic structure of these viruses will be discussed as fully as possible in the ensuing chapters. However, a word of warning should be introduced here. DNA technology ('genetic engineering') techniques are being introduced very rapidly indeed and are expected to revolutionize the previously used biological approaches to development of new viral vaccines. The reader can safely assume that for most viruses discussed in the following chapters, even if it is not indicated in the text, that someone is cloning the particular gene into a eukaryote or prokaryote cell. It cannot be overemphasized that with both new vaccines and antivirals the initial discovery is often made by individuals and single groups. Only during later developments are the large teams of scientists required. Also, with antivirals, a new era has arrived which is seeing the first extended use in the clinic of inhibitory molecules against viral diseases, particularly herpes. So we shall undoubtedly see a plethora of new molecules each with certain biological and pharmacological advantages compared to the parent. Indeed, we are witnessing this trend already with molecular derivatives of acyclovir such as DHPG and DHBG (Chapter 11).

We have tried, in the ensuing chapters to present the reader with a review of the basic scientific knowledge and principles underlying development of vaccines and antivirals. New data should simply enhance interest in the topic and perhaps even encourage a reader to develop a vaccine or antiviral him or herself!

1.12. Economic costs of viral diseases

The impact of viral diseases on society can, to some extent, be expressed in economic terms and this again illustrates the magnitude of the infectious viral disease problem. In the USA, during the period 1972–1978, the annual mortality from influenza was 20 000 deaths, the annual cost of treatment $300 million and the annual loss of productivity $750 million. The cost of less severe respiratory viral diseases, such as common cold occurring on the average more than twice yearly, is probably of the same magnitude. The distress and pain caused by recurrent labial and genital herpes infections is very large, and possibly increasing. The number of patients is difficult to express in economic terms, but an estimated number of 500 000 Americans are contracting genital herpes each year and approximately 100 million episodes of labial herpes will affect the USA population each year. The impact of gastrointestinal syndromes caused by viral infection can be illustrated by an annual mortality of 5–10 million due to rotavirus infections in children in Asia, Africa and South America.

TABLE 1.13.
Examples of viruses infecting humans

Family	Genus/Subfamily	Example
Poxviridae	Ortho pox	Smallpox virus
	Para pox	Orf virus
Herpesviridae	Alphaherpesvirinae	Herpes simplex virus type 1,2
		Varicella zoster virus
	Betaherpesvirinae	Cytomegalovirus
	?	Epstein-Barr virus
Iridoviridae	African swine fever group	African swine fever virus
Adenoviridae	Mammalian adenoviruses	Adenovirus type 2
Papovaviridae	Papilloma virus	Human papilloma virus
	Polyoma virus	BK virus
Parvoviridae	Parvovirus	Norwalk agent?
Reoviridae	Reovirus	Reovirus type 1
	Orbivirus	Colorado tick fever virus
	Rotavirus	Human rotavirus
Togaviridae	Alphavirus	Eastern equine encephalitis virus
	Flavivirus	Yellow fever virus
	Rubivirus	Rubella virus
Coronaviridae	Coronavirus	Human coronavirus
Paramyxoviridae	Paramyxovirus	Parainfluenza virus 1
	Morbillivirus	Measles virus
	Pneumovirus	Respiratory syncytial virus
Orthomyxoviridae	Influenzavirus	Influenza virus A
Rhabdoviridae	Lyssavirus	Rabies virus
Bunyaviridae	Bunyavirus	Bunyamwera virus
	Phlebovirus	Sandfly fever virus
	Nairovirus	Crimean-Congo haemorrhagic fever virus
	Uukuvirus	?
Arenaviridae	Arenavirus	Lassa fever
Retroviridae	Oncovirinae	Human T-cell leukaemia virus
	Spumavirinae	Human foamy virus
Picornaviridae	Enterovirus	Polio virus
	Rhinovirus	Human rhinovirus IA
Calciviridae	Calcivirus?	Norwalk virus?
Unclassified		Hepatitis B virus
		Marburg/Ebola virus
		Kuru
		Creutzfeld-Jacob disease
		AIDS
		Delta agent
		Hepatitis nonA nonB virus

Many chronic conditions are initiated in infectious diseases. Hepatitis B may result in chronic infection in 10% of the cases, leading to chronic cirrhosis. An esti-

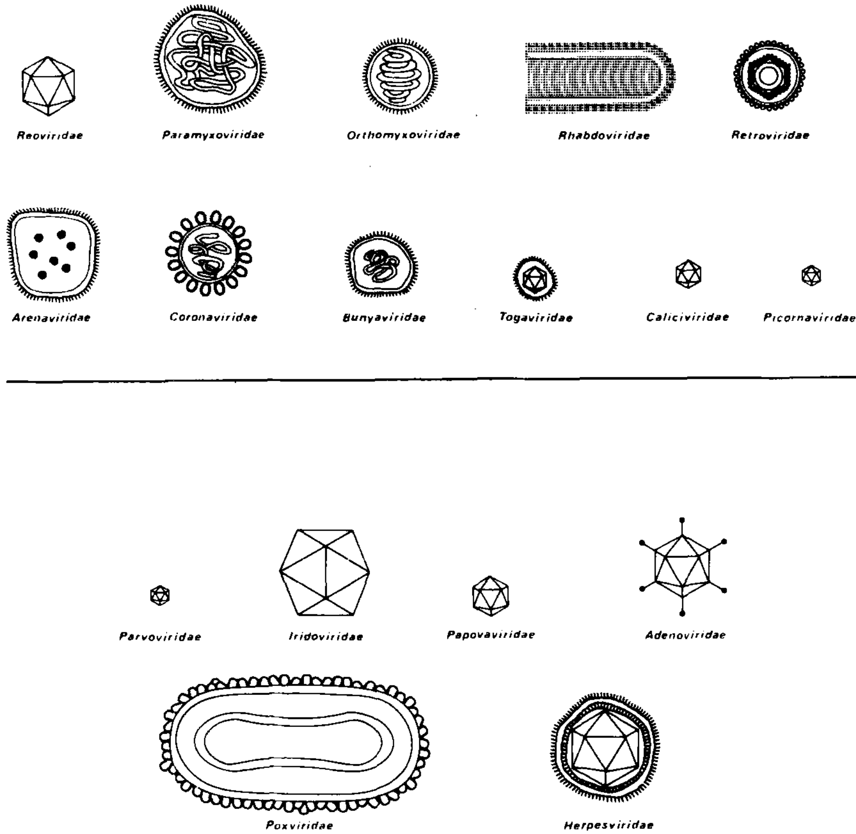

Fig. 1.5. The families of viruses infecting vertebrates. The top part of the figure shows the RNA viruses and the bottom part the DNA viruses. (after Matthews, 1982.)

mated 150–200 million chronic carriers in the world poses a large medical problem. For some communicable diseases such as rabies, although the rate of morbidity is small or non-existent, nevertheless substantial costs are incurred in surveillance, prevention and health education. Also the costs for rabies extend into the agricultural sector of the community. With all communicable diseases allowance must be made for indirect costs such as production losses in the economy and these may sometimes amount to twice as much as the health service costs. Indirect costs again may spread over a number of sectors of the economy, including infected animal stock or food products etc.

1.13. Economic evaluation of programmes to control viral diseases

To date very few detailed studies have been undertaken to establish the cost and

TABLE 1.14.
Important viral diseases of man – prevention by vaccines or chemotherapy

Virus	Disease spectrum	Epidemiology/ distribution	Antiviral agent	Vaccine	Comments
Influenza A	Acute respiratory	Pandemic virus	Amantadine Rimantadine Ribavirin	Inactivated whole virus or split virus and sub unit vaccines. Live attenuated vaccines (*ca* and *ts* mutants and host range mutants).	Both vaccines and amantadine have a comparable degree of efficacy (with 70% protection). Ribavirin aerosol trials unconfirmed at present.
Influenza B	Mild respiratory	Limited epidemics	Interferon	As above	Antiviral agents required
Influenza C	Mild respiratory	Not epidemic	None	None	Antiviral agents required
Parainfluenza viruses	Acute respiratory	Children – world wide	None	None	*ts* mutants under study. Early inactivated vaccines deleterious. Little work with antivirals at present.
Respiratory syncytial virus (RSV)	Acute respiratory	Children – world wide	Ribavirin	None	Early inactivated vaccine deleterious
Measles	Rash and acute infection	World wide	None	Attenuated virus	Successful vaccination programme in many countries. Early inactivated vaccines deleterious.
Mumps	Rash and acute infection	World wide	None	Attenuated virus	Successful vaccine programmes in many countries
Coronavirus	Mild respiratory	World wide	None	Attenuated virus	
Arenaviruses	Acute generalised	Tropical areas	Ribavirin triacetate	None	Ribavirin triacetate is under clinical investigation against Lassa virus.

TABLE 1.14. *(continued)*

Virus	Disease spectrum	Epidemiology/ distribution	Antiviral agent	Vaccine	Comments
Togavirus	Encephalitis, rash Acute illness	Almost world wide	None	Attenuated (17D Yellow Fever) and inactivated viruses	Yellow Fever vaccine is effective. Vaccines against other viruses of the group are used on a small scale (e.g. TBE).
Rhabdo virus	Rabies	Almost world wide	None	Inactivated	Effective new human diploid cell vaccine for immunization pre and post exposure. Range of other vaccines are effective.
rhinoviruses	Common cold	World wide	Envirs unconfirmed prophylactic activity versus rhinovirus tnterferon has mild prophylactic effect.		Antivirals required for rhinoviruses because the multiplicity of serotypes makes immunization improbable unless common antigenic determinants can be isolated.
Poliovirus I, II, III	Enteric infections	World wide	None	Effective live and inactivated Polio virus vaccines.	
Echo	Conjunctivitis (enterovirus type 70), neurological disease (Polio)	World wide	None	None	
Coxsackie		World wide	None	None	
Papilloma viruses	Warts	World wide	None	None	Vaccines or antivirals required

TABLE 1.14. *(continued)*

Virus	Disease spectrum	Epidemiology/ distribution	Antiviral agent	Vaccine	Comments
Hepatitis A	Hepatitis	World wide	None	None	Vaccines or antivirals required.
Hepatitis B	Hepatitis	World wide	Ara−A + interferon	Inactivated 'subunit'	Effective hepatitis B vaccine suitable for small 'at risk' groups. Interferon only active in some persons. Combined therapy a possibility.
Hepatitis non A non B	Hepatitis	World wide	None	None	
Herpes viruses:					
HSV-2	Venereal	World wide	Acyclovir, Ara-A, BVDU, foscarnet, IDU (keratitis only), triflurothymidine (keratitis only), interferon	Experimental	Vaccines may have limited usefulness because of virus latency and the complexity of immune responses. A number of effective antivirals are under trial.
HSV-1	Superficial lesions (including venereal)	World wide	As above	Experimental	
CMV		World wide	Foscarnet?	Experimental	
EBV	Encephalitis, keratitis, infections in immunouncomprised persons	World wide	None		
Herpes zoster	Zoster, varicella	World wide	Acyclovir	None	Mononucleosis

effectiveness of programmes for the prevention and treatment of viral diseases. Moreover, these have been concerned with vaccines and no work has been published as regards specific antivirals. However, the estimated cost to develop an antiviral agent is $20–100 million which, in relation to common diseases such as influenza and herpes, is economically acceptable but with less frequent viral diseases can be a problem for a private company. Such data as there are suggest that improved strategies of prevention of viral disease could make substantial savings and result in better health outcomes.

Some viral diseases such as measles and polio maintain high incidence levels unless immunization levels are constantly maintained, whereas others can be self eliminated when a threshold level of infection is reached. Where the incidence of a disease changes over time, established forms of treatment may lose their justification and should be phased out or altered.

The patterns of some viral diseases have changed due to medical intervention in another sphere and an example is the improvement in treatment of cancers and immunosuppression of transplant patients. This has resulted in an increasingly common situation where a successful, and often very expensive, treatment of a disease is threatened by opportunistic viral infections, mainly by latent herpes viruses. The cost of developing antiviral agents against these types of infections should be considered in the context of the total cost to manage these patients and the risk of an infection.

1.14. Benefit-risk and cost-effectiveness analysis of virus vaccines

An informal weighing of risks and benefits of immunization (e.g. for smallpox) has been carried out in some societies, like the UK, for hundreds of years. However, it is now possible to apply more precise scientific analysis to the problem. Such a scientific analysis of immunization benefits in the early 1960s led to a major alteration in national health policy in the USA — namely the decision that routine smallpox vaccination should be discontinued. The last case of *variola minor* in the USA was in 1949 and by 1963 the risk of death from all smallpox vaccinations was 1 per million for primary vaccinees, rising to 5 per million for children under 1 year of age. In addition, among primary vaccinees the combined rate of post vaccinial encephalitis and vaccinia necrosum was 6.5 per million for infants. On the other hand, the probability of a smallpox importation into the USA in 1970 was 1 importation every 12 years. It would probably have required 15 smallpox importations per year to produce the same mortality which was then associated with smallpox vaccination. This is an excellent example of the direct usefulness of statistics.

In the United States alone more than 50 types of vaccines, both bacterial and viral are used and one may question how worthwhile some of these vaccines are in economic terms. Cost effectiveness analysis and cost benefit analysis aggregate

the net medical care costs and net health benefits from a vaccination programme and thus help to give an economic analysis. Net medical care costs often include the cost of vaccine and its administration, cost of treating vaccine complications and the medical care savings due to prevention of disease. Net health benefits include reduction in morbidity and mortality. Also one may include the gains in productivity resulting from a reduction of absence at work. Other qualitative and more difficult to cost-estimate considerations must be included to place a vaccine programme in an accurate social and medical perspective e.g. pain and anguish of illness, compensation of victims of severe vaccine reactions etc. These days, quite necessarily so, political, social, economic and medical factors are all taken into account (or at least should be by national health authorities). It is an interesting and useful exercise to see how these analyses apply to 4 popular and seemingly useful viral vaccines (Willems and Sanders, 1981). The salient features of this analysis which mainly refers to experience in the USA (and therefore may differ in details in European countries, for example) are presented in Table 1.15.

The crucial issue for a vaccine strategy against rubella is the appropriate age of immunization. Prevention of congenital rubella syndrome is the aim of this programme, because rubella itself is a mild disease scarcely worth considering if it were not for the teratogenic properties of the virus. The teratogenic effect of the virus is unique in its selectivity of action and most embryos of mothers infected during the first trimester would be affected. As an example of the community effects in the 1964–1965 epidemic before vaccine was introduced, 5000 therapeutic abortions were carried out and 20 000 children were born with rubella syndrome in the USA alone. Also 2100 excess prenatal deaths were associated with the epidemic. Of the 20 000 children born with congenital rubella, 8000 were deaf, 3500 deaf and blind and several thousand suffered moderate to severe mental retardation. Estimated direct costs of the epidemic in the USA were $1 billion, mainly (90%) as regards long term care associated with rubella syndrome. In the USA after 1969, when vaccine became widely available, the strategy was to immunize *all* children as a routine, whereas adolescents and women of childbearing age were immunized selectively. In the UK, on the other hand, mainly school girls (not boys) were immunized. The basic strategy in the USA was to displace wild virulent rubella by attenuated non-teratogenic vaccine virus. A reduced objective was aimed for in the UK and the programme to date has not been so successful. In the USA, rubella epidemics have been prevented and cases of rubella have decreased continuously in the under 15 year age group. Most cases of rubella now occur among adolescents. A study of costs and benefits found positive net benefits. In 1972 the direct cost of acute rubella for 1 million persons was estimated at $2.7 million and the costs of congenital rubella syndrome in the offspring of 1 million unprotected females was $35.9 million. The benefit cost ratio for vaccine to 1 million females at 12 years of age was 25:1, assuming 100% immunization of the target population.

Since mumps vaccine was licensed in 1967 in the USA more than 40 million doses

TABLE 1.15.
Results of cost-effectiveness and cost-benefit analyses of some virus vaccines (from Willems and Sanders, 1981)

Vaccine	Efficacy	Duration of immunity	Unit of analysis	Vaccination cost per person	Net health effects	Net medical care costs	Net costs including productivity gains
Poliomyelitis	95%	life	1 million vaccinees	$0.81	Decrease in annual new cases of poliomyelitis (200) and annual deaths (1269)	Decrease of $0.2 million	Decrease of $0.9 million
Measles virus	90%	life	1 million vaccinees	$3.0	Decrease in cases of measles (269 529), death (27), and cases of encephalitis (270) and retardation (90); increase in years of life (8061)	Decrease of $4.6 million	Decrease of $11.3 million
Rubella virus	95%	life	1 million cohort	$3.0	Decrease in cases of acute rubella (72%) and congenital rubella (70%)	Decrease of $9.8 million	Decrease of $17.2 million
Influenza virus	70%	1 year	1 million vaccinees	$3.0	Decrease in deaths (521) and increase in years of life (5423)	Increase of $2.7 million (increase of $491 per year of life gained)	Increase of $1.7 million (increase of $311 per year of life gained) loss of work hours included

have been used and the incidence of mumps has dropped from rates of 90–200 per 100 000 population to 7–10 per 100 000 in the late 1970s. It may be calculated that the use of mumps vaccine for a group of a million persons would prevent 74 000 cases of mumps and 3 deaths. Over a 30 year period mumps vaccine would reduce costs by over 86% and the benefit cost ratio approximates to 7:4:1. Mumps vaccine in Austria has a benefit cost ratio of 3.6:1 and in Switzerland 2.1:1 (data not shown in the table).

Comparable analyses appear to show that influenza vaccine is less effective in economic terms than rubella or measles vaccines but very different considerations pertain. The vaccine is recommended for special risk groups and not particularly for children. Medical care costs of vaccination during 1971–1978 totalled $808 million and 150 million persons were immunized in the USA, giving a cost of $63 per year of healthy life gained. Moreover, the cost effectiveness of vaccination improves with increasing age of the person vaccinated (because the highest mortality occcurs in the over 55 year age groups) so that it costs $258 per vaccination for each year of healthy life gained in the under 3 age group, to $23 per vaccination in the 45–64 age group, to positive cost savings in the 65+ age group. Assuming influenza vaccine is 70% effective, vaccinating a million elderly persons would result in 5400 additional years of life at a net cost of $491 per year of life gained. Most people would agree that this is a reasonable use of medical resources.

Four million cases of measles occurred in the USA each year before vaccine was introduced in 1963, with 4000 cases of encephalitis and 400–500 deaths each year. Net benefits of measles immunization between 1963 and 1972 include savings of 1.4 million hospital days, 75 million school days and 7900 cases of mental retardation. The benefit cost ratio was approximately 10:1. In Finland, as an example of a European country, the benefit cost ratio was 3.7:1. However, as measles declines, marginal reductions in incidence will become increasingly costly. Measles will probably be eliminated as an endemic disease in the USA during 1984.

An excellent recent example of the application of cost effectiveness of viral vaccines is being carried out in several countries at the present moment with the advent of an effective vaccine against hepatitis B (HBV) virus (Szmuness et al., 1981). The vaccine is rather costly because of the technical problems of purifying and inactivating antigen from human sera ($140 per course of 3 doses of vaccine) and, more important, its availability may be limited in the near future. Therefore 'decision analysis' can be used to estimate likely costs and benefits of different immunization approaches in different populations at risk. A decision analysis model was constructed to compare 3 alternatives for prevention, which were:

a. immunizing all persons with no prior screening for indication of previous infection
b. screening all persons for indications of previous infection and then immunizing only those sero or antigen negative persons
c. passive immunization of persons exposed to HBV.

The estimated cost per person of hepatitis vaccination in a 5 year period in a homosexual population with a 60% prevalence of HBV markers and 15% annual attack rate without screening and vaccinations is $96.66. Vaccination of all persons in this group would result in a lowering of HBV incidence from 23 to 4%, costing $105.12 per person. However, screening followed by immunization would cost only $66.35 (because fewer people would be immunized). In contrast, for a group of hospital employees with relatively high exposure (0.5%) and annual attack rate of 6%, vaccination *without* screening is the lowest cost strategy ($104.22). For a low risk population (0.1% annual attack rate) neither vaccination nor screening followed by vaccination would result in a saving in medical care costs. Indeed the net medical costs per case of hepatitis *prevented* by vaccinating the latter population would be $22469. In contrast, net medical care costs per case prevented are negative when the attack rate is greater than 5.6%. A very important caveat of the above discussion, however, is the fact that indirect costs saved by immunization, such as loss of productivity etc, are *not* included. If one is only interested in medical care savings then HBV vaccination should be carried out before or early during a period of unavoidable high risk as with surgical registrars, new dialysis unit patients and staff members, new prisoners, newly institutionalized mentally retarded patients, and promiscuous homosexuals.

We should note that in the example given above, which is rather USA-orientated, extrapolation to conditions in Europe may be questionable. In fact an excellent example of these differences has been highlighted with the current discussion about hepatitis B vaccine. For example, in Greece, the prevalence of anti-HBV among health workers is 40–50% and in medical and nursing students it was 12 and 17%, respectively. Screening costs around $25, whereas vaccine costs approximately $140 and so it seems reasonable to vaccinate health care workers *after* screening, and medical and nursing students without screening. In the UK the problem of finance is very relevant because additional funds may not be made available to health authorities and so again, screening may be resorted to as a cost saving excercise.

1.15. Targets for antiviral drugs

The development of an antiviral drug is a major undertaking and will require, at least for a private company, that the market for a drug is large enough to correspond to the cost and risk of development. Table 1.16 lists some viruses which have been ranked according to different variables in an attempt to select a good candidate for an antiviral drug. The incidence of the virus disease is naturally an important factor, as is the severity of the disease. The incidence can be obtained for diseases being reported in accordance with local regulations, but in many cases viral diseases are not reported and the incidence has to be calculated from different surveys. Also a grading of the severity is not easy and an example is when herpesvirus

infections are handled as a group, which would include both herpes encephalitis and cold sores. Therefore, a rather subjective average has been used in Table 1.16. An additional important factor in deciding the targets for antiviral chemotherapy is the absence or availability of good viral vaccines and the probability of developing vaccines in the future. For viruses like rhino and influenza with many serotypes or antigenic variants, vaccine production may always be a problem especially with influenza when new antigenic types appear rapidly. In the case of HSV-1 and HSV-2 infections the development of a successful vaccine seems unlikely when one considers that patients with frequent episodes of labial or genital herpes have high titres of neutralizing antibodies and that reinfection can occur in spite of circulating antibodies. Aspects of vaccines are discussed in more detail in Chapter 2 and in connection with the different viruses.

Rapid diagnosis (preferably by the patient!) is of importance since an antiviral drug is likely to have a narrow spectrum of activity and the virus to have a short time period of replication. It will be necessary to know exactly which virus is causing the infection and thus which drug should be used. The self-diagnosis of recurrent diseases such as labial and genital herpes is rather easy for the patient and, from that point of view, herpesvirus infections are good targets for antiviral drugs.

Major points of attack when developing new antiviral drugs are viral enzymes. Herpesvirus enzymes are easily accessible and well characterized, as are also influenza virus enzymes, and this feature also makes these two viruses attractive as targets for antiviral inhibitors. The use of viral enzymes as targets for antiviral drugs is discussed in Chapter 3. In essence, we conclude from the considerations in Table 1.16 that herpes and influenza should be major goals for the development of new antiviral drugs. This is also reflected in the literature where anti-herpes compounds are the most flourishing area at present.

TABLE 1.16.
Rank list of candidate viruses for the development of antiviral drugs.
The listing has been made to place the virus most favourable in the development of antiviral drugs on the top of each column. The added rank numbers will then give a crude estimation of the incentive to develop an antiviral drug against each virus. The lower the number the better the target.

Incidence	Severity	Problems with vaccine	Easy diagnosis	Accessible virus enzyme
1. Rhino	1. Hepatitis B	1. Herpes	1. Herpes	1. Herpes
2. Herpes	2. Influenza	2. Corona	2. Hepatitis B	2. Influenza
3. Corona	3. Herpes	3. Rhino	3. Influenza	3. Hepatitis B
4. Influenza	4. Rhino	4. Influenza	4. Rhino	4. Rhino
5. Hepatitis B	5. Corona	5. Hepatitis B	5. Corona	5. Corona

Rank: Herpes (8), influenza (15), rhino (16), hepatitis B (16), corona (20)

TABLE 1.17.
Viruses concerned in 3 WHO Programmes

EPI[a]	CDD	ARI
Measles	Rotavirus	Influenza A
Poliomyelitis	Adenovirus	Influenza B
	Astrovirus	Influenza C
	Calicivirus	Parainfluenza
	Coronavirus	Respiratory syncytial
	Enterovirus	Adenovirus
		Rhinovirus
		Enterovirus

[a] Although not included in the six target diseases, there is a relationship between this programme and the prevention and control of diseases such as yellow fever, hepatitis, rubella and mumps.
EPI, expanded immunization programme; CDD, campaign against diarrhoeal disease; ARI, acute respiratory infections.

1.16. Outlook for the future

The great need for therapy and prophylaxis of viral diseases is obvious. In recent years we have seen a swift expansion in our knowledge of the biological and chemical processes involved in viral diseases. This has made possible rational efforts to manage and prevent viral diseases, and we are now seeing the results in new vaccines and antiviral agents.

The greatest challenges and probably the most difficult and medically important areas for prophylaxis and therapy of viral diseases are those viruses which are rapidly changing in antigenic composition and/or viruses with animal reservoirs (influenza and arboviruses) and also those forming latent infections (herpesviruses). The three major international co-ordinating programmes of WHO (Table 1.17) include both these 'challenge' viruses and also more common viruses such as measles and polio where particular help is required in developing countries. The relative role of vaccines and antiviral drugs is difficult to predict. In cases where cheap and effective vaccines exist or can be developed as exemplified for polio, measles and hepatitis B, this is likely to be the optimum control method. In cases such as influenza, the great variability of the virus probably poses insurmountable difficulties for vaccines, and makes the antiviral drug approach more promising. Finally, for viruses such as the herpesviruses, causing recurrent infections (in spite of both preexisting humoral and cell mediated immunity) vaccines seem an unlikely approach and antiherpes drugs now appear to be more promising.

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CHAPTER 2

Virus vaccines and immunity: general considerations

2.1. Introductory and historical remarks

A rational basis for the prevention of either bacterial or viral infection by immunization could only be established after the germ theory of disease was accepted in the late 19th century. A basic general principle of vaccine design was established quite soon after this, namely by attenuation of virus virulence by serial passage in a new host. Such attenuated viruses are termed 'host range mutants' and an example is rabies virus passaged in the rabbit brain. This is essentially the method used up to the present for the development of many live attenuated vaccines. A rabies vaccine was pioneered by Pasteur in 1884, but attenuated yellow fever vaccine was not prepared until the 1930s, and influenza vaccine until the early 1940s (Table 2.1). The next generation of viral vaccines then had to wait until the late 1950s and 1960s when poliomyelitis, measles, rubella and mumps vaccines were introduced. These later vaccine developments were dependent upon the newly developed technologies of cell culture (Table 2.1).

As a result of mass vaccination campaigns (Table 2.2) childhood viral infections such as polio, measles and rubella have been well controlled in many of the more wealthy countries of the world such as Eastern and Western Europe, USSR, Australia, Japan and the USA, although with certain diseases, such as measles, very significant differences are to be seen in the vigour with which different countries pursue vaccination campaigns. It should also be remembered that in a *global* context infectious diseases (bacterial and viral) still take a heavy toll both in mortality and general human suffering (see Chapter 1). Specifically, paralytic polio is still widespread in certain countries in South America, Asia and Africa, whilst measles

TABLE 2.1.
History of production of human viral and bacterial vaccines

18th Century	19th Century	20th Century
1721 Variolation		1904 Tetanus antitoxin
		1920 Diphtheria TAF
		1930 Diphtheria toxoid
		Pertussis
		1936 Yellow Fever (live)
	1840 Variolation illegal	1940s Influenza
	1853 Vaccination compulsory	1955 Poliomyelitis (Salk)
		1960s Poliomyelitis (Sabin)
	1881 Rabies attenuated	Measles (killed and live)
	1891 Diphtheria antitoxin	Rubella (live)
		Mumps (live)
1798 Jenner's publications		

causes very serious problems indeed in children in these same countries. With these latter diseases, as with smallpox in the 1950s, it is probably correct to deduce that the scientific and medical problems faced in development and production of safe and efficacious vaccines have been overcome: it now requires social organization, political and economic action and a will to use these vaccines in many areas of the world. International cooperation is required because as long as reservoirs of the virus remain, importations into apparently 'virus free' countries will be a constant problem. Smallpox eradication is the example *par excellence* of a vigorous international approach to the elimination of an important infectious disease. The eradication campaign was initiated by the USSR and supported fully by the USA and therefore, with the necessary funds, scientific expertise and influence from the two superpowers the vaccination campaign was pushed forward in some of the poorest nations on earth with dramatic success (Chapter 15). There are no reasons now why similar international cooperation should not result in the demise of polio and measles viruses (Chapters 4 and 8).

Mainly empirical methods have been used for the scientific development of the above mentioned live or killed viral vaccines and biological methods have been used for their large scale production. Thus, following the early successes with inactivated rabies vaccine, virologists followed the seemingly well trodden path of cultivating the causative virus, preferably in cell culture, inactivating with formaldehyde, carefully standardizing the viral product as an immunogen and applying the material as a prophylactic by intramuscular injection. An example of such a vaccine was the inactivated polio virus vaccine which was introduced so successfully in the USA and Europe in the 1950s (Chapter 4). The alternative approach for live viral vaccines, given a lack of knowledge about the genetic determination of virulence and hence any logical way of altering this specifically, was to adapt a virulent virus to an alter-

TABLE 2.2.
Currently used virus vaccines

Virus vaccine and date introduced	Cell substrate	Usage	Inactivated or attenuated	Main problems	Comments on efficacy
Polio 1955 (killed) 1962 (live)	MK cells WI 38 cells	Worldwide	Live and killed vaccine used in different countries	Inactivated vaccine is expensive and more difficult to administer. Live vaccine causes a few cases of paralysis in recipients or contacts	Both vaccines are efficacious but probably could be improved by genetic engineering technology.
Measles (1963)	CEF	Worldwide	Attenuated	Adverse clinical symptoms in low proportion of vaccinees	Successful vaccine with 90% efficacy. Lifelong immunity.
Rubella (1969)	WI 38 cells RK	Worldwide	Attenuated	Adverse clinical symptoms in low proportion of vaccinees	Successful vaccine 95% efficacy. Long immunity. Not teratogenic.
Mumps (1967)	CEF	USA, Sweden, USSR	Attenuated	Sometimes low immunogenicity	Low demand but given with rubella and measles in USA and Sweden as a triple vaccine.
Rabies (19th century)	Animal brain, CEF, duck eggs, WI 38 cells	Worldwide	Inactivated	Older established brain vaccines are cheap but reactogenic. Human diploid vaccines are safer but expensive and so will have limited usage.	All effective and prophylactic or therapeutic. Genetic engineering of glycoprotein antigenic determinants in progress.
Influenza (1940s)	Eggs (UK, USA, Europe) or cell culture	Eastern and Western Europe, USA, Australia, China	Attenuated *ts*, *ca* viruses (USSR, China) and inactivated vaccines used.	Antigenic variation outdates vaccine yearly. Poor longevity of immune response. 'Antigenic sin'	Only 70% effective even against homologous virus. Experiments in progress with *ts*, *ca* mutants and genetic engineering.

TABLE 2.2. *(continued)*

Virus vaccine and date introduced	Cell substrate	Usage	Inactivated or attenuated	Main problems	Comments on efficacy
Hepatitis B (1981)	Human serum	USA, Europe	Inactivated subunit	Expensive and complicated to produce. In theory may be contaminated with unknown human viruses (e.g. AIDS).	Effective in controlled trials in special risk groups in USA, e.g. homosexuals.
Yellow fever (1936)	Eggs	Tropical areas	Attenuated 17D virus	Cheap to produce but some vaccine strains have contaminating avian leukosis viruses.	Long immunity induced. Highly effective.
Smallpox (19th century)	Cells lymph	—	Vaccinia (unknown origin)	Reactions could be serious leading to deaths.	Highly effective vaccine developed empirically. Stocked for emergency use only now.
Experimental vaccines					
Herpes HSV-1 HSV-2 cytomegalovirus	Cells	—	Inactivated	Poorly understood immunity and antigenic structure. Doubt about use in preventing reactivation e.g. genital herpes	Biological and cloning experiments in progress.
Varicella	Cells	—	Attenuated *ts* virus		Limited usefulness for varicella.
Rotavirus	Cells	—	Attenuated	Absence of easy growth in cell cultures of many strains	Recombinants between human and calf viruses under study. Also cloning experiments with human strains.

TABLE 2.2. *(continued)*

Virus vaccine and date introduced	Cell substrate	Usage	Inactivated or attenuated	Main problems	Comments on efficacy
Respiratory syncytial	Cells	—	Attenuated	Previous adverse reactions of inactivated vaccines. Emphasis now on *ts* viruses.	Preliminary data only.
A variety of tick borne and mosquito borne encephalitis viruses	Cells	—	Inactivated	Batches produced for at risk groups e.g. army, laboratory workers.	Not known.
Dengue haemorrhagic fever	Cells	—	Attenuated	Since immunopathology is incompletely understood, work progresses slowly i.e. superinfection with other dengue viruses can cause toxic syndromes.	Not known.

Note: It can be assumed that cloning experiments with all the above and also other viruses (e.g. Norwalk, enterovirus type 70, AIDS, picornaviruses, Non A Non B hepatitis) are now in progress and that a whole new range of experimental vaccines will soon be available for testing as viral antigens. Similarly, site specific mutagenesis should lead to new attenuated vaccines with known and stable mutations.

native host or tissue culture by the multiple passage technique and to hope that by the accumulation of random genome mutations, a new virion population of host range mutants would be selected, attenuated for man. It is now clear that this approach is aided by the extremely high genetic mutation rate in all RNA viruses. Even cloned RNA viruses are probably complex mixtures of genetically and biologically different subpopulations (Holland et al., 1982). Experience has shown this passage technique to be quite a successful method, but by no means perfect, and we shall examine some successes in other chapters with live rubella, measles, polio and mumps virus vaccines (Chapters 4, 8 and 9), all produced in this manner.

However, some serious and unexpected medical problems have been noted over the last decade with both inactivated and attenuated virus vaccines and clearly all has not been plain sailing in the area of development of new vaccines. As an example of a totally unexpected experimental problem, inactivated vaccines against respiratory syncytial virus (RSV) infection resulted in some immunized children, when they were later in contact with virulent RSV and should have been protected, developing a more serious disease than their non-immunized classmates (Fulginiti et al., 1966; Parrott et al., 1967). This warns us that, optimally, the immunopathology of an infection and the antigenic composition of the virus particle itself should be understood before attempts are made to prepare and use an inactivated vaccine. On the other hand, a problem with some attenuated vaccines such as polio and *ts* mutants of influenza virus is reversion to parental-type virulence (reviewed by Meyer et al., 1980) and this warns us that for most viruses we still do not understand the underlying genetic mechanisms which determine virulence. However, a practical person could argue that little detail was known about the immune response to smallpox virus proteins or about the virus itself and yet the vaccine virus was remarkably stable genetically and efficacious in the field. Perhaps, to some extent, we are now left with the difficult unsolved problem viruses: influenza, common-cold viruses, RSV, hepatitis, arboviruses and herpes viruses such as varicella, herpes zoster and herpes labialis. Influenza can undergo rapid antigenic shifts and drifts (see Chapter 7) and so can circumvent not only vaccine induced immunity but natural immunity as well. Rhinoviruses exist as over 150 serotypes with no common antigen, at least, no such antigen has been described to date. Herpes viruses, apart from their antigenic complexity, have a latent phase as part of their biological life cycle and a cell to cell passage which makes it difficult to envisage how or where in such a cycle vaccines could be used to prevent reactivation or symptoms. With hepatitis B virus we face problems of laboratory cultivation of the virus, as well as a dearth of clear knowledge about immune responses and their significance. However, new ideas have evolved recently which open new concepts in vaccine technology (although unfortunately not in philosophy). Thus, viral genes can now be cloned in plasmids or other vectors and can be translated in certain host cells (bacteria, yeasts, plants and human cells) to produce immunologically active viral proteins (Gething et al., 1980, Greenberg et al., 1981, Hardy et al., 1981, Moriarty et al., 1981, Bittle et al., 1982).

In conjunction, the application of rapid DNA sequencing techniques to these DNA clones gives a wealth of sequence data for important viral immunogenic proteins. It is now possible to synthesize short peptides of these proteins to act as artificial antigens (see below, Green et al., 1982) and it might be possible to discover previously obscured common antigens for all the rhinoviruses, shared fusion proteins for myxo- and paramyxoviruses or immunogenic peptides for both herpes type I and type II viruses. So the ideas of immune prophylaxis of viral infections have expanded considerably in recent times and we are entering a new technical era and a time of scientific judgement where vaccines could be *designed*. Nevertheless, we shall note with antivirals (Chapter 3) that the most successful inhibitory compounds have been found by serendipity (but remember the Pasteurian thought 'Chance favours the prepared mind') and similarly, several of the most successful vaccines (smallpox, polio, measles and rubella) were discovered and used well before molecular biology became consolidated as a scientific discipline. It is nevertheless apparent that the technology alluded to above will undoubtedly increase the pace of new developments with both vaccines and antivirals. We shall have to judge in the future if these developments result in fundamental new approaches, or simply add some superficial gilt and technology to an already existing discovery. The reader is referred to a number of excellent reviews giving details of these and other approaches to viral vaccines (Voller and Friedman, 1978, Arnon, 1980, Chanock, 1981, 1982, Meyer et al., 1980, Schiff, 1980, Selby, 1976) and we shall limit ourselves in this chapter to dealing more clearly with a few viruses to illustrate some successes, some failures and important general concepts and future prospects. As is often the case in human endeavour most work on a topic does not lead to most success (e.g. we know more about influenza than any other virus, but it is the least successfully controlled) and conversely single brilliant steps of a moment by one or two persons can lead to tremendous advances (e.g. smallpox and polio vaccines). In this same chapter we would also like to introduce the reader to mathematical-modelling ideas which are designed to help epidemiologists to co-ordinate and design immunization campaigns in a logical way. General principles of viral immunopathology, epidemiology, immune response and virus neutralization will also be discussed. Finally, we should not forget techniques and principles of vaccine production and standardization, and studies of side reactions and efficacy of vaccines in the field. The interrelationship of all these factors is important for the planned demise of any virus disease of man.

2.2. Simple immunological concepts relevant to viral vaccines

Following infection with a virus, two different types of immunological reaction may occur to a varying degree. Firstly, synthesis and release of free antibodies into the blood and other body fluids (humoral immune response) which would neutralize

virus particles themselves or mediate an immune response to a virus infected cell. Secondly, production of sensitized lymphocytes may occur which are responsible for cell mediated immunity (CMI). This latter cell mediated immunity may also be effective against viruses replicating intracellularly because cytotoxic T cells, for example, can lyse virus infected cells, which have virus antigens on the cell surface (reviewed by Mims, 1982).

The small circulating lymphocyte cells which move from the blood into lymph nodes, spleen and tissues and then back to the blood (Fig. 2.1) are of central importance for both types of immune response since, for example, experimental removal of these cells (in animal models) results in gross impairment of the primary antibody response to antigens like influenza HA. The effect of naturally occurring depletions of B or T lymphocytes in humans are summarized in Table 2.3. These small lymphocyte cells can also become antibody synthesizing plasma cells (B cells) as well as effector cells in cell mediated immunity (T cells). In addition, transfer experiments in animals have established that small lymphocytes carry 'memory' of the

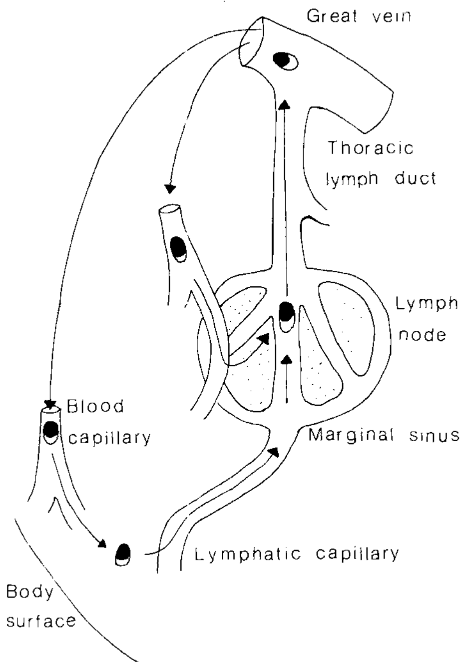


Fig. 2.1. Lymphocyte recirculation (after Mims, 1982). Regional lymph nodes respond to antigens introduced into the tissues they drain, and the spleen to antigens in the blood. The gut and external mucous surfaces also have their own less specialized lymphoid organs. The mixing of lymphocytes, particularly in the spleen and lymph nodes, ensures the maximum contact of cells that have encountered antigen with others potentially able to respond, and ensures the bodywide dissemination of expanded memory populations in readiness for a second encounter with the same viral antigen. Different types of lymphocytes tend to home to different regions in the lymphoid organs. This is presumably due to either chemotactic factors, unique to particular sites, or to recognition of different lymphocytes by local organs.

TABLE 2.3.
The effects of depletion of T or B lymphocytes on recovery from various viral infections (after Fenner and White, 1976)

Animal	Immunodeficiency	Lymph-ocytes involved	Infections aggravated	Infections unaffected
Man	Hypogammaglobulinemia with intact CMI	B	Paralytic poliomyelitis	Smallpox vaccination
Man	Deficient CMI (with or without normal immunoglobulins)	T	Vaccinia Herpes simplex Varicella-zoster Cytomegalovirus Measles	
Mouse	Suppression of antibody by cyclophosphamide	B	Coxsackievirus B	
Mouse	Impairment of CMI by neonatal thymectomy or antilymph-ocyte (or anti-theta) serum	T	Herpes simplex Mousepox Vaccinia	Influenza Sendai Yellow fever

first contact with a virus antigen. Thus, transfer of small lymphocytes from an immunized animal to an immunological 'virgin' and then immunization of the latter will result in a rapid secondary immune response rather than a normal slow 'primary' response. In essence, following an immune stimulus by a viral antigen a small number of lymphocytes specific for the virus antigen proliferate and undergo so-called 'clonal expansion' and produce antibody or cell mediated immunity, together with memory (Fig. 2.2).

An extensive cooperation exists between B cells and T cells and the latter, for example, help the stimulation of B lymphocytes by viral antigens to be more effective. Primed B cells make a secondary antibody response to a hapten bound to a protein carrier only when T 'helper' cells primed to the carrier are also present. Certain antigens which have highly repeating antigenic determinants are thymus independent and stimulate B cells without T cell help, but most viral antigens probably use their other determinants as a carrier to evoke T cell cooperation as outlined above. T-cell assistance will be even more essential where virus determinants appear only once per molecule, thus acting as a monovalent hapten. To some extent, therefore, we can predict that larger viral proteins may be more efficient antigens because they have more antigenic determinants acting as carriers and that intact virus particles have several identical capsomeric subunits acting as antigenic determinants.

Macrophages (mononuclear cells) play a further and pivotal role in the immune response to certain viral infections (Fig. 2.3). On the one hand intimate cytoplasmic contact between macrophages and lymphocytes has been described, and also, when macrophages are depleted antibody response to viral antigens is markedly reduced.

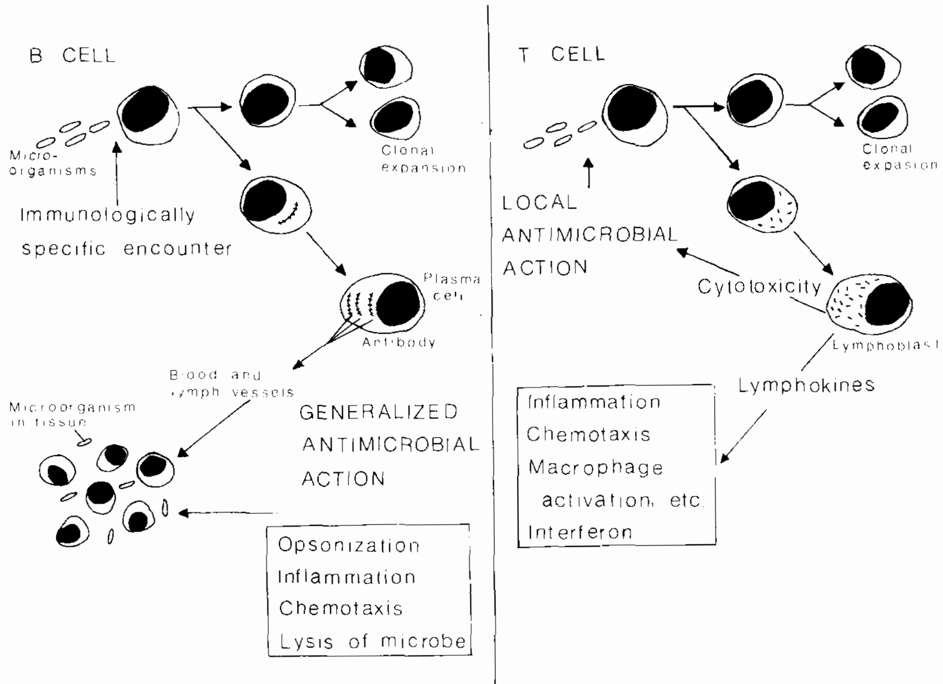
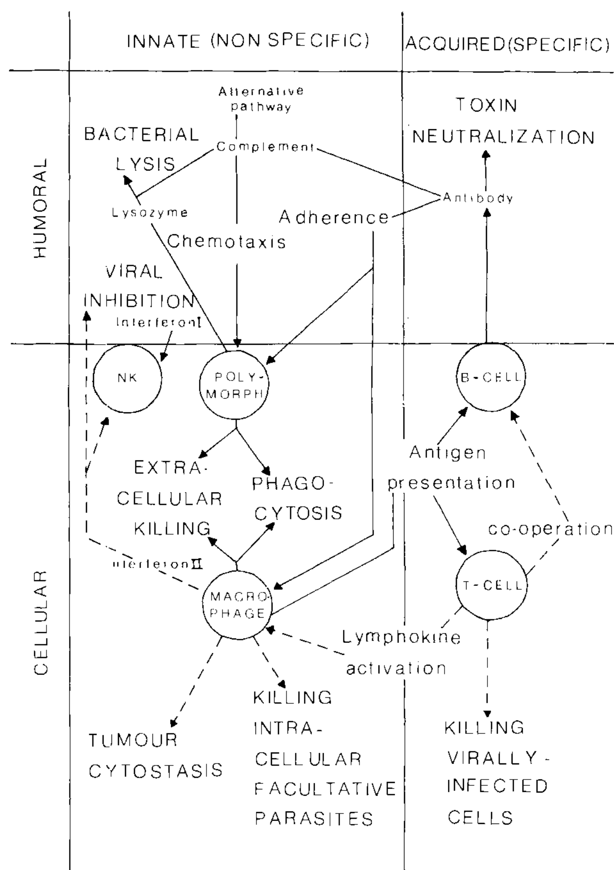


Fig. 2.2. Comparison of antimicrobial action of B and T lymphocytes (after Mims, 1982). The first property of T cells to be distinguished was that of helping B cells to make antibody, but four types of T cell are now recognized namely cytotoxic, suppressor, helper and delayed hypersensitivity T cells. Cells resembling lymphocytes, but without characteristic T or B cell markers are referred to as 'null' cells. This group probably includes early T cells, B cells, and monocytes, as well as the natural killer (NK) cells, possibly important in tumour immunity. In blood and lymphoid organs, up to 10% of lymphocytes are null cells.

Mechanisms proposed for this cooperation include antigen trapping and antigen presentation to lymphocytes. Certain antigens are taken up by macrophages and degraded by proteolysis whilst a portion is presented on the macrophage cell surface, possibly in association with major histocompatibility antigens and in a strongly immunogenic state.

Fig. 2.3. Interactions between natural and specific immunity mechanisms (after Roitt, 1980). Note that lymphokines are a large group of non-antigen specific factors produced by T (and sometimes B) cells, with different biological effects, concerned essentially with mediating interactions between cells. Some of the best known act on macrophages, but there are also interactions in the opposite direction — i.e. by macrophages on lymphocytes and the present tendency is to refer to all such 'interaction' molecules as interleukins. In general these factors are presumed to act only at high concentration, and thus to help localise cell-mediated immune responses to the vicinity of the antigen. Examples of this type of factor are:



Made by lymphocytes: Macrophage migration inhibitory factor (MIF), macrophage activating factor (MAF), eosinophil chemotactic and activating factors, mitogenic factor, T cell growth factor, interferon (type 2 or 'immune' interferon) and proliferation inhibitory factor (PIF).

Made by macrophages: Lymphocyte activating factor, interferon (type 1), Cytoxin. 'Helper' T cells respond to 'altered-self' determinants and help 'cytotoxic' T cells. Most of the proliferation when allogeneic lymphocytes are mixed ('mixed lymphocyte reaction') is due to these cells. Like the antibody-helper T cells, they recognize antigen in association with Ia products of the major histocompatibility complex (MHC). 'Cytotoxic' T cells, recognize major histocompatibility antigens, or antigens (eg viruses) associated with them, and induce irreversible lytic changes in the target cell. They differ from helper T cells both in their surface antigens (eg LyT, and in the MHC products they recognize. Instead of differentiating into antibody-producing plasma cells, some B cells persist as 'memory' cells, whose increased number and rapid response underlies the highly augmented secondary response. Memory B cells differ slightly from their precursors (in surface Ig, adherence, recirculation, etc). The generation of memory cells appears to take place in germinal centres, and to be somehow linked to T cells, since T-independent responses do not show memory. However T-dependent memory cells can be generated in the absence of T cells, so it may be the B cells themselves which are different. Macrophages play a central role, both in triggering T and B cells and in eliminating the antigen. The cells which present antigen may not be the same as the ones that phagocytose it; probably macrophages will turn out, like lymphocytes, to be made up of interesting subpopulations. Reactions influenced by T cells are shown by a broken line.

2.2.1. Cell mediated immune responses to viruses

As well as providing helper activity to bone marrow derived lymphocytes, further T cell subsets are responsible for different effector functions for cell mediated immunity — delayed type hypersensitivity (Ta) and virus infected cell killer activity (cytotoxic or killer T cells). In addition, T cell subpopulations modulate immune responses by either helping B or T cell clonal expansion and maturation (T helper or T_H cells) or suppressing these events (T suppressor or T_S cells) following antigenic stimulation. Cytotoxic T cells are thymus derived lymphocytes that can lyse virus infected 'target' cells. The lytic effect is not complement or antibody mediated but probably results from a fusion event between the two cells. Cytotoxic T cells are specific in their action and recognize virus antigen on the surface of cells. However, for their action to be successful they must also recognize histocompatability antigens on the same cell surface (reviewed by McMichael, 1980, Zinkernagel and Doherty, 1974). These histocompatability antigens are cell surface glycoproteins controlled by a group of cell genes; the major histocompatibility complex. The genes show a considerable degree of polymorphism and the glycoproteins provoke vigorous immune reactions if mismatched, for example, in transplantation experiments.

Since virus sensitized cytotoxic T cells are specifically adsorbed onto virus infected cells they must have surface receptors which recognize virus antigen, although the precise nature of these receptors is not clear. The receptors are not conventional IgG antibodies. An accepted view is that the receptors have a molecular weight of 150 000 and consist of 2 chains, each of which uses an Ig heavy chain variable region linked to a constant region gene but different from those coding for normal heavy chain peptides (reviewed by Roitt, 1980). The T cell membrane becomes activated and the cell transforms into a large blast cell and proliferates, one population releasing soluble factors (lymphokines), another becoming cytotoxic, a third becoming memory cells.

Obviously a very careful and precise balance must be maintained when a normal host resists viral invasion using these complex interdependent methods. We would want to ensure that, in most cases, vaccine-virus induces a similar response to the natural infection and not an aberrant response. Cell mediated immunity aids in recovery from viral infection and is not primarily responsible for prevention of infection.

2.2.2. Natural killer (NK) cells and viral infections

These NK cells which may have an important immunoregulatory role have the ability to kill certain tumour target cells grown in vitro – they are not T or B lymphocytes, or adherent, or phagocytic cells. In man, NK cells are large granular lymphocytes. There is increasing evidence that NK cells play a role in either protection, or more likely, recovery from viral infections and NK cell activity can be stimulated by immunization.

TABLE 2.4.
Role of antibody and cell mediated immunity (CMI) in resistance to systemic infections

Type of resistance	Antibody	CMI
Recovery from primary infection	Yellow fever Poliovirus Coxsackie viruses	Pox viruses e.g. ectromelia (mice) vaccinia (man) Herpes viruses Herpes simplex Varicella-zoster Cytomegalovirus LCM virus (mice) Measles
Resistance to infection	Nearly all viruses	
Resistance to reactivation of latent infection	No effect	Varicella-zoster Cytomegalovirus Herpes simplex

Furthermore, NK activity is increased by the presence of interferons (Ennis and Meager, 1981). NK cells may act as a surveillance mechanism against viruses. The increase in NK activity mediated by interferon may result from recruitment of NK cells, and interferon may be produced by NK cells themselves. Recently some evidence has been published to suggest that NK activity is augmented by interleukin 2. NK cells quite obviously play an important, although as yet still undefined, role in the complex network of immunological interactions (reviewed by Mims, 1982).

2.2.3. Damage mediated via the immune response

It should not be forgotten that the actual expression of the immune response, involving cell infiltration and swelling of lymph nodes, can itself result in a severe pathology, disease or even death (Mims, 1982). Thus the lymph node swelling seen in glandular fever (Chapter 14) is an immunopathological feature of the disease. Different types of immunopathology are summarized in Table 2.5. Of particular relevance to viral vaccines are anaphylactic reactions (type 1) since these are thought to result in *increased* clinical symptoms in some children immunized with experimental RSV or measles vaccines (Chapter 8). Immune complex reactions (type 3) can give rise to disseminated intravascular coagulation, as with yellow fever and haemorrhagic arthropod borne diseases (dengue haemorrhagic fever (Chapter 5) and smallpox (Chapter 15)). Immune complex reactions activate the enzymes of the coagulation cascade and the fibrin formed is deposited in blood vessels in the kidneys, adrenals and pituitary causing multiple thromboses.

TABLE 2.5.
Immunopathological reactions and viral diseases

	Mechanism	Result	Example from infectious disease
Type 1 Anaphylactic	Antigen + Reaginic (IgE) antibody attached to mast cells → histamine etc. release	Anaphylactic shock Bronchospasm Local inflammation	Contribution to certain rashes
Type 2 Cytotoxic	Antibody + antigen on cell surface → complement activation	Lysis of cell bearing microbial antigens	Liver cell necrosis in serum hepatitis
Type 3 Immune complex	Antibody + extracellular antigen → complex → complement activation, generation of inflammatory mediators and endogenous pyrogen	*Extravascular complex* Inflammation ± tissue damage *Intravascular complex* Complex deposition in glomeruli, joints, small skin vessels, choroid plexus → glomerulonephritis vasculitis, etc.	Edge of smallpox vaccination site Glomerulonephritis in LCM virus infection (mice) or Malaria (man) Prodromal rashes Fever
Type 4 Cell-mediated (delayed)	Sensitized T lymphocyte reacts with antigen; lymphokines liberated	*Extracellular antigen* Inflammation, mononuclear accumulation, macrophage activation Tissue damage *Antigen on tissue cell* T-lymphocyte lyses cell	Acute LCM virus disease in mice. Certain virus rashes

2.2.4. Local production of virus neutralizing antibody of the IgA class

Viral infections of the respiratory or intestinal tracts induce the synthesis of neutralizing IgA antibody and this class of antibodies is synthesized locally (Table 2.6). IgA antibody appears selectively in sero-mucous secretions such as saliva, secretions of the lung, genital and gastro-intestinal tracts and nasal fluids, where it can help protect exposed external surfaces of the body to viral invasion. The IgA molecule itself is a dimer and is stabilized against proteolytic degradation by a 'secretory component' synthesized by the local epithelial cells. For example, IgA is found in human nasal secretions and may be of major importance in protecting persons against respiratory viruses, since a marked correlation has been found between titres of local IgA antibodies and resistance to parainfluenza or respiratory syncytial virus (RSV) infection, whereas a poor correlation is often noted between serum IgG levels of neutralizing antibody and protection. Modern ELISA techniques may

TABLE 2.6.
Properties of immunoglobulin classes in man (after Mims, 1982)

Property	IgG	IgM	IgA[a]	IgE
Mol. wt.	145 000	850 000	385 000 (170 000)	200 000
Heavy chain	γ	μ	α	ϵ
Half life (days)	25	5	6[c]	2
Complement fixation	+	++	±	—
Transfer to offspring:	Via placenta	No transfer	Via milk	No transfer
Proportion in:				
blood	40%	90+%	0	V. low
extracellular fluids	60%	<10%	0	—
secretions	0[b]	0[b]	100%	High
Functional significance	Major systemic immuno-globulin	Appears early in immune response. Appears early in development	Present on mucosal surfaces	Allergenic responses e.g. epithelial surfaces

[a] Data for secretory IgA: serum IgA in brackets.
[b] Can be increased in inflammation, IgA deficiency.
[c] The half life of secretory IgA on mucosal surfaces is measured in minutes rather than days, because it is soon carried away in secretions or mucus.

now make this fascinating area of local immunity more easy to investigate, because the interpretation of many earlier studies using conventional and less sensitive serological techniques was extremely difficult (see below).

2.2.5. Virus neutralizing IgM antibodies

The high molecular weight IgM antibodies are polymers of five peptide subunits, and when the molecule is visualized by electron microscopy it appears star shaped with an effective combining valency of 5. These IgM antibodies are of low affinity but of relatively high avidity and are good initiators of agglutinating and cytolytic reactions. IgM antibodies appear early in viral infection and are largely confined to the bloodstream. They can be of diagnostic value in determining recent infections, as is the case for the rapid diagnosis of recent rubella infections in pregnancy, where only a single and early serum sample is available. A high level of IgM antibody reacting with rubella virus antigens is taken to indicate a recent infection with this virus.

2.2.6. Virus neutralizing IgG antibodies

IgG is the major immunoglobulin synthesized during a secondary immune response

and is able to diffuse into extravascular body spaces. Often, where an immune response to a vaccine is investigated, in practical terms the investigator measures levels of virus specific serum IgG antibodies.

2.2.7. IgE antibodies

Only very low levels of IgE are present in serum. IgE may be responsible for primary protection of external mucosa surfaces to viral invasion. Theoretically, viruses which avoided IgA antibodies might react with specific IgE on the surface of mast cells and trigger the release of chemotactic and other factors, so resulting in an influx of IgG, polymorphs and complement.

2.2.8. Original antigenic sin and viral antigens

A peculiarity of the immunological memory which should be noted at this stage is that with certain viruses such as influenza, enteroviruses, paramyxoviruses and togaviruses (where there exist several cross-reacting antigenic subtypes) the first encounter of a person with the virus produces an indelible imprint on the immunological memory. During subsequent encounters with immunologically related but nevertheless antigenically distinguishable viral variants the immune system produces an overwhelming immune response to the *first* viral variant encountered. This phenomenon was called 'original antigenic sin' by T. Francis who investigated the immune response to influenza A viruses in considerable detail (Francis, 1953). Certainly, original antigenic sin imposes an immunological barrier of prime importance when we consider the possibility of immunizing against these viruses. Many persons who are immunized with the most recent antigenic variant of influenza produce their main immune response to previous influenza viruses (which are no longer circulating), whereas a specific immune response against the current HA would produce most neutralizing antibody and make the vaccine most efficacious (Schild et al., 1977, Oxford et al., 1981).

2.2.9. Stimulation and synthesis of viral antibodies

Mice may be selectively bred to produce high or low titres of antibodies to viral antigens and ten or more genes may be involved, representing macrophage function, regions of immunoglobulin etc. In general terms it is understood that we inherit genes which allow us to make particular antibodies and our antibody response may be limited by the repertoire of specificities encoded by these genes. In practical terms we might expect, therefore, that the immune response to a particular virus or viral vaccine may vary from individual to individual.

A 'clonal selection' theory represents the basis of our ideas on the functioning of antigen recognition and antibody production. In essence, the hypothesis is that

the data required for the synthesis of antibodies to different virus proteins is already present in our genes and a particular gene encoding a specific antiviral antibody is switched on by contact of the virus antigen (or vaccine) with the immune cell. An appropriate mRNA is transcribed and translated into an immunoglobulin molecule with an appropriate amino acid sequence to form an antibody to bind specifically with that virus. Burnet's theory of clonal selection suggests that each lymphocyte is capable of making only one particular antibody and molecules of that antibody are built into the cell surface as receptors. Viral antigen will combine with a lymphocyte receptor and stimulate that lymphocyte to divide and expand, synthesizing antibody of the correct antiviral specificity and producing some memory cells. It can be estimated that an individual would have a repertoire of 10^8 or more different antibody molecules, which shows a remarkable diversity!

2.2.10. Virus neutralization

In principle it can be envisaged that antibodies stimulated by immunization with an inactivated virus vaccine would interact directly with an antigenic site on the infecting virus and so prevent the virus from attaching to a susceptible cell. It should be realised, however, that other more complex mechanisms could come into play, including interaction of antibodies with regions contiguous to the active site i.e. steric hindrance, or interaction with other regions of the virion surface so producing conformational changes in the molecule leading to loss of infectivity. Detailed immunochemical studies of interactions of antibodies with enzymes have shown that, following immunization, a very diverse population of antibodies is induced and that antibodies effective in inhibition or neutralization may represent only a minute fraction of the total. We could conclude, then, that if we could immunize with the critical virus neutralizing antigenic determinant, then only virus neutralizing antibodies would be stimulated and a much more efficient protective immunity could be induced. We shall return to this point later in this chapter when we discuss the possibilities of synthetic viral oligopeptides as antigens for viral vaccines.

Meanwhile, however, it must be admitted that our knowledge of precisely how antiviral antibodies inhibit the replication of most viruses at the molecular level is very incomplete and antibodies may have many roles. Recently, for example, Possee et al. (1982) have shown that anti-HA antibodies to influenza do not always prevent virus entry but may, unexpectedly, inhibit intracellular virus RNA transcription. This is a controversial interpretation because one would have to postulate interaction of antibodies with the virus RNA polymerase complex. Antibodies to measles virus have been shown to modulate intracellular polypeptide synthesis, again by unknown mechanisms (Sissons and Oldstone, 1980, Fujinami and Oldstone, 1979). An additional factor with certain viruses is that the presence of a low level of antibody may *enhance* virus replication and this has been studied with, for example, certain flaviviruses (Peiris and Porterfield, 1979). The advent of a range of monoclonal

antibodies to virus epitopes, some of them with virus 'neutralizing' properties and others with additional biological activities, will hopefully lead to new conceptions of virus neutralization. In certain viruses we do not want vaccines to induce only neutralizing antibodies. Thus, with paramyxoviruses, antibodies to the F or fusion protein may be more protective in vivo than antibodies to the HN protein.

Finally, we have examples such as antibodies to influenza NA protein, which do not neutralize virus in the classical sense but, nevertheless, inhibit the spread of virus in vivo by cross-linking budding virions and hence inhibiting spread of virus from cell to cell (see Chapter 7). We may summarize by noting that antibody may neutralize alone or function together with complement to lyse (Radwan et al., 1973, Oroszian and Gilden, 1970, Welsh et al., 1976b, Nemerow and Cooper, 1981), aggregate (Oldstone et al., 1974) or cover viruses in a coating of protein (Daniels et al., 1969, Berry and Almeida, 1968, Linscott and Levinson, 1969, Leddy et al., 1977, Beebe and Cooper, 1981) thus inactivating them. In addition, complement recognizes and inactivates certain viruses directly without the participation of antibody. In these instances complement represents a primary rather than a secondary defence mechanism (Welsh et al., 1976a).

2.2.11. Complement

Complement is able to cause inflammation and tissue damage and has antiviral functions as summarized in Fig. 2.4. The inflammation induced at the site of reaction of antibodies with viruses focuses leukocytes and plasma factors to the site whilst the C_3 component bound to complexes attaches to C_3 receptors on phagocytes and may promote phagocytosis. Where antibody has reacted with budding virus particles, $C_{8\ 9}$ is activated and phospholipase may destroy the cell (Mims, 1982).

2.2.12. Mucosal vaccination

As alluded to above, it might seem logical to enhance the production of specific immunological defence mechanisms in mucosal surfaces, as well as systemically, for viruses which replicate in the upper respiratory tract or enterically. Approximately 20 viral veterinary and human vaccines are used which are administered via the mucosal route although only three of these are against human disease (Table 2.7). It is very possible, however, that new human vaccines against RSV, rotavirus, influenza (C. Bergmann, Berlin, personal communication) and parainfluenza may be developed and administered in this manner (Chanock, 1981). It is well established that most human pathogenic viruses gain entry through the mucous membranes of the respiratory, intestinal and genital tracts and after an initial replication in the mucosal surface invade the bloodstream and produce disease in systemic target tissues (reviewed by Mims, 1982, Ogra et al., 1980). We should not forget that a number of respiratory (influenza, RSV etc.) and enteric viruses (e.g. rotaviruses) produce

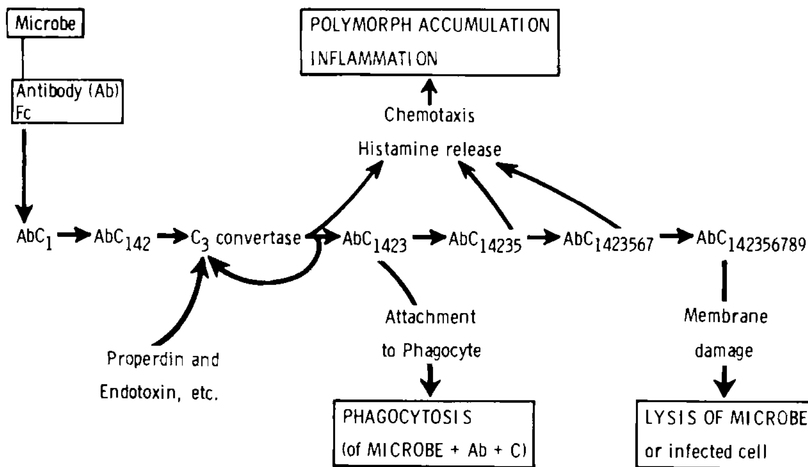


Fig. 2.4. Complement activation sequence and antiviral action (after Mims, 1982). Complement consists of a complicated system of 9 protein components (C_1–C_9) present in normal serum. It functions by mediating and amplifying immune reactions. The first component (C_1) is activated after combining with immune complexes (antibody bound to antigen). The immune complex may be free in the tissues or located on a cell surface following the reaction of specific antibody with a cell surface antigen. The activated first component is an enzyme, and acts on the next component to form a larger number of molecules of the second component's enzyme. This in turn activates larger amounts of the next component, and so on, producing a cascade reaction. A single molecule of activated C_1 generates thousands of molecules of the later components and the final response is thus greatly amplified. The later complement components have various biological activities, including inflammation and cell destruction, so that an immunologically specific reaction at the molecular level can lead to a relatively gross response in the tissues.

disease localized at the site of initial mucosal replication. Furthermore experience with RSV and measles has shown quite clearly (Fulginiti et al., 1966) that the appearance of antibody or cell mediated immune responses systemically may be potentially hazardous.

A striking feature of the mucosal immune system (gut-associated lymphoid tissue, bronchus-associated lymphoid tissue and elements in genital mucosa, salivary glands, respiratory tract, pharynx and mammary glands) is the presence of large quantities of secretory IgA although, in addition, 7sIgA, IgG, IgM and T lymphocytes are also present. Significant amounts of IgG and IgM antibodies in external secretions may be synthesized locally. Secretory IgA is relatively resistant to degradation by proteolytic enzymes and would adhere to the mucuous coat of the epithelial surface by specific interaction with the cystine residues in the mucus. Certainly, protection against mucosal reinfection with a number of respiratory and enteric viruses is better correlated with the level of secretory IgA rather than with serum antibody.

During early trials with attenuated polio vaccine it became apparent that a state

TABLE 2.7.
Viral vaccines that have been successfully used for immunization by the mucosal route (after Ogra et al., 1980)

Animals immunized, virus	Vaccine type(s)	Route
Birds		
Newcastle disease	Live, killed	Inhalation, oral, ocular
Infectious bronchitis	Live	Oral, ocular
Fowl pox	Live	Oral
Infectious laryngotracheitis	Live	Oral, ocular
Encephalomyelitis	Live	Inhalation, oral
Marek's disease	Live	Inhalation, oral
Infectious bursal disease	Live	Ocular, oral
Cattle		
Parainfluenza	Live	Nasal
Infectious rhinotracheitis	Live	Nasal
Adenovirus	Live	Nasal
Rotavirus	Live	Oral
Mink		
Distemper	Live	Oral
Swine		
Transmissible gastroenteritis	Killed	Oral
Duck		
Hepatitis	Live	Oral
Fox, dog and bat		
Rabies	Live	Oral
Humans		
Poliovirus	Live	Oral
Rubella	Live	Nasal
Adenovirus	Live	Oral

of alimentary resistance to subsequent infection with wild virus was achieved, whereas the alimentary tract of individuals immunized with killed vaccine remained susceptible to reinfection (reviewed by Ogra et al., 1980). High levels of secretory antibody can be induced as a result of *intranasal* immunization with killed polio vaccine which completely inhibit nasopharyngeal replication of virus.

In summary, therefore, much remains to be learnt about mechanisms of virus-antibody or virus-immune cell interaction, as well as about interactions of immune cells themselves.

2.3. Virus vaccine production, standardization, and control

In most of Western Europe, the USA, Australia and Japan, for example, human viral vaccines are produced by independent pharmaceutical groups, whilst in certain

Scandinavian countries, in Eastern Europe, USSR and China vaccines are manufactured by state organizations or vaccine institutes. Efforts are made by international organizations such as the WHO to ensure that virus vaccine seed material and standard laboratory reagents are distributed internationally, and that vaccine production facilities in different countries conform to a given standard. Thus, most countries use well investigated stock vaccine viruses, characterized cell cultures for virus growth and well standardized final products for immunization. Most manufacturers use a seed virus technique in production (particularly for attenuated virus vaccines) whereby a large batch of well characterized vaccine virus is frozen at −70°C and the actual virus vaccine is produced by infecting cells with virus that is only 1 or 2 passages removed from the seed virus (British Pharmacopoea, 1980). This reduces the chance of contamination of the vaccine with adventitious viruses and also reduces the opportunity for further random mutations to occur in vaccine virus and reduces the chance of phenotypic change of vaccine virus from attenuation to virulence. The WHO programme on expanded immunization encourages work in many countries (Table 2.8) on nine important uncontrolled infectious dis-

TABLE 2.8.
Countries/areas constituting regions in the expanded programme on immunization of the World Health Organization

Africa	Americas	Eastern Mediterranean	Europe	Southeast Asia	Western Pacific
Angola	Argentina	Afghanistan	Albania	Bangladesh	Australia
Benin	Bahamas	Bahrain	Algeria	Bhutan	China
Botswana	Barbados	Cyprus	Austria	Burma	Democratic Kampuchea
Burundi	Bolivia	Democratic Yemen	Belgium	Democratic People's Republic of Korea	Fiji
Cape Verde	Brazil	Djibouti	Bulgaria	India	Japan
Central African Republic	Canada	Egypt	Czechoslovakia	Indonesia	Lao People's Democratic Republic
Chad	Chile	Iran	Denmark	Maldives	Malaysia
Comoros	Colombia	Iraq	Finland	Mongolia	New Zealand
Congo	Costa Rica	Israel	France	Nepal	Papua New Guinea
Equatorial Guinea	Cuba	Jordan	German Democratic Republic	Sri Lanka	Philippines
Ethiopia	Dominica	Kuwait	Germany, Federal Republic of	Thailand	Republic of Korea
Gabon	Dominican Republic	Lebanon	Greece		Samoa
Gambia	Ecuador	Libyan Arab Jamahiriya	Hungary		Singapore
Ghana	El Salvador	Oman	Iceland		Tonga
Guinea	Grenada	Pakistan	Ireland		Viet Nam
Guinea-Bissau	Guatemala	Qatar	Italy		
Ivory Coast	Guyana	Saudi Arabia	Luxembourg		
Kenya	Haiti	Somalia	Malta		
Lesotho	Honduras	Sudan			
Liberia	Jamaica	Syrian Arab			
	Mexico				

TABLE 2.8. *(continued)*

Africa	Americas	Eastern Mediterranean	Europe	Southeast Asia	Western Pacific
Madagascar	Nicaragua	Republic	Monaco		
Malawi	Panama	Tunisia	Morocco		
Mali	Paraguay	United Arab Emirates	Netherlands		
Mauritania	Peru	Yemen	Norway		
Mauritius	St. Lucia		Poland		
Mozambique	Suriname		Portugal		
Niger	Trinidad and Tobago		Romania		
Nigeria	United States of America		San Marino		
Rwanda	Uruguay		Spain		
Sao Tome and Principe	Venezuela		Sweden		
Senegal			Switzerland		
Seychelles			Turkey		
Sierra Leone			USSR		
South Africa			United Kingdom of Great Britain and Northern Ireland		
Swaziland			Yugoslavia		
Togo					
Uganda					
United Republic of Cameroon					
United Republic of Tanzania					
Upper Volta					
Zaire					
Zambia					
Zimbabwe					
Namibia					

eases. Thus, a supportive administrative and scientific network exists for the development of new vaccines and for more extensive and successful use of existing vaccine. At present no such large scale international encouragement is given to the development of antivirals, with the exception of the Antiviral Screening Programme at the NIH, but this situation may change with the advent of the clinically successful antiherpes compounds (Chapters 11–14) and the possibility of developing antivirals against certain diseases caused by viruses such as influenza, common cold viruses or more exotic tropical viruses (alpha and flaviviruses), where successful vaccines are conspicuous by their absence.

As a specific example of scientific control of vaccine, live polio vaccine is tested for any residual neurovirulent properties of the vaccine virus (which have to be absent, of course) by injection into the spinal cord of 30 rhesus or cynomolgous monkeys. The animals are observed over a period of 30–40 days, and, in the absence

of clinical signs of nervous system damage, are killed and their brains and spinal cords examined histologically for signs of virus induced damage. The monkeys are compared to control animals given standard polio viruses at a known degree of virulence or attenuation. The live polio vaccine is also titrated to ensure correct infectivity titre (Table 2.9).

Inactivated virus vaccines such as influenza are analyzed for antigenic identity and quantity of antigenic HA by standard single radial immunodiffusion techniques (Wood et al., 1977) (Fig. 2.5). Inactivated polio vaccine and rabies vaccine may be similarly standardized by SRD in the near future (Ferguson and Schild, 1982, Schild et al., 1980), although, of course, this test does not indicate how *im-*

TABLE 2.9.
Basic recommendations for biological control of viral vaccines (British Pharmacopaea, 1980)

Virus vaccine	Cell substrate	Tests on final product	General comments
Polio inactivated	Primary monkey kidney cells	1. Immunogenicity test-animals (also potency test) 2. Tests for infective virus in sensitive human and monkey cells 3. Inoculate vaccine i.m. intrathalamically or intraspinally into 20 cynomolgus monkeys 4. Inoculate vaccine into mice, guinea pigs and rabbits for extraneous viruses 5. Bacteriological sterility in culture and in guinea pigs	Virus suspensions of at least 30×10^6 ($TCID_{50}$/ml) of types 1 and 3 and 10×10^6 $TCID_{50}$ of type 2 before inactivation. Vaccine should not contain more than 1 ppm of animal serum from tissue culture medium. May contain small quantities of antibiotics and phenol red indicator. Virus cultivated at 35° or less and harvested within 4 days.
Polio live	Primary monkey kidney cells	Harvests tested for identity, sterility and freedom from adventitious viruses. Harvests may be pooled and filtered to remove bacteria. Virus compared with original seed for viability at 30–38° and 39–41°. Tested for neurovirulence by intraspinal and intrathalamic injection of cynomolgus monkeys. Tested for virus titre (not less than 3×10^5 $TCID_{50}$/ml for type 1 and 3 and not less than 1×10^5 $TCID_{50}$/ml for type 2	Vaccine to be stored at −20°C and used before 3 months after opening and storage at 4°C. Approved seed lot system used i.e. final vaccine represents not more than 3 subcultures of types 1 and 2 and not more than 2 subcultures of type 3 from vaccine on which original laboratory and clinical tests were performed.

TABLE 2.9. *(continued)*

Virus vaccine	Cell substrate	Tests on final product	General comments
Influenza A and B inactivated subunit	Allantoic cavity of 10–13 day fertile hens eggs from healthy flocks	Antigenic characterisation of HA and NA antigens. Quantification of HA using single radial immunodiffusion. Immunogenic in mice or chickens. Virus-free by titration in eggs. Only traces of egg proteins should be demonstrated by polyacrylamide gel electrophoresis.	Virus harvested after 2–3 days and each virus harvested separately. Inactivated with β-propiolactone or formaldehyde. Purified by centrifugation and filtration. Disruption of virus by non-toxic detergents and separation of HA and NA gives 'subunit' vaccine. Splitting of virion with tween-ether gives 'split' vaccine. Virus has to be antigenically updated (usually each year).
Influenza A – live intranasal vaccine (attenuated virus)	Embryonated hens eggs from specific pathogen-free flocks	Virus harvested within 4 days and tested for identity, sterility and freedom from adventitious viruses. Viruses titrated to establish infectivity titre. Inoculated intraperitoneally into guinea pigs.	Virus attenuated by random passage, *ca* mutant, *ts* mutants or host range mutant. Recombinants with multiple genes from attenuated laboratory viruses are often used. Has to be shown to be attenuated and immunogenic in clinical trials involving 50–100 volunteers.

munogenic a vaccine batch may be in man. Such latter data must be obtained by controlled clinical trials, although with existing vaccines (such as influenza) these trials are not usually very extensive. In the UK and Europe such small vaccine efficacy trials may often be carried out in volunteers in the same firms which produce the vaccine. Any larger scale clinical trial has to be approved by a local hospital safety committee or a more national body such as the Committee on Safety in Medicines (UK).

The final stage in this seemingly tortuous process is usage of vaccine in the field. In most countries immunization of children (the main target group of most viral vaccines) is voluntary and dependent upon parental consent. This approach may have disadvantages in view of inaccurate media reporting about adverse reactions to vaccines in some countries. Thus comment about *Bordetella pertussis* (whooping cough) vaccine has eroded public confidence in other vaccines, both bacterial and viral, and acceptance rates of measles and even polio vaccines have declined dramatically in the UK in recent years. In the USA, vaccination is approached with vigour and certain states have obligatory measles vaccination: children must have a measles vaccination certificate before they can attend public school (Chapter 8).

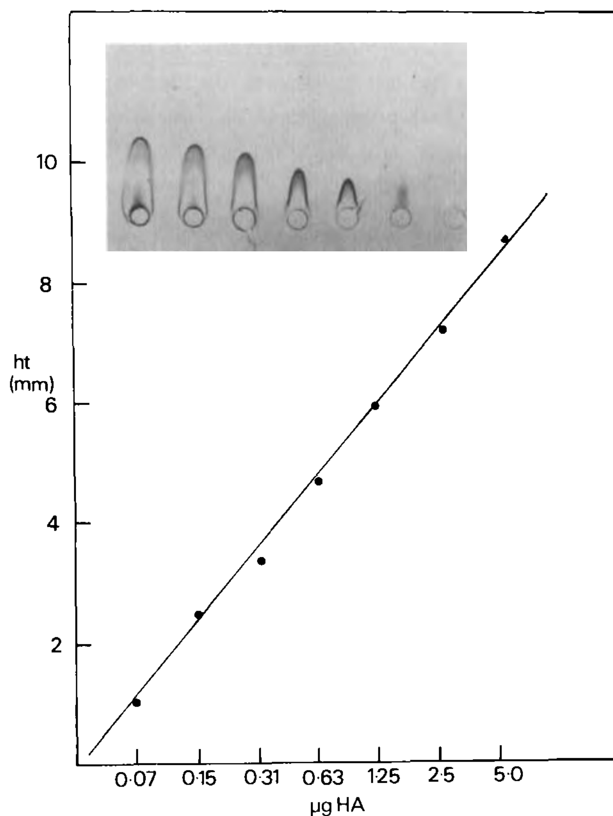


Fig. 2.5. Analysis of HA content of inactivated influenza vaccines by single radial diffusion (SRD) or rocket electrophoresis. Antigen is solubilised with detergent and enters a matrix containing anti-HA antibody. The length of the rocket (above) or size of an SRD ring is then proportional to the HA content of the vaccine.

As another approach, in East Germany, vaccination is not compulsory, but, on completion of a vaccination course of a child, parents are given a financial inducement. This is not paid if the parents decide to opt out of the immunization scheme. Finally, the experience in Scandinavian countries (e.g. Sweden) indicates that high acceptance rates can be obtained by encouragement and education alone.

Some problems encountered during the development and use of viral vaccines are summarized in Table 2.10.

2.4. Choice of cell substrates for virus vaccine production

Enders et al. (1949, see reprinted version in 1980) in a major breakthrough in viro-

TABLE 2.10.
Problems encountered during the development and use of viral vaccines

Vaccine	Problem
Live vaccines	
Poliovirus (Sabin)	Contaminating viruses (e.g. SV40) Back-mutation to virulence (type 3) Interference by endemic enteroviruses in tropical areas. Paralysis in 2–3 children per million immunized.
Smallpox	Inadequate attenuation leading to complications (still noted in military usage). Overattenuation leading to lack of protection.
Measles (early strains e.g. Edmonston)	Inadequate attenuation leading to fever and rash
Rubella (HPV-77)	Inadequate attenuation leading to arthritis in adult females. Other strains are satisfactory.
Yellow fever	Contaminating hepatitis B in 'stabilizing' medium (an earlier problem encountered in the 1940s and hardly since). Over and underattenuation.
Influenza	Reversion to virulence of *ts* vaccines particularly in antibody seronegative children. Lack of 'takes' in adults. Adverse reactions in chronic bronchitics.
Inactivated vaccines	
Poliovirus (Salk)	Residual live virulent polio virus (an earlier problem). Contaminating virus (SV40) resisting formaldehyde inactivation.
Influenza	Pyrogenicity, lack of efficacy, side reactions.
Rabies (Semple)	Allergic encephalomyelitis (not noted in modern diploid cell vaccines).
Measles; respiratory syncytial	Hypersensitivity reactions on subsequent natural infection or live vaccine booster. These vaccines have been discontinued.

logy reported that the Lansing strain of polio could be cultivated in cell cultures of human fibroblasts. The immediate result of this observation (together with the discovery of penicillin, which enabled bacterial contamination to be minimised on large scale viral cultivation) was the development of effective vaccines against polio. Over the succeeding 20–30 years a variety of mammalian cells were used to replicate

human viruses for vaccine production (Tables 2.11, 2.12). In general, the criteria for selection of cells have encompassed the ease of acquisition of the cell, the potential lack of tumourigenicity of the cell for man, its stability and the likelihood that other extraneous, and hence unwanted, viruses might be present. In perhaps an unfortunate beginning, polio vaccine virus was cultivated in kidney cells from the African Green monkey. As a result, this once plentiful animal species has been threatened with extinction. Prior to the discovery of SV40-virus in cells of the rhesus monkey and cynomolgus monkey these animals were also endangered. Moreover, serious problems have arisen as regards the safety of persons handling these monkeys, and over the last two decades nearly two dozen workers have died from causes directly or indirectly related to the preparation of monkey cells. For example, in 1967 seven persons died of Marburg virus (a hitherto unknown virus) caught from handling African Green monkeys. Another 10 persons at least have died from fatal herpes B virus infections from these monkeys. A further 20 adventitious viruses have been discovered in cells cultured from monkeys. In general, the likelihood of encountering extraneous agents in primary cell populations is very high and such agents have been isolated from embryonated hens' eggs (oncogenic avian adenoviruses, sarcoma virus, Mareks Disease virus etc.) and canine kidney cells (e.g. canine hepatitis virus, herpes viruses). Another problem with primary cell cultures from animals is lack of uniformity and stability of the cells. A 'seed cell' system is not possible.

A resolution of the above problem was suggested by Hayflick and Moorhead (1961) who for many years were voices in the wilderness. They proposed the use of a serially propagated cell substrate initiated from normal human embryonic lung tissue (WI-38 cells). In fact, more recently, these and other human diploid cells such as MRC-5 (Jacobs, 1970) have been licensed for virus vaccine production and are now used, for example, for inactivated rabies vaccine and also for attenuated rubella vaccines.

TABLE 2.11.
Cell substrates for production of vaccines and sources of cell substrates

Vaccines						
Polio (killed)	Polio (live)	Measles (live)	Rubella (live)	Mumps (live)	Influenza (killed)	Rabies (killed)
Monkey	Monkey Human diploid cells	Dog Chicken Guinea-pig	Dog Rabbit Duck Human diploid cells	Duck Guinea-pig Eggs	Eggs	WI-38, hamster and monkey cells

The advantages of these human cells (Table 2.12) are that the cell population is derived from a single donor and frozen in liquid nitrogen while exhaustive tests are carried out on karyology, potential viral or bacterial contamination, stability etc. Virtually limitless quantities of vaccine can then be produced from the guaranteed cell stocks (Table 2.12). Human diploid fibroblast cells of this kind can undergo around 40–50 passages in culture before reaching the end of their life span. Earlier worries about the possibility of the cells being contaminated with unknown and/or undetectable human (oncogenic) viruses have not been substantiated. Several million people have now received virus vaccines (e.g. polio, measles, rubella, smallpox, varicella, arboviruses, adenovirus type 4) prepared using WI-38 or other human diploid cell lines without evidence of oncogenic activity. Indeed the scientific and bureaucratic opinion has so changed recently that even 'continuous' cell cultures such as Vero (transformed monkey kidney cells) are being considered for the cultivation of certain viruses such as polio for use in inactivated vaccines.

TABLE 2.12.
Comparative properties of human virus vaccine cell substrates

Property	Cell substrate			
	Primary monkey kidney	Primary canine kidney	Primary avian	Human diploid cell strain e.g. WI 38 or MRC5
Human virus spectrum	Excellent	Poor	Poor	Excellent
Virus titre	Excellent	Excellent	Excellent	Excellent
Cost of tissue	High	High	High	Negligible
Cell propagation	Excellent	Excellent	Excellent	Excellent
Presence of extraneous viruses	High	Undetermined	Moderate	None
Number of cells available from a single tissue source	Small	Small	Small	Virtually unlimited
Cell preservation	Excellent	Excellent	Poor	Excellent
Production of successive vaccine lots in cells from a single source	Not possible	Not possible	Not possible	Easily achieved
Possibility of isolating hitherto undetected agents in successive vaccine lots	Great	Great	Great	Small
Tumorigenicity of cells in man	Not done	Not done	Not done	No activity
Pretesting cells for safety prior to use	Impractical	Impractical	Impractical	Easily achieved
Standardization of cell substrate	Not possible	Not possible	Not possible	Easily achieved
Vaccine production in a cloned cell population	Not possible	Not possible	Not possible	Possible
Oncogenic viruses found in cells	Yes	Yes	Yes	No

More than a decade ago Smith (1970) reviewed current methods of screening virus vaccines and cell cultures for unwanted viruses and, surprisingly, most of the methods are still used today. At the animal and bird colony level, virus-free colonies have now been developed (also primates for monkey kidney cells) and avian leukosis-free chicken flocks and specific pathogen-free dog colonies have been developed. A second level for 'passenger' virus detection is that of the cell culture. The traditional methods of virus induced cytopathic effects (cpe), virus antigen detection by immunofluorescence or ELISA, electron microscopy (including immune electron microscopy using monoclonal antibodies), interference, haemadsorption, nucleic acid hybridization and finally animal inoculation are still used. The third level at which detection of passenger viruses is attempted is during the development of the virus seed, especially of a virus which has been passaged in animal cells during attenuation. As an example, an early attenuated rubella vaccine was developed by passage of virus 77 times in African Green monkey kidney (AGMK) cells (Chapter 9). Over half of AGMK cell lots are now known to be contaminated with simian cytomegalovirus, which is rarely observed unless cultures are kept for periods of several weeks. Another level for detecting passenger virus is, of course, in the final vaccine itself. The live vaccine virus is usually suppressed with antiserum to allow emergence of any adventitious virus, but there are obviously gross limitations here and so priority is always given to control at the first 3 stages described above. We should not forget that, as a last resort, adventitious viruses which have escaped the 'net' can be detected indirectly by antibody surveys in immunized persons. Thus, contaminating SV_{40} virus elicited an antibody response in recipients of early batches of Polio vaccine. The discovery of new viral diseases (e.g. AIDS) should warn us that a continued vigilance against the unknown is necessary.

2.5. Some examples of inactivated whole virus and subunit viral vaccines

The physical structure of most human viruses is complex, with either an outer lipid envelope in which a number of membrane glycoproteins are inserted (e.g. influenza, rabies, measles, herpes viruses) or with a more rigid structure with a regular array of externally situated proteins (e.g. polio, common cold viruses, hepatitis A). The most important immunogenic antigens of the virus are normally the externally situated proteins, and often neutralizing antibody to the virus is induced by such proteins. Good examples are influenza haemagglutinin and polio VP1 protein. However, this may be an oversimplification for many viruses and it cannot be assumed that all external virus structural proteins induce neutralizing antibody or, indeed, necessarily induce antibodies which provide protection against infection. An illustrative example of this problem is respiratory syncytial virus (RSV) and measles virus, where in early vaccine studies children were immunized with the intact inactivated virus particles (Fulginiti et al., 1966). Antibodies were induced which reacted

with the virus in the vaccine but the immune response which developed in these children did not protect them against infection with RSV. Quite the contrary situation happened, because when some immunized children came into contact with the virulent virus naturally they became infected and their clinical response was *enhanced* and resulted in severe illness. At the time this enhanced RSV disease syndrome was attributed to an immune complex of vaccine-induced IgE antibody and RSV. However, presumably immunization had induced antibody to particular determinants on certain viral proteins (e.g. the HN protein) but not to other and more important proteins (e.g. F protein) from the point of view of immunity. We can conclude that, particularly with rather complex viruses, some basic understanding of the antigenic structure of the virus, and the relationship of these antigens to particular virus structural proteins is required if errors of the RSV kind are to be avoided. In this regard, it is now apparent that antibody to fusion (F) protein of paramyxoviruses might be particularly important from the point of view of protection (Chapter 8).

At present the only inactivated whole virus vaccines used widely in man are polio, rabies and influenza and certain arbovirus vaccines. In the former case it has only become clear in recent years that neutralizing antigenic determinants are located on the structural protein VP1 (Minor et al., 1983) although a successful vaccine has in fact been developed in the absence of a clear understanding of the basic antigenic structure of the virus. Similarly with influenza virus, vaccines of formalin-inactivated whole virus were developed as early as the 1940s when no clear details were available about the antigenic structure of the virus.

More recently, and with the advent of technological methods of virus purification on a large scale (such as continuous flow rate–zonal centrifugation) interest has increased in the development of subunit viral vaccines. In theory these should produce fewer side reactions in recipients because unwanted viral proteins, lipids and virus nucleic acid are not present. Essentially these preparations should contain only the main immunogenic protein of the virus which induces protective antibody. Most work has been carried out with influenza subunit vaccine, but the principles could be extended to picornaviruses and also other enveloped viruses such as herpes virus. Early experiments in Australia in the 1960s established that detergent disrupted influenza virus vaccine (which still contained all virus structural proteins, however) produced fewer side reactions (particularly temperature elevation) in animal models and in children and yet was still immunogenic (Webster and Laver, 1966). In the last decade a further refinement has been the separation of the immunogenic membrane glycoproteins (HA and NA) by centrifugation techniques and reconstitution of the two subunits into vaccine by dialysing away the detergent used in the initial solubilization (Fig. 2.6). These vaccines are free of other influenza structural proteins such as MP, NP, polymerase proteins, viral lipids and RNA and have a low reactogenicity in children. Unfortunately the immunogenicity of these subunit vaccines is also reduced in persons such as children with no immunological memory to influenza HA, although they are as immunogenic as whole virus vaccines in ad-

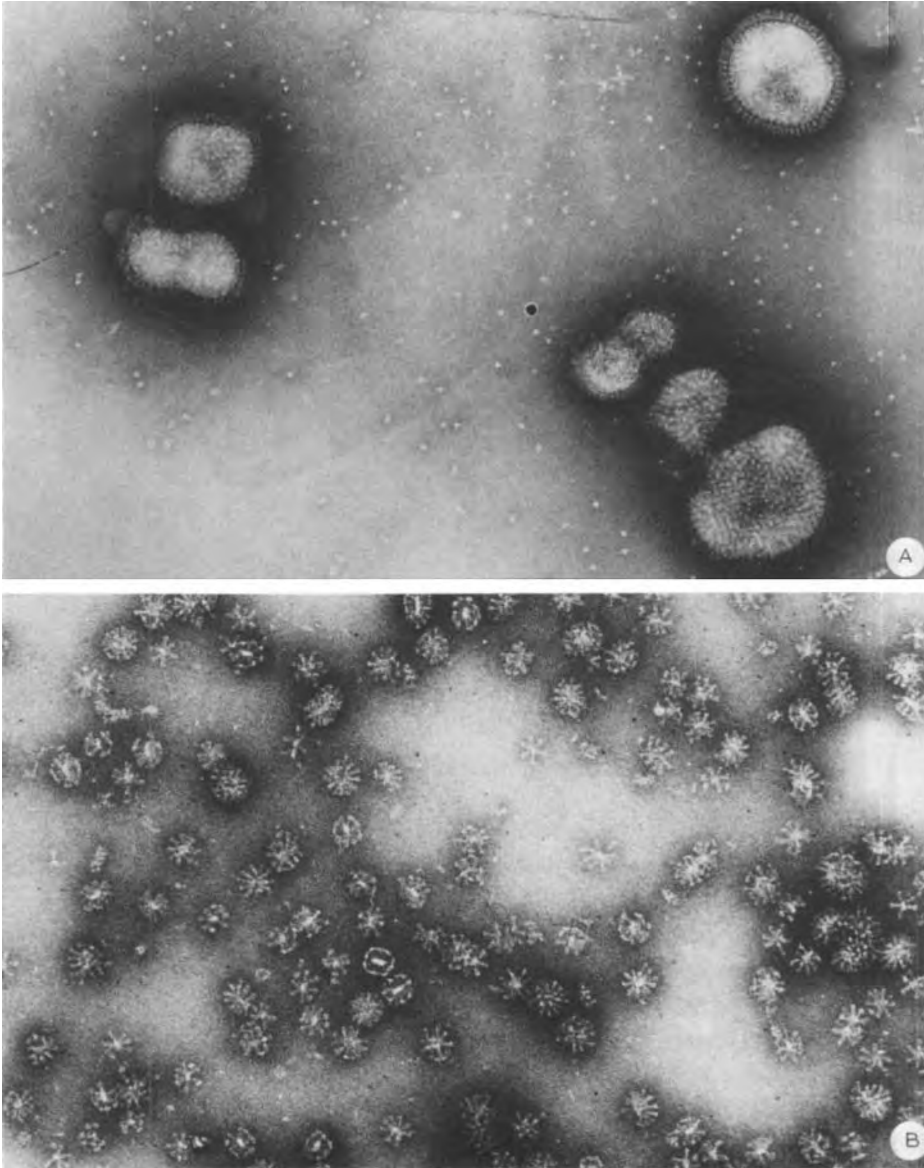


Fig. 2.6. A: Whole influenza virus used in vaccine. B: Subunit influenza vaccine composed of HA or NA subunits. (Courtesy of Dr. D. Hockley).

ults who are already primed by previous contact with influenza viruses. A number of experimental approaches are now being used to 'regain' the immunogenicity of the whole virus e.g. by reconstituting the subunits onto pure lipid liposomes (virosomes) (Oxford et al., 1981) and by re-aggregating the HA and NA rosettes into

larger and hopefully more immunogenic particles (Iscoms). Both these approaches could be used in the future with short peptide viral vaccines.

2.5.1. Usefulness of adjuvants in virus vaccines

As alluded to above, one of the problems with some existing inactivated virus vaccines is the relatively poor immunogenicity, particularly with subunit preparations. With the advent of bacterially-produced but rather poorly immunogenic viral peptides this problem may become even more acute. Oily adjuvants have been used experimentally as adjuvants by immunologists for several decades, but their use in human medicine is restricted because of coinduction of febrile responses, polyarthritis and local abscesses. More recently, some relatively non-toxic 'immune regulators' have been developed including muramyl dipeptides (MDP), liposomes and polynucleotides. MDP is water soluble and consists of a simple sugar and two amino acids (*N*-acetyl-muramyl-L-alanyl-D-isoglutamine), and a number of chemical modifications of MDP have now been studied. Analogues can be synthesized which are immunosuppressive or alternatively increase cell mediated immunity. In a more traditional manner, highly refined peanut oil emulsions with glycerol or lecithin may be useful for increasing immunogenicity of certain viruses. In particular, all the components are readily metabolized. Finally, liposomes (or spheres of phospholipids) may be used to trap viral antigens, or to reconstitute virus glycoproteins into morphological units resembling the original virus (Oxford et al., 1981) (Fig. 2.7).

2.6. Some examples of live attenuated virus vaccines

An eminently (and perhaps the most) successful live attenuated vaccine has been vaccinia against smallpox. Paradoxically it would not be considered a particularly good example of a live virus vaccine if it were newly introduced today. Thus, the precise origin of vaccinia virus used in the vaccine is obscure — the virus may have been a mutant of cowpox or even a recombinant virus. Also methods of production using calf lymph were rather old fashioned and difficult to control (see Chapter 15). But empirical work had discovered that after inoculation of human skin and subsequent replication of vaccinia virus, cross-immunity to variola developed and the vaccine was used to finally eradicate smallpox from the world (see Chapter 15).

Another 'early' attenuated virus vaccine was yellow fever, developed in the 1940s by simply passaging the virus in embryonated hens' eggs (Theiler and Smith, 1937). After the 17th passage in eggs the virus was found to be attenuated for man, induced neutralizing antibodies and induced protection against superinfection with wild yellow fever virus. Thus, by this time the general biological principle was established for live viral vaccine development: adaptation of a virus by serial passage

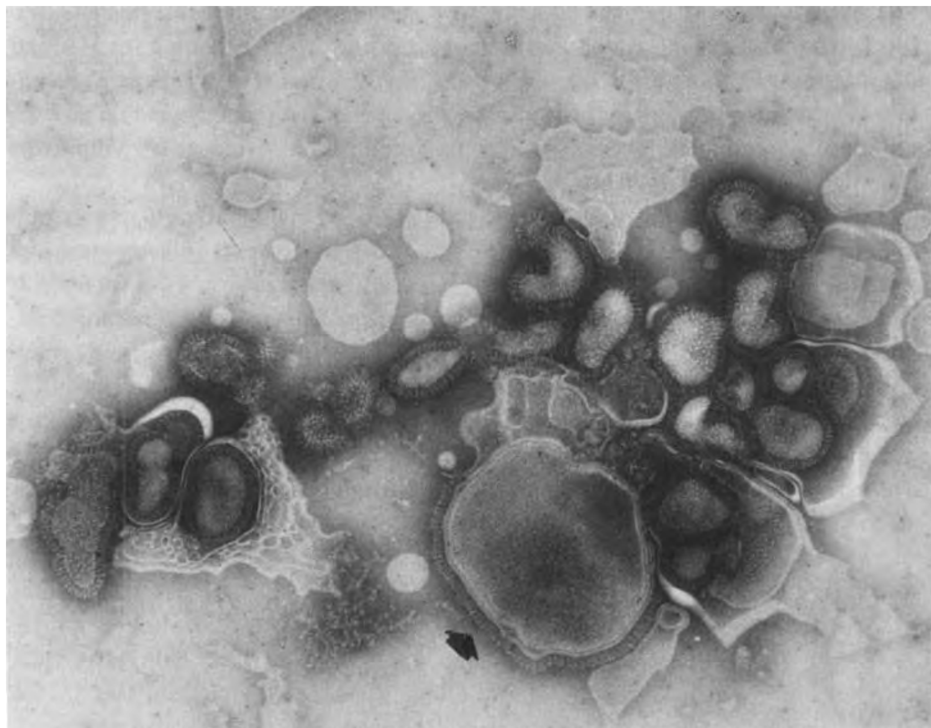


Fig. 2.7. Electron micrograph of influenza virosome (arrowed) mixed with true influenza virus particles. (Courtesy of Dr. D. Hockley.)

in a foreign host and concurrent reduction in virulence for the original host. The genetic basis of attenuation of these viruses is still not known, but presumably the multiple passage selects an already existent subpopulation of virions with different genetic properties, viz. changed biological properties of virulence. Presumably the attenuated virus would have multiple and perhaps cumulative mutations which would be an advantage because the possibility for a reversion to virulence would thereby be lessened. Successful live vaccines produced in this way subsequently have been measles, rubella, polio and mumps. On the other hand, an apparently insurmountable problem with certain viruses such as influenza has been the genetic instability of the passaged virus. If influenza virus is passaged too many times in eggs, for example, then the virus is unable to infect man, whereas administration of underpassaged virus produces influenza-like symptoms in the recipient. These problems led to exploration of the use of genetic approaches to producing live candidate vaccine viruses, particularly since the influenza virus genome is in segments and can be easily manipulated in the laboratory. Three approaches were tried:

1. Recombination between a host range mutant (A/PR/8/34 (H1N1)) which after hundreds of passages in eggs is known to be completely attenuated for man,

and the wild type virus. The concept here is to produce a recombinant with 6 of the 8 genes from the attenuated parent virus and 2 genes (coding for HA and NA) from the wild parent. One hopes that transfer of most of the genetic material from the attenuated parent will result in an attenuated recombinant. However, unfortunately even these recombinants may retain some virulence for volunteers (Beare et al., 1975, Oxford et al., 1978).

2. Induction of genetic lesions in several genes by chemical mutagenesis of a master strain virus (e.g. A/Udorn/72 H3N2) and selection of a temperature sensitive (*ts*) phenotype viz. a virus which would no longer replicate at 37°C. It was considered that such *ts* viruses would a priori be less pathogenic for man, partly because they would be unable to replicate in the lower respiratory tract. However, extensive work with influenza *ts* mutants, particularly in the USA, showed that although they were attenuated for adults and children, in the latter group *ts* revertants were detected (reviewed by Richman and Murphy, 1979). These revertants could possibly spread to other unvaccinated children and initiate an outbreak.

3. Selection of particles with random mutations in any of the viral genes by serial passage at low temperatures (e.g. 25°C). These cold adapted (*ca*) viruses would have similar phenotypic properties to the *ts* viruses mentioned above. Most early work was carried out by the group of virologists in Leningrad (Alexandrova and Smorodintsev, 1965) and later in the USA (Maassab, 1967). Presumably because of the multiple mutations induced and selected for, recent work has shown these viruses to be good candidates for attenuated influenza vaccines.

The *ts* and *ca* mutant approach can be used for viruses other than influenza and experimental vaccines of Varicella, polio, and respiratory syncytial virus are all under investigation at present. (see Chapters 13, 4 and 8, respectively). Finally, some of the advantages and disadvantages of inactivated or live virus vaccines are summarized in Table 2.13.

2.7. Future vaccines: recombinant DNA technology applied to viral vaccines

The 'genetic engineering' technology is essentially one of incorporating fragments of DNA containing viral genes (Fig. 2.8) of interest into a suitable vector (reviewed by Emery, 1981). The vector is then introduced into a host organism such as *Eschericia coli* which produces clones with multiple copies of the incorporated DNA fragment. Initially the DNA fragments are produced using restriction endonucleases (200 or more are now available) which cleave DNA at sequence specific sites, creating double stranded breaks. EcoRI, for example, cleaves between bases G and A in the sequence GAATTC (Table 2.14) so 'staggered ends' are produced:

TABLE 2.13.
Advantages and disadvantages of live and inactivated vaccines

Advantages	Disadvantages
Live vaccines	
Single dose, given ideally by natural route, invokes full range of immunological responses, including local IgA as well as systemic IgG production	Reversion to virulence. Natural spread to contacts. Contaminating viruses. Human cancer viruses.
Possibility of local eradication of wild-type viruses.	Viral interference. Inactivation by heat in tropics.
Inactivated vaccines	
Stability	Multiple doses and boosters needed, given by injection, therefore local IgA fails to develop. High concentration of antigen needed: production difficulties and cost. Wrong immune response may be initiated.

The staggered ends are termed 'sticky ends' because they will combine with complementary sequences produced by the same enzyme on the DNA of a chosen vector. The sticky ends of the new DNA and the DNA of the vector are held together by hydrogen bonding between complementary bases. Finally the ends are sealed and stabilized by a *ligase* enzyme. Staggered ends may be added if necessary in the form of synthetic DNA linker molecules, from which sticky ends may subsequently be generated. Alternatively, complementary bases may be added to the DNA ends such as poly dG to the vector and poly dC to the foreign DNA — *homopolymer*

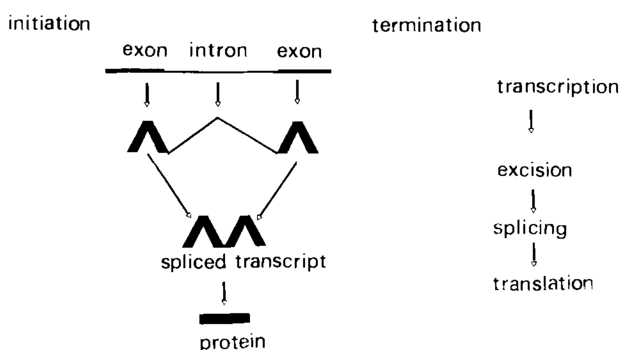


Fig. 2.8. Diagrammatic representation of a gene consisting of exons and introns. Genes are not contiguous elements on a chromosome. With few exceptions genes consist of 'exons' which are transcribed into mRNA and then translated into proteins, and 'introns' from which the precursor RNA is excised and does not contribute to functional mRNA.

TABLE 2.14.
Restriction endonucleases and their cleavage sites

Enzyme	Organism	Cleavage site (*) 5′ → 3′
BamHI	*Bacillus amyloliquefaciens* H	G * G A T C C
EcoRI	*Escherichia coli* RY13	G * A A T T C
HaeIII	*Haemophilus aegyptius*	G G • C C
HindIII	*Haemophilus influenzae* Rd	A * A G C T T
HpaI	*Haemophilus parainfluenza*	G T T * A A C
PstI	*Providencia stuartii*	C T G C A * G
SmaI	*Serratia marcescens*	C C C * G G G
SalI	*Streptomyces albus* G	G * T C G A C
TaqI	*Thermus aquaticus*	T * C G A

tailing. Typical vectors may be plasmids (Fig. 2.9), bacteriophages and cosmids, all of which are replicons, replicating independently of the bacterium. The choice of vector often depends on the size of the piece of foreign DNA to be inserted. Plasmids consist of circular duplex DNA with a limited number of restriction sites and, importantly from the point of view of selecting clones, confer antibiotic resistance to the bacterial host.

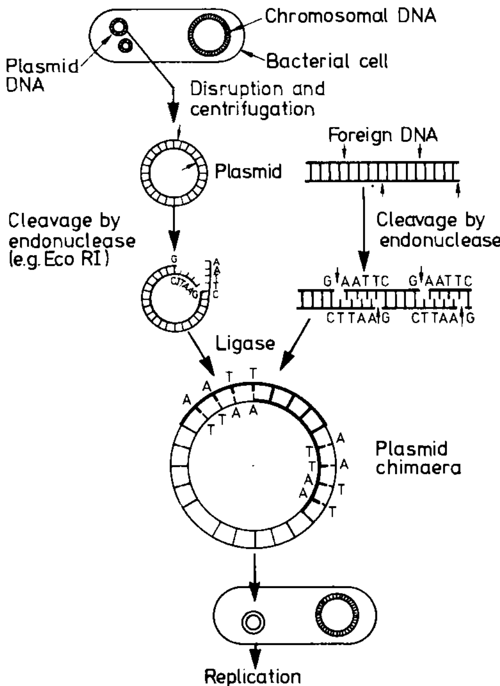


Fig. 2.9. Generation of a plasmid recombinant (after Emery, 1981).

Genetic engineering methodology has advanced to the state that molecular biologists can isolate a viral gene, fuse the gene with a bacterial or yeast gene and reinsert the fused gene into a bacterium or yeast cell. The bacteria multiply and produce millions of copies of their own genes *and* the viral gene. This is essentially a 'gene cloning' experiment. From the point of view of application to viral vaccines there are 3 problems to overcome:

1. identify and separate the viral gene coding for the immunogenic protein of the virus (e.g. HA of influenza, VP1 of polio and FMDV, cross-reacting glycoprotein of HSV1 and HSV2 etc.). With RNA viruses, the RNA gene and gene fragment must be transcribed into DNA before the next step using for example a reverse transcriptase enzyme.
2. clone the gene in a bacterium or eukaryotic cell so it will be maintained as the bacterium or cell grows.
3. manipulate the genetic information (regulatory command of 'start' and 'stop') surrounding the inserted gene so that the DNA sequence is expressed as a protein in large quantities.
4. use a host which will carry out post-translational modifications of the polypeptide if needed.

Often a plasmid, a small circular piece of DNA (reviewed by Day and Burton, 1982) is used as the vehicle for introducing the new DNA into a bacterium (Fig. 2.10). Plasmids carry only a few genes of their own and are maintained in several copies inside the bacterium by the organism itself. Alternatively, the vehicle can be a bacterial virus, perhaps having 10–50 genes of its own or even a human virus such as vaccinia. The DNA genome of a bacterium has some 3×10^6 bases in its structure, whereas a single viral gene may be only a few thousand bases in length. In practical terms the viral gene is often inserted into the DNA of a plasmid (e.g. pBR322) at a position which lies in the middle of a gene coding for penicillinase. The latter enzymatic activity is therefore destroyed and the plasmid no longer conveys resistance to penicillin but still has the gene conferring resistance to tetracycline. When the plasmid DNA is 'inserted' into a population of bacteria, successful 'takes' can

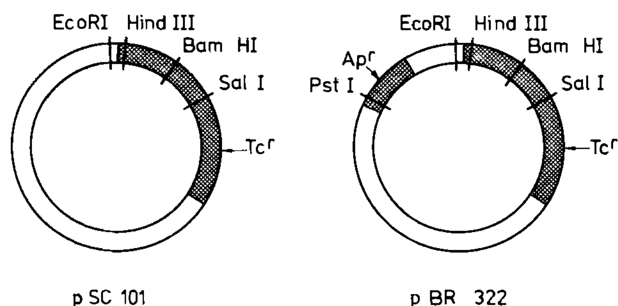


Fig. 2.10. Diagrammatic representation of two plasmids commonly used in recombinant DNA technology: pSC101 and pBR 322.

be identified because progeny bacteria will be sensitive to penicillin and resistant to tetracycline. An important remaining problem is to provide regulatory signals for the bacterium to use. One of these signals signifies a start to synthesis of messenger RNA and a second important signal tells the bacterial ribosome to begin translation into viral protein. Finally, two 'stop' signals are required, one for translation and one for transcription. Regulatory signals must be optimally placed and so tremendous scope exists at this point for ingenuity of the molecular biologists. Even if the bacterium produces the new viral protein, proteolytic enzymes may break it down and, thus, sometimes new proteins are made as a 'hybrid' protein to protect them. Messenger RNA molecules may have to be stabilized to make them more effective, so leading to increased protein synthesis. Bacterial proteins are commonly made from 1×10^3 to 1×10^6 copies per cell and yet newly inserted genetic information may initially yield only 1–2 copies of viral protein per cell. As specific examples in this rapidly growing area of 'vaccinology' we shall examine briefly cloning and genetic expression in the case of influenza and hepatitis B. It should not be forgotten that short synthetic viral peptides (see below) could also be produced on a large scale by genetic engineering rather than by chemical synthesis.

2.7.1. Influenza

In an early study Porter et al., (1979) cloned the HA gene of the avian influenza virus A/FPV in *E. coli*. Later, rather similar conceptual techniques were used to clone HA genes of human influenza viruses and since this is more relevant to the present discussion these later studies will be quoted in more detail. Heiland and Gething (1981) constructed a chimaeric plasmid coding for a fusion protein consisting of the terminal 7 amino acids of β-galactosidase followed by the complete HA amino acid sequence of A/Japan/305/57 (H2N2) virus. *E. coli* HB 101 was transformed by the chimaeric plasmid and ampicillin resistant clones containing HA gene sequences were detected by hybridization with ^{32}P labelled HA DNA. Expression of HA was analyzed by a radio-immune assay technique and approximately 3000 HA molecules per cell were produced (considerably lower than theoretically attainable, and this may be due to inefficient translation, or proteolytic degradation as noted above). In an earlier study, Gething et al. (1980) cloned the HA gene of X.31 virus by insertion of poly (dA) tailed vRNA/cDNA hybrids into the Pst I site of plasmid vector pBR322 after poly (dT) tailing of the linearized plasmid. For the HA of A/Jap/57 virus, dsDNA copies were obtained and inserted into the Pst I site of plasmid pAT153 (a derivative of pBR322). From the subsequently determined nucleotide sequence the complete amino acid sequence of the H2 protein was predicted and compared with the partial sequence of the H3 protein. Surprisingly, 30% homology was detected between the HAs of 3 subtypes (H2, H3 and Havl). We shall refer in more detail to possible common sites in influenza HAs below and their application to new vaccine development.

2.7.2. EXPRESSION OF CLONED INFLUENZA HA IN PRIMATE CELLS

An alternative way of expressing a genetically cloned HA gene is to use SV40 virus to carry the gene into primate cells. Alternatively, the HA gene may be coupled to HSV thymidine kinase (TK) gene and this introduced into a TK^- cell line. Possession of TK enzyme would then identify cells in which the HA gene had been inserted successfully. In addition, it is possible that influenza HA expressed in mammalian cells may be antigenically different from HA expressed in bacterial cells and, indeed, in whole virions cultivated in the traditional manner for influenza vaccines in embryonated hens' eggs (Schild et al., 1983).

Also, site directed mutagenesis is being considered as a possible new option at the present time (see below). If HA genes can be rescued from mammalian cells by co-infection with other influenza viruses then any HA gene with a specific mutation in it could be rescued and used to construct a (hopefully) attenuated virion for the production of live attenuated influenza vaccines. The experiments of Gething and Sambrook (1982) provide a model system for this approach. The HA gene of A/Jap/305/57 cloned as described above in the plasmid vector pAT153 was inserted into the late or early region of the SV40 virus genome. Vectors were designed so that HA was expressed under the control of the SV40 early and late promoter, because the HA gene has no promoter of its own. Thus, in initial experiments the HA gene was inserted into the late region of SV40 between the HpaII site at nucleotide 346 and the Bam HI site at nucleotide 2,533. The recombinant viral genome was cloned into the Bam HI site of pAT153 and propagated in *E. coli*. For transfection into cells the recombinant SV40-HA genome was excised from the plasmid by digestion with Bam HI, purified and ligated to yield the structure SVE HA3. The recombinant viral genome contained the SV40 origin of DNA replication and an intact set of early genes. Because the vector SVE HA3 contains an intact copy of the gene coding for SV40 large T antigen, its DNA will replicate in permissive monkey cells. However, production of infective virus requires complementation by a helper virus. So SVE HA3 and a helper virus DNA (dl 1055) were introduced into CV-1 cells using DEAE-dextran. Virus produced was passaged twice in CV-1 cells and virus stock used to infect further monolayers of CV-1 cells. Influenza HA antigen was produced, and an average of 6×10^6 molecules/cell were produced by 62 h post infection (approx. 80 μg of HA per 9 cm dish of cells. Note that approximately 15 μg of HA are required for a human dose of influenza vaccine). Influenza virus infected CV-1 cells produced 4.5×10^7 HA molecules per cell 10 h after infection. So HA production by the SV40-HA vector was extremely efficient. HA was expressed on the cell surface and could be detected by haemadsorption and by immunofluorescence.

2.7.3. Production of hepatitis B core antigen in *B. subtilis*

Bacillus subtilis has some additional advantages compared to *E. coli* as a host for expressed cloned genes. Thus *B. subtilis* is non pathogenic, does not produce endotoxins and excretes several extracellular proteins in large quantities. Strains of *B. subtilis* are widely used commercially for producing enzymes and antibiotics, and the organism is used in Japan as a source of protein for human consumption. Hepatitis B virus (HBV) DNA was inserted in a plasmid (pBD9) gene for erythromycin resistance. A plasmid vector pKH 80 was made by joining pBD9 to pBR322. The concentration of the HBV core antigen protein produced was less than 0.1% of cellular protein (Hardy et al., 1981).

2.7.4. Expression of Hepatitis B surface antigen gene in cell cultures

An SV40-HBV recombinant was constructed (see Fig. 2.11) which had a 1350 base

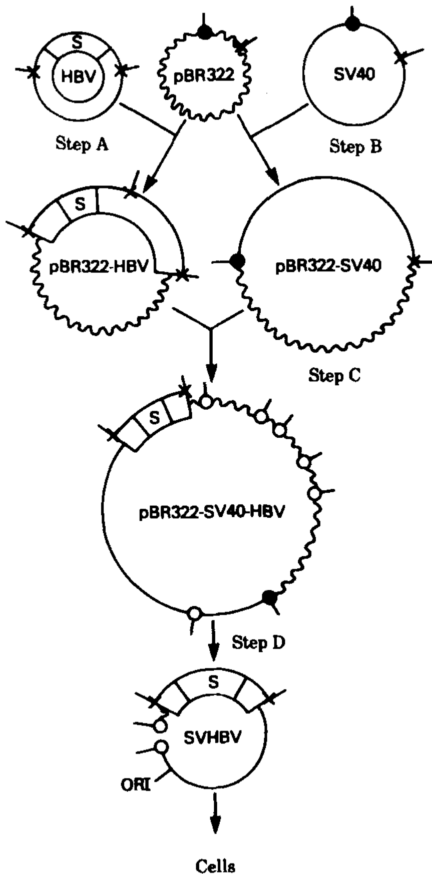


Fig. 2.11. Construction of an SV40 and HBV recombinant (after Moriarty et al., 1981).

pair fragment of HBV DNA (approximately 40% of the viral genome) inserted into the late region of SV40 virus genome (Moriarty et al., 1981). To produce this recombinant, the HB genome was amplified by cloning in an *E. coli* plasmid vector (pBR322). The recombinant retained the SV40 origin of DNA replication and the complete SV40 early gene region but lacked most of the SV40 late gene region and hence was defective. However, it could be packaged into SV40 coats and propagated as virions by making a mixed DNA infection of MK cells with an SV40 *ts* early gene mutant as a helper virus. Mixed infection was carried out at the non-permissive temperature of 39°C so progeny virions would only be produced by cells doubly infected with the SV40-HBV recombinant. The infected cells synthesized HBsAg and 45% of cells had detectable HBsAg immunofluorescence. Approximately 2×10^7 cells produced a total of 2.5 μg of HBsAg (3×10^4 particles/cell/day) and 40% of this antigen was detected in the cell supernatant fluids. The antigen was present in the tissue culture fluid as a 22 nm particle, with physical characteristics the same as those of 22 nm HBsAg particles in human sera.

Others have used the TK co-transformation technique to obtain mouse L cells excreting 22 nm particles, and the surface antigen has also been expressed in *E. coli* as part of a 138,000 dalton HBsAg β-galactosidase fusion polypeptide, although the latter hybrid molecule is not excreted from the cell. The 22 nm particles described above will undoubtedly be useful as a source of diagnostic reagents and perhaps as a source of antigen for hepatitis B vaccine.

2.7.5. Synthetic peptide vaccines

The recent advances in rapid DNA sequencing techniques, coupled with the development of genetic engineering in prokaryotes whereby pieces of viral DNA may be inserted in bacterial plasmids and expanded, has led to a tremendous increase in our knowledge of the precise sequences of viral proteins. If the amino acid sequence of an immunogenic virus protein is known then the way is open for the construction of synthetic peptide vaccines (Arnon, 1980). A pioneering study performed using foot and mouth disease virus (FMDV) is described in more detail below to illustrate the general approach. The basic essence of the method is to analyze the sequence of the major immunogenic protein of a series of antigenic variants of the virus. These should preferably be field variants, but, alternatively 'break through' variants may be selected in the laboratory using classic neutralization techniques and monoclonal antibodies. Virions escaping an excess of neutralizing antibody are often found to be antigenic variants, having single amino acid changes in the most variable part of the sequence of the antigenic protein. In this way the approximate extent of the 'variable' antigenic region is established and short peptides of different parts of this sequence are synthesized. These short peptides are coupled to BSA or keyhole limpet haemocyanin protein to increase their immunogenicity and are used to immunize rabbits and guinea pigs. Certain amino acid

sequences may induce virus neutralizing antibody and presumably these mimic the antigenic determinants on the virus particle.

It can be envisaged, then, that future viral vaccines might be composed of only the necessary short peptides, perhaps coupled to an immunogenic protein also required in a multivalent vaccine such as diphtheria or tetanus toxoid. However, at present the approach is still experimental and a looming problem is the possible cross-reactivity of certain induced antibodies with corresponding short peptide sequences in normal host proteins ('antigenic mimicry'). Already it is known that antibodies to small peptides of SV40 virus cross react, at least in immunofluorescent tests, with uninfected cells. It may be possible to overcome these serological 'mimicry' problems by fixing the normally mobile three dimensional shape of the short peptide to (hopefully) that of the virus antigenic determinant and this might limit cross reactions with normal tissue. With these potential problems in mind it will be useful to examine early approaches described in the literature with FMDV, influenza and polio viruses.

2.7.6. Synthetic peptide vaccines for foot and mouth disease virus (FMDV)

Foot and mouth disease of ruminants and swine is a disease of major concern, and 1000 million doses of inactivated virus vaccine are used each year in attempts to control the disease. The vaccine at present is prepared in a similar manner to many human viral vaccines described above, namely by cultivating the virus in cell cultures and inactivating the virus progeny. However, the two serious drawbacks of this approach include the fact that several strains of the virus cannot be grown to high enough titre to provide sufficient antigenic mass and secondly, the virus particle remains unstable below pH 7.0. Recent biochemical studies have established a major role of the virus structural protein VP1 as a dominant antigen — antibody against this protein neutralizes FMDV infectivity. Recombinant DNA technology has been applied to the problem and 1.2×10^6 copies of VP1 per *E. coli* cell can now be obtained. The primary sequence of VP1 has been deduced for two serotypes (A and O) and in a particularly novel study Bittle et al. (1982) chemically synthesized peptides corresponding to several regions of VP1 and found that they induced neutralizing and protective antibodies against the disease. The techniques in this study will undoubtedly have application to human viral vaccines such as polio. It was reasoned that since considerable variation existed between amino acids in positions 130–160 and 190–213 between different FMDV serotypes, then this could have been due to availability of these regions for reactions with neutralizing antibody, leading to high selective pressure. Seven peptides with amino acid sequences occurring in this area of VP1 were synthesized, coupled to keyhole limpet haemocyanin (KLH) and inoculated into rabbits (Table 2.15). Virus neutralizing antibody was elicited by peptides in the middle region and at the carboxy terminus of VP1

TABLE 2.15.
Antibody response of rabbits to different peptides of VP1 of FMDV, type 0, strain Kaufbeuren (after Bittle et al., 1982)

Rabbit no.	Peptide	Anti-peptide antibody titre	Neutralization index ($\log_{10}$)
1	9–24	80–160	≤ 0.3
2	9–24	40–80	≤ 0.3
3	17–32	80–160	≤ 0.5
4	17–32	20–40	≤ 0.9
5	25–41	40–80	≤ 0.5
6	25–41	640–1280	≤ 0.9
7	1–41	320–640	≤ 0.9
8	1–41	320–640	≤ 0.7
9	141–160	320–640	≥ 3.9
10	141–160	320–640	≥ 3.7
11	151–160	80–160	> 2.9
12	151–160	160–320	1.1
13	200–213	> 1280	3.5
14	200–213	160–320	3.1

(141–160, 200–213). Moreover, serotype specificity of the antibody was similar to that of sera against whole virus. In further experiments, guinea pigs were inoculated with 20 and 200 μg of peptide conjugates with Freunds and aluminium hydroxide adjuvants, or 10 μg of purified virus (Table 2.16). After 35 days antibody levels were quantitated and the animals challenged with virulent FMDV. A high degree of protection was noted in animals immunized with 200 μg peptide. Detailed analysis of the induced antibodies indicated that they precipitated intact 146S virions, and 12S protein subunits (a pentamer of VP1–3). Proteins from purified denatured virus particles were separated by SDS-PAGE transferred to nitrocellulose ('Western blotting') probed with antiserum and the reaction visualized by autoradiography after binding ^{125}I labelled protein A showing reaction between VP1 and antisera prepared against oligopeptides 200–213. In summary, the authors showed that on an equal weight basis, a single peptide has between 1–10% of the activity of the virus particle and the antibody elicited was considerably more active than antibody obtained by immunization with capsid protein VP1, either produced by disruption of virus or expression in *E. coli*. Presumably separated VP1 does not fold properly when deprived of the structural support of other virion proteins. In contrast, a small free peptide may be able to adapt a conformation approximating that which it assumes in the virus particle.

TABLE 2.16.
Protection of guinea pigs against challenge with FMDV by inoculation with inactivated virus particles, VP1 or synthetic peptides 141–160 and 200–213 (after Bittle et al., 1982)

Antigen	Dose (μg)	Adjuvant	Neutralization index ($\log_{10}$)	No. protected/ No. challenged
Virus	1	$Al(OH)_3$	2.7	1/4
	10	$Al(OH)_3$	$\geqslant$4.9	2/2
	1	Freund's	2.5	2/2
	10	Freund's	$\geqslant$5.3	4/4
VP1	20	$Al(OH)_3$	0.5	N.D.
141–160	20	$Al(OH)_3$	2.1	3/4
	200	$Al(OH)_3$	2.7	3/3
	20	Freund's	2.1	1/4
	200	Freund's	$\geqslant$3.3	4/4
200–213	20	$Al(OH)_3$	1.1	1/3
	200	$Al(OH)_3$	0.7	2/4
	20	Freund's	1.1	0/4
	200	Freund's	0.5	0/4

N.D., not determined.

2.7.7. Synthetic peptide vaccines for influenza A virus

Green et al. (1982) synthesized 20 peptides (Table 2.17) corresponding to 75% of the HA1 molecule of X-47 (H3N2) virus. Peptides (200 μg) were coupled with keyhole limpet haemocyanin mixed with Freunds complete adjuvant and injected sub-

TABLE 2.17.
Synthetic influenza HA peptides and their approximate position in the HA molecule (see Green et al., 1982 for details)

Peptide no.	Residue nos.	Position on the HA molecule
2	1–36	Membrane attachment end
3	15–53	Contains a segment of the extended conformation of peptide 2 and the entire laterally orientated loop
4	39–65	Bulge region
7	53–87	External to the HA triangle proposed as an antigenic site
11	105–140	Short external loop parallel to the long axis of the HA molecule — area is possibly involved in antibody binding
12	130–151	Another loop region, thought to be of antigenic significance
17	174–196	α helix at the distal end of the molecule – part of an antigenic site. Lying on the external face of the monomer
20	306–329	Contains carboxyl terminus of HA1 and located in the stem region of the molecule — forms interchain interactions with HA2

cutaneously into rabbits at 3 intervals and bled 4–5 weeks after the first injection. All peptides were immunogenic and titres in an ELISA test against the homologous peptide ranged from 5 to 1280 but were mostly low, around 80–320. Of the 20 anti-HA1 peptide antisera, 15 recognized HA1 determinants on HA released from virus by bromelain enzyme (the exceptional 5 peptides were nos. 8, 9, 13, 14 and 16) and most antisera reacted also with intact virus. None of the antipeptide antibodies reacted with unrelated synthetic peptides and with A/PR/8/34 (H1N1) virus. Immuno-precipitation experiments showed that the anti-peptide antibodies reacted with ^{125}I labelled HA. In essence, the study shows a surprising result — namely that synthetic peptides encompassing 75% of the HAs sequence and corresponding to a diverse array of secondary structures generate antibodies reactive with the HA. All the peptides that elicited antibodies had at least a portion of the length on the surface of the HA molecule. In contrast with peptides, immunization with intact HA presumably results in the preferential recognition of complex determinants by the immune system, a hypothesis supported by the observation that antibodies to intact virions do not recognize the synthetic peptides. It seems likely that short peptides may induce antibodies that could not be raised by the native structure. The chemically synthesized peptides elicit antibodies reactive with the native molecule because they exist for part of the time in a conformation similar to that which occurs in the intact protein. The immunological repertoire of a peptide is presumably larger than the few conformations that are similar to the native form.

2.7.8. Antigenic site for polio virus type III and the possibility of synthetic vaccines

Minor et al. (1983) analyzed antigenic mutants of polio type 3 virus using a panel of monoclonal antibodies. As with FMDV described above, the prime antigenic area was found to be located on the major structural protein VP1 as a six amino acid sequence (nucleotide sequence position 277–294) downstream from the N-terminus of VP1 (Fig. 2.12).

Mutants were selected by incubating polio type 3 viruses with an excess of neutralizing monoclonal antibodies and examining 'breakthrough' viruses for antigenic differences compared to the parent virus (see Table 2.18). Frequencies at which mutants were obtained ranged from 1 in $10^{3.1}$ to 1 in $10^{5.0}$. Plaque purified mutants were than examined to determine how many different antigenic sites and distinct clusters of epitopes were recognized by the 16 monoclones used in the study and, in fact, the viruses could be divided into 10 groups. Subsequent neutralization tests indicated that *all* the different epitopes were interdependent and clustered into a single antigenic area. T1 RNase oligonucleotide maps of representative mutants and parent viruses showed that oligonucleotide 31 was lost in many of the mutants. In addition, maps of the mutants contained an additional spot and the migration position of the new spot was consistent with the idea that it was derived from spot

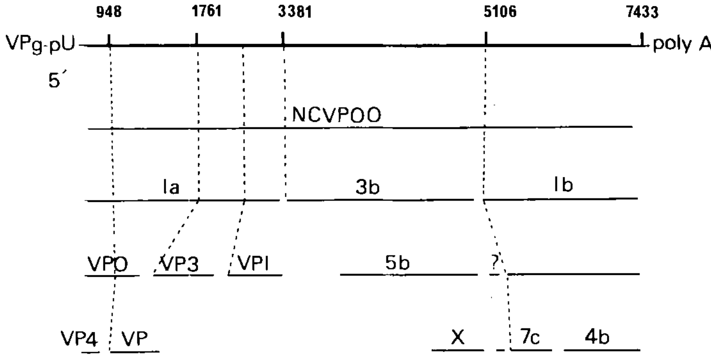


Fig. 2.12. Sequence of VPI of polio type III.

TABLE 2.18.
Sequence of oligonucleotide 31 of parental and mutant polioviruses (after Minor et al., 1983)
Nucleotide sequence and predicted amino acid coding of the region of the genome including oligonucleotide 31. Base changes observed in mutant groups 1, 4 and 8, and the corresponding amino acid substitutions are boxed.

Virus						
Parental	(G)AA Glu	CAA Gln	CCA Pro	ACC Thr	ACC Thr	CGG Arg
Group 1 mutant	(G)AA Glu	CAA Gln	CCA Pro	AUC Ile	ACC Thr	CGG Arg
Group 8 mutant	(G)AA Glu	CAA Gln	CCA Pro	ACC Thr	UCC Ser	CGG Arg
Group 4 mutant	(G)AA Glu	CAA Gln	CCA Pro	ACC Thr	ACC Thr	CAG Gln

31 by single point mutations. Mutations from C→U, A→U and C→A result in increased migration rates culminating in lateral displacement of the spot. The changes in the oligonucleotide map correlated with the antigenic properties of the mutants. Thus, changes in the region of the genome represented by oligonucleotide 31 are implicated in the generation of new antigenic variants. The sequence of the oligonucleotide 31 was determined (see Table 2.18) and its position could be located in the complete sequence of VP1. Synthetic peptides corresponding to this area can now be synthesized and investigated for induction of virus neutralizing antibodies.

2.7.9. Deletion mutants and site-directed in vitro mutagenesis

Changes in cloned viral DNAs may now be introduced at predetermined sites as

almost a routine and these can involve deletions, insertions or point mutations and a whole new technology has been recently introduced. Viable deletion mutants of several DNA animal viruses have been described including adenoviruses and papovaviruses, and some mutants show a reduced capacity for virus replication in vitro which could indicate reduced virulence. One of the most powerful techniques to obtain a single base change is the oligonucleotide directed technique (Smith and Gillam, 1981). An oligonucleotide with a mismatch or small deletion is annealed to a single stranded template (a well characterized synthetic oligonucleotide) and used to prime the synthesis of a complementary strand. In theory up to 50% of the progeny molecules arising after transfection should be mutant and these can be detected using the primer as a probe.

Single base changes can be generated at or near restriction enzyme sites by using a restriction enzyme in the presence of ethidium bromide to nick DNA in one strand only. This nick can be extended to a gap by exonuclease treatment and base transitions introduced into the remaining single stranded DNA by bisulphite treatment which deaminates cytosine to uracil in single stranded nucleic acids, resulting in a transition mutation during repair (reviewed by Harris, 1982). If a restriction site is not conveniently placed for gap or deletion mutagenesis *E. coli* Rec A protein together with small ss DNAs can be used to displace a complementary sequence in a supercoiled ds DNA molecule forming a D loop. The displaced ss DNA loop is then available for mutagenesis using nuclease S1 and bisulphite, so introducing a number of point mutations into a particular area.

Mutations can also be introduced by misincorporation of nucleotides during repair synthesis with DNA polymerase. A restriction site can be nicked and gapped and filled in with DNA polymerase in the absence of a nucleotide or in the presence of a nucleotide analogue. Base exchange during repair can be prevented either by incorporation of α-thio-deoxynucleotides or by using reverse transcriptase which lacks a proof reading activity. After repair, mutations are 'frozen' by sealing across the nick.

With influenza, the problem is to transcribe the genome into DNA and thence back again to an RNA form which can be transferred back to infectious virus. In general, for influenza, a scheme would be to clone individual gene segments in the *E. coli* K12 plasmid system to obtain full length clones and RNA transcribed from such DNA should have all control signals for viral gene replication and expression in eukaryotic cells. Sveda and Lai (1981) produced influenza RNA from cloned DNA in eukaryotic cells using a late region deletion mutant of SV40 to construct influenza DNA—SV40 hybrid molecules. This type of hybrid DNA molecule was propagated in eukaryotic cells that were co-infected with an early *ts* mutant of SV40. When cell cultures were incubated at a restrictive temperature the hybrid influenza DNA—SV40 molecule supplied the function defective in the SV40 *ts* mutant and vice versa, thereby permitting both SV40 molecules to replicate. Replication of influenza DNA—SV40 hybrid molecules in eukaryotic cells resulted in

transcription of influenza messenger RNA and expression of the appropriate functional influenza protein. The final step is to rescue negative strand influenza RNA from cells by co-infecting with an influenza virus and selecting for recombinant virus particles containing the new altered RNA. Construction of deletion mutants with positive strand viruses such as polio and togaviruses should be more simple because altered cloned DNA could be transferred directly into infectious virus by transfection of cells in culture.

2.7.10. Innovative approaches to new viral vaccines

In the recent smallpox eradication campaign vaccinia virus (in spite of its unknown origins) has been amply demonstrated to be a relatively safe vaccine for large scale use in the community. Smith et al. (1983) have exploited these advantages to clone hepatitis B surface antigen into the vaccinia virus genome at a position under the control of vaccinia virus early promoters. Cells infected with these vaccinia virus recombinants synthesize and excrete HBsAg and vaccinated rabbits produce antibodies to HBsAg. The authors noted that stable and infectious vaccinia virus recombinants that contain as much as 2500 bp of foreign DNA have been constructed and thus polyvalent vaccines against a number of agents could be constructed. Of course hepatitis B is an excellent first choice because of the relative absence of alternative sources of antigen except for the vaccines prepared from human serum, and these necessarily are likely to be severely restricted in production quantity. However, widespread vaccination is not without side effects and, before vaccination ceased, deaths occurred in most countries because of vaccinia eczematum etc. Nevertheless it should be possible to select less virulent strains of vaccinia and cultivate them in cell cultures rather than in crude lymph fluid. The cloning technique used by Smith et al. (1983) took into account the large vaccinia virus genome size and transcriptional regulatory signals used by the viral RNA polymerase. A chimaeric gene consisting of a vaccinia promoter fused to a foreign protein coding sequence was flanked by vaccinia virus DNA within a plasmid vector. A 1.35 kilobase pair Bam HI fragment was selected for use. The construction was introduced into vaccinia virus infected cells where homologous recombination with genome viral DNA occurred. Recombinants were selected by their expression of herpes virus thymidine kinase (TK) or by loss of vaccinia virus TK expression. Approximately 60% of TK plaques were shown to contain hepatitis B virus DNA by dot-blot hybridization. Approximately 2.6 μg of HBsAg was detected per 5×10^6 cells infected with recombinants (vHBs2 for example). Approximately 2/3rds of the HBsAg was present in the culture medium 2 h after infection. Finally, HBsAg was detected in cell extracts as early as 1 h after infection which was consistent with the linkage of the hepatitis gene to an early vaccinia virus promoter. 22 nm particles were visualized by electron microscopy. Rabbits were inoculated intradermally with recombinant vHBs4 and wild type vaccinia virus and all rabbits developed virus induced skin

reactions. High levels of anti-HBsAg antibody were detected in the animals infected with the recombinant virus. The technique has tremendous promise for cloned HSV, RSV and hepatitis genes.

2.8. Use of human immunoglobulins (HNIG) in virus infections (passive immunity)

The need for immunoglobulin prophylaxis remains for infections for which there are, as yet, no vaccines or chemoprophylactic drugs and also for individuals who are at risk of infection and have not, for one reason or another, received vaccine. Passive prophylaxis is also important for the increasing number of patients undergoing immunosuppressive therapy and in whom normally trivial viral infections might be life threatening. It is probably correct to conclude that the rather restricted use of HNIG at present might be dramatically changed with the advent of highly virus specific human monoclonal antibodies. At present one of the main problems for the latter approach is the dearth of suitable human myeloma carrier lines to which normal antigen stimulated human B cells can be fused. Naturally occurring human myelomas are not readily cultivable into long term cell lines but nevertheless other human B cell lines exist, representing earlier stages of B cell ontogeny. Also Epstein-Barr virus (EBV) can immortalize human B-cells, and EBV transformed lines secreting specific human antibody to various antigens have been derived. Another advantage of the EBV method is that the cell lines tend to be diploid early in culture and are generally rather stable, while hybridoma lines are aneuploid and tend to lose chromosomes. However, EBV transformed cell lines secrete quantitatively less immunoglobulin (2–20 μg/ml of cell culture) than hybridoma cell lines (10–100 μg/ml).

A further potential method for obtaining human monoclonal antibodies would be recombinant DNA technology, with translation of isolated immunoglobulin genes either in bacterial host cells or in eukaryotic host cells. However, antibodies are large 4 chain molecules and undergo post-translational modification, all of which will complicate production in prokaryotic cells. But studies on artificial antibody-like ligands is likely to progress with identification and characterization of molecular subregions with distinct antigen binding activity.

Meanwhile, HNIG is prepared from the plasma of unselected donors from large plasma pools of 1000 donors (reviewed by Polakoff, 1983). In addition, 'specific' or hyperimmune immunoglobulins for certain viruses are prepared from convalescent plasma or from donors with high levels of a particular antibody predetected by screening for e.g. varicella zoster or vaccinia immunoglobulin. The plasma is fractionated by, in the UK, for example, the modified cold ethanol Cohn process (Nitschmann and Kistler, 1962). The concentration of antibody in the IgG (Cohn fraction II) is about 20 times that of the original plasma and consists almost entirely of IgG antibody. All four subclasses of IgG are present. The IgG fraction is recon-

stituted in sterile solution and can be stored at 4°C with preservative. Any contaminating hepatitis B virus (and hopefully any unknown virus e.g. AIDS) is removed during the fractionation process but, of course, donors are also screened initially for freedom from this former virus. Similarly, non A non B hepatitis appears to be removed during purification. Adverse reactions to HNIG are uncommon in patients with antibody deficiency given repeated intramuscular doses.

HNIG are used in the prevention or amelioration of hepatitis A, B, non A non B, varicella zoster, mumps, polio, vaccinia, rubella, rabies and measles. We shall consider the last 3 viruses as examples here but further data is given in Chapters 5 and 6 where the prevention of infections by the various human pathogenic viruses is dealt with.

The protective efficacy of HNIG against measles was established as early as the 1940s (Stokes et al., 1944). Small doses of HNIG were given at the same time as early live measles vaccines (which were rather under-attenuated) and reduced the incidence of side reactions. In addition, healthy children who have not been vaccinated against measles and who are exposed may be given 250 mg of HNIG to attenuate an attack, followed by active immunization after 3 months when passive antibody would have disappeared. 15 mg HNIG may also be given to reduce reactions to measles vaccine in children with chronic respiratory or cardiac disease or other debility. In children at high risk (e.g. under immunosuppression) 250 mg ($\leqslant$ 1 year), 500 mg (1–2 years) or 750 mg ($\geqslant$ 3 years) may be administered (Polakoff, 1983).

In the UK, human anti-rabies IgG is used in combination with active immunization for post exposure prophylaxis. The recommended dosage is 20 international units/kg given only once at the beginning of anti-rabies prophylaxis to provide antibodies until the patient responds to vaccine.

2.9. Epidemiological models of virus infection and herd immunity

Changes in the incidence of infectious diseases are determined in an important way by the replication potential of the virus (May, 1982). The replicative potential is correlated with the number susceptible in the community and for an infection to persist the density of those susceptible must exceed a critical value, assuming there are no animal reservoirs which will vary between diseases. For measles this critical number would be around 4–4.5 million susceptibles in one area and if this number could be reduced then the disease would die out (The same reasoning would apply to mass chemoprophylaxis programmes). The replication rate of a disease is expressed by 'mathematical modellers' in terms of the average age at which the infection is acquired and there is an inverse correlation between these parameters, i.e. children will be infected at a young age by viruses with a high replication rate. The data for measles would suggest that 95% of each year's new group of children should be immunized at an average age of 2 years to eradicate measles. For polio,

immunization coverages of 80–85% should virtually eliminate the disease. Of course, even when too few persons are immunized to eradicate a disease, immunization still reduces the number of cases. The number of susceptible people does not go down significantly when vaccination is below the critical value for eradication but nevertheless, the chances of being infected are less because the total amount of infection in the population is less.

Directly transmitted virus infections often cause regular epidemics at 2–3 year periods because the number of susceptibles decreases as people develop immunity following an outbreak (inter-epidemic period). The susceptibles then increase again as new children are born and the cycle is reinitiated. Even although immunization may be below a level required to eliminate a viral disease from the community, other aspects of the cycle of the virus may be altered particularly after a number of years of immunization including a prolongation of the inter-epidemic period, and a reduction in the size of epidemics. But while immunization reduces the total number of cases, the number of cases of infection in *older* persons may *increase* as induced immunity wanes. Since many childhood viral diseases are quite serious in adults (e.g. measles, mumps and chickenpox), we could arrive at a difficult and controversial situation. This is already happening to some extent with measles in the USA (Chapter 8).

The mathematical models can be used to explore prospects for the control and elimination of certain infectious diseases. Polio resembles other infectious diseases such as whooping cough and diphtheria in terms of age related morbidity and Cvjetanovic, Grab and Dixon (1982) constructed models for polio which resembled those published for the two bacterial diseases. The natural history of the disease is of central importance in the construction of these epidemiological models and therefore limitation of our knowledge about polio becomes important. For instance, only a fraction of the total number of clinical cases is reported and other paralytic diseases (including those caused by enteroviruses) may disturb the real picture. Also, most epidemiological data comes from developed countries, whereas polio is endemic in underdeveloped countries. Polio has a typical seasonal appearance in temperate areas and the majority of all cases and deaths are in the under 10-year-old age group. Clinical disease may run the gamut from the atypical (with no overt symptoms) mild, through to severe illness with paralysis. Infected persons excrete virus during the incubation period (10 days) and the initial stages of illness. The virus may persist for more than 3 weeks in 50% of cases and two months or more in 10% of cases. Infection with one serotype does not give complete immunity to the other two serotypes. Vaccination gives between 80–90% protection as assessed by serological response. An epidemiological model was constructed and used to investigate the effect of certain vaccination schedules and coverage corresponding to those used in Europe. It was assumed that a coverage of 70% or 90% could be achieved by immunization and the model was then used to identify the disease extinction point with different immunization schedules. The model predicted that

with 90% immunization coverage the disease could be virtually eliminated within 12 years. An incidence rate of 1 per 100 000 persons aged 0–19 years is reached after only 7 years. When the immunization programme covers only 70% of the eligible child population good results are still predicted following immunization of infants at 6–9 months of age, provided the immunization continues for more than 10 years.

Immunization of considerably less than 100% of a population may, nevertheless, result in a reduction in illness among the unvaccinated persons because of the effects of 'herd immunity' (May, 1982). In essence, this overall reduction in disease results from there being less chance of unvaccinated people coming into contact with an infected person: new infections appear at a rate directly proportional to the number of infectious individuals and to the number of susceptibles and are assumed to result from binary encounter within a homogeneous population. Mathematical modelling analysis succinctly summarized by May (1982) shows that if a vaccine programme is initiated that is not intense enough to eradicate an infection, then a new steady state will come into existence in which the number of susceptible individuals is the same as in the unvaccinated population. This might seem surprising at first sight. Furthermore, a possible unwanted outcome of a vaccination programme where less than 100% of individuals are immunized is to delay the time of an initial infection from childhood to adulthood, where the infection might be much more serious. Examples here are polio, measles, rubella, and mumps where fatalities and serious complications increase with age. In the case of rubella, cases in pregnant women can cause rubella syndrome defects whilst with polio, paralysis is a more common sequela in young adults than in young children.

To return to the unexpected result of a vaccination programme where the number of susceptibles remains the same as in the unvaccinated population, the mathematical basis of this prediction is not complex. R_0 is the basic reproductive rate of the infection or the average number of secondary cases produced when an infected person is placed in a homogeneously susceptible population. This factor will depend, of course, on both the nature of the virus and other factors affecting transmission. The average number of secondary infections produced by each infectious person $R = R_0x$ (x is the fraction of the population which is susceptible). At equilibrium each infection must produce 1 secondary infection ($R = 1$) and so for an endemic infection the fraction susceptible is $1/R_0$ and therefore R_0 is a vital factor and is independent of the proportion vaccinated. In more precise terms we can examine an infection where $R_0 = 10$ (e.g. many childhood viral infections) and thus the equilibrium state before vaccine was used gives 10% susceptibles and 90% with acquired natural immunity. If 50% of the population are immunized and we assume no herd effects we would now have a population where 95% were immune and 5% were still susceptible. In the absence of indirect (herd) effects almost 100% immunization would be required to eliminate the infection, but, taking into account herd effects, immunization of more than 90% of the population (or more than a fraction of $1-1/R_0$) leads to eradication of the disease.

Rubella is an example of an infection whose seriousness depends on the age at infection. Usually a mild infection, rubella can cause the damaging congenital rubella syndrome in the offspring of women who acquire the infection in the first trimester of pregnancy. The incidence of congenital rubella syndrome might *increase* after some vaccination programmes.

2.10. Appendix: Current recommendations for viral vaccine usage in some countries

The immunization techniques and procedures currently used in the UK are outlined in 'Immunization Against Infectious Disease', a report prepared by the Joint Committee on Vaccination and Immunization and published by the DHSS in the UK and schedules are summarized in Table 2.19.

Most current viral vaccines with the exception of oral polio are given by deep subcutaneous or intramuscular injection. Oral polio is often given by placing 3 drops on a spoon of syrup or a sugar lump and swallowed immediately. An important parameter is storage temperature of a vaccine because even ambient room temperature of 23°C can cause inactivation of virus. Refrigeration temperatures of 2–8°C are commonly used and are satisfactory. Since administration of vaccines always carries some risk of reaction, in most clinics a sterile syringe and adrenalin injection (1 in 1000 adrenalin) is kept ready for use in emergency treatment for an allergic reaction. In general it is suggested that an immunization should be postponed if the person is already suffering from an acute illness. Also, live virus vaccines should not as a rule be given to pregnant women. The vaccination schemes in Sweden, Holland, Australia and the GDR are given in Tables 2.20, 2.21, 2.22 and 2.23. The general considerations concerning pregnancy, immunosuppressed patients, allergy etc. are similar to the UK recommendations as well as the use of influenza, hepatitis B and rabies vaccines. However, it is well to remember that these policies may change, as has happened recently in Sweden (Table 2.20).

2.11. Summary

Virologists have achieved some successes using empirical methods to develop new vaccines such as rabies, smallpox, polio, measles, rubella, yellow fever and mumps but there have also been some near disasters as for example with RSV and early inactivated measles vaccines. Work must now proceed carefully with herpes viruses in spite of various pressures to develop new products. Optimally the immunopathology of the natural disease should be understood. New immunological methods using monoclonal antibodies to 'fish out' viral proteins and also to purify antigens have stimulated this area of research. New approaches include the investigation of (hopefully) immunogenic oligopeptides prepared to a known formulation from

TABLE 2.19.
Recommended schedule of vaccination and immunization procedures (UK 1982)

Age	Vaccine	Interval	Comments	Route for viral vaccines
During the first year of life	DTPer/Vac/Ads and oral polio vaccine (1st dose) DTPer/Vac/Ads and oral polio vaccine (2nd dose) DTPer/Vac/Ads and oral polio vaccine (3rd dose)	 Preferably after an interval of 6–8 weeks Preferably after an interval of 4–6 months	The first dose of triple vaccine together with oral poliomyelitis vaccine should be given at 3 months of age. If pertussis vaccine is contra-indicated or declined by the parent diphtheria/tetanus vaccine should be given	3 drops of polio vaccine orally
During the second year of life	Measles vaccine	After an interval of not less than 3 weeks following another live vaccine		Deep subcutaneous or 0.5 ml intra-muscular
At school entry or entry to nursery school	DT/Vac/Ads and oral polio vaccine	It is preferable to allow an interval of at least 3 years after completing the basic course		
Between the 10th and 14th birthdays	BCG vaccine	There should be an interval of not less than 3 weeks between BCG and rubella vaccination	For tuberculin-negative children.	
Between the 10th and 14th birthdays	Rubella vaccine, *girls* only		All girls of this age should be offered rubella vaccine whether or not there is a past history of an attack of rubella. (In the USA *all* children are immunized and this is a major difference compared to the UK)	0.5 ml deep subcutaneous

TABLE 2.19. *(continued)*

Age	Vaccine	Interval	Comments	Route for viral vaccines
On leaving school or before employment or entering further education	Polio vaccine (oral or inactivated) and tetanus vaccine			
Adult life	Polio vaccine (oral or inactivated) for previously unvaccinated adults	A course for previously unvaccinated adults consists of: Oral polio vaccine 3 doses with an interval of 6–8 weeks between the first and second doses and of 4–6 months between the second and third; or: Inactivated vaccine: 2 doses at intervals of 6–8 weeks followed by a third dose 4–6 months later	For travellers to countries where poliomyelitis is endemic. Unvaccinated parents of a child being given oral polio vaccine should also be offered a course of oral polio vaccine	0.5 ml inactive polio vaccine by deep subcutaneous or intramuscular route.
	Rubella vaccine for susceptible women of child-bearing age		Adult females of child-bearing age should be tested for rubella antibodies. Sero-negative women should be offered rubella vaccination. Pregnancy must be excluded before vaccination and the patient must be warned not to become pregnant for 3 months after immunization	

TABLE 2.19. *(continued)*

Age	Vaccine	Interval	Comments	Route for viral vaccines
	Active immunization against tetanus for previously unvaccinated adults	A course for previously unvaccinated adults consists of 3 doses with an interval of 6–8 weeks between the first and second dose followed by a third dose 6 months later		

DTP, diphtheria toxoid, tetanus and B. pertussis 'triple' vaccine.
Note that mumps vaccine is not scheduled in the UK and nor is influenza vaccine although the latter is recommended each year by the DHSS in the UK for persons at special risk e.g. diabetics, older persons and people with chronic heart disease or diseases of the respiratory system.
Rabies vaccine is administered immediately after a bite or prophylactically to people at special risk. Hepatitis B vaccine is administered prophylactically to special risk groups e.g. workers in blood transfusion or organ transplantation units, dental surgeons.

TABLE 2.20.
Recommended schedule of vaccination and immunization procedures (Sweden, 1984)

Age	Vaccine	Comments	Route
9–11 months	Polio (types 1, 2, 3)	Killed vaccine	s.c.
10–11 months	Polio (types 1, 2, 3)	Killed vaccine	s.c.
18 months (earliest)	Polio (types 1, 2, 3)	Killed vaccine	s.c.
	Measles, mumps	Attenuated vaccine	s.c.
			s.c.
6 years	Polio (types 1,2,3)	Killed vaccine	s.c.
12–13 years	Rubella	Attenuated vaccine (girls only)	s.c.
Post partum	Rubella	Attenuated vaccine	s.c.

TABLE 2.21.
Recommended schedule of vaccination and immunization procedures (Holland, 1983)

Age	Vaccine	Comments	Route
3 months	Polio (types 1, 2, 3)	Killed vaccine	s.c.
4 months	Polio (types 1, 2, 3)	Killed vaccine	s.c.
5 months	Polio (types 1, 2, 3)	Killed vaccine	s.c.
11 or 14 months	Polio (types 1, 2, 3)	Killed vaccine	s.c.
14 months	Measles	Attenuated vaccine	s.c.
4 years	Polio (types 1, 2, 3)	Killed vaccine	s.c.
9 years	Polio (types 1, 2, 3)	Killed vaccine	s.c.
11 years	Rubella	Attenuated vaccine (girls only)	s.c.

TABLE 2.22.
Recommended schedule of vaccination and immunization procedures (Australia, 1983)

Age	Vaccine	Comments	Route
2 months	Polio (Sabin)	Attenuated vaccine	p.o.
4 months	Polio (Sabin)	Attenuated vaccine	p.o.
6 months	Polio (Sabin)	Attenuated vaccine	p.o.
12 months	Measles, mumps	Attenuated vaccine	s.c.
5–6 years	Polio (Sabin)	Attenuated vaccine	p.o.
10–14 years	Rubella	Attenuated vaccine (girls only)	s.c.

amino acid sequence data, and the deliberate cross-linking of viral proteins to increase immunogenicity. Some work on artificial adjuvants is also proceeding, but rather slowly. Investigations of immunogenicity of new vaccines must include humoral and cellular immunity both generally and at a local level in the body. Recently there has been considerable interest in the investigation of anti-idiotype antibodies as a possible new approach in the development of viral vaccines (Reagan et

TABLE 2.23.
Recommended schedule of vaccination and immunization procedures in the GDR[a] (1983)

Age	Vaccine	Comments	Route
2 months	Polio in intervals of 4 weeks (types 1, 3, 2 respectively)	Live	p.o.
13 months	Measles	Live	s.c.
2 years	Polio (trivalent in a single dose booster)	Live	p.o.
8 years	Polio (trivalent in a single dose booster)	Live	p.o.

[a] Data kindly supplied by Dr. Dittman, GDR Ministry of Health, Berlin.

al., 1983). In general terms, we have noted above that the sequence of amino acids in the variable regions of both heavy and light chains of immunoglobulin molecules produces a conformation in the antigen binding site that allows interaction of the antibody with a specific antigen. Immunization with the specific antiviral immunoglobulin into a heterologous host results in the production of anti-idiotype antibodies, specific for the variable region (as well as anti-xenotypic antibodies specific for species, and anti-isotypic antibodies specific for the class of antibody). The epitopes of virus antigens can be investigated using these reagents and, moreover, the antibodies themselves can be used as an antigen in place of virus, and will stimulate virus neutralizing antibody, just like the virus antigen itself. In the study described by Reagan et al. (1983) anti-idiotype antibodies were prepared against five monoclonal antibodies themselves directed at the major glycoprotein of rabies virus. Two of the anti-idiotype antibodies stimulated rabies neutralizing antibody in mice.

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CHAPTER 3

Antiviral drugs: general considerations

3.1. Background

Since viruses, in contrast to bacteria, replicate inside cells and use part of the cellular machinery for this replication, the development of selective antiviral drugs has been difficult and slow. However, the situation improved about two decades ago when the first virus-coded enzymes were characterized and when a few selective antiviral drugs such as idoxuridine, methisazone and amantadine were discovered. These compounds were found by serendipity but today the scientific situation has changed and a number of virus specific processes are used as targets for a more rational selection and design of antiviral drugs.

Possibilities of inhibiting virus multiplication can be localized to discrete events in the virus replication, separate from normal cellular functions. These steps will be briefly discussed in this chapter. A more detailed discussion of the mechanism of action of different inhibitors will be found in later chapters and, for example, the compounds affecting herpesviruses are discussed at some length in Chapter 11. The use of interferon and immunostimulators as antiviral agents are briefly described in this chapter as well as aspects of the inhibition of myxo and paramyxoviruses.

Inhibitors of virus replication could either change a cellular function so that the virus can no longer replicate, or could interact with a viral function as indicated in Fig. 3.1. In the latter case a compound could act directly as an inhibitor of a viral enzyme (e.g. inhibition of herpesvirus DNA polymerase by foscarnet) or be activated by a viral enzyme (e.g. phosphorylation of acyclovir by a viral thymidine kinase) to form a compound which then affects virus replication and, in some cases,

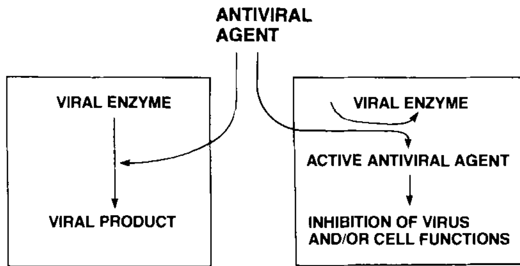

Fig. 3.1. Modes of action of antiviral agents. In the left hand situation the antiviral agent penetrates a cell and selectively blocks a viral function required for the multiplication of the virus. In the other case the antiviral drug penetrates into a cell and is selectively transformed by a viral enzyme into an inhibitor either of the viral multiplication only or of both viral and cellular functions. In the latter case this should result in a toxic effect only on virus-infected cells.

also cellular functions. It is clear from Table 3.1 that most viruses induce RNA or DNA polymerases and also other enzymes which can be used as targets for antiviral compounds.

A large number of authors have reviewed the functions of viral enzymes and their use as targets for antiviral drugs (Kit, 1979, Helgstrand and Öberg, 1980) and have

TABLE 3.1.
Virus specific enzymes which could be useful as 'targets' for antivirals

Virus	RNA or DNA polymerase	Other enzymes
Picorna	+	+
Reo	+	+
Toga	+	+
Orthomyxo	+	+
Paramyxo	+	+
Rhabdo	+	+
Retro	+	+
Arena	+	?
Corona	+	+
Bunya	+	?
Parvo	?	–
Papova	–	+
Adeno	+	+
Herpes	+	+
Irido	+	+
Pox	+	+
Hepatitis B	+	+

discussed different antiviral drugs (Becker, 1976, Swallow, 1978, Chang and Snydman, 1979, Galasso et al., 1979, Shannon and Schabel, 1980, Drach and Sidwell, 1981, Galasso, 1981, Gordon et al., 1981, Oxford, 1981, Sugar, 1981, De Clercq, 1982, Schinazi and Prusoff, 1983). Those aspects mentioned here represent a necessarily small but useful selection of what has been considered by others.

3.2. Virus specific events

The replication of a DNA virus can be schematically outlined as in Fig. 3.2 and several distinct steps can be defined. Apart from the difference due to the type of genome nucleic acid, the replication of an RNA virus is rather similar, as shown in Fig. 3.3. Figure 3.4 outlines the different strategies for replication of positive strand RNA viruses, negative strand RNA viruses and retroviruses. Some properties of these steps will be discussed here with respect to their possible use as targets for antiviral drugs.

3.2.1. Virus adsorption, penetration and uncoating

General considerations

The cellular structures responsible for adsorption of viruses are called receptors. (For reviews see Lonberg-Holm and Philipson, 1974 and Lonberg-Holm and Phi-

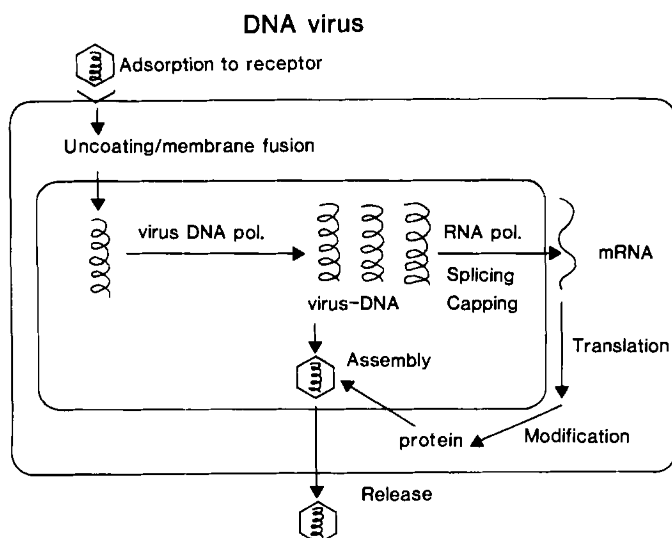

Fig. 3.2. Replication of DNA virus. The figure shows schematically different steps during the multiplication of a DNA virus in a cell.

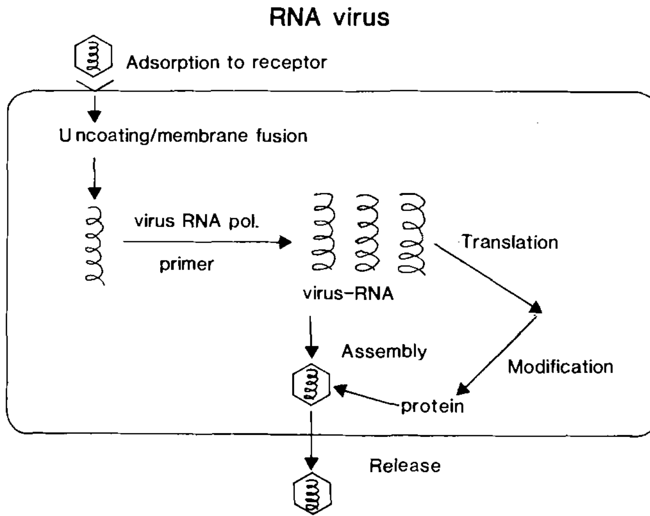

Fig. 3.3. Replication of an RNA virus. The figure shows schematically different steps during the multiplication of an RNA virus in a cell.

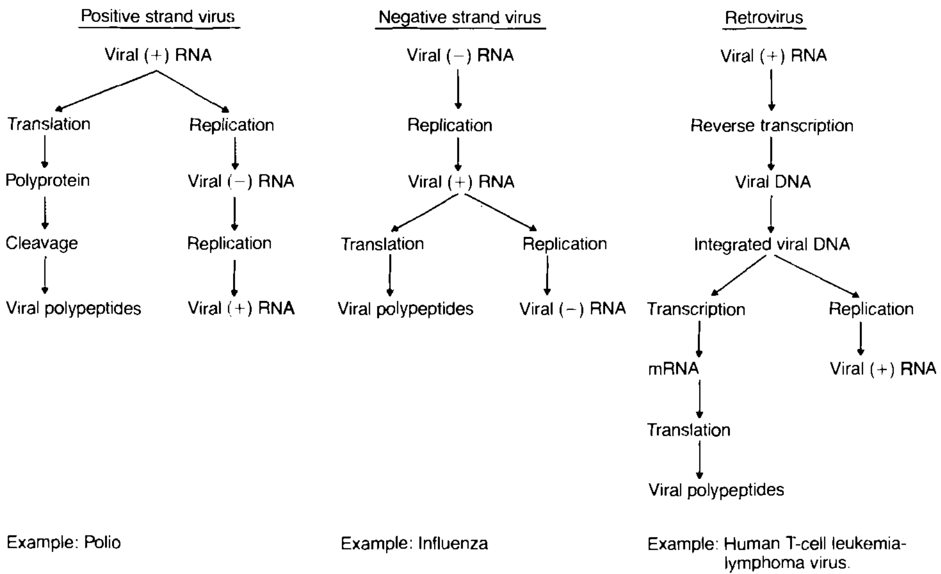

Fig. 3.4. Replication strategies for RNA viruses.

lipson, 1980, Dimmock, 1982). Their functions, apart from the binding of virus particles, are not fully known but for example histocompatibility antigen sites may also be receptors for arboviruses. The receptors seem to be composed of proteins, lipids and often polysaccharides. Different viruses can either share receptors or use differ-

ent receptors. Introduction of receptors in the cell membrane of a cell normally resistant to infection can make it susceptible. The number of receptors for a virus will depend on the type of virus and cell and has been estimated to be $10^4 - 5 \times 10^5$ per cell.

The polypeptide sequence and structure of the protein (HA) in influenza A virus responsible for binding to a receptor is now known and future work in this area will provide detailed information for other viruses about the viral peptide sequences responsible for the binding to a receptor. The possibility of preventing influenza virus adsorption has been investigated using oligopeptides with sequences similar to those of the viral proteins responsible for absorption (see Chapter 8 for details of oligopeptides versus measles virus). An advantage of this type of inhibitor is that it should act on the outside of cells and is not required to penetrate cells. This could be of advantage from a toxicity point of view. A disadvantage is the possibility that these inhibitors are likely to be competitive and might require high concentrations of drug and also that certain of the peptides may be hydrophobic, leading to problems of solubility.

Isolated receptors, or part of them, could possibly be used as antiviral agents by binding to the virus particles and thus preventing their association to receptors on cells. The binding site for vesicular stomatitis virus (VSV) on Vero cells has been characterized in some detail by Schlegel et al. (1983) and was shown to involve phosphatidylserine. The isolated phosphatidylserine binds to VSV particles, but not to HSV, and seems to be an example of a purified receptor structure functioning as an antiviral agent. The plaque formation by VSV is reduced by 80–90% at 1 μm phosphatidylserine.

Viruses such as myxoviruses and paramyxoviruses need to fuse with the cellular membrane at some stage of the life cycle and this process seems to be pH dependent and to involve haemagglutinin (HA) or fusion (F) protein, respectively. Lysosomotropic compounds such as amantadine could possibly act by increasing the lysosomal pH and thus prevent low pH fusion and subsequent infection. This is an important approach which could give inhibitors with a fairly broad range of antiviral specificity. Even though this is an effect on a cell function, the antiviral activity takes place at concentrations not prohibitively toxic to uninfected cells.

Myxoviruses and paramyxoviruses

The combination of the techniques of genetic engineering and rapid DNA sequencing has resulted in extensive new data about the amino acid sequence of influenza proteins. In an early series of experiments Porter et al. (1979) cloned gene 4 (coding for the important haemagglutinin (HA)) protein of an influenza A virus by inserting a DNA copy into an *E. coli* plasmid. Rapid DNA sequencing techniques then allowed a determination of the complete nucleotide sequence of the HA molecule and this classic study has now been extended and applied to the HA of other influenza A viruses. It is apparent by inspection of the amino acid sequences of the HA of

viruses so unrelated antigenically as A/PR/8/34 (H1N1) and an H3N2 virus isolated in the 1970s (See Chapter 7) that certain short amino acid stretches have been conserved over this 45 year period, whereas most of the rest of the molecule is completely different (reviewed by Laver et al., 1980). This might imply a common and important function of this short common sequence, and a resemblance to the amino acid sequence in the fusion (F) protein of the paramyxovirus Sendai led to the suggestion that the sequence at the N terminus of the HA2 polypeptide of influenza HA had a similar function (namely, fusion with cell membranes), allowing exit of nucleic acid from the virus and entry into the cell nucleus. Quite separate biological experiments by White et al. (1981) established that under low pH conditions influenza viruses could lyse red blood cells and cause fusion of tissue culture cells. Richardson et al. (1980) synthesized the short peptides Z-Gly-Leu-L-Phe-Gly and Z-Gly-L-Phe-L-Phe-Gly which mimicked the sequences at the N terminus of the HA^2 polypeptide described above and relatively low concentrations (20–50 μM) of the peptides inhibited influenza virus replication in tissue culture studies (See Chapter 7). The possibility of short peptides acting in vivo as antivirals in a milieu of proteolytic enzymes would seem remote, but it may be possible to synthesize analogues or add groupings to prevent rapid enzyme digestion in vivo.

These biochemical studies have also raised basic questions about the mode of action of amantadine. Since the compound acts early in infection, does it inhibit virus fusion? The interpretation of the data is even more complicated by the results of a recent study (Richman et al., 1982) which demonstrated quite clearly that although amantadine is taken up rapidly by tissue culture cells, it is less rapidly lost when the cells are incubated in amantadine-free medium. On the other hand, the antiviral action is lost almost immediately the cells are placed in the drug free medium. This suggests that intracellular amantadine has little effect on the antiviral state and that perhaps the interaction of amantadine with the external surface of the plasma membrane could explain the antiviral action. Amantadine belongs to a class of compounds termed 'lysosomotropic' which can increase intralysosomal pH (Poole and Ohkuma, 1981). If fusion is an important stage in influenza virus replication, and if such a fusion occurs only at a low pH in cytoplasmic vacuoles, then it is still possible that amantadine could act by raising the intravacuolar pH, thus stopping fusion and blocking virus replication at this stage.

Similarly, the complete sequence of the neuraminidase (NA) gene in influenza A/PR/8/34 has been determined by cloning a DNA copy of the gene into bacteriophage M13 (Fields et al., 1981). Anti-neuraminidase antibodies can inhibit viral replication as also can specific inhibitors of the NA and hence the enzyme is a suitable target for chemotherapy (reviewed by Palese and Schulman, 1977). If sequences of the NA protein are conserved independently of antigenic drift then short oligopeptides could be synthesized and any inhibitory effect investigated as described above for HA protein. The RNA coding for the NA protein is 1413 nucleotides long including 20 nucleotides 5′ to the first AUG and 31 nucleotides 3′ to the last

codon. Only one reading frame is open, beginning with AUG at position 21 and the other two reading frames are blocked by termination codons. The predicated NA protein is co-linear with the RNA and occupies 97% of the coding capacity of the gene and thus the gene codes for a single protein. A major hydrophobic region is located near the N-terminus from residues 7 to 35. In the two human subtypes (N1 and N2) total conservation of the first 12 amino acids has been described and thus some critical role such as interaction with another virus protein or NA subunit might be anticipated for this short sequence. The NA polypeptide may have an extended signal sequence responsible for transferring the NA protein across the membrane and remaining in the bilayer to anchor the NA.

An alternative and relatively new approach to the search for influenza virus inhibitors is to synthesize compounds which compete with virus for receptor binding sites on the cell. Earlier studies of cell surface receptors for paramyxoviruses indicated the importance of sialic acids of cell surface oligosaccharides, since sialidase treatment of host cells prevented virus infection. More recent studies have shown that the host cell receptors for Sendai virus are more complex and that specific gangliosides act as host cell receptors (Holmgren et al., 1980). In an experimental approach Markwell et al. (1981), removed cell receptors to Sendai virus on MDBK cells by sialidase and incubated the cells with individual highly purified gangliosides and determined if the MDBK cells regained their susceptibility to virus absorption and infection. Incubation of the cells with ganglioside containing the sequence NeuAcα2, 3 Gal B1, 3 Gal NAC fully restored susceptibility of infection to the cells. A ganglioside in which the sequence ended in two sialic acids in a NeuAcα2, 8NeuAc linkage instead of a single sialic acid was 100 times more effective as a receptor than other gangliosides. Certain polysialo gangliosides induce cell–cell fusion when incubated with cells and one could speculate that the attachment of Sendai virus to the cell surface may stabilize a clustering of receptor molecules, so facilitating virus-cell fusion (Markwell et al., 1981).

If virus cell fusion is demonstrated to be a central event in the infective process in both para and myxoviruses then a search for fusion inhibitors could result in a broad spectrum antiviral molecule. Certain diamidines have been shown recently to inhibit fusion of respiratory syncytial virus (Dubovi et al., 1980, 1981). This approach would have the added attraction with influenza that the 'fusion' sequence in HA2 is common to many antigenic subtypes, thus potentially allowing broad spectrum antiviral activity.

Comparable receptor binding site studies have been reported with influenza A virus recently and this work additionally emphasizes the phenotypic diversity of these viruses, which is discussed in more detail below (Carroll et al., 1981 and see Chapter 7). Thus, the authors examined absorption to red blood cells of two strains of influenza A virus (A/R1/5−/57 and R1/5+/57). The HAs of the two viruses were found to have totally different specificities binding to the NeuAcα2, 3 Gal and NeuAcα2, 6 Gal linkages, respectively. Examination of the crystal structure of the

HA shows that the host cell receptor cleft is distinct from the antigenic sites (Wiley et al., 1981). So variations in receptor specificity could arise from natural selective pressures by host specified sialyloligosaccharides present on cell surface glycoproteins. In this case inhibitors of this receptor function might have to be rather specific, as mutants circumventing their activity might easily arise.

3.2.2. Virus nucleic acid synthesis

Most viruses code for DNA and/or RNA polymerases as shown in Table 3.1. Some viruses also induce the formation of enzymes involved in the synthesis of nucleosides and nucleotides or are used to degrade RNA and DNA. Details about these enzymes are given in the respective chapter discussing the different viruses. A majority of the antiviral drugs found in recent years have used the viral enzymes involved in nucleic acid synthesis as targets.

Synthesis of nucleosides and nucleotides

Some viral enzymes involved in the formation of nucleosides and nucleotides are listed in Table 3.2. No useful antiviral drugs have yet been found affecting or using the ribonucleotide reductase or the deoxycytidine deaminase. These enzymes will, in the future, be explored in more detail in this respect, possibly using them for a selective activation of drugs. The HSV ribonucleotide reductase has been well characterized and might be required for virus replication. The effect of a few nucleoside analogue triphosphates on cellular and HSV ribonucleotide reductase has been studied and BVDUTP, for example, showed some selectivity, inhibiting the viral enzyme (Nakayama et al., 1982).

Nucleoside kinases, which are enzymes required for the phosphorylation of nucleosides to nucleoside monophosphates, have been found in virus infected cells, especially for members of the herpesvirus family as indicated in Table 3.2. It seems to be a general property of virus induced thymidine kinases (pyrimidine kinases)

TABLE 3.2.
Virus enzymes involved in the synthesis of nucleosides and nucleotides
(See Helgstrand and Öberg, 1980)

Virus	Enzyme
HSV-1, HSV-2	Adenosine diphosphate: thymidine-5′-phosphotransferase
HSV-1	Deoxycytidine deaminase
HSV-1, HSV-2	Ribonucleotide reductase
HSV-1, HSV-2	Thymidine kinase
VZV	Thymidine kinase
Vaccinia	Thymidine kinase
HSV-1	Thymidylate kinase

TABLE 3.3.
Relative rates of phosphorylation of nucleoside analogues

Compound	Thymidine kinase		
	Cytosol	Mitochondrial	HSV-1
dThd	100	100	100
IDU	87	—	115
ara-T	33	82	62
(E)-BVDU	⩽5	35	90
(Z)-BVDU	⩽5	15	112
(E)-BVara-U	0	177	71
FIAC	4.2	201	31
ACG	0	0	28
R-DHBG	0	0	77
S-DHBG	0	0	51

Data from Cheng et al. (1981a and b), Keller et al. (1981), Larsson et al. (1983), and A. Larsson (personal communication).

to have a less strict structural requirement for substrates than similar cell enzymes. Compounds such as acyclovir (Elion et al., 1977), DHBG (Larsson et al., 1983) and BVDU (Cheng et al., 1981a) can be phosphorylated by the viral kinases but not by the cell thymidine kinases, thus leading to a selective activation only in virus infected cells. These phosphorylations are competitive reactions influenced by the concentration of thymidine (Larsson et al., 1983). The further phosphorylation of the nucleoside monophosphates to di- and triphosphates seems to be carried out by cellular enzymes in competitive reactions that might be of importance for the final concentration of a nucleoside triphosphate.

A large number of pyrimidine analogues have been found to be phosphorylated by herpesvirus thymidine kinase and some are discussed later in the chapter on herpesvirus inhibitors. A few are shown in Table 3.3 where for example the difference in phosphorylation between cytosol and viral kinase is obvious. The mitochondrial enzyme has a substrate specificity resembling that of the viral enzyme. The phosphorylations of purine analogues are mediated by cellular enzymes. It should be noted that the acyclic guanosine analogues are recognized by the TKs as pyrimidines but after phosphorylation to monophosphates they seem to be regarded as purine monophosphates and further phosphorylated by GMP kinase.

Polynucleotide synthesis

Since most viruses induce the formation of DNA and/or RNA polymerases (see Table 3.1) a substantial amount of work has been directed towards finding selective inhibitors of these viral polymerases. In general, a polymerase inhibitor could bind to different sites on the polymerase, competing at the sugar, the base or the tri-

phosphate binding sites or affecting the site where pyrophosphate is split off during the polymerization. Some currently known inhibitors are listed in Table 3.4. Triphosphates of nucleoside analogues seem to act as competitive inhibitors/substrates as exemplified by araATP (Müller et al., 1978), acyclovir triphosphate (Furman et al., 1979, Derse et al., 1981) and BVDU triphosphate (Allaudeen et al., 1981, Ruth and Cheng, 1981) while pyrophosphate analogues such as foscarnet and PAA act as non or uncompetitive inhibitors (See Boezi, 1979, Eriksson and Öberg, 1983, Öberg, 1983a). The kinetic parameters for some nucleoside analogue triphosphates are shown in Table 3.5 together with K_m values for the competing substrates.

In earlier studies, several series of molecules reacting with the virion associated RNA transcriptase enzyme of influenza virus were synthesized on the semi-logical basis, that, in common with many DNA polymerase enzymes, the influenza enzyme might be a zinc metalloenzyme. Compounds were synthesized which could bind reversibly with zinc, which would itself be bound reversibly but more firmly to the enzyme – this is the zinc metalloenzyme hypothesis (Perrin and Stunzi, 1980). Compounds with good zinc chelating activity such as 2-acetyl pyridine thiosemicarbazone and selenocystamine had marked inhibitory effects on the virion associated RNA transcriptase enzyme of a number of influenza A and B viruses. However, these compounds had no detectable activity in cells. Subsequently, Helgstrand and Öberg (1978) used the influenza RNA transcriptase enzyme test as the basis for

TABLE 3.4.
DNA and RNA polymerase inhibitors

Substrate analogues	Product analogues	Template base-pairing	Template modification
Base modifications	Foscarnet	d(AATGGTAAAATGG)	ACG
Ribavirin			
IDU			
BVDU			
TFT			
Sugar modifications			
ara-A			
ara-C			
ACG			
DHBG			
Base and sugar modifications			
BVara-U			
FIAC			
AIU			
HPUara			

Note: Most nucleoside analogues are inhibitors as triphosphates.

more extensive in vitro screening for inhibitory molecules, and some compounds active as inhibitors of the polymerase have also shown anti-influenza activity in cells (Stridh et al., 1981).

Since influenza virus is unique in possessing cap recognizing proteins in the RNA transcriptase complex (Kroath and Shatkin, 1982), this could provide an additional target for the selection of inhibitors.

Cloning studies already in progress in several laboratories should eventually delineate RNA transcriptase enzyme binding sites on the RNA and this data may enable the synthesis of specific inhibitors. A recent study of sequence specific contacts of vesicular stomatis virus (VSV) RNA polymerase on the leader RNA gene may therefore provide a model of this approach (Keene et al., 1981). The RNA polymerase complex of VSV consists of the L, NS and N proteins and since transcription starts at the 3′ terminus of the RNA it is assumed that the promoter for the polymerase resides in the leader gene. The authors utilized methylation protection to study sequence specific interactions with VSV RNA. The N protein rendered the RNA resistant to nuclease, although it did not alter the reactivity of the RNA to methylation by dimethyl sulphate (an alkylating agent). The binding of the polymerase L and NS proteins to the nucleocapsid was examined. When the NS protein was present the reactivity of RNA with dimethyl sulphate was altered in the middle

TABLE 3.5.
Inhibition of DNA polymerases by nucleoside triphosphates

Nucleoside triphosphate	DNA polymerase, K_i or K_m (μM)						Reference
	α	β	HSV-1 (Strain)		HSV-2 (Strain)		
dATP	6.4	14.3	13.7	(Lennette)	—		Müller et al. (1978)
β-ara-ATP	7.4	5.6	0.14	(Lennette)	—		Müller et al. (1978)
α-ara-ATP	3.1	>20	>20	(Lennette)	—		Müller et al. (1978)
TTP	5.4	8.6	0.14	(KOS)	0.18	(333)	Ruth and Cheng (1981)
5-Propyl-dUTP	19.7	21	0.24	(KOS)	0.38	(333)	Ruth and Cheng (1981)
E-5-Propenyl-dUTP	5.7	8.7	0.14	(KOS)	0.12	(333)	Ruth and Cheng (1981)
BVDUTP	3.6	6.5	0.068	(KOS)	0.054	(333)	Ruth and Cheng (1981)
BVara-UTP	0.29	12	0.013	(KOS)	0.021	(333)	Ruth and Cheng (1981)
2′-Fluoro-ara-TTP	1.2	18	0.048	(KOS)	0.060	(333)	Ruth and Cheng (1981)
TTP	5.3	17.8	0.66	(HF)	—		Allaudeen et al. (1981)
BVDUTP	3.6	16.4	0.25	(HF)	—		Allaudeen et al. (1981)
dGTP	1.2	4.6	0.15	(KOS)	0.15	(333)	Derse et al. (1981)
ACGTP	0.18	No inhibition	0.0003	(KOS)	0.003	(333)	Derse et al. (1981)
dGTP	1.08	—	0.97	(KOS)	—		Furman et al. (1979)
ACGTP	2.32	—	0.55	(KOS)	—		Furman et al. (1979)
dCTP	3.4	2.4	0.092	(KOS)	0.11	(333)	Ruth and Cheng (1981)
FIACTP	1.25	5.2	0.028	(KOS)	0.041	(333)	Ruth and Cheng (1981)
ara-CTP	10	10	0.15	(KOS)	0.12	(333)	Ruth and Cheng (1981)

of the leader gene. Thus, NS may function as an initiator protein for transcription by recognizing and binding to specific sites on the genome. The NS protein binds to the sequence:

Ribavirin triphosphate has been shown to inhibit influenza RNA polymerase in cell-free assays (Eriksson et al., 1977) but it is unclear to what extent that is important in vivo.

Attempts have been made to use oligonucleotides complementary to viral DNA or RNA as inhibitors, but the results differ for different viruses and the rather extended size of oligonucleotides capable of a stable and specific base-pairing makes the penetration into a cell very difficult and it is not clear whether the viral RNA is freely accessible to base-pairing (Zamecnik and Stephenson, 1978, Stridh et al., 1981).

Smith et al. (1980) and Stridh et al. (1981) have described the RNA transcriptase inhibitory activity of certain polynucleotides. In common with the experiments described above these molecules inhibited a wide range of influenza A and B viruses. Indeed, this is a marked feature of such inhibitors, since, as far as is known, only minimal genetic variation occurs in the virus P and NP polypeptides of influenza viruses which together are considered to constitute RNA transcriptase enzyme activity.

Smith et al. (1980) calculated that 50% inhibition of influenza RNA transcriptase activity occurred with only 4 molecules per RNA molecule and inhibition probably involved direct binding to the transcriptase enzyme. However, the polynucleotide compound has only mild antiviral activity in vivo (Potter et al., 1981).

TABLE 3.6.
Viral nucleases (see Helgstrand and Öberg, 1980)

Virus	Enzyme
Polio	Endonuclease
Reovirus	Nucleoside triphosphatase
Sindbis	Endonuclease
Influenza	Endonuclease, nucleoside triphosphatase
Sendai	Endonuclease
Newcastle disease	ATPase
Vesicular stomatitis	Endonuclease, nucleoside triphosphatase
Avian myeloblastosis	Endonuclease, nucleoside triphosphatase
HSV-1, HSV-2, CMV	DNase
Frog virus 3	DNase, RNase, ATPase
Vaccinia	DNase, nucleoside triphosphatase, polynucleotide 5'-triphosphatase

Degradation of nucleotides and polynucleotides

It is difficult to assess the presence of nucleases in purified virus preparations where contamination with cellular enzymes is often possible. In some cases virus induced nucleases have been identified and they can be part of the viral polymerases. A selection of virus induced or associated nucleases are presented in Table 3.6. The DNase activity associated with HSV-1 DNA polymerase is inhibited by foscarnet (Derse and Cheng, 1981). Interestingly, activation by cleavage of a dinucleotide containing one toxic nucleoside has been suggested as a way to utilize viral nucleases as targets for antiviral drugs (Cheng et al., 1982). Inhibition of the cap dependent endonuclease activity of influenza virus (Ulmanen et al., 1983) should constitute a logical point of attack for an antiviral drug and prevent the utilization of the cellular mRNA cap by the viral polymerase.

3.2.3. Viral protein synthesis and modification

A viral infection often results in a shut-off of cellular protein synthesis. The virus mRNA uses cellular components such as initiation factors, ribosomes and tRNA in the synthesis of viral polypeptides but no effective antiviral drugs have been described which interfere with these steps. Cleavage of viral polypeptides is a common event and in the case of picornaviruses this cleavage is mediated by virus coded proteases. In addition, cleavage of the influenza haemagglutinin polypeptide (HA_0 to HA_1 and HA_2) is necessary for viral infectivity and is a possible target to block by an antiviral drug. Very little has been published in this area but as the sequence determinations and cleavage patterns for viral polypeptides become more clear it is likely that means of inhibiting these cleavages will be found. The use of protease inhibitors against influenza replication has been reported by Zhirnov et al. (1982). Butterworth (1977) and Korant (1981) have reviewed the processing of viral polyproteins to viral proteins.

Several important post-translational events such as glycosylation and phosphorylation of viral polypeptides occur. Compounds such as 2-deoxy-D-glucose and D-glucoseamine interfere with glycosylation and inhibit the multiplication of influenza and herpesvirus, but the compounds studied so far have not resulted in any antiviral drug with therapeutic activity in vivo.

The importance of correct phosphorylation of viral proteins as a way of regulating their functions has been shown with viruses such as influenza. It has also been shown (Collett and Erikson, 1978) that the function of the src gene in avian sarcoma virus is that of a protein kinase. Several other viruses such as corona virus (Siddell et al., 1981) and polyoma virus (Smith et al., 1979) have been reported to contain protein kinases, but their function remains largely unclear. Inhibitors of viral protein phosphorylation and protein kinases have not been reported yet but are likely to come in the future. It is too early to judge the potential usefulness of compounds interfering with protein phosphorylation as possible antiviral drugs, but in

this context it should be noted that one function of interferon is to induce a protein kinase which phosphorylates and impairs the activity of peptide chain initiation factor eIF-2, thus inhibiting protein synthesis (see Lengyel, 1982).

3.2.4. Virus assembly and release

The assembly of viral nucleic acid and viral proteins is a process which appears to be distinct from known cellular functions but no clear-cut inhibitors of viral assembly have been reported. Influenza neuraminidase has been implicated in the release and dispersion of new virus particles (Palese and Schulman, 1977), and several inhibitors of influenza neuraminidase such as FANA (Palese et al., 1974) have been described and have been active in cell cultures but not in infected animals (Palese and Schulman, 1977). These could be fertile areas for future work.

3.2.5. Virucidal effects

Most virucidal compounds (having a direct destructive effect on the intact virus particle) seem to be toxic also to cells, and attempts to find any antiviral selectivity at the present time have to resort to purely trial and error. Virucidal compounds not penetrating into cells could possibly have a lower and hence selective toxicity, but their distribution to the affected organ might be restricted. Unfortunately, from the point of view of this approach, the spread of virus from one cell to another could take place without the virus being exposed extracellularly, as is the case with herpesviruses and certain paramyxoviruses. Butylated hydroxytoluene might act as a virucidal agent disrupting herpesviruses even in infected animals (Keith et al., 1982).

TABLE 3.7.
Examples of antiviral drugs affecting different steps in virus multiplication

Target function	Drug	Type of virus	Reference
Adsorption, penetration and uncoating	Amantadine, rimantadine	Influenza A	Oxford and Galbraith (1980)
	Arildone	Picorna, herpes	Diana et al. (1977)
	FANA	Influenza A	Palese and Schulman (1977)
	Oligopeptides such as Z-Gly-L-Phe-L-Phe-Gly	Influenza, measles	Richardson et al. (1980)
	Polysaccharides, heparin, hyaluronic acid	Picorna, paramyxo, herpes	Becker (1976)
	2-Acetylpyridine thiosemicarbazones	Herpes	Shipman et al. (1981)

TABLE 3.7. *(continued)*

Target function	Drug	Type of virus	Reference
Nucleic acid synthesis	Acyclovir	Herpes	Elion et al. (1977)
	AdThd	Herpes	Pavan-Langston et al. (1982)
	AldUrd	Herpes	Chen et al. (1976)
	AraA	Herpes	North and Cohen (1979)
	AraAMP	Herpes	Preiksaitis et al. (1981)
	AraC	Herpes	North and Cohen (1979)
	AraT	Herpes	Aswell et al. (1977)
	BVaraU	Herpes	Machida et al. (1982)
	BVDU	Herpes	De Clercq et al. (1979)
	Complementary oligonucleotide	Rous	Zamecnick and Stephenson (1978)
	Cyclaradine	Herpes	Vince and Deluge (1977)
	DHBG	Herpes	Larsson et al. (1983)
	DHPG, Biolf 62, 2'NDG	Herpes	Smith et al. (1982)
	Distamycin	Herpes	Hahn (1977) Grehn et al. (1983)
	EHNA	Herpes	North and Cohen (1978)
	EDU	Herpes, vaccinia	Gauri and Malorny (1967)
	FIAC	Herpes	Lopez et al. (1980)
	FMAU	Herpes	Cheng et al. (1981b)
	Foscarnet	Herpes	Öberg (1983a)
	IDU	Herpes	Prusoff and Goz (1973)
	IDC	Herpes	Schildkraut et al. (1975)
	PAA	Herpes	Boezi (1979)
	Pr-dUrd	Herpes	Ruth and Cheng (1982)
	Ribavirin	DNA and RNA viruses	Sidwell et al. (1979)
	TFT	Herpes	Heidelberger and King (1979)
Protein synthesis and modification	Protein synthesis inhibitors penetrating only virus infected cells	Picorna	Carrasco (1978)
	2-Deoxy-D-glucose	Herpes, influenza	Shannon and Schabel (1980)

TABLE 3.7. *(continued)*

Target function	Drug	Type of virus	Reference
	Bis-(5-amidino-2-benzimidazolyl) methane	Respiratory syncytial, influenza	Dubovi et al. (1981)
Virucidal	BTH	Herpes	Keith et al. (1982)
Not conclusive	Carboxylic 3-deaza-adenosine	Broad spectrum	De Clercq and Montgomery (1983)
	3-Deaza-adenosine	Retrovirus	Bader et al. (1978)
	S-DHPA	Broad spectrum	De Clercq et al. (1978)
	Dichloroflavan	Rhino	Tisdale and Selway (1983)
	2-(3,4-Dichloro phenoxy)-5-nitro benzonitrile	Picorna	Torney et al. (1982)
	4′,5-Dihydroxy-3,3′,7-trimethoxyflavone	Picorna	Ishitsuka et al. (1982)
	Enviroxime	Rhino, echo, coxsackie	De Long and Reed (1980)
	HBB	Picorna	Eggers and Tamm (1966)
	Methizasone	Pox	Bauer (1977)
	Sodium 5-aminosulfonyl-2,4-dichlorobenzoate	Myxo, paramyxo, picorna	Ohnishi et al. (1982)

3.2.6. Integrated genomes – latent infections

When a viral genome has been integrated into the cellular genome the replication of the viral genome is probably mediated by cellular enzymes. This severely restricts the possibilities of eliminating the virus from the host cell. Examples of this type of integration are retroviruses, probably hepatitis B virus in chronic infections, and papilloma in warts. If, however, viral enzymes are expressed these could be used for a selective activation of compounds which are then transformed into toxic entities, thus affecting only cells which possess the viral genome. However, no compounds have been reported to be active in such a situation.

In the case of latent infections, where the viral genome has not been integrated, it is possible that a slow viral multiplication takes place. This should then represent a possible target for antiviral compounds. The development in animals of latent infections caused by HSV-1 and HSV-2 have not been affected by antiherpes drugs which block viral replication. A major problem is the lack of data concerning the state in which latent herpes simplex virus exists in the ganglia or elsewhere, and how it is reactivated (Stevens, 1980, Klein, 1982).

3.3. Types of compounds showing antiviral activity

In the search for antiviral drugs a very large number of compounds have been tested in different cell and animal models. The major part of the testing seems to have utilized cell cultures infected with influenza, herpes, rhino or some other virus of interest as a target for antiviral chemotherapy. In most cases the antiviral effects observed in cell cultures have not been followed by any therapeutic effects in infected animals. To illustrate the wide range of compounds showing antiviral efficacy in cell cultures a selection of structures has been compiled in Table 3.7. Interferon and immunomodulators are discussed separately. Some of these compounds are discussed in more detail in later chapters, others have probably no future as antiviral drugs but could serve as useful starting points in the search for better compounds, and a few might require further evaluation. The mode of action is only indicated at one step, although there could be several reactions affected, and only a very limited number of references have been included. Most of these compounds have been discussed in the reviews on antiviral drugs referred to at the beginning of this chapter.

3.4. Interferons

The possibility of using interferon in the clinic, either prophylactically or therapeutically, was limited for a long time by the small amount of interferon available. Because of DNA hybrid technology the situation has now changed and large controlled trials using interferon have now become feasible. The situation has, at the same time, been complicated by the finding of several different human interferons and the need to analyze both different interferons and combinations of interferons for antiviral activity in man. Table 3.8 lists the three main types of human interferons and also indicates that there are several different species within one type of interferon and that the origin of the cell is not enough to define the type and species of interferon.

TABLE 3.8.
Human interferons

Human interferon alpha:	Hu IFN-α. Earlier name: human leukocyte interferon (HLIF) At least 18 different species: HuIFN-α_1, α_2 etc.
Human interferon beta:	Hu IFN-β. Earlier name: human fibroblast interferon (HFIF) At least 2 different species: HuIFN-β_1, β_2
Human interferon gamma:	HuIFN-γ. Earlier name: human immune interferon (HIIF) Unknown number of species.

Addition of (Ly=lymphocyte) or (Le=leukocyte) can be used to specify origin e.g. HuIFN-α(Ly) for human interferon alpha from lymphoblastoid cells.

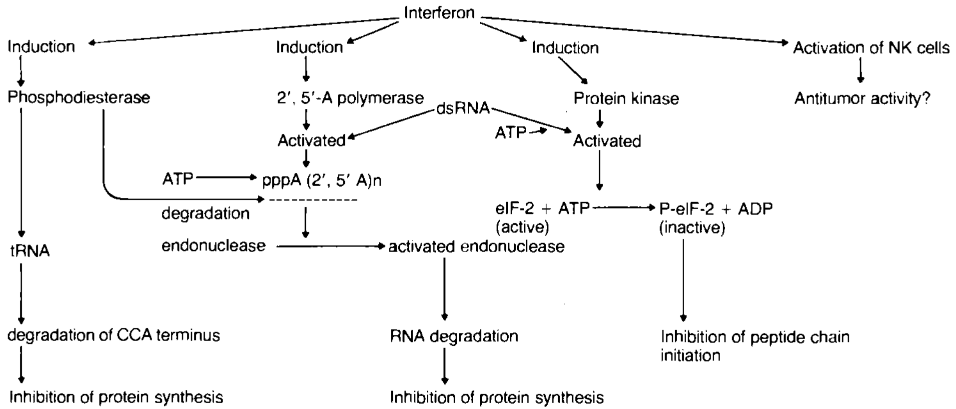

Fig. 3.5. Interferon action.

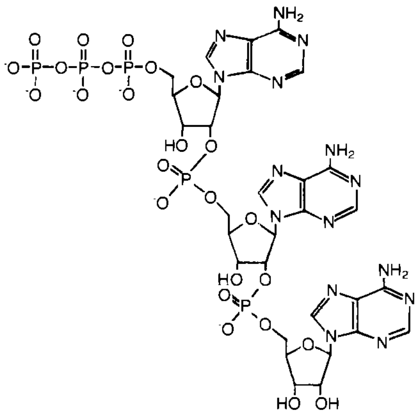

Fig. 3.6. Structure of 2′,5′-A.

Interferon was first detected by Isaacs and Lindenmann (1957) by its antiviral action in cell culture. The synthesis of interferon in a cell can be induced by a number of agents such as viruses, bacteria, nucleic acids, mitogens etc. Several interferon inducers have been investigated and most of these have been polynucleotides such as poly(I) poly(C) or other polymers of rather high toxicity. A few low molecular weight interferon inducers have been developed such as N,N-dioctadecyl-N′,N′-bis(2-hydroxyethyl)-propan-1,3-diamine, carboxyethylgermanium sesquioxide, tilorone, 2-amino-5-bromo-6-methyl-4 pyrimidole and 1,5-diaminoantraquinones. The use of interferon inducers and interferon in virus infected animals has been reviewed by Kern and Glasgow (1981).

When the induced interferon reaches other cells it can exert a number of functions as schematically illustrated in Fig. 3.5 (See Lengyel, 1982), but the relative

importance of the different pathways in interferon action is not known. The induction of 2′,5′-A (Fig. 3.6) seems to have a central role for antiviral activity. The synthesis of 2′,5′-A, by one or more cellular enzymes induced by interferon (Laurent et al., 1983), is balanced by degradation by an interferon induced enzyme (2′-Di) and the resulting 2′,5′-A activates a latent ribonuclease (RNase L) which can degrade both mRNA, rRNA and viral RNA. Since 2′,5′-A is rather labile, efforts have been made to synthesize analogues to 2′,5′-A intended to be more stable and to activate RNase L. Several structural analogues to 2′,5′-A have been synthesized with this intention (Epstein et al., 1982, Kwiatkowski et al., 1982, Sawai et al., 1983, Haugh et al., 1983), and this is a very interesting approach. An increased stability can be obtained but the modified 2′,5′-A is still a compound with a molecular weight too high for easy penetration into cells. This is an active area of research at present and improved compounds are to be expected. Their use would presumably be as broad range drugs against RNA viruses, but it is unclear at present if they will be sufficiently virus-specific and non-toxic to the cell.

Overall et al. (1981) have determined the interferon concentration in herpes vesicles in humans and found it to be high but, in general, interferon production in a virus infected tissue has not been quantitated. The limited knowledge of actual and optimal levels of interferon in tissue during viral infections makes it difficult to predict the therapeutic efficacy of exogenously given interferon. There is a more obvious possibility of interferon as a prophylactic drug assuming that the toxicity is acceptable. Studies on the therapeutic and prophylactic use of interferon will be presented in the following chapters on each type of virus infection and the reader can judge for him/herself. The clinical use of interferon has been reviewed by Dunnick and Galasso (1979, 1980) and Table 3.9 lists some (but not all) clinical trials using interferon. In most cases the limited number of patients preclude an evaluation of clinical efficacy but it seems likely that the hopes that interferon would turn out to be a new wonder drug were grossly exaggerated. However, most likely there will be situations where it can be used both for prophylaxis and therapy, and probably also in combination with other antiviral drugs.

3.5. Immunomodulators

The possibility of modulating the natural immune response has been an obvious way to develop antiviral agents. The rapid progress in our understanding of immunological processes makes it likely that in the future it will be possible to design compounds with therapeutic or prophylactic efficacy against viral infections. However, the compounds tested so far, levamisole and inosiplex, have shown little, if any, effect. The mechanism of action of levamisole is to increase phagocytosis, cell-mediated immune responses and polymorphonuclear leukocyte chemotaxis. The use in herpes and papilloma infections seems doubtful (Russel, 1980) but it might

TABLE 3.9.
Recent clinical trials with interferon (Modified after Dunn and Galasso, 1980)

Subject of study	Preparation, dose, method, schedule, and design (no. of patients)	Results, side effects	Reference
Rhinovirus infection			
Common cold	HuIFN-α2, 5×10^6 U twice daily intranasally	High frequency of nasal side effects, some prophylactic effects of interferon on rhinovirus associated colds	Betts et al. (1983)
	HuIFN-α2, 2×10^7 U/day for 4 days, intranasally (22) or placebo (22)	Protection against challenge infection	Scott et al. (1982)
Influenza virus infection			
Influenza	HuIFN-α2 5×10^6 U twice daily intranasally	Prophylactic effect reducing mean symptom sore and virus shedding	Dolin et al. (1983)
Rubella virus infection			
Congenital rubella	HuIFN-α, $2–7\times10^5$ U/kg/day for 10 days (3)	Transient decrease in pharyngeal virus excretion	Arvin et al. (1982b)
Adenovirus infection			
Adenovirus infections of the eye	HuIFN-β, $1–2\times10^5$ U per day, 6–8 times a day for 6–7 days (20); control patients received either dexamethasone phosphate with neomycin sulfate or sulfacetamide or 2% human albumin drops	Length of disease in treated group reduced to 6.5 days from 27 in controls; complication rate reduced from 50% to 5%	Romano et al. cited by Dunnick and Galasso (1980)
Hepatitis			
Hepatitis B	HuIFN-β, 1×10^6 U per day, i.m., for 82 days	Disappearance of HBcAg and HBeAg; reduction in HBsAg; no visible side effects	Dolen et al. (1979)

TABLE 3.9. *(continued)*

Subject of study	Preparation, dose, method, schedule, and design (no. of patients)	Results, side effects	Reference
Hepatitis B	HuIFN-α, 2–10×10^6 U/day or 5–20×10^6 U/day (16)	Decrease in HBV DNA polymerase during treatment; 4 patients remained polymerase negative and lost HBeAg	Scullard et al. (1981)
Hepatitis	HuIFN-α, 12×10^6 U/day (16)	Transient drop in HBV DNA polymerase. Leucopaenia in 6 of 8 treated	Weimar et al. (1980)
Herpesvirus infections			
Dendritic keratitis (patients virus-positive)	Debridement plus HuIFN-α, 1×10^6 U/ml, 1 drop per day (18); debridement plus HFLF, 1×10^6 U/ml; 1 drop per day (20)	Both preparations equally beneficial at increasing healing rate	Sundmacher et al. (1978a)
Dendritic keratitis	HuIFN-α, 6×10^6 U/ml, 2 drops per day, with either thermocautery (18) or minimal wiping debridement (24)	Best treatment was with debridement	Sundmacher et al. (1978b)
Dendritic keratitis	Trifluorothymidine and HuIFN-α drops, 30×10^6 U/ml	Reduced healing to 2,9 days	Sundmacher et al. (1978c)
Dendritic keratitis	Trifluorothymidine and HuIFN-α drops 100×10^6 U/ml	Better effect than 30×10^6 U/ml	Sundmacher et al. (1983)
Herpes reactivation after surgery for tic douloreaux	HuIFN-α, 7×10^4 U/kg per day, for 5 days	Reduced incidence of viral shedding from 42% to 9% and reactivation from 83% to 47%	Pazin et al. (1979)
Transplant recipients			
CMV infections after renal transplantation	HuIFN-α, 3×10^6 U, i.m., on day of transplant and on day 1, then twice a week	Reduced incidence of CMV viraemia and excretion; depression in white cell and platelet count	Cheeseman et al. (1979)

TABLE 3.9. *(continued)*

Subject of study	Preparation, dose, method, schedule, and design (no. of patients)	Results, side effects	Reference
EBV infections after renal transplantation	HuIFN-β, 3×10^6 U i.m., twice a week for 7 weeks (21) or placebo (20)	Reduced virus shedding	Cheeseman et al. (1980)
Prevention of virus infections after renal transplants	HuIFN-β, 3×10^6 U, i.m., twice a week, for 3 weeks	No effect	Weimar et al. (1979)
CMV pneumonia after marrow transplant	HuIFN-α, 2×10^4–6.4×10^5 U/kg per day (8)	All 8 patients died of pneumonia; no marrow toxicity at doses of $<1.6\times10^5$ U/kg per day	Meyers et al. (1980)
CMV infection after marrow transplant	HuIFN-α and araA	No effect on infection; neurotoxicity and decreasing neutrophil counts	Meyers et al. (1982)
Patients with cancer			
Varicella in children	HuIFN-α, 4.2×10^4–2.5×10^5 U/kg (18)	Varicella complications in 6 of 9 placebo and 2 of 9 HuIFN-α recipients	Arvin et al. (1978)
Varicella in children	HuIFN-α up to 3.5×10^5 U/kg (44)	Reduced formation of new vesicles and less visceral complication in HuIFN-α recipients	Arvin et al. (1982a)
Zoster	HuIFN-α 5.1×10^5 U/kg	No cutaneous dissemination; no effect with lower doses of HuIFN-α	Merigan et al. (1981)
Human laryngeal papilloma	HuIFN-α, 3×10^6 U, i.m., 3 times a week	Disease modified by treatment in 7 of 7 patients	Haglund et al. (1981)

be effective in, for example, children with frequent upper respiratory tract infections (Van Eygen et al., 1979).

Inosiplex (isoprinisone) seems to restore T-lymphocyte function which has been depressed by a viral infection and can possibly also augment interferon action

(Werner, 1979). The usefulness of inosiplex is at present unclear and will have to await further double-blind placebo-controlled trials (Chang and Heel, 1981, Editorial, Lancet 1982). A combination of immunomodulators and selective antiviral drugs is a possibility that has not been explored.

3.6. Drug delivery

The problem of drug delivery is not unique to antiviral drugs but because of their novel character very little data has been reported. For example, it is likely that topical application for herpetic infections will pose more penetration problems on dry skin than on mucous membranes or the cornea of the eye. It is also likely that the vehicle and method of application could be as important as the drug used. A general problem is, and will be, the need to use an antiviral drug at the very onset of the infection. Because of the often very rapid course of the disease, this will require a rapid diagnosis and preferably a direct delivery of the medication to the affected skin by a topical application or to the infected upper airways by an aerosol. A correct diagnosis by the patient and the appropriate drug at hand is a goal as far as rapidly progressing viral diseases like cold sores, genital herpes, common colds and influenza are concerned. Infections with a more prolonged course of virus replication, such as those in immunocompromised patients, might be easier targets for antiviral drugs.

3.7. Development of drug resistance

In spite of the limited use of antiviral drugs so far, concern has been expressed for the possible development of drug resistance (Field and Wildy, 1982). The problems concerning drug-induced resistance have recently been reviewed by Field (1983) and are discussed in detail in Chapter 17. It is clear that use of antiviral drugs in cell cultures results in a selection of drug resistant virus. It is equally clear that the selection of drug resistant bacteria is a more rapid process, perhaps not surprisingly since the replication time of a virus is in the order of several hours and that of bacteria is less than half an hour. This certainly implies that penicillins should not be used indiscriminately but also that the concern for virus resistance should not be exaggerated.

In protocol terms we observe that the wide-spread use of amantadine and rimantadine in the USSR has not resulted in development of resistance although a few resistant viruses have been reported in the GDR (Heider et al., 1981). In the case of recurrent disease, such as a labial and genital herpes, it is not clear whether a possible development of resistance during one episode of the disease will result in emergence of a resistant virus during the next episode. In generalized herpes infec-

tions in immunocompromised patients, long term systemic treatment has resulted in development of resistance to acyclovir (Sibrack et al., 1981, Burns et al., 1982, Crumpacker et al., 1982, Wade et al., 1982) but this situation is clearly different from the treatment of a local recurrent herpes disease in an immunologically normal person. Should development of drug resistance become a problem for herpes infections there remains the possibility of combining drugs with different modes of action and thus decreasing the risk of resistant strains becoming established.

3.8. Prediction of clinical efficacy

Most of the development of antiviral drugs has started with cell culture experiments to determine the inhibition of virus multiplication in vitro. This method has certain advantages, such as low cost and ease of use. However, it can give quite misleading results when substrate analogues, for example, are investigated, since these are often competitive inhibitors or are being activated in a reaction where they have to compete with the natural substrate. If the natural substrate has a higher concentration in the infected human organ than in a cell culture, the compound will have a lower efficacy in the infected organ than in the cell culture. It is also possible that experiments in animal cells or intact animals could be misleading if for example the phosphorylation of an antiviral drug involves cellular enzymes which could have species differences in their substrate specificity.

At present most would agree that the combined use of cell-free assay systems, cell cultures and animal models seem required to make a reasonable prediction of clinical efficacy of an antiviral. An example of this type of correlation between different levels of analysis is shown in Table 3.10 where the lack of correlation between

TABLE 3.10.
Comparison of antiviral activity at the level of enzymes, cell cultures and infected animals (after Öberg, 1983b)

Compound	HSV-1 TK		Cell TK	50% inhibition of HSV-1 in cell culture (μM)	% Reduction in HSV-1 lesion score, guinea pigs
	Relative rate of phosphorylation	K_i (μM)	K_i (μM)		
R-DHBG	77	1.4	>250	4	55 (5% ointment, 20 treatments topically, R, S)
S-DHBG	51	1.5	>250	12	
Acyclovir	28	97	>250	0.3	54 (5% ointment, 20 treatments topically)
Foscarnet	—	—	—	20	90 (2% cream, 6 treatments topically)

cell culture and animal experiments possibly could be explained by the different affinities to thymidine kinase for the nucleoside analogues and the presence of thymidine in guinea pig skin (Harmenberg, 1983). Forscarnet on the other hand is unaffected by thymidine. As more antiviral drugs appear and pass clinical tests it is fair to conclude that we will be in a better position to predict the efficacy of future drugs. The number of antiviral drugs tried so far in proper double-blind placebo-controlled clinical trials is still too small for precise correlations of preclinical and clinical efficacy.

3.9. Development of antiviral drugs

The development of an antiviral drug is, as for other drugs, a long process. The first part of this is outlined in Fig. 3.7, where a stepwise evaluation and modification of structures to optimize antiviral activity is indicated. In most programmes the screening and evaluation start using inhibition of virus multiplication in cell cultures, but in some instances the screening starts in animal models of different viral diseases. In these cases the mechanism of action has to be analyzed after the in vivo effect has been found. When viral enzymes are the targets, a screening can use these cell-free enzymes as the first step and in this way, hopefully, be more rational, defining the mechanism of action from the start.

It is not possible to specify precisely the time and resources required in a newly started project to find a compound active against a virus infection but 5–10 years is a reasonable estimation. For some viruses such as herpesviruses, where a number of active inhibitors are already known, the task is simpler than it is to find inhibitors

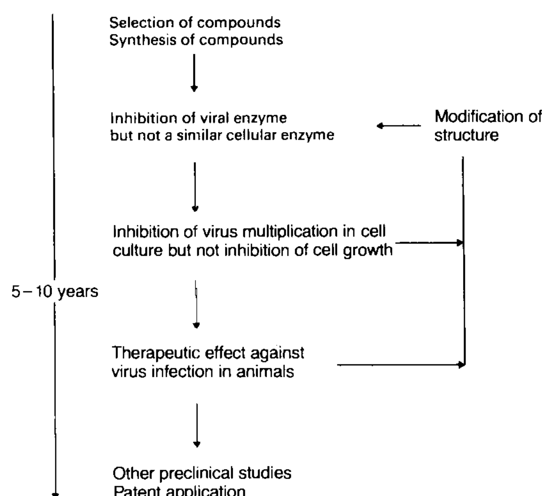

Fig. 3.7. Development of antiviral drugs I.

of a virus such as influenza against which only a few active inhibitors have been reported. Information about inactive structures is helpful in the search for active inhibitors, but unfortunately, mostly the active inhibitors are reported in the scientific literature, and not the compounds tested and found to be inactive!

In some screening programmes a thorough evaluation of the cell-toxic properties is carried out before animal experiments start (Stenberg, 1981). By this procedure a considerable number of toxic inhibitors can be eliminated at an early stage and this reduces the number of animal experiments required. This is an advantage both from an ethical and an economical point of view. It should also be pointed out that human cells should be used in these studies.

The further evaluation of an inhibitor to a registered drug is outlined in Fig. 3.8. The actual scheme for a specific drug could deviate considerably from Fig. 3.8 but the time and effort involved are probably not less. In this evaluation, as in that outlined in Fig. 3.7, toxicity or other problems can at any step involve a restart and synthesis of a new compound. The safety evaluation in animals is very expensive but it should be realized that the animal studies are models and in the first human trial a limited number of volunteers has to be carefully monitored for any adverse effects. Evaluation of clinical efficacy in humans is a large and difficult part of the development of an antiviral drug. The number of uncontrolled clinical studies

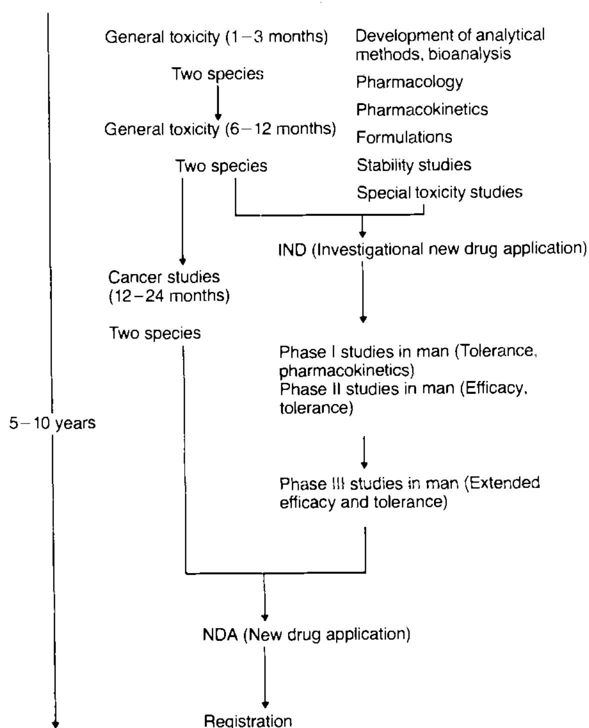

Fig. 3.8. Development of antiviral drugs II.

claiming efficacy of different drugs against viral diseases is depressingly large. It is essential to perform double-blind, placebo-controlled and statistically well evaluated studies to be able to judge the clinical efficacy of an antiviral drug. A praiseworthy high standard in these respects has been shown in the studies carried out in the US by the National Institute of Allergy and Infectious Diseases (NIAID) programme on antiviral substances. These studies could in many respects serve as guidelines for proper clinical evaluation, especially of antiherpes drugs (see Chapters 11, 12 for further details).

3.10. Prospects for the future

As the knowledge of the detailed natural history and molecular biology of viral diseases and viruses themselves increases we will obviously have better opportunities to find new drugs. Methods such as X-ray diffraction measurement and NMR determinations will probably lead to a detailed understanding of the structures and interactions taking place at the active site of viral enzymes and their cellular counterparts. This could hopefully result in a rational, computer aided design of compounds with high selectivity for the viral enzymes. We also need a more detailed insight into the natural history of some viral diseases to be able to choose the right approach for inhibition. The development of rapid diagnosis of viral infections is an important complement to the development of new antiviral drugs. Ideally the method should make a correct diagnosis possible within half an hour. Sensitive methods using immunological principles and monoclonal antibodies or nucleic acid hybridization are being developed but will require simplification to be generally accessible.

Finally, the development of antiviral drugs against uncommon viral diseases is not likely to be pursued by pharmaceutical companies for economic reasons, but could appear as spin-off products in the search of drugs against more common diseases. In this important area international agencies or countries themselves should consider a cost-benefit analysis for developing an antiviral drug. If this consideration turns out in favour of the development of a drug then the actual research work is probably most efficiently carried out by contract to an existing pharmaceutical company or institute. This type of arrangement has not been tried to date for antiviral drugs but should have obvious applications for virus infections in developing countries as well as for rare infections in the industrialized world. Ironically it is also possible that the more or less secret development of antiviral agents against viruses potentially useful in biological warfare or of other military importance could result in therapies against infections such as yellow fever, Japanese B encephalitis and other togavirus infections.

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CHAPTER 4

Picornavirus infections

4.1. Picornaviruses

The family consists of a very large number of serologically distinct viruses (Table 4.1) with differing biological properties but which have, nevertheless, a very close relationship as regards morphology and physical and chemical properties (Table 4.2). Clinical illness caused by members of the group varies from asymptomatic infection, to common cold, pneumonia, aseptic meningitis, myopericarditis and paralysis (reviewed by Douglas, 1977 and Table 4.3).

The 'oldest' member of the group is polio virus which was recognized early by clinicians because of its characteristic paralytic disease and this virus will be examined in some detail below as regards virology, disease and prevention by vaccines and chemoprophylaxis. In the 1940s viruses with similar morphology and physico-

TABLE 4.1.
Picornaviruses of man

Picornaviruses	Serological types
Enteroviruses	
Poliovirus	1–3
Coxsackievirus group A	1–22, 24
Coxsackievirus group B	1–6
Echovirus	1–9, 11–27, 29–33
Enterovirus	68–70
Rhinovirus	1–150

Note that the enterovirus Hepatitis A is discussed in Chapter 16.

TABLE 4.2.
Morphological and physical properties of picornaviruses

Picornaviruses	Size (nm)	Morphology	Buoyant density (g/cm^3)	Heat stability (50°C, 30 min)	pH stability
Polioviruses	27–30	Icosahedron	1.32–1.34	No	Yes
Echoviruses	18–25	Icosahedron	1.32–1.34	No	Yes
Coxsackievirus A	18–25	Icosahedron	1.32–1.34	No	Yes
Coxsackievirus B	18–25	Icosahedron	1.32–1.34	No	Yes
Rhinoviruses	15–50	Icosahedron	1.38–1.43	Yes	No

chemical characteristics to polio were isolated, which paralysed infant mice, and they were divided into 2 groups dependent upon the different pathology in mice, namely Coxsackie A and B viruses. Thereafter, with the extensive use of tissue cultures in the 1950s, more enteroviruses were isolated which were not pathogenic for animals, or humans such as ECHO viruses (*e*nteric *c*ytopathogenic *h*uman *o*rphan) (reviewed by McLean, 1966, Artenstein et al., 1965, Karzon et al., 1956, Grist et al., 1975). Finally, in the late 1950s viruses were at last isolated from patients with common colds and these were classified as rhinoviruses (Tyrrell, 1965). Probably in excess of 150 viruses of the latter group circulate in man. Nowadays, all these viruses are designated as 'enterovirus' serotypes (Table 4.4).

4.2. Virus structure and replication of picornaviruses

Electron microscopy reveals the enteroviruses to be small spheres with a regular protein capsid structure 18–30 nm in diameter, although only a few members have been studied in any detail. The capsid is composed of 32 capsomeres in a pattern of cubic symmetry (Fig. 4.1) of an icosahedron with 180 polypeptide molecules per capsid. The molecular weight of the RNA approximates to $2–2.8 \times 10^6$ (7500 nucleotides) and sedimentation coefficients of the viruses range from 140 to 160 S. Most of the protein is encompassed in 4 polypeptides existing in equimolar amounts (VP1, 2, 3, 4) and an RNA capping peptide. In certain of the viruses, which have been studied in more detail, a single large protein VPO is synthesized and then the 4 capsid proteins are derived by a series of proteolytic cleavages (see below, and Cooper, 1977).

After infection and uncoating, the viral RNA is translated to form the giant polyprotein which is cleaved to yield capsid proteins and the viral RNA polymerase. This enzyme, which may exist in two forms – to synthesize plus- and minus-strands respectively – is unique to virus-infected cells, and is responsible for the synthesis of virus-specified RNA using an RNA template.

TABLE 4.3.
Picornaviruses associated with specific clinical syndromes

Syndrome	Virus group				
	Poliovirus	Coxsackievirus A	Coxsackievirus B	Echovirus	Rhinovirus
Paralytic poliomyelitis	Types 1–3	Types 2, 7, and 9	Types 3–5	Types 2 and 4	—
Aseptic meningitis	Types 1–3	Types 1, 2, 4, 5, 7, 9, 10, 14, 16, 22, and 24	Types 1–6	Types 1–9, 11, 12, 14–19, 21, 22, 25, 30, 31, and 33	—
Encephalitis	—	Types 9 and 16	Types 1–5	Types 2, 6, 9, 19, 11, and 25	—
Exanthemata	—	Types 2, 4, 9, 16, and 23	Types 1–5	Types 1–6, 9, 14, 16, 18, and 19	—
Herpangina	—	Types 1–6, 8, 10, 16, and 22	—	—	—
Hand, foot, and mouth disease	—	Types 5, 9, and 16	—	—	—
Acute lymphonodular pharyngitis	—	Type 10	—	—	—
Acute haemorrhagic conjunctivitis	—	Enterovirus 70	—	—	—
Epidemic pleurodynia	—	—	Types 1–5	—	—
Myocarditis/pericarditis	—	—	Types 1–5	—	—
Orchitis	—	—	Types 1–5	—	—
Gastroenteritis	—	—	—	Type 18	—
Common cold	All types	All types	All types	All types	All types
Acute febrile undifferentiated illness	All types	All types	All types	All types	—

(for additional details see Mirkovic et al. 1973, Carter 1933, and Ranzenhofer et al., 1958)

TABLE 4.4.
Antigenic differentiation of picornaviruses

Picornaviruses	Hemagglutination inhibition (HI)	Cross-reactions occur among indicated types
Poliovirus	None	1 and 2
		1 and 3
Coxsackievirus A	A20, A21, A24, A7	A3 and A8
		A11 and A15
		A13 and A18
Coxsackievirus B	B1, B3, B5, B6	12 and 29
	3, 6, 7, 11, 12, 13, 19, 20, 21, 24, 25, 29, 30, 33	23 and 22
		11 and 19
		6 and 30
		1 and 8
Rhinovirus	None	Many

Note that more modern analytical techniques such as Western blotting may demonstrate hitherto unknown serological relationships (Thorpe et al., 1982)

The 3′ end of all picorna virus genomic RNA contains a stretch of adenosine residues (poly A) averaging 40–100 nucleotides. The function of poly A is not clear, although it is not important for the infectivity of genomic RNA. The 5′ end of the RNA is unique, uncapped and covalently linked to a protein (Lee et al., 1977, Wimmer, 1979). The protein, VPg (6–12 000 M.W. basic hydrophobic protein) and a comparable protein have been found on several picornavirus RNAs, including FMDV, EMC and rhinovirus. The significance of VPg is not known and genomic RNA without VPg is still infectious. It may act as a primer for the initiation of viral RNA synthesis and be covalently bound to the first nucleotide of the nascent strand, or may have a regulatory role.

A poliovirus specific Poly (A), oligo (U) dependent poly (U) polymerase has been purified from infected cell cytoplasm which can copy polio RNA to make complementary negative strands (Flanegan and Baltimore, 1977).

The initial transcription of input RNA produces a complementary (minus-strand) RNA whose only function is to act as template for plus-strand RNA synthesis, which may itself either be transcribed to form more complementary RNA, translated to form virus proteins or packaged to form completed virions. Much more plus- than minus-strand RNA must be produced, and the mechanism by which this is brought about is a major unsolved problem. Three RNA species have been isolated: virus RNA, a double-stranded RNA molecule known as replicative

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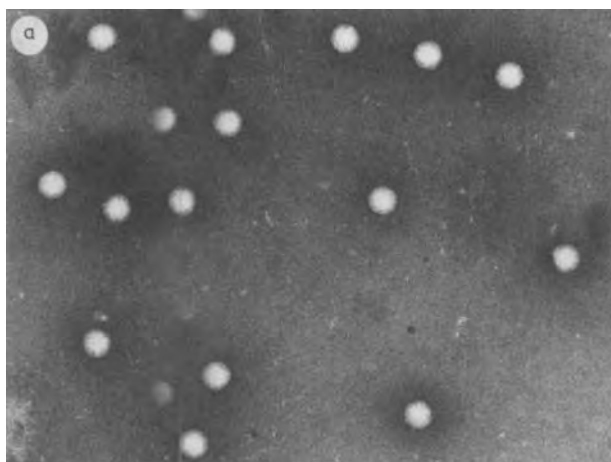
Fig. 4.1. Electron micrograph of a typical picornavirus. a, poliovirus (× 195 000); b, poliovirus (× 170 000); c, poliovirus reacted with monoclonal antibodies (× 170 000). (Courtesy of Dr. D. Hockley, NBSC.)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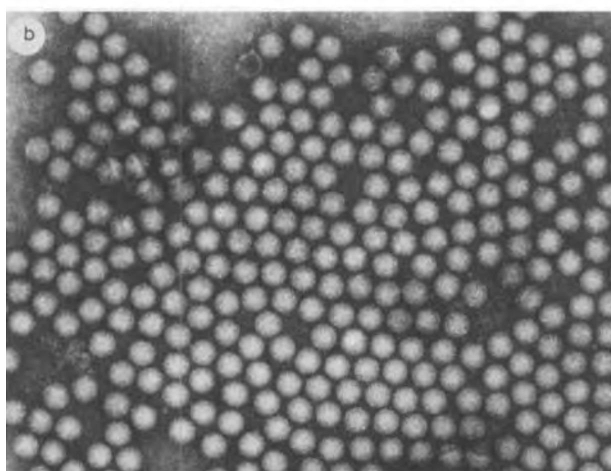
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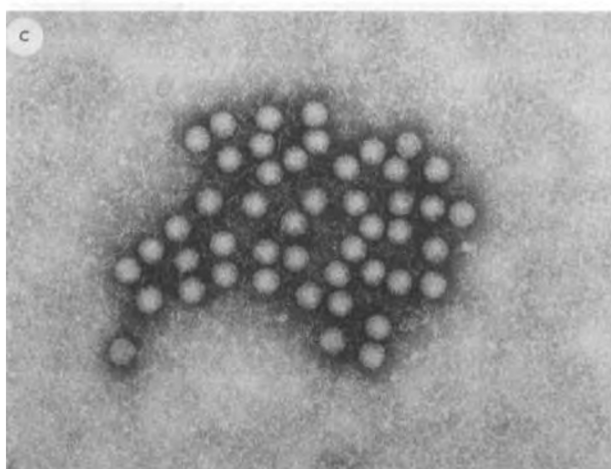
c

form (RF) and a multi-stranded RNA species known as replicative intermediate (RI). There is evidence that the RI is an intermediate in virus RNA synthesis. It consists of an RNA molecule which serves as a template for the simultaneous semi-conservative synthesis of several RNA molecules (between six and seven for poliovirus) with nucleotide sequences complementary to that of the template. Two types of RI could exist – that using a minus-strand RNA to produce plus-strands (positive RI) and that using a plus-strand RNA to produce minus-strands (negative RI). Unlike RI, which is partially sensitive to the action of ribonuclease, RF is highly resistant to this enzyme, and since it is infective it must contain an intact plus-strand, which is hydrogen-bonded to a minus-strand.

RNA synthesis switches from exponential to linear synthesis early in infection, the time varying between 1 and 3.5 h after infection and depending upon input multiplicity. This switch implies that the concentration of positive RI (the structure responsible for the synthesis of the major product) is constant throughout the latter period of infection. This could most readily be explained if synthesis of the template of positive RI (the minus-strand) ceased at the switch point, or alternatively was produced at a rate equal to its degradation. It may be important that the switch occurs at the time when the virus RNA is beginning to be encapsidated to form progeny virus.

4.3. Polio – epidemiology and clinical aspects

To develop a sensible and effective strategy of immunization or chemotherapy for a particular disease, we need to know features of the virus epidemiology, how the virus is spread in the community, reservoirs, infectivity and how and where the virus replicates and spreads in the host.

The term 'poliomyelitis' comes from polios (grey) and muelos (marrow) and 'polio' has come to be used as a shortened version meaning paralysis. The virus exists as 3 serotypes originally called Brunhilde, Lansing and Leon but now known as types 1, 2 and 3 which are easily distinguished by neutralization tests, although some cross-immunity between them occurs (see Fig. 4.2 and Table 4.5). Second attacks of polio are rare (Bodian, 1951) and, as far as is known, vaccination with one serotype (e.g. type 1) will protect against all members of these type 1 serotypes, although individual viruses may differ in virulence (Boulger et al., 1979), biochemical properties (Romanova et al., 1981) and antigen composition (Nakano et al., 1963, Schild et al., 1980).

Polio is a highly infectious disease and infection rates may reach 100% in infant classes. Personal contact is the main method of spread. A seasonal incidence is marked in temperate countries but outbreaks may still occur in winter, whereas in tropical areas it occurs throughout the entire year (Paul, 1955).

Following initial infection of humans, virus is detected in the throat secretions

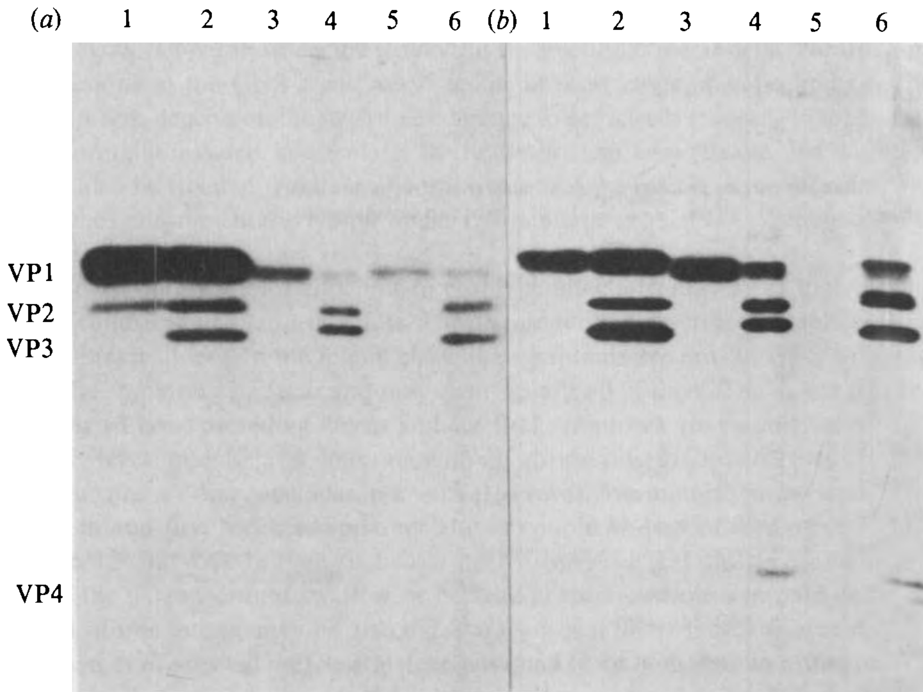

Fig. 4.2. Immunoblot experiments showing serological cross reactions between polioviruses (after Thorpe et al., 1982). (a) Immunoblot processed with rabbit antiserum 115B. Lanes 1, 3 and 5, antibody-labelled proteins; lanes 2, 4 and 6, biosynthetically labelled proteins. Lanes 1 and 2, poliovirus type 3; lanes 3 and 4, poliovirus type 2; lanes 5 and 6, poliovirus type 1. (b) Immunoblot processed with rabbit antiserum R51. Lanes 1, 3 and 5, antibody-labelled proteins; lanes 2, 4 and 6, biosynthetically labelled proteins. Lanes 1 and 2, poliovirus type 3; lanes 3 and 4, poliovirus type 2; lanes 5 and 6, poliovirus type 1. Antiserum R50 produced an identical blot (see also Table 4.5).

and faeces as virus replication continues in the tonsil and intestinal wall regions. Most patients excrete virus for 2–3 weeks and more rarely for 6 weeks. The virus then passes to the regional lymph nodes and probably then reaches the bloodstream. At this stage there is no clinical evidence of invasion of the central nervous system (CNS). There is little doubt that the virus travels along neural pathways from the periphery to the CNS and spread becomes neural once the CNS is reached. Certainly, if antibody is present in the blood the virus does not reach the CNS. A basis for a successful immunization is thus apparent already – induction of neutralizing antibody in the serum and local IgA antibody in the gut. The incubation period is as long as 2–3 weeks.

The most remarkable feature of the pathology of polio is the very high selectivity of the virus for nervous tissue, where it can cause rapid and widespread damage to the central nervous system. The primary changes are to the neurones which can be destroyed. As a response to this nerve cell damage an inflammatory reaction oc-

TABLE 4.5.
Characteristics of the antibodies to poliovirus 3 antigens used in the 'blotting' test (after Thorpe et al., 1982)

Antibody preparations		Virus[a] neutralization titre	Blocking antigen titre with poliovirus 3 antigens		Poliovirus proteins reacting with antibody on the blot								
					Type 1			Type 2			Type 3		
Designation	Specificity		D particles	C particles	VP1	VP2	VP3	VP1	VP2	VP3	VP1	VP2	VP3
115B	Polyclonal antiviral	100 000	30 000	30 000	++[b]	—	—	+++	—	—	++++	+++	+
GP35	Polyclonal anti-D	30 000	5000	20	++	—	—	+++	—	—	+++	+	—
R51, R50	Polyclonal anti-C	<2	3	3000	+	—	—	+++	—	—	+++	+	—
	Monoclonal anti-C	<2	1	50	—	—	—	—	—	—	+++	—	—
	Monoclonal anti-C	<2	1	50	—	—	—	—	—	—	+++	—	—
	Monoclonal anti-C	<2	1	50	—	—	—	—	—	—	—	—	—
	Monoclonal anti-D	<2	300	<10	—	—	—	—	—	—	—	—	—

[a] Against homologous virus.
[b] Autoradiographic bands were scored in order of increasing intensity from + (weak) to ++++ (very intense).
See also Fig. 4.2 for extra details of serological cross-reactivity.
See also Ferguson et al., 1982.

curs, with infiltration of surrounding tissue with polymorphonuclear cells and lymphocytes, but paralysis occurs from virus destruction of nerve cells. The distribution of lesions in the CNS seems very similar in most cases of polio and the clinical symptoms depend on the severity of damage to nerve cells (Lassen, 1956). The brain is normally invaded, particularly in the brain stem area (Baker, 1949). The virus can also be isolated from extraneural tissues e.g. lymphoid tissue including Peyers patches and mesenteric lymph nodes (Horstmann et al., 1954, Wenner and Rabe, 1951).

The proportion of inapparent to paralytic infections may be as high as 1000 to 1 in children and 75 to 1 in adults. The disease is often described as biphasic – minor and major illness. In the minor phase the symptoms are non-specific, with malaise as the common symptom and may clear up after 3–4 days. The major illness may occur without preceding illness and the first symptoms are meningitis, with headache, fever, malaise and vomiting with an abrupt onset (Christie, 1948). The meningitic phase often concludes in a week. However, in a minority of persons paralysis sets in and first becomes apparent after a couple of days of meningitis symptoms (Fig. 4.3 and 4.4). In the worst cases nearly every skeletal muscle can be paralysed and the person cannot swallow or breathe properly, whereas in mild cases only a part of one muscle may be affected. Paralysis is a lower motor neurone type with flaccidity of affected muscle and develops quickly. In bulbar poliomyelitis, paralysis of cranial nerves occurs and paralysis of the pharynx is a feature. In fact, the inability to swallow is the main clinical characteristic of bulbar poliomyelitis. Signs of other cranial nerve involvement may be present, facial weakness and paralysis of the flexor muscles of the neck being the commonest. Respiration may be deeply disordered and there may be pharyngeal paralysis (Baker, 1949).

In cases of paralytic polio, the full extent of paralysis is reached by 72 hours although not until 4 weeks have passed can the clinician make a reasonable prognosis, since by this time most reversible neuronal damage will have disappeared and residual irreversible damage can be assessed. Often great improvement takes place in these weeks but by the end of one month muscles still paralysed will probably remain paralysed. In bulbar polio the outlook is good if the patient is still alive by the 10th or so day since the pharyngeal muscles then begin to show signs of recovery. The basic principles for clinical care are outlined by Christie (1980) with his characteristic flair "to accept into ones care a patient stricken with a disease that may cut him off for all time from a return to normal life, but to ensure at every stage of his illness whether his life is at risk from respiratory failure or a limb is made useless by paralysis, that the exact nature of his disability is analysed as accurately as the medical sciences and clinical skill can measure it and, when that has been done, to bring to his aid all the sovereign art of modern medicine." Fortunately, the development of polio vaccines was given considerable priority in the late 1950s following the new developments of tissue culture technology (reviewed by Enders et al., 1980). Few clinicians, at least in Europe, USA, Japan, Australia and

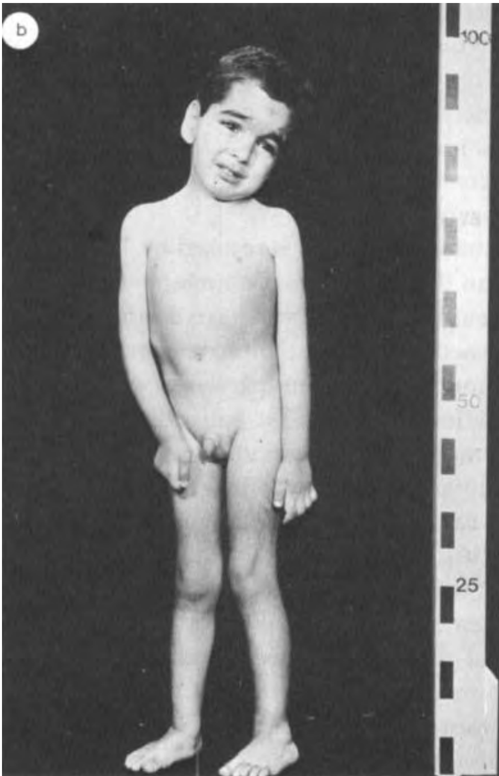

Fig. 4.3a and b. For legend see opposite page.

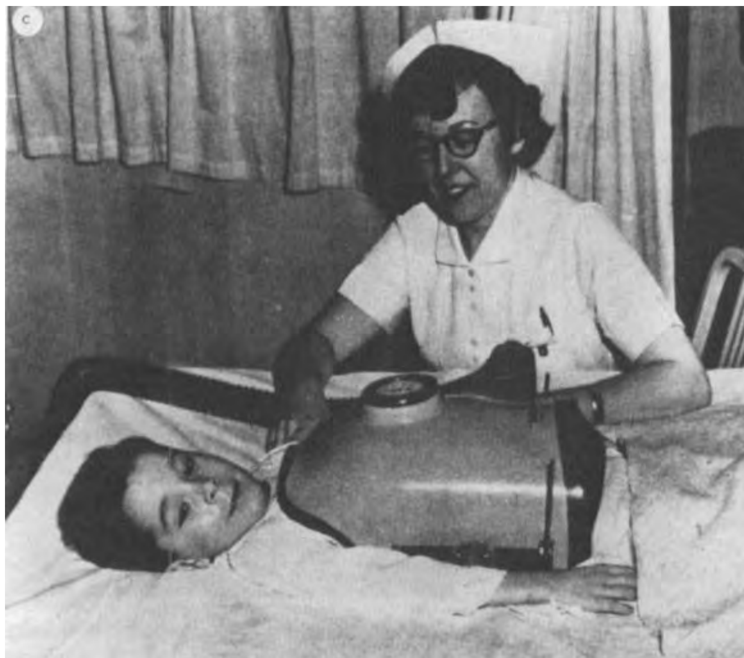

Fig. 4.3. Clinical poliomyelitis. a, children with polio (African continent); b, polio case in London (Courtesy of the late Dr. W.C. Marshall, Hospital for Sick Children, Great Ormond Street); c, artificial respiration.

the USSR have now to diagnose polio or to care for the ravages of the virus in children. The dramatic effects of vaccination on the incidence of polio is illustrated for European countries in Table 4.6.

Although the clincial effects of polio have been known since 1000 BC and a concise discussion of the disease was published by Underwood in 1789, surprisingly *epidemics* of polio have only been described in the last 100 years or so. It would seem in part that when sanitation improved, infection was encountered later in life and paralysis became more prominent. Polio vaccine (Melnick, 1954, Koprowski et al., 1956, Sabin, 1959, Salk, 1960, Salk and Salk, 1967) has been used so successfully in many countries that the virus and vaccine can be used as an example of a situation where the remote risks of vaccination itself begin to assume major importance (see below). In the US before the introduction of vaccine approximately 20 000 cases of polio-caused paralysis were recorded annually and now this number approximates to 10.

4.4 Polio vaccines – general comments

Polio has been controlled successfully by vaccines in many temperate countries of

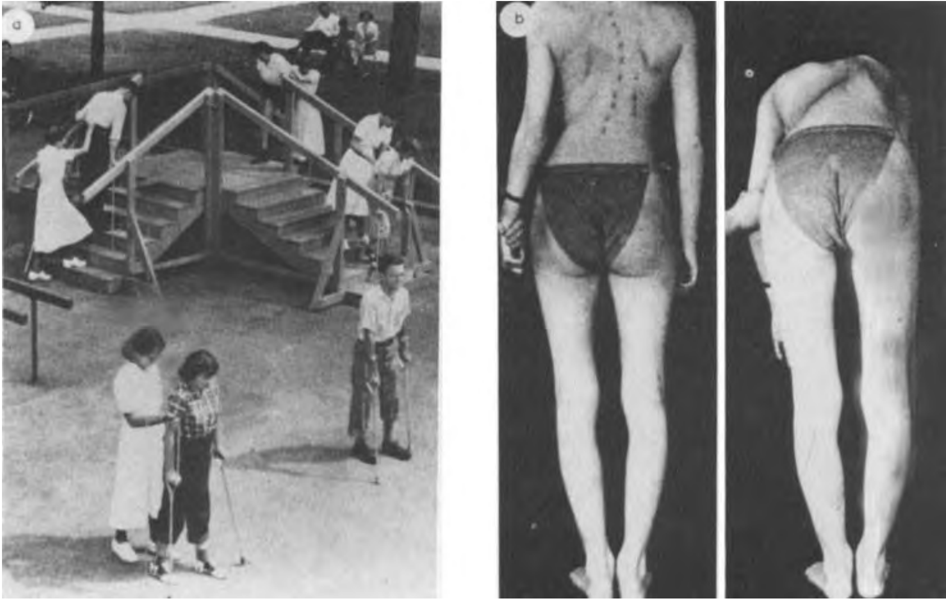

Fig. 4.4. Common residual clinical problems after recovery from acute poliomyelitis. a, physiotherapy for paralysed limbs (Third International Poliomyelitis Conference, 1958); b, scoliosis (from Ottolenghi 1958).

the world (Böttiger et al, 1966, Nathanson, 1982) but not in many tropical, third world countries (Sabin, 1982, John and Christopher, 1975, Swartz et al., 1972).

There is little precise information concerning the antigenic determinants of polioviruses and their involvement in virus structure and function. Polioviruses have been classified into three serotypes (1, 2 and 3) according to the ability of type-specific antisera to neutralize infectivity. The virus exists in two different antigenic forms: the D antigen, which is associated with the infectious particles, and the C antigen, which is associated with non-infectious particles (Mayer et al., 1957, le Bouvier et al., 1957, Roizman et al., 1959, Minor et al., 1980). As infectivity is associated with D antigen particles, it has been assumed that this immunogen is largely responsible for the production of neutralizing antibodies. Most immunochemical work with polioviruses, as well as with other picornaviruses, has been carried out with intact virus particles in which the capsid proteins are arranged in complex structural configurations, so that the immunological and antigenic properties of individual virus proteins could not be shown. In contrast, for foot-and-mouth disease virus it has been possible to establish that immunization with the trypsin-sensitive protein (VP1) induces neutralizing antibodies (Meloen et al., 1979). (see section 4.12 on immunogenic peptides of polio VP1.)

Relevant to vaccines, no single common antigen has yet been described for picornaviruses, but, as stated above, we should remember that very little detailed bio-

TABLE 4.6.
Poliomyelitis in European countries in 1960 and 1969 (i.e. before and after extensive immunization campaigns)

Country	No. of poliomyelitis cases	
	1960	1969
Austria	404	—
Belgium	300	2
Denmark	22	1
Finland	273	—
France	1663	69
West Germany	4139	25
Hungary	38	2
Ireland	183	7
Italy	3555	56
Netherlands	29	15
Norway	59	1
Poland	301	6
Portugal	244	1
Spain	1632	387
Sweden	18	—
Switzerland	139	1
United Kingdom	378	15
Yugoslavia	1680	24
Total	15057	612

chemistry has been carried out with many of these viruses. Indeed RNA hybridization shows a high degree of homology between certain members and so common amino acid sequences with corresponding antigenic relationships may exist. Certainly, antigenic cross-reactions have been noted among certain serotypes such as coxsackie A3 and A8 for example, between A9 and 11 coxsackie B viruses. Again with the ECHO viruses, heterotypic cross reactions occur between some viruses, e.g. types 1 and 8, and between 12, 29, 23 and 22. Thorpe et al. (1982) using the sensitive immunoblot technique have described antigenic relationships between VP1 of polio types 1, 2 and 3. Furthermore, certain monoclonal antibodies directed towards the C antigen of polio type 3 reacted with VP1 of this virus. VP1 seems to be immunodominant regardless of the form of the immunizing antigen. A recent report (Blondel et al., 1982) suggests that immunization with VP1 causes the production of neutralizing antibodies and in this respect the immune response to poliovirus VP1 may be similar to that to VP1 of foot-and-mouth disease virus and to VP2 of coxsackie virus (Meloen et al., 1979, Beatrice et al., 1980). Although homotypic antibodies binding to VP2 were detected with hyperimmune sera, no heterotypic cross-reactivity was observed for this protein.

To some extent views about the use of either killed or live polio vaccines are in a state of flux at present (Melnick, 1978, Sabin, 1982). Perhaps not unexpectedly the virologists who hold dominating and contrasting views are Drs. Salk and Sabin (originators of inactivated and attenuated polio virus strains respectively). Also, perhaps not unexpectedly Sabin enthusiastically supports the widespread use of live vaccine whilst Salk confines his interest to killed polio vaccine. Both vaccines have their merits and weaknesses and it may not be possible to decide between them (Tables 4.7 and 4.8). To some extent a judgement on their relative efficacy and usefulness may depend on the purpose of the vaccination campaign and where in the world it is being conducted. If our goal is total eradication of virulent polio virus from the world community (which should be possible, there being no animal reservoir of the virus, and the virus exists as a limited number of 3 serotypes) then inacti-

TABLE 4.7.
Killed poliovaccine: advantages and disadvantages (after Melnick, 1980)

Advantages

- Confers humoral immunity in satisfactory proportion of vaccinees if sufficient numbers of doses are given
- Can be incorporated into regular paediatric immunization, with other vaccines (DPT)
- Absence of living virus excludes potential for mutation and reversion to virulence
- Absence of living virus permits use in immunodeficient or immunosuppressed individuals and their households
- Appears to have greatly reduced the spread of polioviruses in small countries where it has been properly used (wide and frequent coverage)
- May prove especially useful in certain tropical areas where live vaccine has failed to 'take' in young infants

Disadvantages

- Several studies have indicated a disappointing record in percentage of vaccinees developing antibody after three doses[a]
- Generally, repeated boosters have been required to maintain detectable antibody levels[a]
- Does not induce local (intestinal) immunity in the vaccinee; hence vaccinees do not serve as a block to infection with wild polioviruses
- More costly than live vaccine, in single-dose cost, administration expense, and total amount required, including boosters
- Subject to problems from present and growing scarcity of monkeys (but could be resolved if high-titer virus could be grown in human diploid cells and shown, in field tests with adequate numbers of persons, to be free of any problems resulting from *injection* of virus grown in human cells
- Use of antigenically potent but virulent polioviruses as vaccine seed creates potential for tragedy if a single failure in virus inactivation were to occur in a batch of released vaccine

[a] Some of the disappointing results in the decade after killed vaccine was introduced may have been due in part to problems that may now have been corrected. Present antigens are stronger than the older antigens. (See also Marsden et al., 1980 and Boulger et al., 1979 for a further discussion of assessment of neurovirulence of poliovirus vaccine.)

TABLE 4.8.
Live poliovaccine: advantages and disadvantages (after Melnick, 1980)

Advantages

Confers both humoral and intestinal immunity, like natural infection
Immunity induced may be lifelong
Induces antibody very quickly in a large proportion of vaccinees
Oral administration is more acceptable to vaccinees than injection, and is easier to accomplish
Administration does not require use of highly trained personnel
When stabilized, can retain potency under difficult field conditions with little refrigeration and no freezers[b]
Under epidemic conditions, not only induces antibody quickly but also rapidly infects the alimentary tract, blocking spread of the epidemic virus
Is relatively inexpensive, both to produce the vaccine itself and to administer it, and does not require continued booster doses
Can be prepared in human cells, thus is not dependent on continuing large supplies of scarce monkeys (this also eliminates theoretical risk of including monkey virus contaminants in the vaccine)

Disadvantages

Being living viruses, the vaccine viruses do mutate, and in rare instances have reverted toward neurovirulence sufficiently to cause paralytic polio in recipients or their contacts
Vaccine progeny virus spreads to household contacts[a]
Vaccine progeny virus also spreads to persons in the community who have not agreed to be vaccinated[a]
In certain warm-climate countries, induction of antibodies in a satisfactorily high proportion of vaccinees has been difficult to accomplish unless repeated doses are administered. In some areas, even repeated administration has not been effective.
Contraindicated in those with immunodeficiency diseases, and in their household associates, as well as in persons undergoing immunosuppressive therapy

[a] Some people consider this spread into the community to be an advantage, but the progeny virus excreted and spread by vaccinees often is a mutated virus. Obviously it cannot be a safety-tested vaccine, licensed for use in the general population.
[b] see Magrath, 1976.

vated vaccine must be used in the final thrust, although much earlier work could be carried out with live vaccine or a combination of live and killed vaccine. This is because attenuated viruses may (and do) revert to virulence on occasion (Nathanson, 1982). As mentioned above the genetic determinants of virulence or attenuation for polio virus are not known and it will require extensive biochemical analysis of the genome of a number of attenuated and virulent viruses, mutants and recombinants to establish the genetic basis of virulence. At this stage one could envisage that an attenuated and *stable* variant could be constructed by using DNA technology (see Chapter 2). On the other hand, it may not be practicable to produce large enough quantities of inactivated vaccine cheaply enough for a total eradication campaign and moreover, if the manufacture of vaccine is extended more widely

then escapes of virulent virus in incompletely inactivated vaccine may occur. An early example of such an accident was the Cutter affair in the USA where 92 children contracted paralytic poliomyelitis following immunization with insufficiently inactivated vaccine (Nathanson and Langmuir, 1963). It could be added however that nowadays, should large scale manufacture of inactivated polio vaccine be reinitiated then viruses would be chosen with less than full virulence to avoid potential problems of incomplete inactivation (Salk et al., 1981). Genetic engineering techniques could now be explored to produce immunogenic polio proteins and synthetic peptides which would side step any 'failure of inactivation' problems although these synthetic techniques are likely to produce problems of their own (see Chapter 2).

We can conclude that both vaccines have had their successes and failures, both vaccines are not in evolutionary blind alleys and can be improved in the near future, and both vaccines probably have a long and useful life and function ahead of them.

4.5. Early history of laboratory studies with polio virus

Until the 1930s polio virus could only be identified by infection of monkeys and subsequent production of paralytic disease. This property of neurotropism of polio in vivo and early studies in cell culture which were wrongly interpreted as indicating that polio could only replicate in neural tissues delayed work with the virus and, moreover, erroneously led to deductions that droplet infection and subsequent invasion of the CNS via the olfactory nerve endings was the major point of entry of the virus in humans. In fact it could be said that the early studies of 'chemotherapy' were initiated by these observations and sodium alum, zinc sulphate and picric acid were all used unsuccessfully to prevent paralysis (Armstrong and Harrison, 1935, Sabin et al., 1936, Olitsky and Sabin, 1937, Schultz and Gebhardt, 1937, Armstrong, 1937). Sabin and Ward (1941) were among the first to appreciate that the alimentary tract was a major site for polio virus replication in man. Studies in the 1940s and 1950s established that although polio virus could be isolated in flies and cockroaches, and antibodies detected in cattle and chimpanzees (Sabin and Ward, 1942, Melnick and Penner, 1952, Sabin and Fieldsteel, 1952), nevertheless the viruses are maintained in nature by human to human transmission. Epidemiological studies in Eskimoes demonstrated that antibodies to polio persisted for at least 40 years in the absence of re-infection and that natural infection gave rise to complete or partial resistance in the intestine. The scientific ground work was therefore laid for the production and application of polio vaccines. Theiler (1941) reported laboratory modification of the neurotropic property of polio and Koprowski et al. (1952) described for the first time that an immune response could be produced in humans following infection with an attenuated (mouse adapted) type 2 polio virus which had reduced intra-cerebral virulence for monkeys. Finally, the clear demonstration by Enders et al. (1952) that all three polio virus types could replicate in

TABLE 4.9.
Results of titrations in monkeys and in tissue cultures of materials from a tissue culture line of Type 1 virus (Brunhilde strain). (after Enders et al., 1980)

Material titrated	Monkey infectivity (PD_{50}/1 ml)	Tissue culture infectivity (ID_{50}/1 ml)	Ratio: TC infectivity / Monkey infectivity
Monkey cord	10^5	10^5	(1)
Susp. cell cult. pass. 2	10^3	10^5	100
Susp. cell cult. pass. 10	19^1	10^5	1000
Roller cult. pass. 1	<1	10^4	$>10\,000$
Roller cult. pass. 2	<1	10^5	$>100\,000$

cell cultures of non-nervous tissue (see below) and simultaneously lose monkey virulence (Table 4.9.) opened the way to the extensive laboratory studies leading to the development of successful formalin inactivated and also live attenuated polio vaccines.

4.6. Early development of inactivated polio vaccine

Salk commenced work on producing an inactivated polio vaccine with the premise that circulating serum IgG antibody provided protection against the clinical signs of polio and therefore as long as a killed vaccine induced such antibody in sufficient quantity it would be effective. (Earlier work in his laboratory had been orientated towards developing live attenuated viruses, but this ceased as soon as it was apparent that tissue culture cultivated formalin-inactivated virus was immunogenic.) His group demonstrated that virus concentration (and hence antigen concentration) was the single most important variable factor in vaccine production, rather than the inactivation process per se. Inactivation with 1/4000 formalin at 35°C at pH 7.0 resulted in a constant rate decline of virus infectivity for the first $2\frac{1}{2}$ days. However, even at this early stage inactivation of lots of vaccine varying from 30 to 50 litres was showing some variation between manufacturers, with satisfactory 'base line' virus inactivation in one manufacturer detected at 6–9 days but 13 days at another production plant (12 days was later chosen as safer after the Cutter incident). If inactivation proceeded for too lengthy a period then immunogenicity decreased, and hence the time period was critical.

Early experiments in monkeys established that vaccine given in three divided doses was more immunogenic than a single large dose. Table 4.10 summarizes the data from some of the first trials in children who had no antibody prior to immunization with polio vaccine and the effect of boosting at 7 months and at 20 months (Table 4.11). Quite clearly the era of successful immunization against polio had commenc-

TABLE 4.10.
Antibody levels before and 7 months after primary vaccination and after 'booster' injection in persons inoculated with an aqueous poliomyelitis vaccine (inactivated). (after Salk, 1955)

Subject no.	Age in years	Type 1			Type 2			Type 3		
		Pre-vacc.	7 mos. later	12 days after 'booster'	Pre-vacc.	7 mos. later	12 days after 'booster'	Pre-vacc.	7 mos. later	12 days after 'booster'
Group A										
F-332	3	0	8	256	0	128	1024	0	0	512
F-4	6	0	64	1024	0	4	1024	0	4	512
F-2	9	0	32	512	0	8	128	0	4	256
F-45	9	0	32	4096	0	32	512	0	16	1024
F-47	10	0	16	1024	0	4	256	0	8	1024
F-328	10	0	64	4096	0	4	256	0	4	256
F-335	11	0	32	2048	0	0	128	0	8	1024
Group B										
F-80	6	0	4	512	0	0	256	0	4	512
F-77	7	0	4	512	0	4	128	0	8	512
F-79	10	0	4	128	0	4	256	0	4	128
F-336	12	0	16	1024	0	16	256	0	16	512

Primary vaccination – 3 doses, 1 week apart.
Group A – 1 ml of trivalent vaccine, intramuscularly.
Group B – 0.1 ml of each type of monovalent vaccine, intradermally.

ed, although problems of incomplete and variable inactivation of virus were to be noted immediately the vaccine process was scaled up to produce the tens of millions of doses required for mass immunization (Nathanson and Langmuir, 1963).

As regards production of polio vaccine nowadays, human diploid cells are used by some manufacturers and still other cell substrates are being considered for polio vaccine production mainly because of the immediate shortage of monkeys and safety considerations but additional advantages might be increased yields of virus (Van Wezel et al., 1978, Marel et al., 1981). Possible new cell substrates are:

1: subcultured monkey cells rather than primary cells. In addition, newly developed techniques of perfusion with trypsin gives increased yields of cells.
2: primate embryo diploid cells (in addition to human diploid cells).
3: continuous cell lines e.g. Vero (these cells have been suggested because of evidence of absence of tumourigenicity).
4: 'suspension' cultures of continuous cell lines using Sephadex beads.

Captive bred monkeys have fewer adventitious 'foamy' viruses and so are more suitable as a source of kidney cells than captured animals. Good cell growth of MK

TABLE 4.11.
2-Year follow-up in first group of subjects in whom polio vaccination studies were undertaken (after Salk, 1955)

Age at time of first vacc. (years)	Identification no.	Type 2 antibody titre vs. 100 ID_{50} virus			
		Before vacc.[a]	2 months	Booster at 20 months[b]	22 months
2	W-44	0	32	8	2048
4	W-31	0	64	8	1024
5	W-18	0	16	8	512
6	W-27	0	8	2	512
8	W-20	0	128	64	256
9	W-33	0	8	0	256
10	W-8	0	4	0	512
10	W-26	0	16	8	256
11	W-74	0	32	4	512
14	W-34	0	16	8	256
17	W-1	0	32	16	128
17	W-32	0	32	16	N.T.
31	W-3	0	16	8	256

[a] 2 doses I.D. of 0.1 ml each 6 weeks apart monovalent vaccine.
[b] 1 dose I.M. of 1 ml trivalent vaccine.
N.T., not tested.

cells is obtained in monolayers and in microcarrier cultures for up to 3 passages (approx. 12 cell generations) although the cells stop growing after 7–8 passages. 15–20 monkeys per year will then be required to produce $3–4 \times 10^6$ doses of vaccine. Although diploid cells have many favourable properties (absence of viruses, strict control on karyotype (Chapter 2)) they require high quality serum for growth and are less suitable for large scale cultivation. Finally, Vero cells may be suitable particularly since polio virus will be inactivated with formalin at the final stages (so inactivating any adventitious or unknown virus) and these cells can be cultivated in microcarrier systems.

Criteria for seed polio viruses for inactivated vaccine include stability, immunogenicity, and replication rate but the most important factor is immunogenicity. Type 2 strains (MEF1) with type 3 (Saukett) induce much higher antibody levels than a comparable antigenic mass of type 1 (G. Mahoney) polio. It has also been recognized that antigenic differences exist within polio types and so the antigenic structure of the vaccine strain should be representative of field strains. Type I virus still gives problems of antigenic instability after formalin inactivation. For final vaccine production, cell debris are removed by filtration and the virus suspension concentrated by ultrafiltration and standardized using D antigen concentration, or by single radial diffusion techniques. Gel filtration on Sepharose (to remove remaining

serum proteins), followed by ion exchange on DEAE Sephadex is used to remove further impurities. The original inactivation techniques developed by Salk (1955) using formaldehyde at elevated temperatures has proved successful also for purified polio virus suspensions (Van Wezel et al., 1978). It could be added that attenuated polio viruses could be used nowadays for production of inactivated polio vaccine thus avoiding potential problems of virus escaping inactivation and infecting and paralysing recipients.

4.7. Early development of attenuated polio viruses

During 1953–1954 Sabin and co-workers attempted to attenuate highly virulent polio virus (Mahoney, Y-SK and Leon) by cultivation in cynomolgus kidney tissue cultures. However, it should be realised that several other groups were actively working at the same problem and several potential 'candidate' vaccine strains had been selected (Koprowski et al., 1952, Enders et al., 1952, Melnick, 1954). They soon found that mere passage had no attenuating effect but, significantly, use of *high* inocula together with *rapid* passage favoured the overgrowth of virions able to replicate well in non-nervous tissue and the harvests had diminished virulence for monkeys (Table 4.12). This suggested a mixed population originally and therefore terminal cloning was carried out and non-virulent variants of all three polio types obtained (Sabin, 1955). A marked diminution in intramuscular and oral infective capacity for cynomolgus monkeys was noted for these strains (Table 4.13) and these monkeys produced antibodies in response to infection. Sabin had selected a variant of the mouse passaged Mahoney strain (type 1) which was potentially useful

TABLE 4.12.
Intracerebral virulence in cynomolgus monkeys of type 1 poliomyelitis virus (Mahoney strain) propagated in different ways in cynomolgus kidney tissue cultures (after Sabin, 1955)

Kidney passage 10 Serial passages with minimal inocula		Kidney passage 33 Rapid passages with large inocula followed by purification by terminal dilution technique		
No. of TCD_{50} inoculated	No. of monkeys paralyzed	No. of TCD_{50} inoculated	No. of monkeys paralyzed	No. showing CNS lesions
		16 000 000	0/4	0/4
		1 600 000	0/4	0/4
500 000	5/5	160 000	0/4	0/4
50 000	5/5	16 000	0/4	0/4
5000	5/5	1600	0/4	0/4
500	5/5	160	0/4	0/4
50	3/5	16	0/4	0/4
0.5	1/5			

TABLE 4.13.
Intracerebral activity in cynomolgus monkeys of optimum single-plaque strains of each type of polio virus grown in monkey kidney tissue culture (after Sabin, 1955)

Type	Strain	PFU virus per ml	Paralytogenic effect of 1 ml of indicated dilution	
			Undiluted	10^{-1}
1	LSc, 2 ab	4.2×10^7	0/10	0/5
2	P-712, Ch, 2 ab	3.6×10^7	0/10	0/5
3	Leon, 12 a_1b	4.3×10^7	0/10	0/5

as a vaccine strain, and early tentative trials in volunteers confirmed attenuation of these viruses (Table 4.14).

4.8. Early large scale immunization trials with inactivated and attenuated polio vaccines

The WHO Expert Committee on Poliomyelitis in 1957 recommended large scale trials of the newly developed formalin-inactivated vaccine and particularly well controlled trials in the USA (Francis et al., 1957) demonstrated efficacy and safety of the new vaccine. However, Sabin (1955) had by now developed several live attenuated candidate vaccine viruses and since he did not want to interfere with the still progressing trials of Salk vaccine in the USA, he initiated large scale trials in Eur-

TABLE 4.14.
Resistance of alimentary tract to infection with attenuated strains in nonimmune and naturally immune adults as well as in adults who received single feedings of attenuated virus or 2 doses of Salk vaccine intramuscularly (after Sabin, 1958)

Category	Type 1 LSc	Type 2 P-712	Type 3 Leon
No homotypic low-avidity or high-avidity antibody	17/19[a]	15/15	17/17
Naturally acquired low-avidity antibody *only* (pH test) (with few exceptions may be result of heterotypic infection)	15/19	2/3	7/7
Naturally acquired low-avidity *and* high-avidity antibody present	1/11	3/11	6/12
Ingested homotypic attenuated virus 8 to 15 months before test. No homotypic low- or high-avidity antibody before Salk vaccine.	1/10	1/7	1/6
Received 2 doses Salk vaccine – tested 2 weeks to 3½ months after 2nd dose	9/9	6/7	9/9

[a] Number of infections detected.

ope. By this stage the genetic basis of virulence of these new polio strains had been established, at least tentatively, and several phenotypic virological markers which co-segregated with virulence had been observed. Thus, attenuated polio strains often possessed *ts* or *ca* properties. In fact it appeared, in retrospect, that since the early tissue culture passage experiments to 'attenuate' these viruses had been carried out at low temperatures of 35°–36°C this may have also increased the selection pressure for *ts* attenuated virus subpopulations (Sabin, 1958).

Large scale polio immunization was carried out in Europe in collaboration with the large and active virology group in Leningrad and these represent, in retrospect, a classic series of experiments both scientifically and for the benefit of mankind. Initially small groups of children were immunized, but as increasing clinical and laboratory experience showed no adverse effects the campaign was widened. Thus, some of the early clinical trials were carried out with Sabin live vaccine virus in the baltic republics of Latvia, Lithuania and Esthonia (Chumakov et al., 1959). Twenty seven thousand individuals were immunized with vaccine provided by Sabin but a further 10 million people were fed with first passage material grown in the USSR. The first cycle of immunizations was carried out in January–March 1959 (Table 4.15 and 4.16). Almost half the susceptible population in Lithuania received live virus vaccine and convincing proof of the highly immunogenic characteristics of the virus was obtained. Trivalent mixtures were also used effectively with children developing antibody to all three types.

In separate studies, Smorodintsev et al. (1959) also used a cautious approach and between April 1957 and April 1958 observed 150 vaccinated children and contacts before vaccinating a further 822 children in May 1958 and 12 000 children in January 1959. By March 1959 a further 1.8×10^6 children were fed vaccine virus. Table 4.16 shows illnesses in 10 000 immunized children and non-immunized controls in Riga. No clinical polio was detected in the vaccinated group or the internal control group.

Early experiments by Smorodintsev in Leningrad also established that no marked increase in virulence occurred on human–human passage of these attenuated virus-

TABLE 4.15.
Scope of vaccination with Sabin's polio vaccine of the population in Lithuanian SSR, by age groups (up to May 10, 1959) (after Chumakov et al., 1959)

Age group	Population (in thousands)	No. vaccinated (in thousands)	% vaccinated
0–7	390	164	42
7–15	450	256	57
15–19	270	147	54
Total	1110	547	49.7

TABLE 4.16.
Analysis of the reaction-causing properties of live poliomyelitis vaccine according to data concerning vaccinations in schools in Riga (Latvian SSR) (after Smorodintsev et al., 1959)

Recorded diseases	Rate of sickness among the groups mentioned (per 10000 persons for 4 months)		
	Live vaccine Schoolchildren 8311 Children of pre-school age 1346	Internal control Schoolchildren 4188 Children of pre-school age 768	External control Schoolchildren 3300 Children of pre-school age 1062
I. Poliomyelitis and similar diseases	—	—	9.3
	—	—	—
II. Acute infectious diseases	3697.6	3662.3	3506.0
	2526.0	2556.4	2824.8
III. Diseases of the respiratory organs and ear,	474.5	496.0	472.7
nose and throat	1315.0	1119.8	1468.1
IV. Diseases of the digestive organs	21.5	23.4	18.1
	74.3	78.4	75.3
V. Other diseases (pyelitis, nephritis, bronchial asthma and lymph-	10.3	22.3	21.1
adenitis)	29.7	26.0	28.2
VI. Injuries	2.4	2.5	12.1
	—	—	—

es (Smorodintsev et al., 1959) and the vaccines began to be widely used (Table 4.17 and 18). However, in these early studies it soon became apparent that problems of vaccine 'take' could occur in countries with poor hygiene and where many other enteroviruses were present in the gut which could interfere with the replication of the polio vaccine virus (Sabin, 1959, 1963).

4.9. National campaigns to eradicate polio

Several developed countries have now virtually eradicated polio and these include, for example, the USA, USSR, UK, Scandinavia (Böttiger, 1969) (Fig. 4.5) and certain other Western and Eastern European countries. However, polio is still com-

TABLE 4.17.
Distribution of live poliovirus vaccine in the USSR up to December 31, 1960 (after Weissfeiler, 1961)

Republic	Distributed doses of live vaccine	Estimated number of the vaccinated persons
RSFSR	166 590 800	42 604 150
Ukraine	47 752 300	14 152 778
Kazakh	6 438 700	3 385 184
Uzbek	8 030 000	3 872 000
Byelorussia	12 495 600	2 888 000
Georgia	3 199 200	1 320 000
Azerbaijan	2 991 000	1 261 920
Moldavia	1 545 000	1 007 600
Lithuania	2 750 000	1 760 000
Latvia	2 608 000	880 000
Kirghiz	1 902 000	968 000
Tajik	1 852 200	968 000
Armenia	1 702 000	792 000
Turkmenia	1 478 000	792 000
Estonia	1 948 000	827 240
Total	263 282 800	77 478 872

mon in underdeveloped countries and a quandary for eradication here is that live polio vaccine often works erratically and so is less than satisfactory in these countries (Chandra, 1975, Swartz et al., 1972, Hale et al., 1959, Hale et al., 1961). Also inactivated polio vaccine may not produce herd immunity which is assumed to be

TABLE 4.18.
Data on the use outside the USSR of live vaccine prepared in the USSR from Sabin's attenuated strains (after Weissfeiler, 1961)

Country	Amount of vaccine doses	Calculated number of vaccinees
Albania	803 000	450 000
Bulgaria	6 850 000	2 000 000
People's Republic of China	9000	3000
Czechoslovakia	3 500 000 (types 2 and 3)	2 000 000
German Democratic Republic	18 100 000	5 000 000
Hungary	7 700 000	2 400 000
Japan	600	
Korean People's Democratic Republic	750 000	350 000
People's Republic of Viet Nam	4 500 000	950 000
Total	42 712 600	13 153 000

Fig. 4.5a, b and c. Illustrations of early studies with polio vaccine production, control and usage. a, polio vaccine production in roller bottles containing monkey kidney cell; b and c, children in the first 3 school grades took part in the nation-wide vaccine trials in the USA. In some areas, only second-grade children received the vaccine, with first- and third-grade children serving as uninjected controls. In others, half the children in all 3 grades received vaccine and the other half medium No. 199 without virus. (illustrations from Third International Poliomyelitis Conference, 1955).

Fig. 4.5d, e: The scientists, Dr. A. Sabin and Dr. J. Salk, who initially developed live and inactivated polio vaccines.

so important, since in these countries, the faecal-oral route of transmission is most common and inactivated polio may only reduce transmission via the pharyngeal route (Gelfand et al., 1959, Chin et al., 1961). Therefore, further research is clearly needed before a decision is made on the strategy for the control and eradication of poliomyelitis in developing countries. Meanwhile, however, we might learn from the very useful data on the experience of eradication of polio in certain countries like the USA using live vaccine, or in Scandinavia using inactivated vaccine.

From 1951 to 1977 the incidence of polio in the USA dropped steeply from a level of 10 000–20 000 cases a year to 10 cases a year. Similar figures were obtained in the UK and Scandinavia (Fig. 4.6). Naturally occurring poliomyelitis due to indigenous wild poliovirus essentially disappeared from the USA after 1972 (Nathanson, 1982). Nevertheless, 5–10% of children under 16 years of age in the USA are considered to remain susceptible to polio because vaccine coverage is not 100% (Table 4.19). This success in eradication of polio in the USA was achieved mainly by the use of attenuated vaccine (Table 4.20), but the continuing problem with this vaccine is the, albeit small, number of vaccine-associated cases of poliomyelitis (Schonberger et al., 1976) which approximate to 2 paralytic cases per million vaccinees. In the USA with 3.5 million children being immunized yearly 7 paralytic cases are expected, caused by the vaccine virus, of which one third occur in recipients of vaccine and two thirds in contacts, who are mainly adults.

At present it is estimated that 2–5 million children in the USA are still susceptible to polio type I, providing a substantial pool of susceptibles for epidemics to occur.

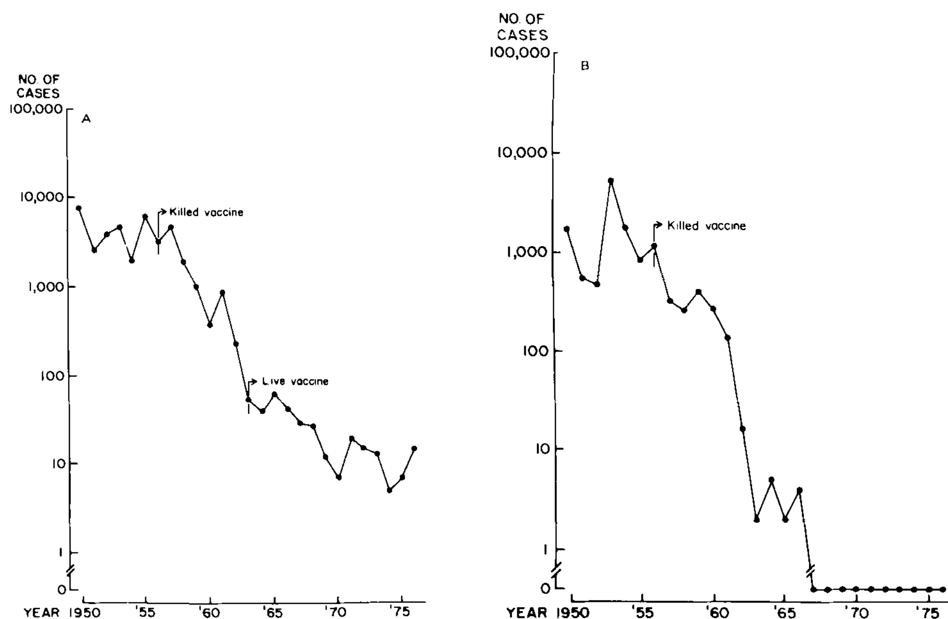

Fig. 4.6. Total reported cases of poliomyelitis – UK (1950–76), Sweden and Finland (1950–1976).

TABLE 4.19.
Poliomyelitis vaccination status by age group in the United States, 1965–1981 (after CDC Poliomyelitis Surveillance, 1982)

Year	Percentage of children who received 3 doses of OPV		
	Age group		
	1–4	5–9	10–14
1965	73.9	89.9	92.1
1966	70.2	88.2	90.0
1967	70.9	88.3	89.7
1968	68.3	84.9	87.8
1969	67.7	83.6	85.7
1970	65.9	82.3	85.3
1971	67.3	81.2	83.9
1972	62.9	78.9	81.8
1973	60.4	71.4	69.3
1974	63.1	73.5	69.8
1975	64.8	76.7	71.5
1976	61.6	71.8	65.2
1977	60.1	69.9	62.8
1978	61.4	74.2	67.7
1979	59.1 (76.0)	71.8 (88.5)	67.2 (84.5)
1980	58.8 (77.7)	71.5 (89.8)	68.7 (87.6)
1981	60.0 (78.3)	73.1 (89.5)	70.2 (87.5)

An example of the problem was the outbreak of polio in an unimmunized pocket of Amish fundamentalist protestants, where 13 cases of polio occurred (Chapter 17). In the post-eradication era in the USA there is a constant risk of introduction of wild type virus. It would seem unnecessary to change vaccine at present in the USA, but inactivated polio vaccines could be used with benefit in immunocompromised individuals (Davis et al., 1977), for adult contacts of children immunized with live vaccine, or for boosting immunity in adults travelling abroad.

An important, and as yet incompletely answered question, is whether inactivated polio vaccine could reduce the spread of virus in the alimentary tract or circulation of virus in the community. Early experience with inactivated polio vaccines in the USA in 1960 would suggest that inactivated polio vaccine *did* inhibit virus circulation (Marine et al., 1962). Thus, studies of polio outbreaks in single towns with high and low socio-economic groups showed clustering in the latter groups and few cases in the former groups. Also polio virus isolations from sewage were infrequent in the upper class areas but were frequent in the lower class areas. In an outbreak in 1960 on Rhode Island polio clustered in two lower class areas in Pawtucket (a high attack rate of 12.8%), whereas other areas remained free from polio. Sewage from

TABLE 4.20.
Doses (millions) of poliomyelitis vaccines distributed by year, United States, 1962–1981 (CDC Poliomyelitis Surveillance, 1982)

Poliomyelitis vaccine	1962	1963	1964	1965	1966	1967	1968	1969	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981
Inactivated (IPV)	15.3	19.0	8.8	7.5	5.5	4.0	2.7	—	—	—	—	—	—	—	—	—	—	0.04	0.03	0.02
Live, oral (OPV)																				
Monovalent (MOPV)																				
Type 1	33.1	38.7	24.9	4.7	1.4	1.3	0.5	0.4	0.3	0.2										
Type 2	37.0	34.2	29.8	3.4	1.3	0.9	0.5	0.4	0.2	0.1										
Type 3	13.7	54.2	28.4	3.7	1.4	1.0	0.6	0.4	0.3	0.2										
Trivalent (OPV)	—	4.2	24.0	17.4	24.0	18.0	23.9	22.5	25.8	25.5	24.7	24.9	25.2	24.8	19.4	23.2	24.6	24.5	23.7	22.8
Total	99.1	150.3	115.9	36.7	33.6	25.2	28.2	23.7	26.6	26.0	24.7	24.9	25.2	24.8	19.4	23.2	24.6	24.5	23.7	22.8

the lower class area contained type I polio. The most likely interpretation is that the better vaccinated affluent neighbourhoods were not subject to the silent ecological spread of virus at a time when there was an intense epidemic in a low class area. More recently there has been some evidence from the Netherlands polio outbreak that even without 100% immunization a barrier is provided to the transmission of polio by inactivated vaccine. Although there was some evidence of infection in unimmunized classmates of cases, widespread transmission of virus did not occur.

As mentioned above, it seems quite possible that inactivated polio vaccine has an inhibitory effect on pharyngeal excretion of polio virus. Gelfand et al. (1959) followed families who had received inactivated polio vaccine. When wild polio was introduced into the household previous immunization of children had no influence on virus spread and faecal excretion of virus was the same in vaccinated and unvaccinated persons. In contrast, spread of polio *was* limited in upper economic households and a possible explanation is that inactivated vaccine reduced excretion of *pharyngeal* virus, whilst the level of hygiene in these homes was high enough to limit spread of virus by the faecal oral routes.

4.10. Future control of polio in developed and underdeveloped countries

Paralytic polio still occurs in all countries of the world but the incidence rates vary from a minute 2.6 per 100 million persons per year in the USA to over 200 cases of persisting paralysis per million persons per year in certain African countries. Recent 'Lameness surveys', a technique pioneered by WHO in the Expanded Programme on Immunization (Chapter 2) have changed previous ideas about poliomyelitis in tropical areas. It is now clear that residual paralytic polio in Burma, Egypt, Thailand, Indonesia, Ivory Coast and Brazil, India, Yemen and Niger is higher now than it was during the prevaccine era in the USA and Sweden. The old dogma that in the tropics the infection rate with polio is high but the paralysis rate is low, and that an increased incidence of the paralytic disease was related to emerging epidemics and an improvement in the standard of living reflected in diminishing infant mortality rates, may no longer hold (Sabin, 1982). Between 50% and 75% of lameness in certain developing countries has been found to be caused by poliomyelitis virus. In the absence of immunization programmes some 4 out of 1000 school age children will be disabled by polio. This estimate does not take into account those who have been afflicted and died, and those who have recovered (Tarantola, 1982). Obviously, on a world scale there is a serious imbalance as regards incidence of polio which requires correction as a high priority. The complete list of reasons for the calamatous differences between countries are not absolutely clear and disagreement exists in the current scientific literature. But, an important reason is the absence of a modern medical and social infrastructure in certain developing countries.

Even although the scientific debate may and will continue about the merits and drawbacks in underdeveloped countries of inactivated versus attenuated polio vaccines it is, nevertheless, clear from the studies of Sabin and others that a vigorous and concerted *rapid* immunization campaign (Sabin, 1982) using oral attenuated polio vaccine will stop polio in tropical and semi-tropical underdeveloped countries. As early as the 1960s Cuba showed the way with annual mass vaccination campaigns on two Sundays of the year for *all* children under five years of age regardless of the number of doses they might have had previously. Only such annual mass vaccinations make it possible to reach the extremely high proportions of children necessary to break the chain of infection. When vaccination is more spread out in time, wild polio and other enteroviruses remain dominant in the community. As another example, Brazil in 1980 and 1981, organized an army of volunteers to vaccinate almost all children (18 million) under 5 years of age twice a year on a single day (June and August in 1980 and August and October in 1981). A precipitous drop in reported cases followed (Sabin, 1982). An alternative option is to use more doses of vaccine. John (1976) found that multiple doses of conventional oral vaccine containing 10^5 and $10^{5.5}$ $TCID_{50}$ for types 1, 2 and 3 (five or more doses) were required to give more than 90% seroconversion, but such an approach requires good organization and mass campaigns.

Fang-chou et al. (1982) have described how polio has been controlled recently in sub-tropical areas in China using oral attenuated vaccine. In Guangxi autonomous region, with a population of 20 million persons, apparently healthy children were found to be heavily infected with non-polio enteroviruses. Even as late as 1971 several polio epidemics were detected since the polio vaccination rates were still low, especially in rural areas. An intensive campaign was initiated in 1973 using 'barefoot doctors' and special immunization teams in rural areas and the incidence of polio has since declined from 0.41 (1973) to 0.05 (1977) and 0.02 (1979) per 100 000 persons. Fang-chou et al. (1982) conclude "the key to elimination of paralytic poliomyelitis from a community is to have a powerful leadership and authority in public health that can provide effective administrational guidance, and measures to train and organize health personnel."

The alternative approach is to use killed polio vaccine. In an early study Beale (1969) showed that vaccine given at 3, 4 and 5 months with a booster at one year gave a high seroconversion rate. The polio was mixed with diphtheria, tetanus and pertussis vaccine, thus protecting against 4 diseases in one immunization course. Immunization in Mali or Indonesia with 40 D units of currently used polio vaccine can result in essentially 100% seroconversion and it is probable that a single such dose at 6 months will provide immunological memory; subsequent contact with natural virus would boost immunity (reviewed by Beale, 1982 and see also Table 4.21). Krishnan et al. (1983) evaluated the immunogenic efficacy of inactivated polio vaccine in India in infants given a quadruple vaccine incorporating polio with diptheria-pertussis-tetanus. A total of 150 babies aged 6–45 weeks were immunized with

three doses of vaccine containing 20, 2 and 3.5 D antigen units of polio types 1, 2 and 3 respectively at intervals of 4–8 weeks and the overall sero-conversion rates to poliovirus types 1, 2 and 3 were 99%, 89% and 91% respectively. An otherwise adverse effect of maternal antibody on the immune response is reduced by increasing the intervals between doses from 4 to 8 weeks.

4.11. Characterization of polio viruses isolated in the USA between 1969–1981: vaccine associated cases

A total of 203 cases of paralytic polio (most with pure spinal paralysis and 39 cases of spinal involvement although in 93% of cases one or both lower limbs were paralysed) were reported in the USA during this period and this represents a dramatic decrease from the 10 000 cases or so reported each year in the 1950s. Therefore the attack rate of paralytic polio has declined from a high of 13.7 cases per 100 000 population (1952) to 0.003 cases per 100 000 population in 1981. Of these 203 cases, 40 were associated with epidemics, 127 were endemic, 22 were imported and 14 were in immune deficient persons. Of particular interest is the group of 86 vaccine-associated cases which were examined: 26 had occurred in recipients of oral polio vaccine, while 45 occurred in household contacts of vaccinees and 15 in non-household contacts. Perhaps, as to be expected, there was no seasonal distribution of cases and most (99 cases) were under 5 years of age. Compared to the 1960s more adult cases were being observed. Analysis of polio virus isolates by oligonucleotide mapping showed that most isolates from vaccine cases were vaccine-like in oligonucleotide spot pattern (see Chapter 17 for more details).

Of the 167 polio cases analyzed carefully, 77 involved polio type 1, 45 type 2 and 45 type 3. Type I was responsible for all 38 epidemic associated cases but was responsible for only 4 vaccine associated cases, which were more often associated with

TABLE 4.21.
Antibody response to inactivated polio vaccine (IPV) in India (after Jacob John, 1982)

Maternal antibody	Interval between doses	Number of infants showing seroconversion to poliovirus		
		Type 1	Type 2	Type 3
Absent	4 weeks	44/44 (100)[a]	47/52 (90)	57/63 (90)
Absent	8 weeks	29/29 (100)	26/26 (100)	41/43 (95)
Present	4 weeks	25/25 (100)	7/17 (41)	4/6 (67)
Present	8 weeks	21/21 (100)	23/24 (96)	6/7 (86)
Both groups	4 weeks	69/69 (100)	54/69 (78)	61/69 (88)
	8 weeks	50/50 (100)	49/50 (98)	47/50 (94)
Both groups	4 and 8 wks	119/119 (100)	103/119 (86)	108/119 (91)

[a] No. responding/no. tested, with seroconversion rate as percent within brackets.

type 3 or type 2 viruses. A more recent analysis of the epidemiological characteristics of paralytic polio cases in the USA is illustrated in Table 4.22.

We have left to last in this chapter any indication of potential new developments in polio vaccines, either as regards live or inactivated vaccine. It has taken many years to establish the safety of existing polio vaccines and therefore it is unlikely that any new technical developments will be introduced quickly, unless (and this is unlikely) they represent a striking scientific breakthrough in, for example, immunogenicity (i.e. producing cross-protection against a variety of enteroviruses) or a marked reduction in cost (it should be remembered that live polio vaccines are very cheap). A more extensive discussion, with new data, is presented in Chapter 2 and we shall content ourselves here with a brief review of typical recent data with polio immunogenic peptides since this is an area of intense investigation at present.

TABLE 4.22.
Paralytic poliomyelitis cases by epidemiological characteristics, United States, 1980–1981 (CDC Poliomyelitis Surveillance, 1982)

Year	Epidemiologic classification	State	Age	Sex	Month of onset	Prior polio vaccination	Poliovirus type implicated[c]
1980	Endemic, not VA[a] (IIA)	CA	11 months	M	June	None	2
	Recipient, VA (IIB)	NJ	3 months	M	February	None[b]	3
	Contact, VA (IIC1)	LA	6 months	F	January	1 TOPV	1, 2, 3
	Contact, VA (IIC1)	WA	25	F	May	1 TOPV, 3 IPV	?
	Contact, VA (IIC2)	MI	28	M	October	None	2
	Imported (III)	OR	67	F	February	None	3
	Imported (III)	CA	4 months	F	January	None	1
	Immune deficient (IV)	WY	2 months	M	March	1 TOPV	2
	Immune deficient (IV)	NJ	1	F	December	4 TOPV	2
1981	Recipient, VA (IIB)	CA	3 months	F	October	None[b]	?
	Contact, VA (IIC1)	NB	41	M	August	None	2
	Contact, VA (IIC1)	MN	29	M	August	None	2
	Contact, VA (IIC1)	WV	8 months	F	April	None	2
	Contact, VA (IIC2)	MD	14	F	January	1 TOPV	2
	Contact, VA (IIC2)	WA	27	M	June	None	2
	Immune deficient (IV)	MO	17	M	July	3 TOPV, 4 IPV	1

[a] VA = vaccine associated
[b] Excludes OPV administered within 30 days prior to onset of illness
[c] By virus isolation and/or fourfold rise in antibody titer

4.12. Immunogenic synthetic peptides corresponding to antigenic areas of polio VP1 protein

Recently Emini et al. (1983) have synthesized five peptides containing amino acid

sequences from type 1 poliovirus structural protein VP1. Each of the peptides was able to prime the immune system of rabbits, which on subsequent challenge with a single inoculation of whole polio virus produced a long lasting virus neutralizing IgG antibody response. The essential data is summarized in Table 4.23 and 4.24. The sequences were chosen after a hydrophilicity analysis of protein sequence and a sequence variation analysis obtained by comparing the amino acid sequence of VP1 of the three polio serotypes. It was assumed that hydrophilic regions of VP1 were more likely to be exposed at the virus surface. Four of the five peptides were recognized by neutralizing monoclonal antibodies raised to the intact type I poliovirus. A single peptide (No. 3) elicited virus neutralizing antibodies when coupled to a carrier protein. Finally, the peptides were able to prime animals who subsequently reacted rapidly when immunized with whole polio virus. The priming was specific in the sense that priming only occurred for the homologous type I virus. It is just possible that priming alone might give sufficient protection against poliovirus infection. A major study of poliovirus antigenic sites has been reported by Minor et al. (1983).

TABLE 4.23.
Priming of anti-poliovirus immune response by peptides 2, 3, 4 and 5 (after Emini et al., 1983)

Peptide	Rabbit	Inoculated poliovirus type	Weeks after virus inoculation	Plaque titre reduction ($\log_{10}$ PFU ml^{-1})			Antiserum		
				Type 1	Type 2	Type 3	Type 1	Type 2	Type 3
2	A	1	1	>8.5	3.6	3.3	+	–	–
	B	1, 2, 3	1	5.5	3.2	1.4	+	+	–
			5	6.3	2.7	<1.0	+	+	–
3	B	1, 2, 3	1	7.8	1.9	<1.0	+	–	–
			5	8.0	<1.0	<1.0	+	–	–
4	A	1	1	6.7	<1.0	<1.0	+	–	–
			5	6.2	<1.0	<1.0	+	–	–
	B	1, 2, 3	1	5.8	1.3	<1.0	+	–	–
			5	6.4	1.1	<1.0	+	–	–
5	A	1	1	>8.5	1.7	<1.0	+	–	–
			5	>8.5	<1.0	<1.0	+	–	–
	B	1, 2, 3	1	6.7	1.8	<1.0	+	–	–
			5	6.5	<1.0	<1.0	+	–	–

All rabbits were inoculated with 1.0 mg per inoculation of carrier protein-linked peptide (diluted 1:1 with CFA) by the i.d., s.c. and i.m. routes at 0, 4 and 5 weeks, respectively. Approximately 5.0 $\log_{10}$ PFU of the appropriate poliovirus type(s) (1:1 with CFA) was inoculated, i.m., 10 days following the final peptide inoculation.

TABLE 4.24.
Sequences of immunologic peptides of Polio type I (after Emini et al., 1983)

Peptide	Sequence
Peptide 1	H_2N-TYR-GLY-GLY-ARG-SER-ARG-SER-GLU-SER-GLY-COOH
Peptide 4	H_2N-CYS-GLY-GLY-ARG-SER-ARG-SER-GLU-SER-SER-ILE-GLU-SER-PHE-COOH
Peptide 2	H_2N-TYR-GLY-GLY-SER-THR-THR-ASN-LYS-ASP-LYS-GLY-COOH
Peptide 3	H_2N-TYR-GLY-GLY-ASP-ASN-PRO-ALA-SER-THR-THR-ASN-LYS-ASP-LYS-COOH
Peptide 5	H_2N-TYR-GLY-GLY-ASP-ASN-THR-VAL-ARG-GLU-THR-GLY-COOH

4.13. Specific inhibitors of polio

Since the vaccine has been so successful in preventing the disease of polio, rather little new work has been initiated in recent years to study specific inhibitors of polio virus replication, although important studies were carried out earlier with guanidine and hydroxybenzimidazole compounds (see below). In contrast, inhibitors against rhinoviruses have been sought extensively, although without success until recently (reviewed by Tyrrell et al., 1983, Reed, 1980). Little work has been carried out with rhinovirus vaccines, mainly because of the awesome problems of multiplicity of serotypes.

4.13.1. Benzimidazoles

Early work by Hollinshead and Smith (1958) described the inhibitory effects of 2 (α - hydroxybenzyl)benzimidazole (HBB) (Fig. 4.7) on polio type 2 replication in mice: death of the animals was prevented. Early tissue culture experiments established that the compound exerted an antiviral effect in vitro, was not simply virucidal and had no toxic effect on uninfected tissue culture cells (Tamm and Nemes, 1959). This compound was one of the first antiviral molecules to be discovered. However, HBB showed some unwanted characteristics which were to become familiar to specialists working with later antivirals – drug resistant virus mutants could be isolated with relative ease, and the compound had a very narrow antiviral spectrum more or less limited to enteroviruses (Table 4.25).

HBB acts during the first 2–5 hours after polio virus infection of cells, although the precise point of action is not known. Virus adsorption, penetration and uncoating, however, are not affected and virus RNA and protein synthesis are not inhibited. Virus macromolecular synthesis is thought to be the target site for inhibition, and cessation of virus RNA synthesis eventually stops viral protein synthesis. An interpretation of the rather conflicting data is that HBB inhibits the synthesis of a virus directed RNA polymerase, which in turn halts replication of virus RNA leading to inhibition of synthesis of viral capsid polypeptides. Alternatively, other experiments suggest that HBB inhibits viral RNA synthesis, thereby inhibiting replication of infectious virus.

HBB significantly delays the development of enterovirus CPE in MK cells, although the cells ultimately succumb to virus induced CPE, presumably because of

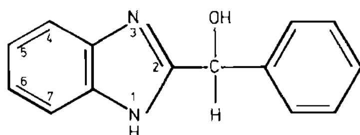

Fig. 4.7. Molecular structure of 2-(α-hydroxy-benzyl)benzimidazole.

TABLE 4.25.
Antiviral spectrum of HBB

Viruses inhibited in vitro	Viruses not inhibited in vitro
Polio 1–3	Echovirus 22, 23
Coxsackie B 1–6	
Coxsackie A 9	Other Coxsackie viruses
Echovirus 9, 11–21, 24–27	Rhinoviruses
Porcine and bovine enteroviruses	FMDV
Arenavirus (e.g. LCM)	

virus induced cell protein and RNA shut off. Production of infectious virus is very significantly inhibited by HBB in vitro with $>8.0 \log_{10} LD_{50}$/ml inhibition of certain enteroviruses. However, as with many antivirals much less antiviral effect is noted in vivo.

A large number of derivatives of HBB have been investigated for virus inhibitory effects (Tamm et al., 1969) and it has been found that the hydroxybenzyl group at position 2 of the benzimidazole ring is of critical importance. Also, replacement of the benzimidazole nucleus of HBB by an imidazole ring results in complete loss of antiviral activity. Certain substituted derivatives such as 1-propyl, 1-β-methylpropyl and 1-phenyl-HBB are more active and selective in vitro than HBB itself. Thus, the selective virus inhibitory activity of HBB is dependent upon the overall geometry of the molecule and most structural modifications lead to a loss of viral inhibitory activity and selectivity.

4.13.2. Guanidine

As early as 1961 Rightsel et al. published details of the inhibition of polio virus by guanidine and soon afterwards the by now familiar drug resistant or even drug dependent mutants were detected (Loddo et al., 1963).

Many enteroviruses are inhibited in vitro by guanidine (see Table 4.26 and Fig. 4.8) but nevertheless the antiviral spectrum of the compound is severely restricted to RNA viruses.

Guanidine has no direct virucidal effect and enterovirus attachment and penetration are not inhibited. (Crowther and Melnick, 1961). Inhibiting effects begin midway through the virus latent phase and continue through the reproduction cycle. The principal site of guanidine action appears to be the synthesis of viral RNA and more than one step may be affected (reviewed by Tershak et al., 1982, Caliguiri and Tamm, 1968). Polio RNA polymerase is not inhibited in vitro, presumably because initiation of viral RNA synthesis does not occur in cell free extracts but only results in completion of RNA chains in the process of synthesis during extraction of the

TABLE 4.26.
Antiviral spectrum of guanidine (after Tershak et al., 1982)

Viruses inhibited in vitro	Viruses not inhibited in vitro
Polio	Orthomyxoviruses
Coxsackieviruses	Coxsackie B6
Echoviruses	Echo 6, 7, 8, 12
Rhinoviruses	Paramyxoviruses
FMDV	Reoviruses
Togavirus (Sindbis and SVF)	Togaviruses
Tobacco necrosis	Herpes
Tobacco mosaic	Vaccinia
	Adenovirus

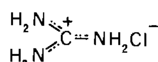

Fig. 4.8. Molecular structure of guanidine.

crude enzyme (although more recently pure preparations of picornavirus polymerase that utilize exogenous viral RNA have been obtained and could now be tested). Inititation of synthesis of viral RNA seems to be depressed by guanidine, and release of completed RNA chains is restricted. Current data would point to the initiation step of viral RNA synthesis as the site of action of guanidine (Tershak et al., 1982). In addition, guanidine exerts mild conformational changes in viral proteins and specifity could be due to a specific amino acid sequence in picornaviruses compared to cell proteins or proteins of other viruses. It is possible that synthesis and encapsidation of viral RNA both require capsid subunits and Vpg_1 and any interaction with guanidine would cause multiple aberrations.

Guanidine, in combination with HBB has been used to successfully treat echovirus type 9 and coxsackie A9 infections in newborn mice, although neither compound alone was effective (Eggers, 1982). Theoretical levels of inhibitor were 200 μM HBB and 2mM guanidine.

4.13.3. Arildone

Several acyclic β-diketones were shown to inhibit the replication of equine picornaviruses (Diana et al., 1977). Arildone, 4-[(2-chloro-4-methoxy)phenoxy]hexyl-3,5-heptadione, emerged as a promising candidate antiviral, having broad spectrum in vitro antiviral activity against equine rhinovirus and interestingly HSV (Table 4.27, 28). The compound arildone is composed of a β-diketone separated from the substituted benzene ring by an alkyl chain of 6 carbons. It is stable to heat but virtually

insoluble in water, which is a drawback. Substituents of the benzene ring contributed lipophilicity and substitution and addition of more hydrophobic substituents decreased the antiviral activity of the compound. Shorter and longer alkyl chains decreased antiviral activity (reviewed by McSharry and Pancic, 1982).

The compound is thought to inhibit polio replication by preventing intra-cellular uncoating of the virion (McSharry et al., 1979) perhaps by stabilizing capsid proteins so that they cannot undergo conformational changes required for uncoating and release of virion RNA. The isolation of drug resistant mutants of polio also suggests a direct antiviral effect on the virus. Certainly arildone appears to stabilize polio in the presence of heat and alkali (Table 4.29).

Arildone was assessed for its ability to prevent paralysis and death in mice infected intracerebrally with a lethal dose of human poliovirus type 2 (McKinlay et al., 1982). Intraperitoneal administration of arildone suspended in gum tragacanth prevented paralysis and death in a dose-dependent manner (minimal inhibitory dose = 32 mg/kg, twice daily) and protected animals from virus challenges in excess of twenty 50% lethal doses (Table 4.30). Oral medication with arildone solubilized in corn oil was similarly effective in preventing poliovirus-induced paralysis and death. Arildone was therapeutically effective even when intraperitoneal medication

TABLE 4.27.
Effect of arildone on virus infectivity

Virus	MIC (μM)[a]	
	CPE	Plaque reduction
Poliovirus 2	0.8	$<$ 0.27
Murine cytomegalovirus		10.8
Herpes simplex virus 1		
Sheely strain	16.2	$<$ 1.35
Robinson strain		$<$ 5.4
McKrae strain		$<$ 5.4
Herpes simplex virus 2		
Curtis strain	16.2	$<$ 5.4
75–1000 strain		$<$ 5.4
Varicella zoster virus		$<$ 2.7
Corona virus A 59		$>$27
Vesicular stomatitis virus		
Indiana serotype	1.9	$>$27
Influenza A_0/WSN/(H_0N_1) virus		$>$27
Vaccinia virus	8.1	$>$13.5
Adeno virus		$>$27
Sindbis virus		$>$27

[a] Minimal inhibitory concentration expressed as μM arildone required to reduce CPE or plaque formation by 50%

TABLE 4.28.
Effect of arildone on virus replication

Virus	MIC (μM)
Murine cytomegalovirus	<8.1
Semliki forest virus	<8.1
Vesicular stomatitis virus	2.7
Poliovirus 2	<2.7
Herpes simplex virus 1	2.7
Herpes simplex virus 2	<2.7
Coxsackievirus A 9	<5.4

was delayed for 48 h postinfection. Analysis of the virus titres in the central nervous system tissues of animals infected with 200 50% lethal doses demonstrated that arildone reduced titres in the brain and spine by approximately 3 and 4 $\log_{10}$ PFU per g of tissue, respectively, implying that direct inhibition of virus replication was responsible for host survival. However, this animal model depends on direct inoculation of polio into the CNS and therefore the relevance of the study to human polio must await further study. It should be noted that the acute oral LD_{50} of arildone in mice is in excess of 8 g/kg and monkeys and rats medicated daily for 5 weeks with 1000 mg/kg showed no toxic reactions.

In summary, these compounds excite little interest with clinicians because of the success of polio vaccines and relative lack of interest in other enteroviruses. Their main use at present appears to be in laboratory studies of enterovirus replication, including genetic markers.

TABLE 4.29.
Effect of arildone on thermal inactivation of poliovirus

Time (min)[a]	Poliovirus titre (PFU/ml)		
	Eagle's MEM	Eagle's MEM +0.01% DMSO	Eagle's MEM +2.7 μM arildone +0.01% DMSO
0	3.4×10^9	4.3×10^9	4.0×10^9
2.5	2.5×10^9	2.3×10^9	4.5×10^9
5	2.2×10^8	2.4×10^8	2.3×10^9
10	2.3×10^7	6.1×10^7	2.5×10^9
20	1.3×10^6	3.4×10^6	2.2×10^9

[a] Time of incubation of poliovirus 2 at 47°C.

TABLE 4.30.
In vivo activity of arildone (after McKinlay et al., 1982)

Expt. no. (size of inoculum)	Arildone treatment regimen			No. of expts × no. of mice challenged per expt	% Survivors[a]		
	Route of administration/ vehicle	Treatment schedule	Individual dose (mg/kg)		Day after challenge		
					5	10	20
1 (2 LD_{50})	i.p./GT[b]	Doses delivered 4 h before challenge, 2 h postchallenge, and twice daily for 13 days thereafter	0	5×10	98± 2.0	46± 5.1	26± 5.1
			32	5×10	100± 0	88± 3.8	42± 2.5
			63	5×10	95± 2.0	90± 3.2	78± 3.8
			125	5×10	100± 0	100± 0	94± 2.4
			250	5×10	100± 0	100± 0	100± 0
2 (20 LD_{50})	i.p./GT	Doses delivered 4 h before challenge, 2 h postchallenge, and twice daily for 13 days thereafter	0	3×10	97± 3.3	23±12.0	3± 3.3
			250	3×10	100± 0	100± 0	100± 0
3 (2 LD_{50})	i.p./GT	Doses delivered 4 h before challenge, 2 h postchallenge, and twice daily for 5 days thereafter	0	3×10	100± 0	43± 3.3	20± 1.0
			250	3×10	100± 0	100± 0	93± 6.6
4 (2 LD_{50})	i.p./GT	Doses delivered 48 h postchallenge and twice daily for 14 days thereafter	0	3×10	97± 3.3	50± 5.8	30±10.0
			250	3×10	100± 0	97± 3.3	87± 3.3
5 (2 LD_{50})	Oral/GT	Doses delivered 4 h before challenge, 2 h postchallenge, and twice daily for 13 days thereafter	0	4×10	92± 4.8	52± 4.8	25± 9.5
			250	4×10	100± 0	87± 6.3	59± 6.3
6 (2 LD_{50})	Oral/corn oil	Doses delivered 4 h before challenge, 2 h postchallenge, and twice daily for 13 days thereafter	0	3×10	90±10.0	40± 5.8	30±10.0
			250	3×10	100± 0	100± 0	93± 3.3

[a] Geometric mean ± standard error of the mean.
[b] Arildone suspended in 1% gum tragacanth plus 2% Tween 80.

4.14. Rhinoviruses

Minor respiratory infection (common cold) is one of the commonest viral diseases in the UK and other temperate and even sub-tropical countries. In addition, they cause exacerbation of chronic conditions such as asthma, chronic bronchitis or cardiac failure (reviewed by Andrewes, 1962, Hope-Simpson, 1958). We have mentioned above that, at present, few cross-reacting antigenic determinants have been detected among this group of 150 viruses and hence development of effective vaccines would be difficult. Since the viruses are all closely related as regards physico-chemical properties and presumably biochemistry receptor sites etc., it would appear more logical to place an emphasis on development of antiviral drugs or interferon. Most pharmaceutical groups searching for new antivirals include typical rhinoviruses in their antiviral screening programme and most of the latter work is done in tissue culture systems because of the absence of an animal model of human rhinoviruses. Suitable compounds are tested in volunteers for pharmacological properties and any nontoxic compounds are then tested at, for example, the Common Cold Research Unit at Harvard Hospital, Salisbury, or in comparable units in Leningrad and Houston. Volunteers in the UK unit are aged from 18–50 years and on arrival at the unit are given a medical examination and a preliminary serum sample is taken and subsequently used to estimate the level of any anti-virus antibody (Beare and Reed, 1977). During the experimental period volunteers are isolated in pairs in their own self-contained apartments and no physical contact is allowed with other persons (Fig. 4.9). Subjective symptoms and objective criteria are used to grade reactions induced by experimental viruses ranging from very mild (trivial reactions), mild (local symptoms), moderate (both local and constitutional symptoms) and severe (febrile and other unpleasant reactions necessitating bed rest). The number of paper handkerchiefs used each day is counted and may often increase from a baseline of 3 per day to 35 per day during the symptoms of a cold. After 3 days quarantine, antiviral medication is started and the volunteers are then infected with rhinovirus intranasally (Fig. 4.10). Nasal washings are taken for several days thereafter for virus reisolation studies. Drugs may be administered locally by spray or orally. In the latter case there must be evidence that enough compound reaches the secretions of the upper respiratory tract. On the other hand, mucociliary clearance mechanisms are efficient and may rapidly reduce the local concentration of a nasally administered drug.

Reed (1980) has summarized earlier trials in volunteers with both specific antivirals and interferon (Tables 4.31 and 4.32) (see also Chapter 3). In general, guanidine-type molecules have given disappointing results in volunteers but more recently a benzimidazole has been shown to have mild antiviral effects (enviroxime or 2-amino-1-(isopropyl sulphonyl)-6-benzimidazole phenyl ketone oxime (Table 4.33, Fig. 4.11). The compound completely suppressed plaque formation in vitro by rhinoviruses types 3, 9, 14 and 31 (Table 4.33). Moreover, enviroxime inhibited a fur-

Fig. 4.9. Accommodation at the Salisbury Common Cold Unit (after Beare and Reed, 1977).

ther 12 rhinovirus serotypes by at least 3.0 $\log_{10}$ units at 0.5 μg/ml. Enviroxime was equally effective in human embryo fibroblasts and HeLa cells and also in human embryo nasal trachea organ culture at concentrations of 0.2 μg/ml. In clinical trials (Table 4.34) both intranasal and oral administration of the compound was used, and the severity of colds was reduced (Fig. 4.12) although at the borderline of statistical significance. We should remember also that volunteers were given a minimum virus challenge in the presence of a maximum (near toxic) level of drug. Even under these conditions the antiviral effect was not striking, but it nevertheless represents the first synthesized antiviral coumpound to have detectable anti-rhinovirus effects in man.

However, trials of enviroxime have also been carried out by Hayden and Gwaltney (1982), who were unable to detect any antiviral effect (Table 4.35). Intranasal administration of enviroxime by aerosol spray was associated with drug levels in nasal secretions that 1 h later averaged 750-fold higher than those inhibitory for rhinoviruses in vitro (0.2 μg/ml). However, administration of intranasal enviroxime (one spray per nostril, five times per day) to susceptible volunteers, beginning 1 day before and continuing for 4 days after virus exposure, did not significantly reduce infection or illness due to experimentally induced rhinovirus type 39 infection. The combined results of two separate trials yielded an infection rate of 100% for 21 pla-

Fig. 4.10. Method of administration of virus inoculum in the form of nasal drops (after Beare and Reed, 1977).

cebo-treated and 89% for 19 enviroxime-treated subjects. Approximately one-half of the volunteers in each group had seroconversion to the challenge virus. Overall, 52% of the placebo-treated and 53% of the enviroxime-treated subjects developed colds. No significant differences in symptom scores, nasal mucus weights, or numbers of nasal tissues used were observed between the two groups. Two-thirds of the enviroxime-treated volunteers noted intranasal irritation immediately after sprays, as compared with only one-third of the placebo-treated subjects.

Another recently investigated compound, dichloroflavan, (Fig. 4.13) was shown to have no detectable anti-rhinovirus effects in volunteers (Fig. 4.14, Table 4.36) although the compound has anti-rhinovirus activity in vitro (Table 4.37), but this could be attributed to pharmacology problems with perhaps only low concentrations reaching the nasal mucosa.

4.15. Some recently discovered inhibitors of picornaviruses

A nitrobenzene derivative, MDL-860, was found to inhibit plaque formation, cyto-

TABLE 4.31.
Broad spectrum antiviral agents tested in humans experimentally infected with rhinoviruses

Preparation	Dosage	Virus	Effect[a] of medication on:		
			Symptoms	Virus shedding	Antibody response
Human leukocyte interferon	1.4×10^7 U spread over 4 days 9–12 doses/day[b]	Rhinovirus type 4	+	+	±
Human fibroblast interferon	Between 6×10^5 and 4×10^7 U, spread over 4 days 3 doses/day[b]	Rhinovirus type 4	–	–	–
Polyriboinosinic polyribocytidylic acid	7.0 mg/day for 1 day, 3.5 mg/day for 6 days, given as 4 or 7 doses/day[b]	Rhinovirus type 13	+	+	–
Polynucleotide from fungal virus (BRL 5907)	5.0 mg/day for 3 days 5 doses/day[b]	Rhinovirus type 4	±	±	–
Synthetic low M.Wt. inducers CP 20 961 and CP 20 888	In most studies, 5 doses of 50 mg, 3 before virus challenge and 2 after it[b]	Rhinovirus types 13, 14 and 21	(Significant effects on symptoms and virus shedding in some but not all studies.)		
Isoprinosine	4–6 g/day[c]	9, 31, 44, 32 and 21	(Significant effects, mainly on symptoms, in some but not all studies.)		

[a] + indicates a definitely favourable effect, usually statistically significant; ± indicates minor beneficial effects, not statistically significant
[b] Intranasal medication
[c] Oral medication

pathic effect, or both in 11 of 12 picornaviruses (Table 4.38) at concentrations which did not affect cell growth (Torney et al., 1982). The compound did not directly inactivate the virus and inhibited actinomycin D-resistant ^{3}H-uridine uptake in cells infected with coxsackie virus A_{21} or rhinovirus 1-A, whereas incorporation into uninfected cells was not inhibited. With three picornaviruses (echovirus type 12, poliovirus type 2, and rhinovirus type 1-A) made photosensitive with neutral red, MDL-860 did not appear to cause a significant reduction in their loss of photosensitivity (uncoating) during the first 3 h of infection. The authors concluded that MDL-860 appeared to inhibit some early event in virus replication, after uncoating, which is required for synthesis of viral RNA.

In multiple growth cycle experiments, 1 μg of MDL-860 per ml caused a reduction in virus yield of at least 1.0 $\log_{10}$ 50% tissue culture infectious doses per 0.2

TABLE 4.32.
Some synthetic antiviral compounds tested against rhinovirus infection in vivo. Experiments were done in humans except where indicated (after Reed, 1980)

Preparation	Route	Effect[a] of medication on:			
		Rhinovirus	Symptoms	Virus shedding	Antibody response
SKF 21687 (triazinoindole)	Intranasal	44	–	–	–
SKF 40491[b] (triazinoindole)	Intranasal	2	ND	+	+
As above[b]	Oral	1A	ND	–	–
As above	Intranasal	3	–	±	–
19326 RP (imidazo-thiazole)	Intranasal	9	±	+	–
Abbott 36683[c] (bis-benzimidazole)	Oral	30, 49 or 44	Side effect at high dose	+ at high dose only	+ at high dose
ICI 73602 (ureidoguanidine)	Intranasal	3	–	–	–
CL 88, 277 (substituted guanidine) (-phenyl-3-(4-phenyl-2-thiazolyl)guanidine	Intranasal	44	–	±	±

[a] +indicates a definitely favourable effect, usually statistically significant, ± indicates minor beneficial effects, not statistically significant
[b] In gibbons
[c] In chimpanzees

TABLE 4.33.
Effect of incorporation of antiviral compounds in the overlay medium of HeLa cells on the titre of four preparations of rhinovirus (after Reed, 1980)

Compound	Concentration (μg/ml)	Reduction in titre ($\log_{10}$ PFU) using rhinovirus serotypes			
		3	9	14	31
SKF 40491	2.0	2.0	1.4	2.0	3.0
RP 19326	2.0	0.7	2.0	1.0	1.0
CP-196J	0.5	>4.7	>3.3	>4.0	>4.4
Enviroxime	0.6	>5.3	>6.6	>4.5	>5.9

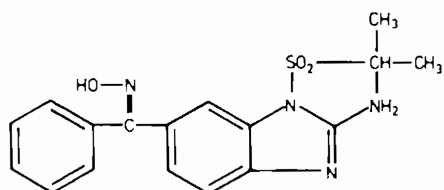

Fig. 4.11. Molecular structure of enviroxime.

TABLE 4.34.
Prophylactic effect of enviroxime versus rhinovirus type 9 (after Phillpotts et al., 1981)

Treatment	Clinical colds				Virus shedding
	Total	Severe or moderate	Mild	None or insignificant	
Enviroxime	18	0	4	14	10
Placebo	18	3	3	12	15

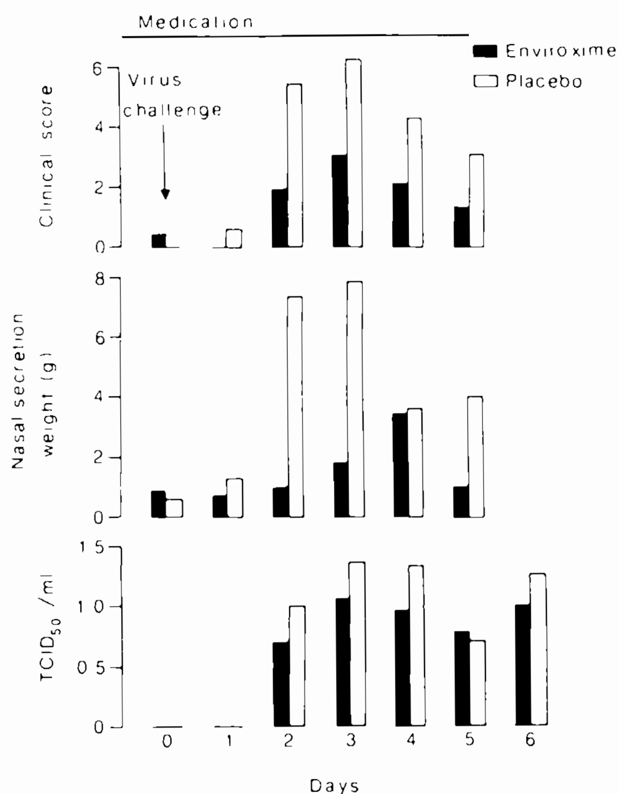

Fig. 4.12. Clinical antiviral effects of enviroxime. (after Tyrell et al., 1983)

TABLE 4.35.
Frequency and severity of illness in enviroxime- and placebo-treated volunteers with experimental rhinovirus type 39 infection (after Hayden and Gwattney, 1982)

Trial	Treatment	No. of colds/ no. of volunteers (%)	Total symptom score	Wt of nasal secretions (g)	No. of tissues used
First	Placebo	3/7 (43)	6.0±2.4	11.9±6.3	32±12
	Enviroxime	4/7 (57)	6.1±2.1	12.9±4.2	39±11
Second	Placebo	8/14 (57)	8.1±1.4	21.2±5.4	42± 9
	Enviroxime	6/12 (50)	10.8±3.1	17.1±4.5	37± 9
Combined	Placebo	11/21 (52)	7.4±1.3	18.1±4.2	39± 7
	Enviroxime	10/19 (53)	9.3±2.5	15.5±3.2	38± 7

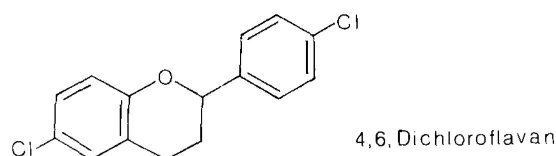

Fig. 4.13. Molecular structure of 4,6 dichloroflavan.

TABLE 4.36.
Results of Rhinovirus type 9 challenge – dichloroflavan trial (after Tyrrell et al., 1983)

Treatment	Clinical colds				Virus shedding
	Total	Severe or moderate	Mild	None or insignificant	
Dichloroflavan	26	5	4	17	22
Placebo	23	5	5	13	17

ml for 8 of 10 enteroviruses and 72 of 90 rhinovirus serotypes (Table 4.39). This antiviral activity was dependent on both compound concentration and virus inoculum size. At concentrations that had no toxic effects on cell cultures, MDL-860 did not inhibit cytopathic effect or hemadsorption activity due to coronavirus 229-E, vesicular stomatitis virus, herpes simplex virus type 1, adenovirus, influenza virus A, or parainfluenza virus 1. Compound concentrations up to 25 μg/ml did not cause cytopathic effect in short-term incubated cultures of rhesus monkey, WI-38, or HeLa cells and 10 μg/ml did not inhibit the replication of HeLa cells (Powers et al., 1982).

TABLE 4.37.
Sensitivity of rhinoviruses to dichloroflavan (after Bauer et al., 1981)

Serotype	IC_{50} (μM)	Serotype	IC_{50} (μM)
1A	0.013	13	0.66
1B	0.007	14	Inactive
2	0.04	15	0.17
3	10.10	16	0.02
4	Inactive	18	0.29
5	Inactive	19	0.81
8	8.00	21	1.70
9	0.011	29	0.008
12	0.15	30	52.00
		31	0.013

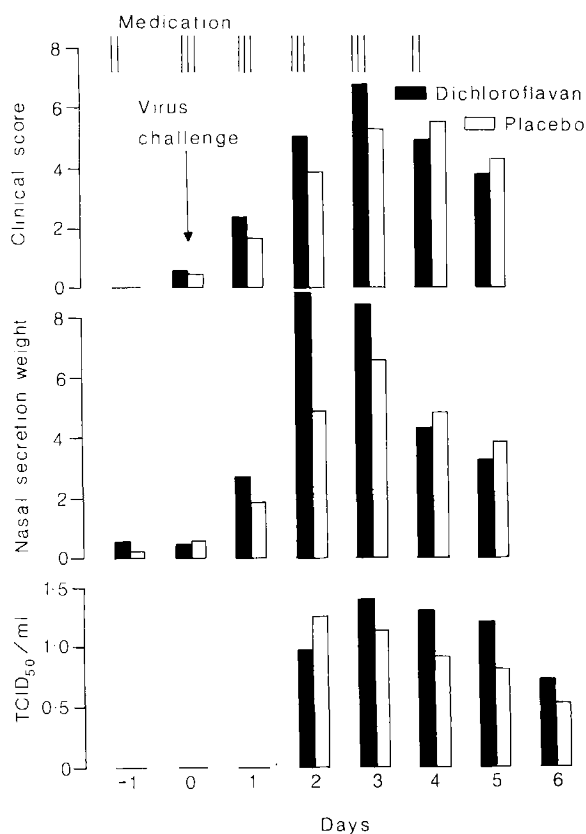

Fig. 4.14. Dichloroflavan has no prophylactic effect against rhinovirus type 9 in volunteers (after Tyrrell et al., 1983).

TABLE 4.38.
Effect of MDL-860 on virus plaque formation (after Torney et al., 1982)

MDL-860[a] (μg/ml)	No. of PFU		
	RV Hank	Echo 12	Coxsackie B_4
0	38	37	44
0.06	37	33	45
0.125	16	21	40
0.25	9	13	30
0.5	6	2	17
1.0	0	0	3
2.0	0	0	3

[a] MDL-860 was present only in the overlay medium

Ro 09-0179 (4′,5-dihydroxy-3,3′,7-trimethoxyflavone), isolated from a Chinese medicinal herb, was found to have potent antiviral activity by Ishitsuka et al. (1982). It selectively inhibited the replication of human picornaviruses, such as rhinoviruses and Coxsackie viruses in tissue culture (Table 4.40), but not other DNA and RNA viruses. A further compound, Ro 09-0179, prevented coxsackie virus (B1) infection in mice (Table 4.41). The critical time for the inhibition of rhinovirus replication by the compound was 2 to 4 h after virus adsorption, i.e., in the early stages of virus replication. It markedly inhibited coxsackie virus and rhinovirus RNA synthesis in infected HeLa cells, but not in a cell-free system using the RNA polymerase complex isolated from the infected cells. In the infected cells, the RNA polymerase complex was not formed in the presence of the drug and therefore, it was suggested that Ro 09-0179 interferes with some process of viral replication which occurs between viral uncoating and the initiation of viral RNA synthesis.

Furthermore, studies of various analogs related to 4′,5-dihydroxy-3,3′,7-trimethoxyflavone led to the identification of 4′-ethoxy-2′hydroxy-4,6′-dimethoxychalcone

TABLE 4.39.
Inhibitory effect of MDL-860 (1 μg/ml) on picornavirus replication in vitro (after Powers et al., 1982)

Virus (no. of strains)	No. (%) of strains with mean decrease in yield ($\log_{10} TCID_{50}$/0.2 ml) of:		
	⩾1.0	0.5–0.9	<0.5
Rhinovirus (90)	72 (80)	12 (13)	6 (7)
Enterovirus (10)	8 (80)	1 (10)	1 (10)

(Ro 09-0410), a new and different type of antiviral agent (Ishitsuka et al., 1982). Ro 09-0410 had a high activity against rhinoviruses but no activity against other picornaviruses. Of 53 rhinovirus serotypes tested, 46 were susceptible to the compound in HeLa cell cultures. The concentration of Ro 09-0410 inhibiting 50% of the types of rhinovirus was about 0.03 μg/ml, whereas the 50% cytotoxic concentration was 30 μg/ml. Ro 09-0410 inactivated rhinoviruses in direct dose-, time-, and temperature-dependent fashion. We can conclude that the inactivation may be associated with the binding of the agent to some specific site of the rhinovirus capsid.

Finally, we end up near the beginning of attempts at chemotherapy with rhinoviruses, namely with interferon. Merigan et al. (1973) showed that 14×10^6 units (50 μg approximately) of partially purified human leukocyte interferon could protect volunteers against infection with rhinovirus type 4. At the time this experiment used up a great proportion of the total world supply of interferon and the pretreatment and treatment was prolonged! This classic experiment has now been repeated but

TABLE 4.40.
Reduction of yield of echovirus from Ro 09-0179-treated cells in culture (after Ishitsuka et al., 1982)

Ro 09-0179 added (μg/ml)	Reduction of yield ($\log_{10}$) of echovirus type:			
	7	11	12	19
0.5	2.8	2.5	>3.6	3.1
2.0	3.7	3.3	>3.6	3.6

TABLE 4.41.
Inhibition of viraemia caused by Coxsackievirus B1[a] (after Ishitsuka et al., 1982)

Treatment	Virus titre ($\log_{10}$ PFU/ml of blood) at day after infection:		
	1	2	3
None	5.7	6.5	7.0
	6.2	6.7	6.3
	6.4	7.3	6.7
Ro 09-0298 (10 mg/kg)	<4	<4	<4
	<4	<4	6
	<4	<4	6.7
Ro 09-0298 (20 mg/kg)	<4	<4	<4
	<4	<4	<4
	<4	<4	5.7

[a] Groups of three mice were infected intraperitoneally with about 10 LD_{50} of coxsackievirus B1 and were treated orally four times with Ro 09-0298 at 1, 2, 5, and 19h after infection

using highly purified and ten-fold more interferon, and using rhinovirus type 9 (Scott et al., 1982). The data is summarized in Table 4.42. Symptoms were markedly reduced but virus infection and seroconversion were not prevented and it was possible that interferon only *delayed* the onset of the cold. There is still a long way to go before the common cold is vanquished!

4.16. Acute haemorrhagic conjunctivitis (AHC or Apollo 11 disease)

This infection caused by enterovirus 70 now has a unique position in virology as one of the two human viruses which are pandemic in their epidemiology (with influenza still heading the 'record' as the classic example). The virus infection was first described by Chatterjee et al. (1970) in Ghana as a disease with an incubation period as short as 24 h and was called Apollo 11 disease in the locality because the outbreak coincided with the time of Apollo 11 landings on the moon. Within 3 years outbreaks had occurred in Japan (Kono, 1975) and a new picornavirus was isolated from the conjunctival scrapings. Enterovirus 70 has now spread almost around the world. (Hierholzer et al., 1975, Likar et al., 1975, Mirkovic et al., 1973).

Although Apollo 11 disease is most usually rather benign with complete recovery within 2 weeks, some neurological complications have nevertheless been described affecting about 1 in 10 000 cases. Most of the paralysed cases are men and typically the neurological phase begins 2–3 weeks after the onset of conjunctivitis with an acute hypotonic asymmetrical proximal paralysis of the lower limbs with fever, malaise and nerve root pains. Cranial nerve paralysis is often noted. Paralysis may be

TABLE 4.42.
Clinical grades of colds, rhinovirus 9 shedding on any day, and seroconversion (rise in serum neutralizing antibody of over fourfold) in volunteers treated with interferon or placebo and challenged with rhinovirus 9 (after Scott et al., 1982)

Grade of cold	No. of volunteers receiving:					
	Interferon			Placebo		
	Total	Virus secretion	Sero-conversion	Total	Virus secretion	Sero-conversion
Nil	5	3	2	1	1	0
Doubtful or very mild	3	2	1	2	1	1
Definite colds:						
Mild	0	0	0	1	1	1
Moderate	0	0	0	7	7	4
Total	8	5	3	11	10	6

permanent in up to 50% of those affected and hence in the near future the virus may be an important candidate for vaccine development or chemoprophylaxis. Epidemics have reached enormous proportions (Table 4.43) with over a million cases, for example, in Calcutta in 1971. The disease is most frequently seen in young adults and is not common in children, although serological evidence shows that infection occurs no less frequently in children (Table 4.44). Under conditions of poor hygiene and overcrowding rapid and extensive spread can occur.

Enterovirus 70 is seldom found in the faeces of patients. The viruses are rather fastidious from a cultivation point of view but may be isolated in human diploid fibroblasts and organ cultures of human embryonic conjunctiva. They are not pathogenic for suckling mice. Sensitive kinetic neutralization tests have shown that serological differences occur among isolates of enterovirus 70 (Higgins, 1982).

Apollo 11 disease is an excellent example of how a new disease can occur in man not only as a result of a previously unknown virus (e.g. Marburg virus) but also when members of a group of viruses behave in a previously undescribed fashion, with coxsackie A24 and enterovirus 70 causing disease not previously recognized as characteristic of the enteroviruses.

Prevention

At present no vaccine or specific treatment exists. In fact even if a live or inactivated vaccine were to be developed it is not clear which route of administration, for exam-

TABLE 4.43.
Number of patients with AHC reported or treated and estimates of actual number of cases in different parts of the world (after Kono, 1975)

Country or city	Period	No. of cases	
		Reported or treated	Estimates of the whole
Africa			
Accra, Ghana	Jun–Oct 1969	13 664	
Lagos, Nigeria	Sep–Dec 1969	12 799	over 100 000
Morocco	Dec 1970–May 1971	137 991	
Tunisia	Feb 1971		over 50 000
Sana, Yemen	Feb 1972		30 000
Asia			
Singapore	Sep–Oct 1970	60 180	
Singapore	Jun–Dec 1971	38 150	
Hong Kong	Aug 1971	6420	
Bangkok, Thailand	Jun–Nov 1971	2105	
Japan	Aug 1971–Feb 1972		30 000
Bombay, India	Mar–Sep 1971		500 000
Calcutta, India	May–Jul 1971		1 000 000

TABLE 4.44.
Age distribution of patients with AHC under treatment at ophthalmologic clinics (after Kono, 1975)

Age groups	Singapore	Bangkok	India	Nairobi	Age groups	Tokyo
10 or under	2.5	13.1	6.4	8	0–9	4.0
11–20	33	30.5	21.8	9.3	10–19	9.5
21–80	20	24.4	38.1	40	20–29	21.0
31–40	20	14.2	19.8	16	30–39	18.5
41–50	12.5	9.3	8.5	16	40–49	18.5
51–60	10.6	4.5	3.7	4	50–59	9.5
61–70	1.2	2.9	1.0	5.3	60–69	10.5
71 or over		1.0	1.0	1.3	70 or over	8.5

ple, would be most effective. Specific antivirals could prevent the secondary paralysis syndrome, especially when the rather long interval between conjunctivitis and paralysis is considered, but a large number of persons would have to be treated to prevent a single case of paralysis. As with certain arboviruses (see Chapter 5) resources might be more usefully diverted to altering and improving hygiene and living conditions in developing countries where the highest attack rates have been recorded.

4.17. Summary

Polio has been well controlled in most developed countries using live or (in a few countries) inactivated vaccines. Research work has intensified using genetic engineering techniques to produce live attenuated viruses with defined and stable mutations so as to prevent reversion to virulence, and also to produce immunogenic oligopeptides or proteins for a new generation of inactivated polio vaccines. Chemotherapy is therefore not required for polio infections.

In contrast, no vaccines have been developed against rhinovirus infections and nor are vaccines thought to have a use, unless broadly reacting antigenic determinants can be located. Several interesting but only weakly effective antiviral compounds have been selected against rhinoviruses and this is a major research area at present. Studies continue also with interferon, but because of toxicity problems these look less interesting at present.

Sequence and biochemical data is now available for several additional enterovirus strains (including the pandemic enterovirus type 70 and hepatitis A) and this could open new possibilities both with antivirals or vaccines (e.g. synthetic peptides) in the near future. At present little attention has been paid to preventing the spread of many important enteroviruses of the coxsackie and ECHO virus group, although

some of these viruses can result in serious neurological sequelae, resembling polio viruses in this regard.

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CHAPTER 5

Arbovirus infections

5.1. Arboviruses

This is a very heterogeneous group of RNA containing viruses (Porterfield, 1980), but a dominant characteristic of members is that the virus is maintained in nature principally through biological transmission between vertebrate hosts by arthropods (Miles, 1964; Komio et al., 1966). The viruses replicate and produce a viraemia in vertebrates including man (who is not, however, the principle host) and thus cause disease (Fig. 5.1). The viruses are world wide in their distribution (Table 5.1) from Finland in the north to Australia in the south (reviewed by McLean, 1983, Simpson, 1972).

Around 446 arboviruses have been documented and at least 60 groups are distinguished on a serological basis (McLean, 1983). The more important groups for humans are the Togaviruses consisting of Group A (alphaviruses) containing (at least) 20 mosquito borne viruses and group B (flaviviruses) containing 61 viruses, approximately half of which are mosquito borne. Other viruses loosely grouped as 'arboviruses' in many publications are the negative stranded viruses such as the bunyaviruses and arenaviruses (see Table 5.14). New viruses are still being discovered and characterized (Clerx et al., 1983).

All arboviruses are thought to be zoonoses since they are maintained in nature by animal hosts and not man, whom they infect coincidentally. Indeed, in their natural host arboviruses are so well adapted that more often than not they cause no overt disease. In man the diseases caused by these viruses range from mild febrile illnesses with skin rashes to fatal encephalitis or haemorrhagic fever.

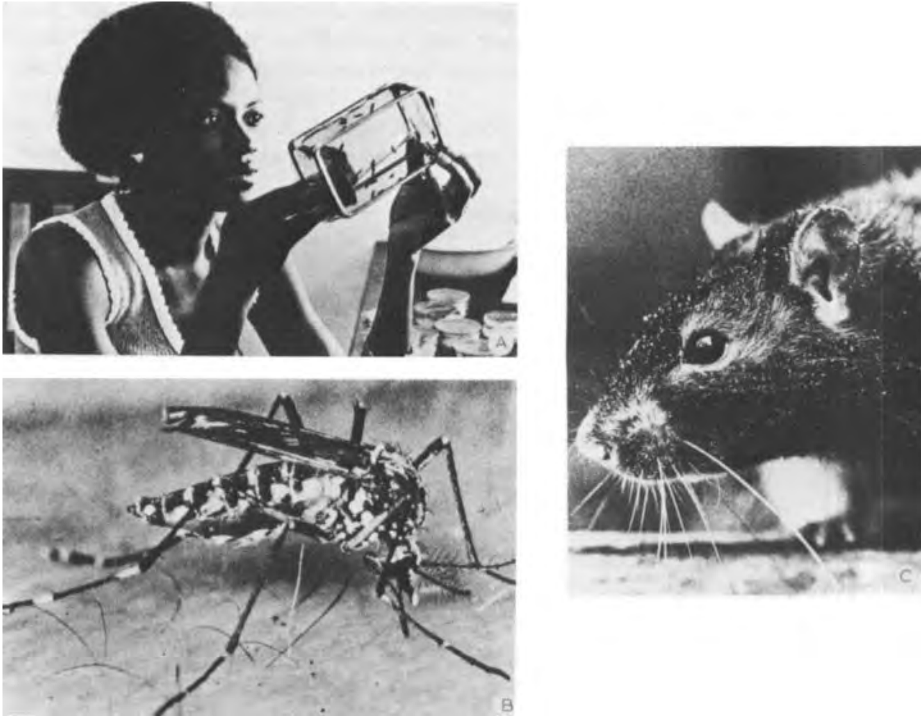


Fig. 5.1. Insects and animals implicated in the spread of arboviruses. Constant surveillance of virus carrying insect populations is a necessary adjunct to vaccine programmes (A). *Aedes aegypti* (B) carries urban yellow fever and the rat (C) is suspected of playing an important role in the transmission to humans of Ebola and Lassa viruses. (from World Health, a WHO publication.)

5.2. Togavirus structure and replication

The togaviruses (such as Sindbis and Semliki Forest viruses) consist of a nucleoprotein core, containing a single species of plus-strand RNA, known as 42 S RNA (M.W. 4×10^6), and a single polypeptide (Table 5.2). This core is surrounded by a lipoprotein envelope, containing two glycoproteins E1 and E2 (Roehrig et al., 1982, Dalrymple et al., 1976, Garoff et al., 1974, Burke and Keegstra, 1976). Since the 42 S RNA is infective, the input strand must initially be translated to give virus proteins, which include the virus RNA polymerase (see Fig. 5.2) (reviewed by Schlesinger, 1980).

During infection by these viruses, two polyadenylated viral messenger RNAs, a 42 S and a 26 S RNA species, can be isolated from the polysomes of infected cells. The 42 S polysomal mRNA, M.W. $4.3 \pm 0.3 \times 10^6$ is identical to the genome RNA that is encapsidated in infectious virus particles, except for quantitative differences in the methylation of some internal cytidine residues. The RNA retains infectivity

TABLE 5.1.
Arthropod-borne viruses causing CNS disease

Family / Genus / Complex / Virus species	Vector	Animal reservoir	Geographic location
Togaviruses			
Alphaviruses[a]			
Eastern equine encephalitis virus	Mosquitoes	Birds	Eastern and Gulf coasts of U.S. Caribbean
Western equine encephalitis virus	Mosquitoes	Birds	Widespread; but disease in West and Southwest U.S.
Venezuelan equine encephalitis virus	Mosquitoes	Horses and small mammals	South and Central America; Florida and Southwest U.S.
Flaviviruses[b]			
St. Louis complex			
St. Louis virus	Mosquitoes	Birds	Widespread in U.S.
Japanese virus	Mosquitoes	Birds	Japan, China, Southeast Asia, and India
Murray Valley virus	Mosquitoes	Birds	Australia and New Guinea
West Nile virus	Mosquitoes	Birds	Africa and Middle East
Ilheus virus	Mosquitoes	Birds	South and Central America
Tick-borne complex			
Far Eastern tick-borne encephalitis virus	Ticks	Small mammals and birds	Eastern U.S.S.R.
Central European tick-borne encephalitis virus	Ticks	Small mammals and birds	Central Europe
Kyasanur Forest disease virus	Ticks	Small mammals and birds	India
Louping-ill virus	Ticks	Small mammals and birds	Northern England, Scotland, and Northern Ireland
Powassan virus	Ticks	Small mammals and birds	Canada and Northern U.S.
Negishi virus	Ticks	Small mammals and birds	Japan
Bunyaviruses			
California group			
California encephalitis virus; La Crosse virus	Mosquitoes	Small mammals	Widespread in U.S., mainly in north central states
Tahyna virus	Mosquitoes	Small mammals	Czechoslavakia, Yugoslavia, Italy, south France
Reoviruses			
Orbivirus			
Colorado tick fever virus	Ticks	Small mammals	Rocky Mountain area

[a] Formerly group A arboviruses.
[b] Formerly group B viruses.
[c] Formerly Russian spring-summer encephalitis.

TABLE 5.2.
Proposed system of nomenclature for flavivirus-specified proteins

Present designation	Proposed designation	
Structural		
V3	E	Envelope glycoprotein of virion; MW range for flavivirus 50 000–60 000
V2	C	Core protein associated with genome; MW 14 000
V1	M	Membrane-like protein, apparently located between E and C in virion; MW 8500
Nonstructural		
NV5	P98[3]	
NV4	P71	The two largest nonstructural proteins are apparently conserved in size
V3	P51 (E)	Comigrates with E during electrophoresis but differs slightly by peptide mapping
NV3	gp44	Minor glycoprotein, difficult to resolve
NVX	p32	Variable in production; probably is a complex of at least two polypeptides
NV2½	P21	
NV2	GP19	The smallest glycoprotein; stable after post-translational processing but exhibits microheterogeneity
NV1½	P14(C)	Originally designated V2, but lacks one tryptic peptide of V2
NV1	P10	Not easily resolved from P14(C)

Nonstructural proteins and glycoproteins which are stable end-products and unique by peptide mapping are designated 'P' or 'GP', respectively. The designations of other products which are known variants or precursors, or which are yet to be characterized, are prefixed by 'p' or 'gp'. The E or C in parentheses signifies that a definite relationship to the envelope or core protein, respectively, has been demonstrated.

when deproteinized. The 26 S mRNA, M.W. 1.8×10^6, is an exact copy of the 3'-terminal third of the 42 S RNA. Synthesis of the 42 S and 26 S mRNAs results from transcription initiating at separate sites on a 42 S negative-stranded RNA template (Simmons and Strauss, 1972, Clegg and Kennedy, 1974, reviewed by Kennedy, 1980).

The 26 S mRNA directs the synthesis of a single polyprotein that is processed by proteolysis, transmembrane insertion, glycosylation, and fatty acid acylation to generate the structural proteins of the virus: the capsid protein and the envelope glycoproteins (reviewed by Schlesinger and Kääriäinen, 1980). The interrelationships of proteins and relative map positions have been firmly established (Rice and Strauss, 1981). Other polypeptides, which are presumed to be translation products of the 42 S mRNA, have been identified in cells infected with Semliki forest virus (SFV) and biochemical and genetic evidence indicates that these products include components of the viral RNA-dependent RNA polymerase(s) (Gomatos et al., 1980). The nonstructural polypeptides are generated by proteolytic cleavage of larg-

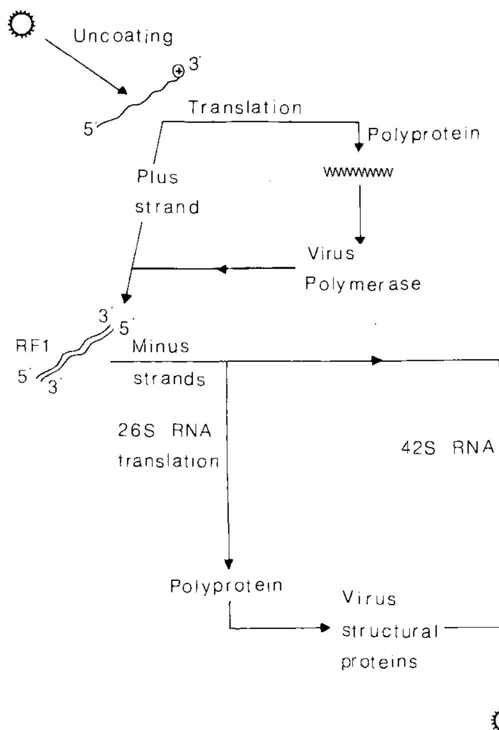


Fig. 5.2. A possible scheme for the multiplication of togaviruses (after Martin and Burke, 1974).

er precursors (Lehtovaara et al., 1980, Collins et al., 1982), but their interrelationships are unclear.

5.3. Clinical aspects of arbovirus infections

Arboviruses cause some of the most disabling epidemic diseases of man. A wide range of clinical manifestations may be noted including encephalitis (Skinner, 1963), yellow fever, febrile conditions, and haemorrhagic signs (Simpson, 1972, Christie, 1980, McLean, 1983) or, alternatively the infection may be subclinical (in a ratio as high as 1000:1). Certainly, each clinical syndrome may be caused by many different virus serotypes (e.g. haemorrhagic fever caused by dengue virus, chikungunya or Omsk haemorrhagic fever virus (Cohen, 1982, Pattyn, 1978)) but it should be remembered that, with the exception of dengue, most arboviruses are localized to a particular continent or geographic zone (Table 5.3). Christie (1980) notes that "in no other area of medicine in the present century has the laboratory worker offered so much to the clinician as in the study of the encephalitides".

A common clinical sign is encephalitis, characterized by neck stiffness, nausea

TABLE 5.3.
Clinical syndromes associated with selected arboviruses (after McLean, 1983)

Syndrome	Serogroup	Causative Arbovirus serotype	Geographic distribution
Encephalitis or Aseptic meningitis	A	Venezuelan equine encephalitis	Central and South America
		western equine encephalitis	Western Canada and USA, Caribbean
	B (mosq. borne)	Japanese B encephalitis	Orient (Japan to Malaysia)
			Australia
		Murray Valley encephalitis	
		St. Louis encephalitis	Canada, USA, Central America
	B (tick-borne)	Louping ill	Scotland, Northern Ireland
		Powassan	Canada, Northern USA
		tickborne encephalitis	Central and northern Europe, Siberia
	CAL	La Crosse[a]	USA
		snowshoe hare[a]	Canada
Yellow Fever	B	yellow fever	Tropical Africa, Caribbean, tropical South America
Dengue	A	chikungunya	East Africa, India, Southeast Asia
	B	dengue (4 types)	Entire tropical zone
Hemorrhagic fever	A	chikungunya	East Africa, India, Southeast Asia
	B (mosq.)	dengue (4 types)	India, Philippines, Southeast Asia, Oceania
	B (tick)	Kyasanur Forest disease	India
		Omsk haemorrhagic fever	Siberia
	CHF-CON (tick)	Congo[a]	Central and southern Africa
Undifferentiated tropical fever	A	chikungunya	East Africa, India, Southeast Asia
		Ross River	Australia, Oceania
	B	Ilheus	Caribbean, South America
		West Nile	Central and northern Africa
	PHL (mosq. borne)	Rift Valley fever[a]	Northern, eastern and southern Africa
	PHL sandfly-borne	Punta Toro[a]	Central America
		sandfly-fever – Naples[a]	Mediterranean
	SIM	Oropouche[a]	Caribbean, South America

[a]Bunyaviruses.
(see also Bennett, 1976, Farber et al. 1940, Likar and Dane, 1958, Smorodintsev, 1958)

and vomiting, fever, headache, drowsiness and general disorientation. Incubation periods may extend to two weeks and symptoms peak at 2 – 5 days after onset. Other patients may linger for weeks or months in coma, but the majority of infected persons recover in the first week or two and regain their previous health.

The essential pathological lesion is destruction of nerve cells in the grey matter by viral replication (Quong, 1942, Mijake, 1964). In the earliest stage there may be only oedema and swelling of the nerve cell but this may be followed by necrosis of the cell. Areas of infiltrating cells, mainly polymorphonuclear, surround the dying cells, but later polymorphonuclear cells are replaced by monocytes. Perivascular cuffing may be prominent and there may be petechial haemorrhages in the pons, medulla and cord. In yellow fever, after an incubation period of 3–6 days the patient develops a sudden headache and fever, accompanied by generalized myalgia, nausea, and vomiting. Jaundice may appear on the third day of illness, and in severe cases death may occur as soon as 3 days after the onset of illness. The mortality rate is 5–10%. Laboratory diagnosis is helpful to distinguish yellow fever from viral hepatitis, leptospirosis or malaria.

In dengue infections persons suddenly develop fever, severe frontal headache, pain in the back and limbs ('Break bone fever') perversion of taste and lymphadenopathy, whilst a maculopapular rash lasting 2–7 days may appear in up to 2/3rds of patients. Fever persists for 3–5 days and may show a remission, followed by a relapse in about half the patients.

Haemorrhagic manifestations (purpura, gastrointestinal bleeding) accompanied by varying degrees of shock have been observed in Southeast Asia in association with typical symptoms of dengue (Halstead, 1980) with a fatality rate of 4–12% (Dengue haemorrhagic shock syndrome), and this is discussed in more detail below.

5.4. Control of arbovirus infections

Vaccination of humans (Edwards, 1948, Kenyon et al., 1975, Ascher et al., 1978) has no effect on the maintenance of arbovirus infections in nature since humans are only an incidental host of the virus, although immunization would give *individuals* protection against the disease. Therefore, most control efforts are orientated towards breaking the links between man and the natural maintenance cycle by controlling arthropod and vertebrate hosts. Mosquito control is attempted on a large scale using insecticide, while simple measures such as introduction of piped water supplies can be most effective. Artificial breeding sites of mosquitoes in urban areas have to be destroyed. It is fair to say that large urban development, deforestation, irrigation and resettlement programmes all contribute towards outbreaks of arbovirus infections in man.

Apart from the classical studies with Yellow Fever Virus (see below), tick borne encephalitis virus and Japanese encephalitis, experimental live attenuated vaccines

of dengue types 2 and 4 have been tested, but work has proceeded cautiously, and one of the major problems is whether vaccine would protect or alternatively sensitize and thus increase the chance of serious complications of shock syndrome (see below). In initial studies a dengue type 2 virus was plaqued in monkey kidney cells and a small plaque *Es* variant obtained with reduced mouse and monkey virulence and which replicated poorly in human monocyte cultures. A further 3 passages were carried out in rhesus lung diploid cells and virus was inoculated into 6 volunteers who had been immunized previously with yellow fever vaccine. Two of the volunteers developed a short fever and one had accompanying headache and myalgia, whilst a third had a rash. All 6 volunteers developed antibody and five of them had a viraemia. In a subsequent study 19 volunteers who had no antibody to flaviviruses were infected with varying doses of the dengue vaccine and 8 of them seroconverted.

Unfortunately (and in particular as regards the future development of vaccines) the role of the immune response in recovery from infection with certain alpha and flaviviruses including Japanese encephalitis virus (JEV) is poorly understood. Haemagglutination-inhibiting (HAI) antibody may play a protective role against JEV in mice and neutralizing antibody in gibbons. However, some recent studies have shown that HAI antibodies do not appear to be the major determinant of recovery after primary JEV infection in mice, nor do they prevent subsequent infection. HAI antibodies were produced after primary infection of pregnant mice but JEV nevertheless persisted and was transmitted transplacentally in consecutive pregnancies (Mathur et al., 1982b). The precise role of antibodies in protection against JEV infection is therefore not clear. Fatal encephalitis is seen in horses and pigs in the presence of neutralizing and complement-fixing antibodies. The co-existence of JEV with its HAI antibodies is seen in the blood of vaccinated pigs. Mathur et al. (1982) have observed that JEV is transmitted to the foetus even when the mother had JEV HAI and neutralizing antibodies. The HAI and neutralizing antibodies against JEV in man are both IgM and IgG in nature. The findings of another recent study (Mathur et al., 1983) indicate that IgM antibodies are protective in JEV infection of mice, and this observation is supported by clinical studies which reported disappearance of the neurological signs in patients of Japanese encephalitis in the presence of IgM antibodies.

The half-life of IgM antibodies in mice is less than 4 days and transfer experiments show that immune cells are effective for 2 weeks only. If JEV escapes immune surveillance during this period, a persistent infection may result. In the absence of IgM antibodies JEV is not eliminated in the mouse model. Boosting and sustaining the production of IgM antibodies against the virus may be considered therefore as the main host defence mechanism, and this may have wider implications as regards vaccine strategies in humans.

Experimental studies with Semliki forest virus (Helenius et al., 1976) and tick borne encephalitis virus (Heinz and Kunz, 1980) have shown that multimeric forms

of the surface glycoproteins (protein micelles) are particularly immunogenic, and certainly more so than the viral subunits or single protein entities. Also pertinent to the design of vaccines against these viruses, we note that Sindbis virus (SV), the prototype of the alphaviruses, contains two distinct envelope glycoproteins, E1 and E2, which exist as a complex, and a nucleocapsid protein, NC. Antibodies raised in rabbits against each of the individually isolated and purified glycoproteins of SV possess distinctly separable biological activities in vitro. Antibodies to E1 (the viral haemagglutinin) inhibit haemagglutination by SV, whereas only antibodies to E2 significantly neutralize viral infectivity. Antibodies specific for the internal NC do not react with intact virions. Both HAI and neutralizing antibodies are induced during productive infections with SV as well as with other alphaviruses, but their respective roles in mediating host recovery are not clear as noted above. Among the heterogeneous population of antibodies specifically induced during many acute viral infections, those having virus-neutralizing activity in vitro are generally considered to be most important for recovery and immunity to reinfection. Similarly, the ability to stimulate production of circulating neutralizing antibodies is a major criterion for evaluating the immunoprophylactic potential of many antiviral vaccines as we have noted above. Although there is obviously an association between neutralizing antibody induction and host resistance, Schmaljohn et al. (1982) have presented data which indicate that other virus-specific antibodies lacking neutralizing

TABLE 5.4.
Properties of anti-SV_p monoclonal antibodies (after Schmaljohn et al., 1982)

Monoclonal antibody			Reactivity with SV_{na}	
Specificity	Isotype	IF	NT titre	% C′-dependent cytolysis
Protective in vivo				
E2	IgG2b	+	3200	55.3
E2	IgG2a	+	800	21.1
E2	IgG2a	+	1000	47.7
E1	IgG2a	+	<10	29.4
E1	IgG2a	+	<10	16.8
E1	IgG2a	+	<10	29.1
E1	IgG2a	+	<10	32.6
E1	IgG2b	+	<10	28.5
Non-protective in vivo				
E2	IgG3	+	<10	2.1
E2	IgG2a	−	<10	N.D.
E2	IgG2a	−	<10	N.D.
E1	IgG1	+	<10	−1.3
NC	IgG3	+	<10	0.3

N.D., not determined.

function may be equally important in conferring protective immunity to alphaviruses. They used monoclonal antibodies against SV to demonstrate that passive protection of SV-infected mice from fatal paralytic CNS disease may be mediated not only by antibodies which neutralize the infectivity of extracellular virus particles but also by those lacking this capacity, which react preferentially with virus-infected cells.

To initially screen monoclonal antibodies for their protective capacity, each monoclone was given intraperitoneally to mice 24 h before challenge i.c. with 1,000 PFU of SN_{na}. All, or most mice in eight such groups resisted challenge, whereas all of a control group of infected non-immune mice died. Passive protection by three anti-E2 monoclonal antibodies correlated with their having measurable neutralizing activity against SV_{na}, although all such antibodies were capable of neutralizing SV_p. In contrast, although *none* of the six anti-E1 monoclonal antibodies had NT activity against either SV_p or SV_{na}, five were protective. Sindbis virions acquire their envelopes by budding from host-cell plasma membranes which express both E1 and E2 during viral morphogenesis. Thus, irrespective of their glycoprotein specificity, all protective monoclonal antibodies shared an ability to bind to SV_{na} infected cells as shown by immunofluorescence and, as might be expected from their IgG2a and IgG2b isotypes, by C′-dependent cytolysis. These data suggest two specific but functionally different humoral mechanisms of SV elimination in vivo: (1) neutralization of infectivity by antibodies which bind to E2 determinants on extracellular virions, a process which can occur in vitro in the absence of C′; and (2) C′-dependent lysis of SV-infected cells mediated by either neutralizing or non-neutralizing antibodies (of restricted isotypes) which bind, respectively, to E2 and E1 determinants on the cell surfaces. It is likely that one or both of these mechanisms involve(s) the recognition of more than one viral glycoprotein determinant. For example, all six anti-E1 monoclonal antibodies were capable of binding to SV-infected cells but only two (1 and 31) had significant HAI activity.

The ability of five anti-E1 monoclonal antibodies to effect cytolysis, but not neutralization, in the presence of C′ suggests that one or more E1 determinants expressed on the plasma membrane of infected cells are not similarly expressed on infectious SV particles.

5.5. Human arbovirus infections in the USA

The most complete information regarding incidence of arbovirus encephalitis has been gathered in the USA (Table 5.5) because of the high level of surveillance and accompanying laboratory diagnosis (Kappus et al., 1982, Monath, 1979). Between 1975 and 1978, 6970 human cases of arbovirus encephalitis were reported in the USA, but this represents a fraction of the true incidence. St Louis encephalitis (4824 cases), California encephalitis (1035 cases) and Western equine encephalitis (967 cases) accounted for 98.5% of all the reported infections.

TABLE 5.5.
Number of reported cases of arboviral encephalitis[a] in the United States of America (after Monath, 1979)

Year	SLE	WEE	EEE	CE[a]	Other	Total
1955	107	37	15			159
1956	563	47	15			625
1957	147	35	5			187
1958	94	141	2			237
1959	118	14	36			168
1960	21	21	3			45
1961	42	27	1			70
1962	253	17	0			270
1963	19	56	0	1		76
1964	470	64	5	42	1	582
1965	58	172	8	59		297
1966	323	47	4	64		438
1967	11	18	1	53		83
1968	35	17	12	66	1	131
1969	16	21	3	67	1	108
1970	15	4	2	89		110
1971	57	11	4	58	20	150
1972	13	8	0	46	3	70
1973	5	4	7	75		91
1974	72	2	4	30		108
1975	1815	133	3	160	2	2113
1976	379	1	0	47		427
1977	169	46	1	69	4	285
1978	22	4	5	109	1	140
Total	4824	947	136	1035	33	6970

SLE=St Louis encephalitis; EEE=eastern equine encephalitis; WEE=western equine encephalitis; CE=California encephalitis.
[a] Bunyavirus

Meanwhile endemic diseases such as California encephalitis (a bunyavirus) have continued to occur with unabated incidence and other major encephalitis outbreaks have occurred, some with unprecedented morbidity rates and geographical spread (e.g. VEE and SLE). 1980 saw the first indigenous transmission of dengue virus in the USA since 1945. 58 cases of dengue type 1 virus infection were documented in the USA in 1980 and it is most likely that 21 persons acquired the disease in the USA itself. Fortunately illness in all the cases was mild and no-one required hospitalization (Kappus et al., 1982). A total of 115 cases of SLE were reported from 13 states, which is a marked increase compared to the previous 2 years. The largest outbreak centred on Houston, where 52 cases were reported. A total of 50 cases of California group encephalitis (bunyavirus) was reported by 7 states, mainly caus-

ed by La Crosse virus. Nine cases of EEE were reported from 5 states in 1980 and the cases in Michigan and New Hampshire were the first to be reported in residents of these states. Finally, for the first time since national surveillance began, no cases of WEE were reported in any state in 1980.

5.6. Dengue haemorrhagic fever (DHF)

The enormous territory occupied by the group of flaviviruses, much of it newly won, is a tribute to the resourcefulness of its main vector, the yellow fever mosquito *Aedes aegypti* (Halstead, 1980). While yellow fever has been confined to forested foci in Africa and South America, dengue viruses have kept pace with urban encroachments of the mosquito and by 1979 had girdled the globe in the tropical zones (Table 5.6).

TABLE 5.6.
Number of reported cases of dengue haemorrhagic fever (and in parentheses dengue shock syndrome) (after Halstead, 1980)

Year	Country							
	Philippines	Thailand	South Viet Nam[a]	Malaysia	Indonesia	Burma	Sri Lanka	Singapore
1956	1207 (72)							
1958	94 (34)	2706 (296)						
1959	40 (11)	160 (21)						
1960	551 (40)	1851 (65)	100					
1961	1459 (33)	561 (36)						
1962	134 (62)	5947 (308)	283					42 (12)
1963	189 (74)	2215 (173)	374 (127)	41 (1)				
1964	759 (169)	7763 (385)	559 (177)					
1965	652 (109)	4094 (193)	171 (39)				4 (2)	
1966	9384 (250)	5816 (137)	53				19 (5)	630
1967	1371 (105)	2060 (65)					29 (8)	826
1968	1116 (115)	6430 (71)					9 (2)	848
1969	1336 (103)	8670 (109)			198 (50)		1	189
1970	922 (83)	2767 (47)			400 (69)	1654 (81)	2	71
1971	438 (34)	11540 (299)			174 (13)	691 (34)	3	116
1972	1570 (83)	23786 (682)	763 (215)		970 (25)	1013 (32)	8	64
1973	591 (62)	8280 (315)	14320 (986)	969 (54)	9947 (454)	349 (15)		1324 (27)
1974	1665 (153)	8160 (328)	4261 (438)	1482 (104)	3667 (188)	2477 (159)		229 (4)
1975	603 (42)	17771 (441)		735 (57)	4160 (259)	6750 (363)		59 (2)
1976	460	9561 (359)	21361	773 (71)	2620 (109)	3153 (98)		30 (0)
1977	376	38768 (756)	45011 (736)	341	7388 (301)	5364 (236)	4	92 (1)
1978	—	12547 (308)			6395 (283)	2029 (82)		352 (2)

[a] The figures for 1976 and 1977 are for the Socialist Republic of Vietnam

It was not realised until the 1950s that dengue could cause a shock syndrome of dramatic swiftness, often complicated by gastro-intestinal haemorrhage followed by death (DSS). Dengue haemorrhagic fever and its complications are among the leading causes of morbidity and mortality in children in areas with at least 1500 million people. Thus in Thailand in 1977 DHF and DSS were the second leading causes of hospitalization of children, and the leading cause of death due to infectious disease. Unanswered are questions of whether DHF/DSS will remain restricted to South East Asia or will spread more widely, and indeed exactly how dengue viruses produce the disease. Dengue viruses are included among pathogens of man which thrive during troubled times. War, population explosions, deterioration of urban environments and sanitation, and migration of refugees all lead to conditions which increase the endemic transmission of several types of dengue viruses.

In normal DHF, infants and children often have an undifferentiated febrile illness with rash (classical dengue fever) whilst older children and adults may have a more severe illness with fever, headache, myalgia and gastrointestinal symptoms with rash. In contrast, DSS as seen in Asian children usually has two stages. Firstly, the illness may begin abruptly with fever and classical dengue symptoms. However, during or shortly after the fall in temperature the patient deteriorates and presents with a cold skin and lethargy. The child may become hypotensive and die in as little as 4 hours. In short, DSS is defined as an acute vascular permeability syndrome accompanied by activation of the blood clotting and complement systems. DSS is rarely seen in malnourished children and, moreover, the syndrome normally occurs in persons with pre-infection dengue antibody actively and passively acquired. However, not all secondary infections result in DSS. Dengue 2 viruses are often isolated from DSS cases and this association might be explained if one or more of the infection sequences, dengue type 1-2, 3-2 or 4-2 were more pathogenic than other sequences of dengue infection. Dengue viruses replicate in human peripheral blood monocyte cultures if the donor is dengue-immune and in cultures from non-immune persons if such neutralizing concentrations of antibody are added to the culture. This 'immune enhancement' (also noted with other flaviviruses, Peiris and Porterfield, 1979) requires the attachment of anti-dengue virus complexes to cellular Fc receptor, which mediate the internalization of the immune complex, resulting in infection of the monocyte. Toxic shock syndrome may be produced by the generation of vascular permeability factor and the activation of complement and blood clotting systems. It is possible that the immune elimination response directed against dengue infected mononuclear phagocytes activates these cells.

5.7. Yellow fever

Epidemics of the flavivirus yellow fever are outstanding both in their medical effects and their effects on the course of history in many countries (Downs, 1982). Follow-

ing the classical studies of Walter Reed, whereby the spread of virus by the bite of the infected *Aedes aegypti* was established, it was widely thought that the disease could be vanquished. This has not proven to be the case. Explosive outbreaks as well as sporadic cases occur whenever mosquitoes and unvaccinated people co-exist (Woodall, 1981). Yellow fever is present in Africa south of the Sahara and north of the tropic of Capricorn and in the equatorial and sub-equatorial regions of South and Central America. In several of the immense virus endemic regions in Africa and South America epidemic outbreaks have occurred after long intervals of apparent freedom from virus activity. It is even possible that a permanent reservoir exists in certain mosquito species. More recently transovarial passage of virus has been demonstrated, male mosquitoes have been found infected in the field and virus has been isolated from certain ticks.

French workers developed a mouse brain vaccine in the 1930s and this was given by scarification and later the vaccine was co-administered with smallpox vaccine to millions of persons in French West African colonies. However, later development of the famous 17D attenuated vaccine in embryonated chicken eggs by Theiler and Smith (1937) supplanted the mouse brain vaccine. This was undoubtedly an American medical triumph. Side reactions to the 17D vaccine are uncommon and the immunity induced is very long lasting, perhaps even for a lifetime. However, the vaccine is not used on a very wide scale in endemic areas for several reasons. The vaccine is not heat stable and a 'cold chain' is required, and a compromise now often reached is to introduce mass immunization only when an outbreak is spotted in an urban area. At this time immunization is commenced immediately and a thorough mosquito clean up is instigated to destroy all breeding places. Insecticide fogs from aircraft are used to kill infected mosquitoes. However, if early diagnosis is not made the epidemic could easily be in the second or third wave of transmission before control is started. The potential usefulness of an antiviral agent is obvious but there are few candidates at present (see below).

5.8. Early development of the 17D yellow fever virus vaccine

Theiler and Smith (1937 a and b) in a now classic study demonstrated the propagation of yellow fever virus (17D) in tissue cultures of chick embryos, which contained minimal amounts of nervous tissue, and selected a virus subpopulation which produced only mild reactions when inoculated into rhesus monkeys (Table 5.7). Moreover, seven of the animals were subsequently challenged with virulent French neurotropic virus and six survived, whereas 3 control animals similarly challenged died. Also the passaged virus lost the ability to produce fatal encephalitis when injected intracerebrally into monkeys (lost between the 89th and 114th in vitro passage). Virus at the 176th passage level was considerably less neurotropic for mice. Finally, the high passage material was immunogenic for monkeys following subcutaneous injection.

TABLE 5.7.

The response of rhesus monkeys to a subcutaneous inoculation of yellow fever virus 17D (after Theiler and Smith, 1937)

Monkey no.	Inoculum: Subculture	Inoculum: Estimated no. of mouse M.L.D.	Inoculum: Route	Test for virus in circulating blood: mortality ratio in mice inoculated with serum — Days after inoculation: 1	2	3	4	5	6	7	8	9	10	Fever on days after inoculation	Results	Antibody titer 30 days after inoculation	Immunity test: French neurotropic virus intracerebrally — Fever (days)	Immunity test: Results
1	216	73 000 000	i.p.	3/7	0/5	0/4	0/6	0/6	0/6	0/6	0/6	0/4	0/6	5	Lived	1:19	—	Lived
2	216	30 000 000	i.v.	6/6	6/6	0/6	0/5	0/6	0/6	0/6	0/6	0/5	0/6	—	Lived	1:32	5, 6, 7, 8	Lived
3	217	13 000	s.c.	0/5	0/7	0/7	0/6	0/5	0/6	0/6	0/6	0/6	0/6	—	Lived	1:2	6, 7	Encephalitis, lived
4	217	1300	s.c.	0/5	0/6	0/6	0/6	2/5	2/5	5/6	0/6	0/6	0/6	—	Lived	1:134	—	Lived
5	217	130	s.c.	0/7	0/6	0/6	0/5	0/6	0/6	0/6	0/5	0/5	0/6	—	Lived	1:5	—	Lived
6	217	13	s.c.	0/6	0/5	0/7	0/6	0/6	0/6	0/6	0/6	0/6	0/6	—	Lived	Negative	Not tested	
7	217	1.3	s.c.	0/5	0/6	0/6	0/6	0/6	0/6	0/6	0/5	0/6	0/6	—	Lived	1:37	5, 6, 7, 8	Lived
8	217	0.13	s.c.	0/7	0/6	0/4	0/6	0/6	0/6	0/6	0/6	0/6	0/5	1	Lived	1:2	4, 5, 6	Died of encephalitis

In contrast, the French yellow fever virus (which had been used up to that time as a vaccine virus with reasonable success) modified by serial mouse brain passage was invariably fatal after i.c. injection in monkeys and also killed 1/3rd of the animals even when administered by extraneural routes (Table 5.8).

In a crucial experiment Theiler and Smith (1937) inoculated four individuals (including themselves) with the new 'candidate' yellow fever vaccine virus at the 227th passage in chick embryo tissue (up to 330 000 mouse lethal doses were administered s.c.). A marked antibody response was noted (Table 5.9) and, apart from a slight local reaction at the site of inoculation, no adverse signs or symptoms were noticed. Eight further volunteers who had no pre-existing antibody to yellow fever virus were then inoculated and 5 had a febrile reaction with slight headache but "not severe enough to prevent the subject from following his normal occupation." More extensive clinical trials quickly followed but the basic results were encapsulated in the 1937 paper: a safer vaccine had been successfully produced and marked a significant scientific advance. We should note that once again a successful attenuated virus vaccine had been produced by empirical methods and with little or no knowledge of the biochemistry, antigenic structure or genetic basis of virulence of the virus. In fact, the genetic basis of virulence of yellow fever virus is only now being investigated (see below).

Soon after live attenuated yellow fever vaccines were widely introduced in the 1940s and 1950s problems became apparent of both over-attenuation and under-

TABLE 5.8.
A comparison of the pathogenicity of French neurotropic virus and virus 17D for experimental animals (after Theiler and Smith, 1937)

Virus	Mice	Rhesus monkeys		Hedgehogs
	Average time of death after intracerebral inoculation (days)	Intracerebral inoculation	Extraneural inoculation	Subcutaneous inoculation
French neurotropic	4–10	Fatal encephalitis	Fever in approximately 50 per cent of animals. Virus present in circulating blood for a period of 2–6 days. Fatal encephalitis in approximately 30 per cent of animals	Death from encephalitis with liver necrosis
Tissue culture virus 17D	8–20	Non-fatal encephalitis	Occasional fever. Minimal amounts of virus in the circulation: No deaths	Animals survive

TABLE 5.9.
Antibody response in immune persons to a subcutaneous inoculation of tissue culture virus 17D (after Theiler and Smith, 1937)

Immune persons inoculated	Inoculum			Serum antibody titre										
	Amount	Subculture	No. of mouse M.L.D.	Before inoculation	Weeks after inoculation									
					1	2	3	4	5	6	7	8	9	10
M.T.	1.0	227	330000	1:32		1:90		1:125		1:110		1:80		1:96
H.S.	1.0	227	330000	1:2	1:2	1:4	1:3	1:8		1:5		1:21	1:8	
T.F.	0.8	227	70000	1:2	1:6	1:100	1:100	1:100						
R.L.	0.5	227	44000	1:2	1:2	1:25	1:20	1:34						

attenuation of virus. Thus, vaccine produced in Brazil from the 17DD strain which had been passed over 300 times in tissue culture was over-attenuated. In contrast, other vaccines originating from both 17DD and 17D – 204 substrains were too neurovirulent in man and monkeys (Fox et al., 1941, 1942, 1943). These problems were soon resolved by the establishment of a virus seed lot system in 1945 (see Chapter 2, WHO 1945, 1956, 1980) but probably as a result of these historical difficulties many derived strains of somewhat varying passage history are still used today for vaccine manufacture (Liprandi, 1981, Monath et al., 1983), although no problems have occurred recently as regards excessive neurovirulence of administered yellow fever vaccines. We have described already (Chapter 2) two additional problems of yellow fever vaccine in earlier times – namely contamination with avian leukosis virus (Waters et al., 1972) and hepatitis B virus (not a present problem) (Sawyer et al., 1944).

5.9. Current status of yellow fever (17D) virus vaccine

Production techniques have, in general, remained unchanged for the past four decades for 17D virus vaccine (Tannock et al., 1980). A recent paper has discussed assay of yellow fever vaccines (Seagroatt and Magrath, 1983). Between 1970 and 1979 75.5 million doses of vaccine were produced by the two main manufacturers, the Columbian National Institute of Health, Bogota and the Oswaldo Cruz Foundation, Rio de Janeiro (Woodall, 1981). Antibody induced by the vaccine is long lasting and, for example, 60% of 109 persons given a live attenuated virus vaccine (17D) in 1940 had significant levels of HAI antibody when tested 40 years later (Woodall, 1981).

Yellow fever 17D vaccines are currently manufactured with approval of the

World Health Organization in 11 countries (the United States, England, Federal Republic of Germany, France, the Netherlands, India, Senegal, South Africa, Australia, Brazil and Columbia) and these vaccines have proven both highly efficacious and safe. Despite this record, several theoretical problems remain. There is a disparity between vaccines and their substrain origin alluded to above. Vaccines presently in use are derived from two distinct substrains which represent independently maintained series of passages from the original 17D virus developed by Theiler and Smith (1937a). Some manufacturers use seed lots derived from substrain 17DD at the 284th to 286th passage level, whereas others use lots derived from substrain 17D-204 at the 233rd to 237th passage level. Additionally certain producers have used various techniques to free vaccine seed lots from the avian leukosis virus (ALV) contaminant. Both ALV-free and ALV-contaminated vaccines are currently used. Full biochemical and biological characterization of 17D yellow fever vaccines has not been accomplished to date, despite clear indications for heterogeneity and the demonstrated presence of plaque mixtures with variable mouse neurotropism (Liprandi, 1981). Finally, there are difficulties in large-scale vaccine production, resulting from shortages of suitable embryonated eggs and short shelf-life of vaccines. In the light of these problems Monath et al. (1983) have published the first detailed biochemical study of genetic and antigenic variation among these different 17D virus vaccines using the biochemical techniques of oligonucleotide mapping and monoclonal antibody analysis (Fig. 5.3). For example, an understanding of the molecular biology of the virus could, as with polio and FMDV (see Chapter 2) lead to an exploration of modern methods of gene cloning in *E.coli* for future vaccine production. Surprisingly, despite the very varied origins and passage levels of the viruses used for vaccine production throughout the world a high degree of genetic and antigenic similarity was found between the vaccines and an RNA sequence homology of 98–100% was noted. In essence, this provided direct experimental confirmation of the usefulness of the virus seed system introduced because of the original problems of certain yellow fever vaccines being over-attenuated or under-attenuated (see above). Vaccines derived from the 17DD substrain were genetically distinguishable from substrains 17D-204, and could also be distinguished using monoclonal antibodies (Table 5.10). Although some oligonucleotide differences were detected between the parental Asibi virus and the 17D vaccine viruses, the relationship between the genetic structure and virulence or attenuation could not be established. This will require detailed comparison of nucleotide sequences between defined virulent and attenuated viruses, and it is quite possible that only a single or very few mutations are required to change a virulent into a non-virulent virus.

Schlesinger et al. (1983) have reported an analysis of 19 monoclonal antibodies to the 17D strain of yellow fever virus (17D YF) and the monoclonal antibodies distinguish clearly between the wild and vaccine strain of virus. It should now be possible to derive antigenic variants and hence identify antigenic determinants for subsequent sequencing and also to analyze virus variants of differing virulence and

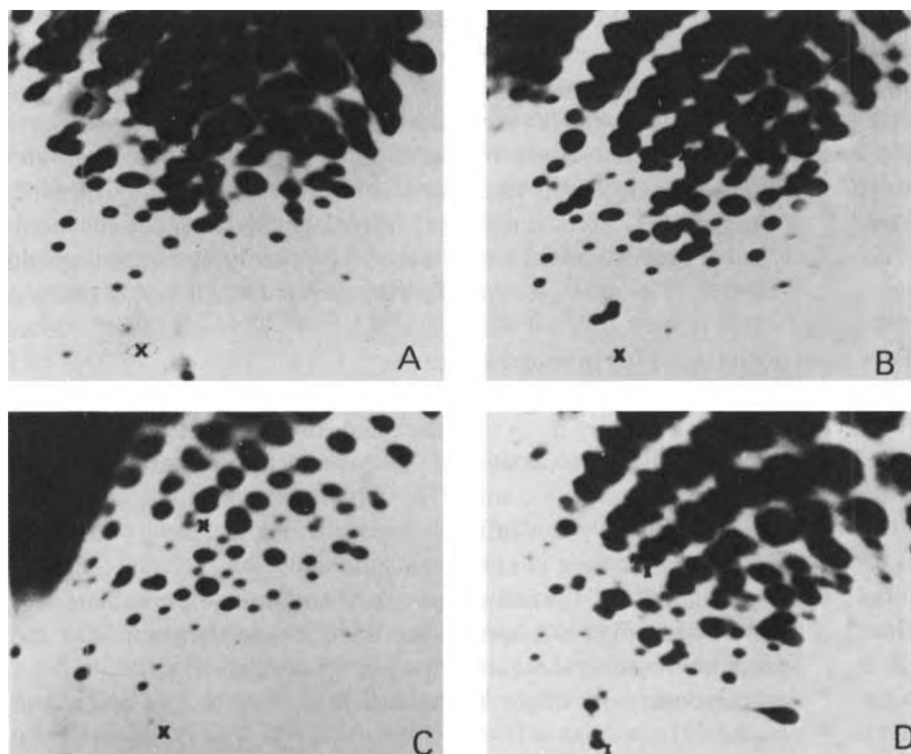


Fig. 5.3. Oligonucleotide maps of RNA of parent Yellow fever virus and derived vaccine strains (after Monath et al., 1983). A, 17D vaccine strain (USA) (reference virus); B, 17D-204 substrain produced in South Africa; C, 17DD substrain produced in Brazil; D, parent Asibi virus.

TABLE 5.10.
Neutralization (N) test results using monoclonal antibody to 17D vaccines (after Monath et al., 1983)

Virus	Substrain origin	N titre monoclone 8A3[a]
YF 17D Connaught 2848	17D-204	81 920
YF 17D Wellcome YF/1/189	17D-204	81 920
YF 17D R. Koch 213/A/80	17D-204	81 920
YF 17D S. Africa primary	17D-204	40960
YF 17D S. Africa 10802	17D-204	40960
YF 17D Osw. Cruz S-101	17DD	<20
YF 17D Bogota 284	17DD	40
YF 17D Dakar 18A	17DD	20
YF Asibi	—	<20

[a] Monoclonal antibody produced to YF 17D Connaught

hence to determine the genetic basis of this factor. Monoclonal antibodies against the flavivirus West Nile (Peiris et al., 1982) and Sindbis virus (Roehrig et al., 1980, 1982) have also been characterized.

Since reactions to yellow fever 17D vaccine are very mild (2–5% of vaccinees have mild headaches, myalgia and low grade fever 5–10 days post-immunization) and the vaccine is highly efficacious, it is recommended for persons living in or travelling to endemic areas and for persons travelling internationally. A single subcutaneous dose of 0.5 ml of reconstituted vaccine is used for primary immunization and boosters every 10 years. For example, the following recommendations are made in the USA:

A. Persons living or travelling in endemic areas:

1. Persons 6 months of age or older, travelling or living in areas where yellow fever infection exists – currently parts of Africa and South America – should be vaccinated. Vaccination is also recommended for travel outside the urban areas of countries in the yellow fever endemic zone. The actual areas of yellow fever virus activity far exceed the infected zones officially reported and, in recent years, fatal cases of yellow fever have occurred in unvaccinated tourists.

2. Infants under 6 months of age and pregnant women should be considered for vaccination if travelling to high-risk areas when travel cannot be postponed and a high level of prevention against mosquito exposure is not feasible.

3. Laboratory personnel who might be exposed to virulent yellow fever virus should be vaccinated.

B. Vaccination for international travel:

For purposes of international travel, yellow fever vaccines produced by different manufacturers worldwide must be approved by WHO and administered at an approved Yellow Fever Vaccinating Centre. Vaccinees should have an International Certificate of Vaccination filled in, signed, and validated with the centre's stamp where the vaccine is given.

As another example of the complexity and unpredictability of the immune response to Flaviviruses, the presence of antibody to yellow fever virus does not induce cross protection against dengue viruses for example but *augments* the response to dengue. Thus Scott et al. (1983) described small scale experiments where volunteers either with or without neutralizing antibody to yellow fever virus were inoculated with an attenuated dengue type 2 candidate vaccine. The virus was a clone of a naturally occurring *ts* small plaque variant of wild type dengue virus. The volunteers with pre-existing antibody to yellow fever virus all sero-converted whereas no relation between virus dose and sero-conversion was detected in volunteers who had no preexisting antibody to yellow fever virus. It would be postulated that dengue virus attachment is achieved by association of the Fc portion of the virus-complexed (yellow fever) antibody molecule with Fc specific receptor sites on the surface of permissive cells. Apart from this rather unnerving observation, the authors concluded that the vaccine itself was attenuated, although with leukopenia in 55%

of volunteers and rash and fever in 15% and 10% respectively, there is clearly a lot of opportunity for improvement.

With the advent of new antivirals, particularly those compounds with antiviral activity perhaps restricted to certain flavi or alpha viruses, rapid and precise diagnosis will be of the utmost importance. It could be anticipated that monoclonal antibodies reacting with individual members of a genus, and not with other arboviruses, could be extremely useful. Table 5.11 shows additional data of the unique serological specificity of certain monoclonal antibodies. Thus monoclone 86.64 reacts only with the 17D vaccine strain of yellow fever and not with any other flavivirus so far tested. The reactivity of this monoclonal with virus is further illustrated in Fig. 5.4.

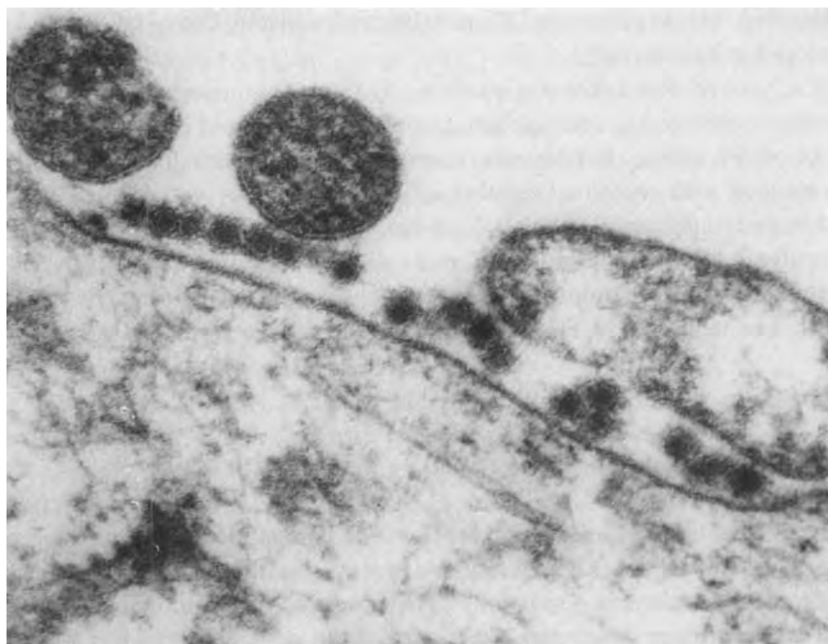
5.10. Highly purified vaccines against tick borne encephalitis (flavivirus)

Tick borne encephalitis (TBE) causes severe illness in many central and eastern European countries and in Austria, for example, several hundred hospitalized cases of TBE are recorded each year. A partially purified vaccine containing formalin inactivated whole virus was developed in the early 1970s which, although immunogenic, nevertheless produced side reactions such as headache and fever (reviewed by Heinz et al., 1980). Therefore, a more pure virus vaccine has now been produced using rate-zonal centrifugation techniques (Heinz et al., 1980, Kunz et al., 1980). Two vaccinations are given, 1–3 months apart, followed by a third dose after 9–12 months and serological studies have shown that the sero-conversion rate is 100% after the second vaccination. Local and systemic reactions are still noted with the

TABLE 5.11.
Characteristics of three monoclonal antibodies prepared against either yellow fever – 17D or Japanese encephalitis virus (unpublished data, courtesy of Dr. E. Gould, London School of Hygiene and Tropical Medicine)

Monoclonal antibody	Virus[a] specificity	IgG subclass	Molecular specificity	HAI activity	Neutralization activity
86.64	YF-17D	IgG2a	54K	+	+
94.79	YF[b]	IgG2a	47K	–	–
98.95	JEV	IgG2a	N.T.	–	–

[a] Tested against several representatives (total 37) of every subgroup of flavivirus. Tested both by indirect immunofluorescence on acetone fixed infected Vero cells and also by ELISA using the monoclonal antibody as the solid phase.
[b] Twenty different wild type isolates from Africa, Brazil or Trinidad and additionally 7 different seed pools of YF-17D.
N.T., not tested.



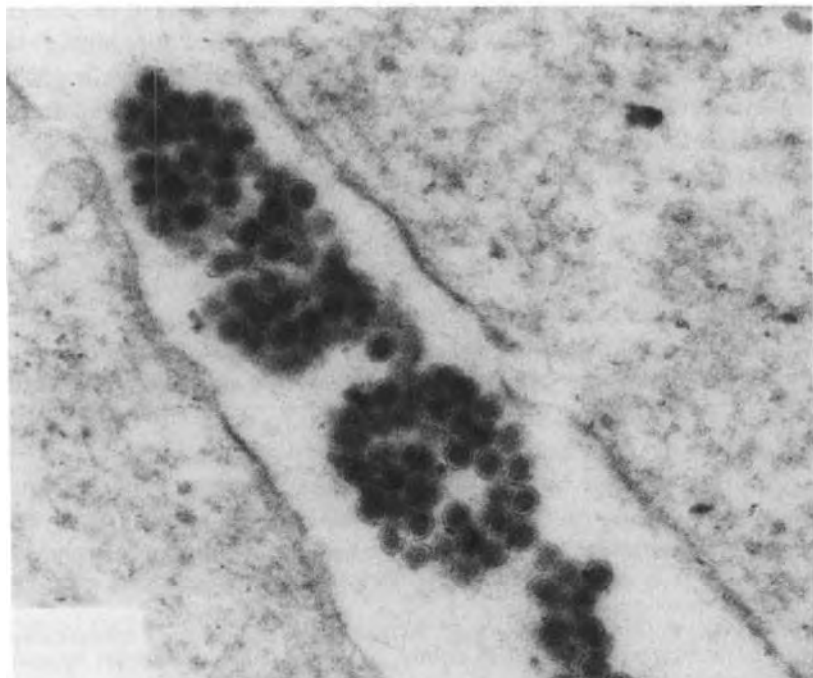


TABLE 5.12.
Local and systemic reactions in adults (>14 years) after vaccination with purified TBE vaccine (after Kunz et al., 1980)

Side reactions (%)	Vaccination (0.5 ml)			Vaccination (1.0 ml)
	1st (n=101)	2nd (n=70)	3rd (n=113)	1st (n=33)
Local				
Pain	36	23	28	58
Redness	6	3	4	27
Swelling	12	3	4	12
Systemic				
Malaise	33	19	22	76
Headache	34	14	19	55
Fever (>37.3°C)	9	1	2	39
37.3–38°C	8	1	2	27
38–39°C	1	0	0	12

purified vaccine in adults and children but are significantly reduced compared to the more impure and earlier vaccine (Table 5.12). Of course, even minor side reactions may strongly restrict the level of acceptance of a vaccine by the population (as noted, for example, with inactivated influenza vaccines, see Chapter 7) and so this aspect of vaccine development is extremely important.

5.11. Bunyaviruses

The Bunyaviridae include more than 200 serologically distinguishable virus isolates (Bishop et al., 1981) (Table 5.13). Infection of vertebrates by many of the viruses in the family leads to the development of inapparent infections, although some viruses cause an occasionally fatal meningoencephalitis, while others produce a haemorrhagic fever. Some 150 viruses in the family have been assigned to the Bunyavirus genus. Another 30 or more viruses are assigned to the Phlebovirus genus (e.g., Karimabad, Punta Toro and Rift Valley fever viruses). Some 18 viruses have been placed in the Nairovirus genus (serogroups: Crimean-Congo haemorrhagic fever (2), Nairobi sheep disease (3), Hughes (4), Dera Ghazi Khan (6) and Qalyub (2); and 1 unassigned virus). Another 7 viruses constitute the Uukuvirus genus. In addi-

Fig. 5.4. Reaction of monoclonal antibody 86–64 with 17D Yellow fever virus (courtesy of Dr. E. Gould, London School of Hygiene and Tropical Medicine). Antibody has clumped budded viruses (lower electron micrograph).

TABLE 5.13.
Proposed serological classification of viruses of family Bunyaviridae, genus *Bunyavirus*

Anopheles A Group
- Anopheles A
  - CoAr 3624
  - ColAn 57389
  - Las Maloyas
  - Lukuni
  - Trombetas
- Tacaiuma
    - II-32580
    - SPAr 2317 (Virgin River)
  - CoAr 1071 (CoAr 3627)

Anopheles B Group
- Anopheles B
  - Boraceia

Bunyamwera Group
- Bunyamwera
  - Batai (Calovo)
  - Birao
  - Cache Valley (Tlacotalpan)
    - Maguari
    - Playas
    - Xingu
  - Germiston
  - Ilesha
  - Lokern
  - Northway
  - Santa Rosa
  - Shokwe
  - Tensaw
- Kairi
- Main Drain
- Wyeomyia
  - Anhembi
  - Macaua
  - Sororoca
  - Taiassui

Bwamba Group
- Bwamba
  - Pongola

C Group
- Caraparu
    - Caraparu
    - Ossa
  - Apeu
  - Vinces
  - Bruconha
- Madrid
- Marituba
    - Murutucu
    - Restan
  - Nepuyo
    - Gumbo Limbo
- Oriboca
    - Itaqui

California Group
- California encephalitis
    - Inkoo
    - La Crosse (snowshoe hare)
    - San Angelo
    - Tahyna
  - Melao
    - Keystone
    - Jamestown Canyon (South River Jerry Slough)
    - Serra do Navio
  - trivittatus
- Guaroa

Capim Group
- Capim
- Acara
  - Moriche
- Benevides
- BushBush
    - Benfica
  - Juan Diaz
  - Guajara

Gamboa Group
- Gamboa
  - Pueblo Viejo
- Alajuela
  - San Juan

Guama Group
- Guama
  - Ananindeua
  - Moju
  - Mahogany Hammock
- Bertioga
  - Cananeia
  - Guaratuba
  - Itimirim
  - Mirim
- Bimiti
- Catu
- Timboteua

Koongol Group
- Koongol
  - Wongal

Minatitlan Group
- Minatitlan
  - Palestina

Olifantsvlei Group
- Olifantsvlei
- Botambi

Patois Group
- Patois
  - Abras
  - Babahoyo
  - Shark River
- Zegla
  - Pahayokee

Simbu Group
- Simbu
- Akabane
  - Yaba-7
- Manzanilla
    - Ingwavuma
    - Inini
    - Mermet
  - Buttonwillow
- Nola
- Oropouche
  - Facey's Paddock
  - Utinga
  - Utive
- Sabo
  - Tinaroo
- Sathuperi (Douglas)
- Shamonda
  - Sango
    - Peaton
- Shuni
  - Aino (Kaikalur, Samford)
- Thimiri

Tete Group
- Tete
    - Bahig
    - Matruh
    - Tsuruse
- Batama

Turlock Group
- Turlock
  - Lednice
  - Umbre
- M'Poko
    - Yaba-1

Viruses are classified in three steps indicated by degrees of indentation – complex, virus, and subtype; viruses in parentheses are varieties. (D. Bishop, personal communication.)

tion to these 179 viruses there are more than 30 viruses that have not yet been assigned to a genus (Bishop et al., 1981).

Most of the bunyaviruses, as well as a few of the phleboviruses, have been isolated from mosquitoes. Some have also been shown to replicate in, and be transmitted by, mosquitoes. The nairoviruses and uukuviruses have come from ticks, while many of the phleboviruses have been obtained from phlebotomines.

5.11.1. Virus structure and replication of bunyaviruses

The genome complexity of bunyaviruses appears to be the sum of one L, one M and one S-RNA molecule (viz. segmental RNA genome), which can code for approx. 350K to 450K daltons of protein (assuming a coding ratio of RNA to protein of 10:1) (Table 5.14). The total M.W. of virus proteins (in virus particles and infected cells) is the sum of the major nucleocapsid protein, N (approx. M.W. = 20K to 25K), the two glycoproteins, G1 and G2 (approx. M.W. 150K to 160K, without regard to the contribution of carbohydrate to the apparent M.W.) and the minor nucleocapsid protein, L (approx. M.W. = 170K to 200K).

From genetic studies the nucleocapsid protein, N, appears to be a primary gene product of the S-RNA, which leaves for the other two RNA segments (i.e. L and M RNA) the coding functions for three proteins i.e. G1, G2 and L. This consideration implies either that one of these RNA species is first translated into a 'polyprotein' which is subsequently cleaved to yield functional proteins, or that one (or both) of the RNAs has internal initiation sites for translation. The latter hypothesis is unlikely since multiple initiation sites have not been found in mammalian messenger RNA. Messenger RNAs isolated from the polysomes of cells infected with

TABLE 5.14.
Properties of four virus families containing arboviruses

Family	RNA genome structure					virion-associated		Capsid symmetry
	Linear	Segmented	Single-stranded	Double-stranded	Polarity	RNA polyolymerase	Envelope	
Marburg/ Ebola viruses		?	X		–	?	Yes	Helical
Bunyaviridae		X	X		–	Yes	Yes	Helical
Arenaviridae		X	X					
					–	Yes	Yes	Helical
Togaviridae	X		X		+	No	Yes	Cubic
Reoviridae		X		X	+/–	Yes	No	Cubic
Rhabdoviridae	X		X		–	Yes	Yes	Helical

LUM (Lumbo) virus have the same size as the virion L, M, and S RNA species, which implies that each virus RNA is transcribed separately into complementary species. The mRNA species may code for the two glycoproteins, G1 and G2, and the L-RNA segment may code for the minor nucleocapsid protein, L. The presence of polymerase activity associated with each RNA segment and the finding of intracellular virus complementary RNA molecules makes it likely that each segment of this negative stranded virus is replicated separately. The viruses are unique in their site of maturation viz. Golgi apparatus.

Few preventative measures have been developed against these viruses, although certain antivirals may have promise (see below).

5.11.2. Epidemiology and control of bunyavirus infection

At present a considerable amount of effort is directed towards an understanding of the epidemiology of this complex group of viruses. Recombination among viruses of a certain serotype can easily be demonstrated in the laboratory in a way very similar to that observed with the segmented genome virus influenza (Chapter 7). However, whether recombination occurs naturally in the field with bunyaviruses is not known. Certainly, different serotypes seem to be restricted to particular continents. Little attention has been paid to date to vaccine development but some work is proceeding with chemoprophylactic agents (e.g. versus Rift Valley Fever) as detailed below. La Crosse and LAC virus are two additional causes of human disease.

Korean haemorrhagic fever virus is recognized to cause outbreaks of haemorrhagic fever with renal syndrome in the Soviet Union, Scandinavia and much of Asia and, more recently a study has shown that the virus infected laboratory rats and laboratory workers in Belgium (Desmyter et al., 1983). It is believed that the virus is maintained in wild rats, mice and voles and is thereby transmitted to humans. A prototype of the group is Hantaan virus and Schmaljohn et al. (1983) have published the first biochemical details of the virus, which is probably a bunyavirus (see Table 5.16). Thus, the sedimentation properties of Hantaan virions resemble those of Bunyaviridae and, moreover, three distinct nucleocapsid species and three RNA species (27, 1.2 and 0.6×10^6) are present in Hantaan virus.

5.12. Arenaviruses

The arenaviruses are primarily agents causing acute or persistent infections in rodents, although some of them can cause severe haemorrhagic disease in man. The prototype, lymphocytic choriomeningitis (LCM) virus, is distributed almost worldwide (Table 5.15), but, of the remainder, the Tacaribe complex is confined to the New World (mainly South America), Lassa virus is found in West Africa, and viruses closely related to it have been isolated in Mozambique and Zimbabwe (Rawls

and Leung, 1979). All these 13 viruses are related antigenically by virtue of the crossreacting nucleoprotein but are distinguished easily by neutralization tests. Therefore a common protective vaccine is unlikely to be developed.

5.12.1. Virus structure and replication of arenaviruses

These enveloped viruses contain a single-stranded RNA genome of total M.W. 4×10^6 in two segments of negative polarity (Vezza et al., 1978a,b). The major structural proteins of the viruses comprise a nucleocapsid protein (N) and, in most cases, two glycoproteins (G1 and G2) which are components of the virus envelope, although in some viruses only a single glycoprotein has been found (reviewed by Howard and Simpson, 1980, Compans et al., 1981, Pederson, 1979, Young et al., 1981). Other minor proteins have been found in purified arenavirus preparations, but their status as true components of the virion is at present unclear (Fig. 5.5). In addition, an RNA polymerase activity, which would be expected in negative-strand RNA virus (see Chapter 2), has been found in purified preparations of Pichinde virus, as well as a protein kinase activity in purified LCM virus (Fig. 5.6).

A further virus-specific protein has been found in immunoprecipitates from cells

TABLE 5.15.
The arenaviridae

Virus	Distribution	Principal vertebrate host
Old world species:		
LCM	Worldwide	*Mus Musculus*
Lassa	West Africa	*Mastomys natalensis*
Mobala	Central African Republic	*Praomys jacksonii*
Mopeia	Mozambique	*Mastomys natalensis*
New world species: Tacaribe complex		
Amapari	Brazil	*Oryzomys goeldi*
		Neacomys guianae
Flexal	Brazil	*Oryzomys* species
Junin	Argentina	*Calomys laucha*
		Calomys musculinus
		Akodon azarae
Latino	Bolivia	*Calomys callosus*
Machupo	Bolivia	*Calomys callosus*
Parana	Paraguay	*Oryzomys buccinatus*
Pichinde	Colombia	*Oryzomys albigularis*
		Thomasomys fuscatus
Tacaribe	Trinidad	*Artibeus lituratus*
		Artibeus jamaicensis
Tamiami	Florida	*Sigmodon hispidus*

infected with LCM, Tacaribe or Pichinde viruses namely a glycoprotein of M.W. 70K to 79K which appears to be processed by proteolytic cleavage and carbohydrate modification to give the mature envelope glycoproteins.

One of the most notorious members of the group, from the point of view of human infection, is Lassa virus. It is of some interest, therefore, to see if this virus is a typical member of the group because, if this is proven to be the case, then further detailed work can be carried out on less pathogenic members, and the data extrapolated with some confidence to Lassa virus itself. In such a study Clegg and

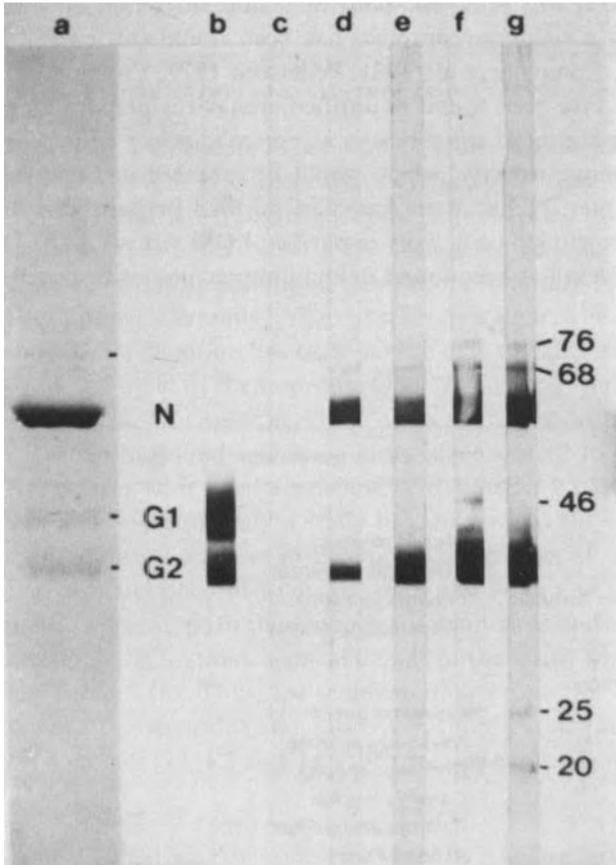


Fig. 5.5. SDS-gel electrophoresis of proteins of purified Lassa virus (after Clegg and Lloyd, 1983). Purified virus was disrupted in 1% SDS, 1% 2-mercaptoethanol and analyzed on 8 to 15% polyacrylamide gradient gels 0.75 mm (lane *a*) or 1.5 mm (lanes *b* to *g*) thick. (*a*) 8 μg protein in an 8 mm slot stained with Kenacid blue. Lanes *b* to *g* were strips cut from a 3.5 cm slot containing 15 μg protein after transfer to nitrocellulose: (*b*) incubated with concanavalin A; *c* to *g* incubated 4 h with (*c*) normal guinea-pig serum s/100 dilution, (*d*) guinea-pig serum after one injection of Lassa virus, 1/100 dilution, (*e*) guinea-pig serum after two injections of Lassa virus, 1/100 dilution, (*f*) guinea-pig serum after two injections of Lassa virus, 1/50 dilution, (*g*) human serum from a Lassa fever patient, 1/100 dilution.

Lloyd (1983) purified Lassa virus from culture fluids of infected CV-1 monkey kidney cells and analyzed structural proteins by polyacrylamide gel electrophoresis. Stained gels (Fig. 5.5) showed a typical arenavirus profile, with a prominent protein of M.W. 60K, corresponding to the nucleocapsid protein N, and two faint broad bands with M.W. of 45K and 38K, the envelope glycoproteins G1 and G2. G1 and G2 were shown to be glycosylated by their ability to bind concanavalin A. When immunoblots of proteins of infected cells which had been lysed in SDS were probed with anti-Lassa virus serum or stained for glycoproteins, four virus-specific bands were apparent: the N, G1 and G2 proteins seen in purified virus, and a glycoprotein of M.W. 72K which probably corresponds to the envelope protein precursor seen in other arenavirus systems. Immunoprecipitates from infected CV-1 cells labelled with (^{35}S)-methionine contained three major virus-specific proteins: the nucleocapsid protein N and proteins of M.W. 36K and 24K (designated fN1 and fN2). Similar immunoprecipitates from Vero cells contained fN1 and fN2 and only very low levels of N. The polypeptides fN1 and fN2 are most probably fragments of N.

5.12.2. Control of arenavirus infections

Only a very few studies have been published of any attempts to develop vaccines against these viruses (Johnson, 1975, Kiley et al., 1979). Genetic engineering techniques could be usefully applied in the future. As regards chemotherapy, anecdotal reports have described the use of interferon and also ribavirin (see below) to treat cases of Lassa Fever, and such studies could usefully be extended. A summary of characteristics of these infections relevant to the development of strategies for control is given in Table 5.16.

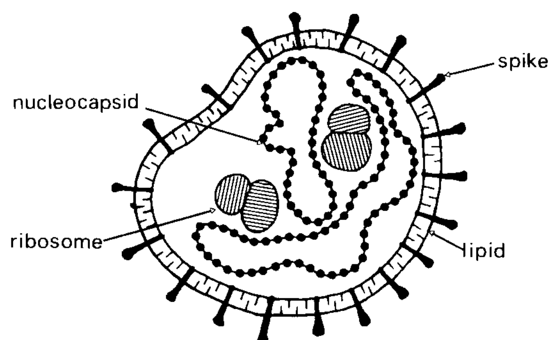


Fig. 5.6. Arenavirus structure. The nucleocapsid is depicted in the form of two circular strands corresponding to the L and S RNA segments, in association with the N and P proteins. Ribosomes are usually present in the virion and give a 'sandy' appearance to sections of the virus; hence the derivation of the name from *Arenosus* (sand). The glycoprotein spikes are composed of either 1 or 2 glycosylated polypeptides; their arrangement and the extent of their penetration through the viral lipid layer are uncertain.

TABLE 5.16.
Clinical and epidemiological characteristics of viral haemorrhagic fevers

Characteristic	Lassa fever	Ebola haemorrhagic fever	Marburg virus disease	Crimean-Congo haemorrhagic fever
Endemic areas	West Africa (Guinea to Central Africa)	East Africa (Zaire, Sudan, Central African Republic, Kenya)	East Africa, South Africa	Eastern Europe, Asia, Africa
Aetiological-agent classification	Arenaviridae	Filoviridae (proposed)	Filoviridae (proposed)	Bunyaviridae
Reservoir in nature	Rodents *(Mastomys natalensis)*	?	?	Ticks (*Hyalomma* genus and others), wild and domesticated mammals
Modes of transmission	Rodent-to-human (virus excreted in urine); person-to-person	Person-to-person	Person-to-person	Person-to-person
Incubation period	6–21 days	2–21 days	3–9 days	3–6 days
Symptoms	**% of cases**	**% of cases**	**% of cases**	**% of cases**
Headache	50–75	75–100	75–100	75–100
Myalgia	25–50	75–100	50–75	50–75
Sore throat	75–100	75–100	50–75	25–50
Cough	50–75	25–50	5–25	25–50
Dysphagia	5–25	5–25	25–50	
Vomiting	75–100	50–75	75–100	75–100
Diarrhoea	25–50	75–100	75–100	25–50
Chest pain	25–50	50–75	5–25	5–25
Abdominal pain	50–75	75–100	5–25	75–100

5.12.3. Clinical syndrome and epidemiological characteristics

One of the relatively well characterized viruses of the group, and certainly the most notorious, is Lassa fever (Table 5.16).

Lassa fever first came to medical attention in 1969 when three nurses working in missionary hospitals in Nigeria became ill. Two died in Nigeria, and the third patient, who was transported to the United States while still ill, survived. Two persons who worked in the laboratory where virological studies were being done also became ill; one had worked with tissue cultures and infected mice, while the other had no known contact with the virus. Lassa fever has been shown to be endemic in many areas of West and Central Africa. The reservoir of infection, is the multimammate rat *Mastomys natalensis*. This rodent inhabits rural areas in sub-Saharan Africa and lives in and around human dwellings. Persons presumably acquire nat-

urally occurring infections by contact with *M. natalensis*, either through handling the animal directly or by inhaling aerosolized excretions, such as urine. Subsequently, person-to-person transmission may occur within households and hospitals. Although airborne transmission may occur, direct contact with a patient or overt exposure to infective tissues, secretions, or excretions is probably necessary to transmit the infection from person to person.

The incubation period of Lassa fever ranges from 6 to 21 days. Illness usually begins with fever, headache, myalgia, sore throat, and cough; chest and abdominal pain are also frequent complaints. In severe cases encephalopathy, haemorrhage, and shock may occur.

Treatment of Lassa fever is supportive and includes restoration of blood losses and maintenance of plasma volume, blood pressure, and electrolyte balance. Although immune plasma obtained from survivors of the disease has been used in severe cases, there are no data to confirm its efficacy. Preliminary data suggest that ribavirin may be useful in the early stage of the illness (see below). No Lassa fever vaccine is available.

5.13. Marburg and Ebola viruses

Marburg and Ebola viruses are recently isolated agents that can cause severe, often fatal, fevers in man (Martini and Siegert, 1971). There is general similarity between Marburg-Ebola and rhabdoviruses (Pattyn, 1978). However, the length of infectious virions, the diameter of the central virus core, and the size of the surface glycoproteins of Ebola and Marburg viruses are all different from any known rhabdovirus. Long flexious particles of varying lengths, toroids, and simple rods have been visualized in electronmicrographs of these former viruses. Viral infectivity of both viruses co-sediments with discrete particle populations which have sedimentation coefficients of approximately 1300–1400 S in rate-zonal gradients. The viral structures in these populations are bacilliform in outline, and have average lengths of 790 nm (Marburg virus) and 970 nm (Ebola virus). Particles of both viruses frequently exhibit asymmetries exemplified by one nearly hemispherical end and one blunt or ragged end. The virion structures have narrow axial cores approximately 20 nm in diameter and have striations perpendicular to the long axis of the particle with a periodicity of approximately 5 nm; both of these structures are visible in only some virus preparations (Regnery et al., 1981).

The sensitivity of Ebola genome nucleic acid to ribonuclease established that this moiety is single-stranded RNA. Electrophoresis of glyoxal denatured virion RNA demonstrated that the molecular weights of Ebola and Marburg genome RNAs were approximately 4.2×10^6. The isolated virion RNA of Ebola virus did not bind to oligo(dT)-cellulose and was non-infectious, suggesting that the genome is a negative strand RNA (see Chapter 3 for details of replication of typical negative-stranded viruses).

5.13.1. Control of Marburg and Ebola virus infections

To date very little work has been directed towards the development of vaccines or antiviral compounds partly because of the restricted geographical nature of the infections and partly because of the biohazards created by working with these viruses. Therefore they are excellent candidate viruses for vaccine development using modern genetic cloning techniques in prokaryotes.

5.13.2. Clinical syndrome and epidemiological characteristics

Serological studies suggest that Ebola fever is endemic in limited areas of Sudan and Zaire, as well as the Central African Republic and Kenya. Both the reservoir of the virus in nature and the source of human infection remain unknown.

Ebola haemorrhagic fever came to widespread attention in 1976 when successive outbreaks occurred in Sudan and Zaire, comprising over 500 cases. The Sudan outbreak involved workers at a cotton factory, with subsequent spread in a hospital. Hospital transmission was associated with direct patient contact, and particularly with nursing a patient. The Zaire outbreak centred around an outpatient department; contaminated needles were involved in disseminating infection in nearly half the cases. The case-fatality rates in these two outbreaks were 53% and 88%, respectively.

The case-to-infection ratio of Ebola fever is unknown, but serologic studies suggest that mild or inapparent infection may be common in areas with endemic disease. Person-to-person transmission in medical units may result in a higher case-to-infection ratio.

The average incubation period of Ebola fever is estimated to be 6–9 days, with a range of 2–21 days. Ebola illness begins with sudden onset of fever, accompanied by headache, myalgia, sore throat, abdominal pain, and diarrhoea. A maculopapular skin rash is commonly seen in fair-skinned patients. Haemorrhage, usually from the gastrointestinal tract, is very common. The diagnosis can be made serologically by immunofluorescence or, preferably, by isolation of Ebola virus from the blood in the acute phase of illness. As with Lassa fever, the diagnosis of Ebola fever is unlikely if virus is not isolated from blood obtained during the first 7 days of illness, or if antibody is not present by the 14th day of illness (Table 5.16).

Treatment of Ebola illness is supportive. Immune plasma may be effective in reducing the level of viraemia but controlled studies to evaluate its effect on the outcome of illness have not been done.

Marburg virus disease first came to medical attention in 1967 when 31 persons became ill in Europe following the importation of a group of African green monkeys from Uganda. Twenty-five of these patients were exposed directly to tissues from the monkeys. Six secondary cases occurred, all in persons who had direct contact with patients or their tissues. Despite intensive investigation of these outbreaks,

no natural reservoir of the Marburg virus has been identified, and the area of endemicity has not been well defined. Morphologically, Marburg virus resembles the Ebola agent, but it is antigenically distinct.

Person-to-person transmission of Marburg disease has occurred in three of the four outbreaks that have been investigated. In each of these situations, transmission resulted from direct contact with an infected animal, an infected human, or infected tissues; there has been no evidence of airborne person-to-person transmission. The case-to-infection ratio of Marburg disease is unknown, but the case-fatality rate in the reported outbreaks has been 26%.

After an incubation period of 3–9 days, Marburg disease begins with fever, headache, myalgia, sore throat, dysphagia, vomiting, and diarrhoea. A maculopapular skin rash is extremely common. Haemorrhage, usually from the gastro-intestinal tract, is a frequent finding, and disseminated intravascular coagulation has been implicated in its pathogenesis. Diagnosis is made by immunofluorescent testing of serum specimens or by isolation of the virus from blood.

Treatment of Marburg virus disease is supportive. Immune plasma has been used, but its efficacy is unknown.

Since the original Marburg disease outbreak, there have been no known cases of Marburg disease, either imported or laboratory acquired, in Europe or the United States.

5.14. Chemoprophylaxis of arbovirus infections (including bunya and arenaviruses)

Because of the extreme diversity and multiplicity of viruses in this group (we include here togaviruses, arenaviruses, bunyaviruses), and a dearth of vaccines, new and effective antivirals are urgently required. However, two particular problems, alluded to in the first chapter of the book, are the difficulties in working with this group of infective agents (extensive use of safety cabinets, primate animal models often needed etc.) and the low return in potential sales because countries most affected are in the developing areas of the world. However, an antiviral drug development programme has been initiated at the US Army Medical Research Institute of Infectious Diseases of Fort Detrick. The laboratory has biohazard containment facilities at the P-3 and P-4 level, beyond the capabilities of most university and pharmaceutical companies. Initially the viruses used in the tissue culture screen for new antivirals are Venezuelan equine encephalitis and yellow fever (Togaviruses), pichinde (arenavirus) and Rift Valley Fever (a bunyavirus). Already some interesting studies with ribavirin have been published.

An overall summary of the antiviral effects of ribavirin is shown in Table 5.17. Especially in the alpha and flavivirus groups, in vitro data did not correlate with in vivo data (Stephen et al., 1980) but this could be explained by the lack of high concentrations of ribavirin in the CNS in some in vivo model systems. In more de-

TABLE 5.17.
Antiviral activity of ribavirin (after Stephen et al., 1980)

Virus group	In vitro[a]			In vivo	
	Virus	5–25 μg	100 μg	Rodents	Monkeys
Alpha-	VEE	+	+	− (mice)	Not done
	CHIK	+		No model	−
Flavi-	JE	+		− (mice)	Not done
	YF	+		− (mice)	±
	DEN-1	+		No model	Not done
	DEN-2	+		No model	Not done
Arena-	PIC	+		+ (MHA hamsters) + (guinea pigs)	No model
	MAC	+		+ (guinea pigs)	+
	LAS	+		+ (guinea pigs)	+
Bunya-	RVF	+	+	+ (mice)	+
	SFS	+		No model	No model

[a] Ribavirin concentration in μg/ml.

tail, Table 5.18 shows the effect of treatment of hamsters infected with the arenavirus, pichinde virus. Treatment could be delayed up to 4 days after infection with some delay of death and increased survival compared to the untreated group. Ribavirin or ribavirin triacetate also prevented death in monkeys infected with the arenavirus, Bolivian haemorrhagic fever virus (MAC) (Table 5.19).

TABLE 5.18.
Effect of ribavirin on survival of hamsters inoculated with arenavirus (PIC) (10000 PFU, s.c.) (after Stephen et al., 1980)

Days after inoculation	% Survival		
	Virus only (n=27)	Ribavirin (15 mg/kg, s.c. 2×daily)	
		Days 0–14 (n=20)	Days 4–14 (n=10)
7	100	100	100
8	95	100	100
9	60	100	100
10	54	100	100
11	38	100	90
12	34	100	60
13	25	100	50
14	20	100	50
15	11	100	50
21	11	100	50

TABLE 5.19.
Effect of treatment on survival and time to death of arenavirus (MAC) infected monkeys (n=4/group) (after Stephen et al., 1980)

Study number	Group	% Survival		MTD (days±SE)
		Day 35	Day 90	
1	Saline	0	0	26± 3
	Ribavirin triacetate			
	10 mg/kg/injection	100	25	71±17[a]
	20 mg/kg/injection	100	0	63+15[a]
2	Saline	0	0	23± 4
	Ribavirin	100	25	43± 6
3	Saline	25	0	36±21
	Ribavirin	100	25	39± 2[a]

[a] Sacrificed when paralyzed.

A series of didemnins, a class of depsipeptides isolated from Caribbean tunicates have been investigated for anti-arbovirus activity (Canonico et al., 1982). The antiviral activity of didemnins A and B was studied in vitro with a plaque reduction assay on Vero-76 cells for RVF, Venezuelan equine encephalomyelitis virus (Trinidad donkey), and pichinde virus and, on LLC-MK_2 cells, for yellow fever virus (Asibi).

Didemnins A and B were both found to exhibit significant activity against RVF (median inhibition dose (ID_{50}) for didemnins A and B was 1.37 and 0.04 μg/ml, respectively), Venezuelan equine encephalomyelitis virus (ID_{50}, 0.43 and 0.08 μg/ml, respectively), and yellow fever virus (ID_{50}, 0.4 and 0.08 μg/ml), respectively). A concentration of 0.1 μg of didemnin B per ml inhibited plaque formation by these three viruses by more than 80%. Didemnin A, on the other hand, was less efficient, requiring a 25-fold increase in concentration to achieve the same level of virus plaque inhibition. As compared with the other three test viruses, pichinde virus (ID_{50} for didemnins A and B was 2.9 and 0.22 μg/ml, respectively), a representative arenavirus, was less sensitive to the in vitro antiviral effect of both didemnins.

Studies were also conducted with RVF-infected mice. Treatment with didemnin A at a dose of 1.25 to 5 mg/kg per day resulted in 50% survival for mice challenged with RVF (Table 5.21). Higher dosages given to inoculated, but not to control mice, appeared to be toxic. Treatment of RVF-infected mice with didemnin B (0.25 mg/kg per day) resulted in a 90% survival rate, although some drug-related deaths were observed. There were no drug-related deaths when the dose was lowered to 0.20 mg/kg, although at this dose the survival rate was decreased to 40%. Didemnin B was toxic and uniformly lethal to mice when administered at 1.0 mg/kg per day for 5 days. These studies therefore represent only a beginning to the search for anti-

TABLE 5.20.
Relative interferon sensitivity of RNA viruses (from Canonico, P., personal communication, 1983)

Virus	Assay cell line	Interferon sensitivity[a]		
		Alpha	Beta	Gamma
Vesicular stomatitis	MRC-5	1	2	1
Togaviridae				
Alphavirus				
Eastern equine encephalitis	MRC-5		2.5	0.2
Western equine encephalitis	MRC-5		2.5	
Sindbis	MRC-5		3.2	
Venezuelan equine encephalitis	MRC-5	0.2	0.5	
O'nyong-nyong	MRC-5		1	
Mayaro			1	
Chikungunya			2.5	
Flavivirus				
West Nile	LLC-MK-2		0.1	
Yellow fever	LLC-MK2		0.004	
Japanese encephalitis	LLC-MK2		10	
St. Louis encephalitis	VERO		2.5	
Langat	LLC-MK2		0.6	
Dengue type 1	LLC-MK2		0.03	
Dengue type 2	LLC-MK2		0.01	
Unclassified				
Ebola	VERO	0.001		
Bunyaviridae				
Oropouche	MRC-5		10	
California encephalitis (La Cross)	MRC-5		0.5	
Rift valley fever	MRC-5		3	0.001
Korean haemorrhagic fever	VERO-E6	0.1	0.3	0.001
Congo-Crimean haemorrhagic fever	MRC-5		1.3	
Arenaviridae				
Lymphocytic choriomeningitis	BSC-1		0.03	
Lassa	VERO		0.0003	
Pichinde	BSC-1	0.003	0.001	
Machupo (BHF)	BSC-1		0.02	
Junin (AHF)	BSC-1		0.13	

[a] Concentration of IFN causing a 50% plaque reduction with VSV / Concentration of IFN causing a 50% plaque reduction with virus.

virals against this important group of viruses. Guanidine inhibits certain togaviruses (Chapter 4, Table 26) and benzimidazoles inhibit arenaviruses in vitro (Chapter 4, Table 25).

TABLE 5.21.
Survival of Rift Valley Fever (bunyavirus) infected mice treated with didemnin A or B[a] (after Canonico et al., 1982)

Mouse group	Treatment regimen		No. of 21-day survivors	Mean no. of days until death (±SD)
	Didemnin	Daily dose (mg/kg)		
Inoculated	None	0	0	3.8±0.8
Inoculated	A	1.25	5	3.8±0.9
		5.0	5	6.0±1.4
		10.0	0	3.7±0.8
Control	A	1.25	10	
		10.0	10	
Inoculated	B	0.1	0	6.2±4.2
		0.15	1	7.0±2.0
		0.2	4	7.0±2.0
		0.25	9	6.0
		1.0	0	2.8±0.6
Control	B	0.2	10	
		0.25	8	10.0±5.7
		1.0	0	2.5±0.6

[a] Didemnins, dissolved in ethanol and reconstituted with Hanks balanced salt solution buffered to pH 7.2 with 10 mM HEPES (4% ethanol, final concentration), were given daily beginning on day −1 for 5 consecutive days.
[b] Ten mice per group, inoculated subcutaneously with 250 to 350 PFU of RVF.

Finally, given the very varied biological and antigenic nature of these viruses it might seem an appropriate group for attack using interferon. Alphaviruses, at least in vitro, are quite sensitive to IFN-β, whereas the flaviviruses and arenaviruses are relatively insensitive. Certain of the bunyaviruses are sensitive, such as Rift Valley Fever (see Table 5.20).

5.15. Summary

This 'arbovirus' group is composed of such a vast array of viruses of diverse biochemical and antigenic structure that little hope is extended for major developments for prevention in the near future. Yellow fever can be prevented using the 'old' empirically selected vaccine, but little progress has been made with vaccines against other flaviviruses or alphaviruses (with the exception of tick borne encephalitis and Japanese encephalitis). A particularly unexplored area is the arenavirus and bunyavirus groups of viruses. Chemoprophylaxis could be investigated more intensively than at present with the proviso that in vivo experimental investigation is limited

in some cases by the infectivity of the viruses for man. A modest start has been made with the nucleoside analogue ribavirin.

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CHAPTER 6

Rhabdovirus infections

6.1. Rabies (Hydrophobia)

When fully developed clinically, rabies presents as one of the most dramatic illnesses of humans (Bhatt et al., 1974, Christie, 1980). The disease has been recognized since the dawn of history and references appear in the Eshnunna Code before 2300 BC. Celsius first described hydrophobia as such in 100 AD and recommended cautery of animal bites with a hot iron and this remained the treatment of rabid animal bites until 1884–1885 when Pasteur introduced his famous rabies vaccine (Pasteur et al., 1884, 1885).

Rabies virus belongs to the rhabdovirus (Greek Rhabdos: a rod) family of characteristically bullet-shaped viruses and shares a position here with 60 other animal and insect viruses and 16 plant viruses (Crick et al., 1981). Together these viruses constitute the genus Lyssavirus (Lyssa = madness).

Five viruses related to rabies occur in Africa (Shope, 1982). Obodhiang virus was isolated in Sudan and Kotonkan virus in Nigeria from insects and are only distantly related to rabies but three further viruses are more closely related, namely Mokola isolated from shrews in Nigeria, Lagos bat virus isolated from fruit bats in Nigeria and Duvenhage virus isolated from the brain of a man bitten by a bat in South Africa. These latter 3 viruses and rabies virus itself are classified together as the genus Lyssavirus. There has now been a clear demonstration of epizootic spread of rabies-related viruses in domestic animals in Zimbabwe and these viruses were isolated from rabid cats and a rabid dog. The isolates were distinct from rabies virus by neutralization tests and these viruses could pose a serious problem in Africa, since rabies vaccine do not always protect. Minor antigenic differences are clearly

recognized among rabies viruses (Wiktor and Clark, 1973, Flamand et al., 1980, Wiktor and Koprowski, 1980) although a *potent* rabies vaccine will still protect against challenge with these minor variants. However, the rabies-related viruses noted above are even more distantly related antigenically. Shope (1982) has suggested that since Africa is the site of the greatest antigenic diversity of rabies, the virus may have evolved originally in this continent and may have moved with man from Africa in the form of infected domestic animals. The long incubation period of rabies makes this a plausible event despite lengthy ship voyages to the New World.

6.2. The virus and mode of replication

Analyses of members of the *Vesiculovirus* genus of rhabdoviruses (for example, vesicular stomatitis virus, VSV, Indiana serotype) have established that they have three major polypeptides, a glycoprotein, G, a nucleocapsid protein, N, and a matrix (or membrane) protein, M (McSharry, 1979). In addition, there are two minor virion polypeptides, a large polypeptide, L, and a phosphoprotein, NS, which are transcriptase components (Kawai, 1977).

These viruses contain a single-stranded RNA genome whose nucleotide sequence is complementary to that of the mRNA (negative strand viruses (see Chapter 3)). Thus, viruses in this class contain a virion associated transcriptase (the L protein) which is responsible for the production of virus mRNA in infected cells. Although the rhabdoviruses look very different from paramyxoviruses (Fig. 6.1) they show considerable structural and replicative similarities. Both groups of viruses have a central nucleoprotein and an outer lipoprotein envelope, in which several glycoproteins are embedded (Dietzschold et al., 1983) (Table 6.1). Between the nucleoprotein and the envelope lies a matrix protein.

RNA of the rhabdoviruses sediments at 40 S and has a molecular weight of 3.8×10^6. In cells infected with VSV several new RNA species are found. These are 40 S RNA, (both plus- and minus-strands being found), a number of low molecular weight RNAs of about 13–15 S, and other species with S values in the 20–30 S range (Fig. 6.2). The low molecular weight RNAs contain three major components of molecular weight approximately $0.75–0.35 \times 10^6$, thus corresponding well with the molecular weight of the three most abundant viral polypeptides (0.69, 0.50 and 0.29×10^5). Indeed, these RNAs have been translated in vitro to produce the expected polypeptides.

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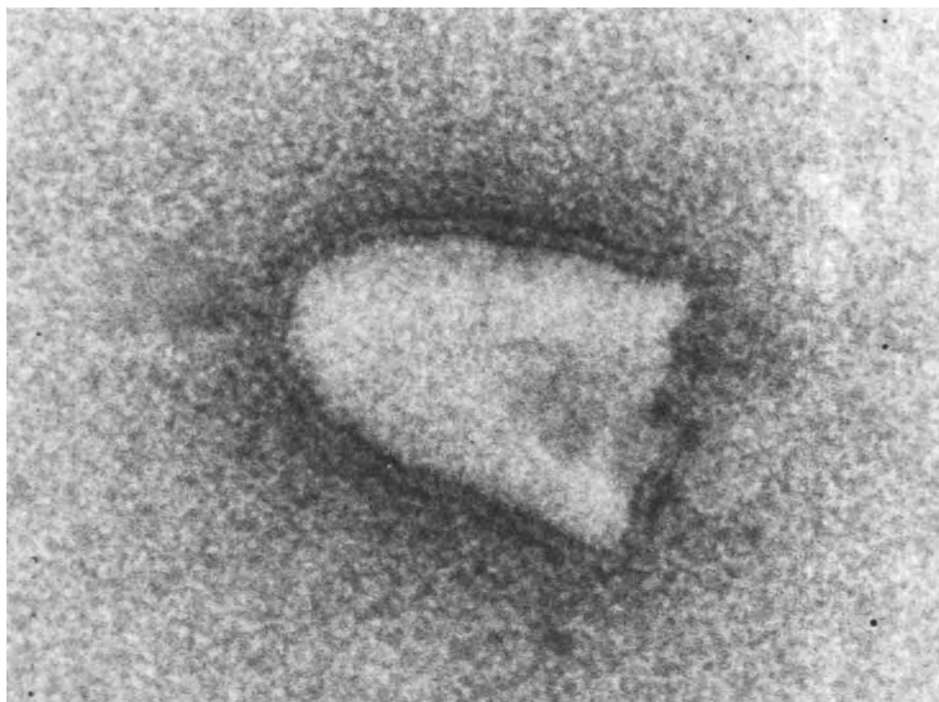
Fig. 6.1. Electron micrograph of rabies virus (courtesy of Dr. D. Hockley).



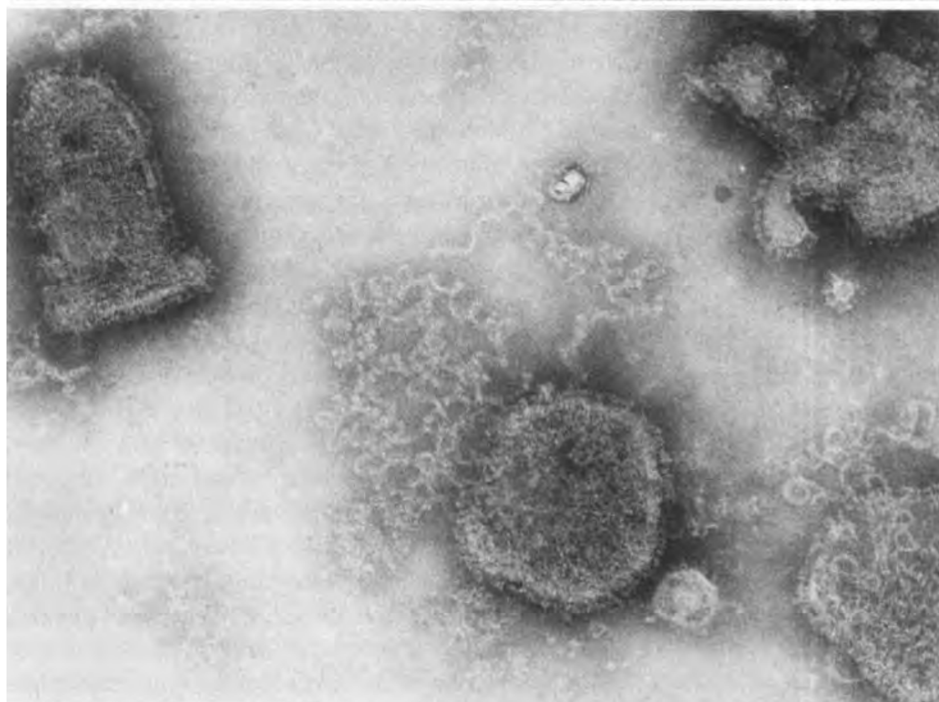


TABLE 6.1.
Rabies virus components

Component (symbol)	Molecular weight	Location	Function
RNA	3.8×10^6	Core	Genetic information
Nucleocapsid proteins (N)	54 000–62 000	Core	Associated with RNA
L protein (L)	170 000–200 000	Core	RNA-directed RNA polymerase
Membrane protein (1/NS)	37 000–40 000	Core	Attaches to nucleocapsid protein and to lipid in envelope
Lipid	?	Envelope	1% of virus
Membrane protein 2 (M)	21 000–25 000	Envelope	Structural protein of membrane
Glycoproteins (G)	65 000–80 000	Spikes on surface of envelope	Induces production of neutralizing antibodies. Critical protein for virus vaccines.

6.3. Epidemiology

Rabies is primarily a disease of mammals (Steck, 1982, Kaplan and Koprowski, 1980) and several wild animal species act as a natural reservoir of the virus, including skunks, foxes, racoons, bats, mongooses and wolves (Baer, 1975). Human infection with rabies virus is nearly always secondary to bites, although it is possible to infect laboratory personnel when virus is aerosolized and exists in high concentration. In most parts of the world a major reservoir of the virus for man is the domestic dog and cat (Fig. 6.3) but in some areas of the world such as the UK no animal reservoir exists. About 700 rabies deaths are *reported* each year worldwide, but this must represent only a small fraction of the actual number of cases. About 40% of human cases occur in children aged 5–14 years, although all age groups are

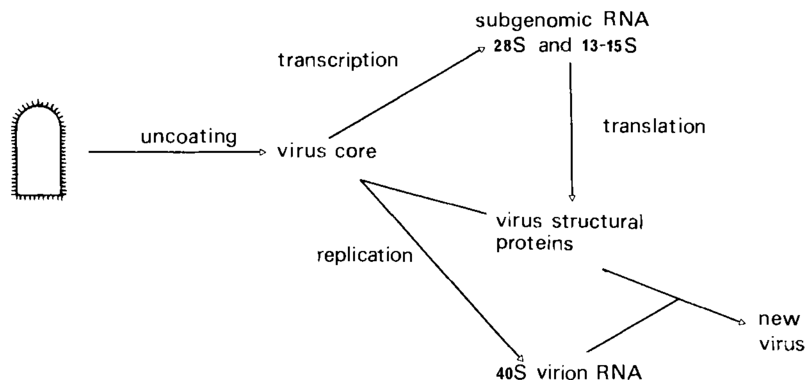


Fig. 6.2. A scheme for the multiplication of the rhabdovirus VSV.

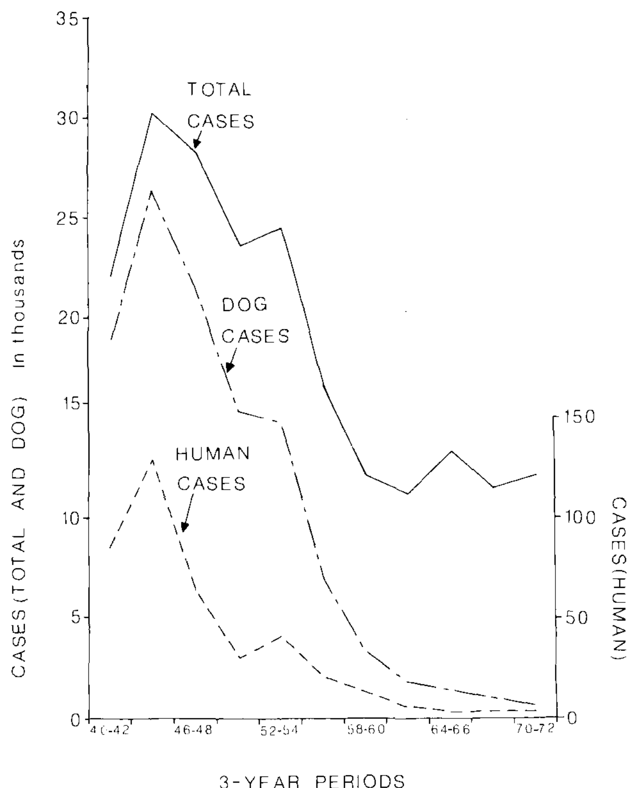


Fig. 6.3. Rabies cases by 3 year periods, United States, 1940–1972 (after Harwick and Gregg, 1975).

susceptible. The majority of cases are male, presumably because of greater contact with infected animals. The epidemiology of the 4 rabies related viruses is not clear and only Mokola virus is considered to cause aseptic meningitis in humans.

Certain areas of the world are rabies free, such as the UK, Norway, Sweden, Australia, New Zealand, Japan, Antarctica, Hong Kong, Singapore and many islands of the Pacific including Hawaii, Fiji, New Cadedonia and Samoa; but a rabies free area is certainly no guarantee against future problems since the virus is not excluded from any area by specific ecological factors (reviewed by Murphy, 1983). To date, rabies has never been eliminated from a complex multiple host ecosystem involving wildlife although it was eliminated from the UK in 1903 by controlling stray dogs. But there was no evidence of wild life rabies in the UK at this time. Murphy (1983) describes the costs and problems of a single introduction of a rabid dog in California, emphasizing the value of strict control in rabies free areas. Only three persons were actually bitten but 70 persons required post-exposure treatment (cost £45 000) and a stray dog control programme was initiated (cost £6000). In Amsterdam in 1962 an episode involving a primary dog case resulted in post exposure treatment

of 500 people and immunization of 650 000 pets. Where rabies is present a control programme is essential, involving control of animal movement (especially stray dogs), immunization of dogs and cats, public education, surveillance based on laboratory diagnosis and programmes to interrupt wild life transmission.

6.4. Pathogenesis and clinical picture

Following an animal bite, rabies virus is considered to reach the central nervous system in man by way of peripheral nerves (Murphy, 1983). Initial virus replication occurs in the striated muscle cells at the site of the bite. Deep in the muscles the peripheral nervous system is exposed in neuromuscular spindles. The rate of centripetal progress along the peripheral nerve has been estimated experimentally in mice as 3 mm per hour. It has been evident for many years by clinical observation that the incubation period of rabies is shorter when rabies virus enters via a bite in the neck compared to the leg. Thus incubation periods vary from 10 days to 2 years but average 1–3 months. Once the virus has replicated in the central nervous system it may spread centrifugally along the peripheral nerves to other tissues, and so reaches the salivary glands where it must replicate for further transmission to new individuals. This site is very productive sometimes producing 10^6 ID_{50}/ml of infective virus. In persons infected by the aerosol route (e.g. in bat inhabited caves and in the laboratory) it is assumed that the virus reaches the CNS via the upper respiratory tract or the conjunctiva. The olfactory end organ where neuroepithelial cells are exposed to the body surface and extend directly to the olfactory bulbs of the brain is easily infected, for example, by aerosols of virus.

The incubation period in human cases varies from 10 days to 2 years but an average would be 1–3 months depending to some extent on the quantity of virus deposited and the distance of the bite from the head (Bhatt et al., 1974, Shah and Jaswal, 1976). Although the slow course of virus movement to the CNS would appear to give ample opportunity for host immune responses to develop, this clearly does not occur to any helpful degree (Murphy, 1983). Presumably very little virus antigen is present in the early infected cells and once rabies replicates more extensively in the CNS the virus is safely sequestered in an immunologically privileged area. The onset of the disease is usually quiet, with 2–4 days of malaise, fever and headache and the first symptom to suggest an affected nervous system may be pain and tingling in the bitten limbs, including small jerky movements (Christie, 1980). The patient may then pass into the so called 'stage of excitement' with anxious and apprehensive expression, fast pulse and rapid breathing. Local paralysis can be noted at this stage especially in the muscles of the legs, face, throat leading to difficulty in swallowing or a choking sensation. This fear may be such as to induce terror at the mention of water, giving the name 'hydrophobia' to the disease. However, in many cases this hydrophobia may not be marked. The patients may die in a con-

vulsive state although often paralysis develops with cardiovascular collapse and coma. The average duration of the illness is less than a week. Rabies is invariably fatal although three well characterized cases have been recorded of persons surviving. Once the symptoms of the disease have appeared the only treatment is intensive care and the prognosis is almost hopeless (Hattwick and Gregg, 1975).

6.5. Person to person transmission of rabies

The first recorded case of person to person transmission occurred by a corneal transplant and because of a missed diagnosis of rabies (Houff et al., 1979). A male patient was admitted to hospital six days after onset of a progressively increasing paralysis and after a series of seizures he died on the 16th day and a cornea was given to a female patient who was discharged. Approximately one month later she was readmitted to the hospital with severe headaches and died 50 days after the transplant and rabies was diagnosed retrospectively. Three subsequent deaths have occurred but a fourth grafted patient was treated with antirabies globulin, human diploid cell vaccine and interferon and survived.

6.6. Immunization against rabies

A remarkable feature of immunization against this virus was that until very recently regimens had changed little from those originally proposed in the late 19th Century and so it is worthwhile to review the early work by Pasteur in some detail (Pasteur et al., 1881, 1884, Pasteur, 1885). Indeed the original method used by Pasteur was still used in Paris until the early 1950s. The technique also illustrates how viruses can be altered biologically in the laboratory without knowledge of biochemistry or immunology. The rabies virus strain which Pasteur employed for his now famous 'experiment' (Fig. 6.4) was isolated from the brain of a rabid cow in 1882. The virus was passaged 90 times intracerebrally in rabbits, by which time the incubation period had become shorter and 'fixed' or stabilized at 6–7 days. The spinal cords of infected rabbits were dried in air at room temperature and the virulence of virus-containing tissue suspensions was found to decrease rapidly with successive days of desiccation and was often lost completely by 15 days. In July 1885 a nine year old alsacian, Joseph Meister, was admitted with multiple and severe bite wounds from a presumed rabid dog. For the next 10 days Pasteur administered a course of 13 injections of rabies infected cord suspensions, the earliest preparation having been desiccated for 15 days, the subsequent ones for decreasing periods down to one day. Jospeh Meister survived. Pasteur noted "The death of this child seemed inevitable and I decided not without lively and cruel doubts, as one can believe, to try in Joseph Meister the method which has been successful in dogs". A year

Fig. 6.4. Pasteur in his laboratory (after Atanasiu, 1975).

later Pasteur reported the result of treatment of 350 cases and only one person developed rabies, a child bitten nearly 4 weeks before treatment commenced. (contemporary figures showed that 50% of those bitten should have developed rabies). "The prophylaxis of rabies is established. It is time to create a centre for vaccination against rabies". Within a decade there were Pasteur Institutes around the world and by 1898, for example, 20 166 persons had been treated, with a mortality of only 0.46%. Of course, no one knew about viruses at the time and since Pasteur was unable to cultivate bacteria from the rabbit spinal cord he concluded "one is tempted to believe that a microbe of infinite smallness having the form neither of bacillus nor a micrococcus is the cause".

Rabies vaccines derived from infected animal nervous tissues continue to be widely used today but it was recognized that neurological reactions in approximately one in every 1630 recipients limited the acceptability of the vaccines (Applebaum et al., 1953, Briggs and Brown, 1960, Crick and Brown, 1976, Held and Adaros, 1972, Trejos et al., 1974). In the 1950s a rabies vaccine grown in duck embryos and later in cells also became available and replaced nervous tissue vaccine in the USA and some European countries (Greenberg and Childress, 1960, Lavender and Van Frank, 1971, Peck et al., 1956). However, even these products induced side reactions (Rubin et al., 1973).

An extensive survey of efficacy of early rabies vaccines was carried out by McKendrick in 1940, who analyzed post-exposure treatment in 1 062 707 cases. The overall mortality in this group was only 0.33% and remarkably little difference was detected between the efficacy of the different vaccines used although they had been produced in many different laboratories throughout the world. He also noted that many persons must possess a high degree of natural resistance to rabies because the highest mortality rates noted were 6.78% in persons bitten by rabid wolves and where, for one reason or another immunization had been delayed for 14 days or more, at which time it would not be expected to be efficacious. However, in another very large study in Southern India much higher mortality rates were noted in bitten but untreated individuals (Table 6.2).

An early rabies vaccine of cell culture origin was developed in the USSR by Selimov et al. (1967, 1978) who adapted the SAD strain of 'fixed' rabies virus to Syrian hamster kidney cells and inactivated the virus with phenol.

Most recently a major advance has been the cultivation of rabies virus in human diploid WI-38 cells which appears to be a more potent product than other vaccines and considerably less reactogenic (Wiktor and Koprowski, 1965, Cabasso et al., 1974, Aoki et al., 1975, Bahmanyar, 1978, Bahmanyar et al., 1976, Cox and Schneider, 1976, Kuwert et al., 1976, 1977, 1978a, 1978b, Nicholson and Turner, 1978, Turner et al, 1982). This will probably become the vaccine of choice worldwide (but too expensive for certain third world countries), particularly for prophylactic use in high risk groups of people. (It should be noted that $1\frac{1}{2}$ million people are still immunized yearly throughout the world, often with Semple type brain derived vaccine.)

TABLE 6.2.
Deaths from rabies in exposed persons, treated and untreated, at the Pasteur Institute of Southern India, 1946–1968 (after Veeraraghavan, 1969)

Category	No. persons	No. deaths[a]
A. Persons bitten by dogs causing at least one human rabies infection		
Completely treated[b]	844	62 (7.35)
Incompletely treated	69	7 (13.43)
Untreated	215	122 (56.74)
B. All persons bitten by dogs proved or presumed to be rabid		
Completely treated	16 098	62 (0.38)
Incompletely treated	1323	9 (0.68)
Untreated	730	122 (16.70)

[a] Percent given in parentheses.
[b] Complete treatment consisted of 14 daily inoculations of Semple-type vaccine.

6.7. Historical events in the development of a rabies vaccine for humans

The milestones in the long history of progressive step by step improvement of rabies vaccines are summarized in Table 6.3 and virus strains in Table 6.4. In general, once the efficacy of the vaccine had been established the main problem was to reduce the neurological side reactions.

6.7.1. Nervous tissue rabies vaccines

Probably more nervous tissue rabies vaccines are still used worldwide than any oth-

TABLE 6.3.
Development of rabies vaccines for humans

Date	Originator	Technique
1884	L. Pasteur	Virus passage from brain of cow to rabbits reduced virulence. Desiccation also reduced virulence.
1908	Fermi	Partial inactivation of virus infected brain tissue with phenol.
1911	Semple	Modified phenol treatment leads to complete inactivation of virus. Prepared in brain of rabbit, sheep or goat. Most widely used rabies vaccine in developing countries.
1925	Hempt	Ether treatment of brain tissue prior to phenol.
1955	Fuenzalida and Palacios	Brain of suckling mice – less reactogenic vaccine. (Nervous tissue vaccine).
1956	Peck et al.	Duck embryo vaccine. Virus inactivated with β-propiolactone. Reduced neurological reactions.
1960	Fenje	Hamster kidney cell culture vaccine.
1964	Wiktor, Fernandes and Koprowski	Human diploid cell virus (HDCV) Virus infected cells subcultured 47 times followed by cell free passage of virus in WI-38 cells. Virus inactivated with β-propiolactone.
1967	Selimov et al.	Development of cell culture rabies vaccine.
1983	Fangtao et al.	Other more easily cultivable cells (hamster kidney) used instead of WI-38 which are fastidious in growth characteristics.

TABLE 6.4.
Rabies virus strains used for vaccine and antiserum production

Strain	Origin
Pasteur	Bovine-Europe 1882
Pitman-Moore (P-M)	
CVS	
Flury	Human-U.S.A. 1939
LEP	
HEP	
HEP 675	Plaque isolate from HEP

er type because of their potency, ease of manufacture and cheapness, although in developed countries human diploid cell vaccine is the preparation of choice. The most widely used is the Semple type vaccine (Semple, 1911) prepared from the brains of infected animals such as sheep, goats, rabbits or mice (Fuenzalida and Palacios, 1955), where the virus infected tissue is fully inactivated with phenol. The brains of very young animals are almost free from encephalitogenic factors and are used in a number of countries for the preparation of inactivated rabies vaccines. Thus, suckling mouse brain vaccine or suckling rat or rabbit brain vaccine have become the most widely produced rabies vaccine. They are used also for immunizing cats and dogs (Table 6.5).

6.7.2. Avian embryo rabies vaccines

Live virus vaccines prepared by growing rabies virus in chick or duck embryos are available (Greenberg and Childress, 1960, Schell et al., 1980) and two, Flury LEP and HEP vaccines are used as veterinary vaccines (Table 6.4, 6.5). The LEP (low egg passage) vaccine is used at the 40–50th egg passage level and is used in veterinary situations throughout the world, but may retain some pathogenicity for young puppies, cats and cattle. The Flury HEP vaccine is virus at the 180th passage level and is safe for puppies and cattle.

An advance in man was the use of β-propiolactone-inactivated rabies virus cultivated in duck embryos (Peck et al., 1956) and relatively high antibody levels to this vaccine are produced rapidly in humans.

6.7.3. Human diploid cell rabies vaccine

By the early 1960s the general trend towards the use of tissue cultures for viral vaccine production had increased and most new rabies vaccines since that date have utilized varieties of cultured cells. A significant breakthrough as noted above came with the use of human diploid WI-38 cells to cultivate the virus. The HEP attenuated strain of rabies virus was found to be 10 times more antigenic for animals after adaptation to WI-38 cells (Wiktor and Koprowski, 1965). Further technical developments and improvements included the use of ultrafiltration to concentrate the virus for vaccine (Wiktor et al., 1969) or the use of zinc acetate or ammonium sulphate (Schlumberger et al., 1970). A split product vaccine was tested with virions disrupted using tri-(n)-butyl phosphate and several different vaccines are in fact made.

In the best traditions of vaccine development (see also Yellow Fever Vaccine, Chapter 5) initial experiments were carried out on the originators of the vaccine! The vaccine was highly immunogenic and only two doses were required to produce a strong antibody response. Subsequent investigation of different dose schedules showed that the best responses were obtained when the first two doses of vaccine were given one week apart and a third dose on day 14 (reviewed by Plotkin, 1980).

In summary, with USA, German and French vaccine, 100% of vaccinees who received 3 or more doses of diploid cell vaccine sero-converted, and allergic reactions were few, with 7–15% of vaccinees showing local symptoms and 2–9% systemic symptoms. Importantly, combinations of diploid vaccine and human rabies im-

TABLE 6.5.
Some rabies vaccines marketed in United States for use in animals

Vaccine	Product name Marketed by	For use in	Dosage[a]	Age at primary vaccination[b]	Booster recommended
A) Modified live virus					
Canine cell line origin	ENDURALL-R			3 months and	
	Norden	Dogs	1 ml	1 yr later	Triennially
High egg passage		Cats	1 ml	3 months	Annually
		Dogs	1 ml	3 mos and 1 yr later	Triennially
Porcine cell line origin	ERA STRAIN	Cattle	1 ml	4 months	Annually
	RABIES VACCINE	Horses	1 ml	4 months	Annually
	Wellcome				
High cell passage	(Jensen-Salsbery)	Sheep	1 ml	4 months	Annually
		Goats	1 ml	4 months	Annually
Canine tissue culture origin	NEUROGEN-TC			3 mos and	
	Bio-Ceutic	Dogs	1 ml	1 yr later	Triennially
High cell passage					
Canine cell tissue culture origin	UNIRAB				
	Bio-Ceutic	Dogs	1 ml	3 months	Annually
High cell passage					
B) Inactivated					
Murine origin	TRIMUNE			3 mos and	
	Ft. Dodge	Dogs	1 ml	1 yr later	Triennially
		Cats	1 ml	3 months	Annually
Hamster cell line origin	RABCINE-FELINE				
	Beecham	Cats	1 ml	3 months	Annually
Porcine cell line origin	ENDURALL-K	Dogs	1 ml	3 months	Annually
	Norden	Cats	1 ml	3 months	Annually
Monkey cell line origin	CYTORAB	Dogs	1 ml	3 months	Annually
	Wellcome	Cats	1 ml	3 months	Annually
Feline cell line origin	RABVAC	Dogs	1 ml	3 months	Annually
	Fromm	Cats	1 ml	3 months	Annually

TABLE 6.5. *(continued)*

Vaccine	Product name Marketed by	For use in	Dosage[a]	Age at primary vaccination[b]	Booster recommended
C) Combination					
Murine origin	PAN-RAB Douglas	Cats	1 ml	3 months	Annually
Feline cell origin	ECLIPSE III KP-R Fromm	Cats	1 ml	3 months	Annually
Feline cell origin	ECLIPSE IVKP-R Fromm	Cats	1 ml	3 months	Annually

[a] All vaccines must be administered intramuscularly at one site in the thigh.
[b] Three months is the earliest age recommended. Dogs and cats vaccinated between 3 and 12 months should be revaccinated 1 year later.

mune globulin (given for post-exposure treatment of rabies) had no suppressive effect on the immune response (Hattwick et al., 1976). Trials of the rabies diploid cell vaccine for post exposure treatment has established the protective effect under field conditions in Germany, Iran and the USA. In the trials in Iran, for example, all persons survived and remained free of rabies in an area where normally around 35% of bite victims would be expected to die.

Plotkin (1980) has summarized clinical experience with the human diploid cell rabies vaccine. Relatively high levels of neutralizing antibody were detected following immunization of volunteers with a variety of dosage schedules of whole virion vaccine. Moreover the antibody titres were markedly higher than in volunteers given duck embryo vaccine. Finally, in a number of persons immunized in Iran after exposure to rabies from dog bites a rapid antibody response was detected and no-one died of rabies (Fig. 6.5). Very recently some failures have been noted when vaccine was administered intradermally under field conditions.

6.7.4. Rhesus monkey diploid cell rabies vaccine

This vaccine has been developed as an alternative to human diploid cell vaccine where, for any reason, injection of human cell material is to be avoided (Berlin et al., 1983). Thus, allergic reactions and Guillain Barré syndrome which have been noted following use of the human cell vaccine (MMWR, 1980, Boe and Nyland, 1980) may be circumvented using the rhesus monkey cell material. A fibroblast cell line derived from foetal rhesus monkey lung (the rhesus analogue to the WI-38 cell line) was inoculated with the Kissling rabies virus strain adapted to grow in monkey fibroblasts. Supernatant fluid containing virus was inactivated with β-propiolac-

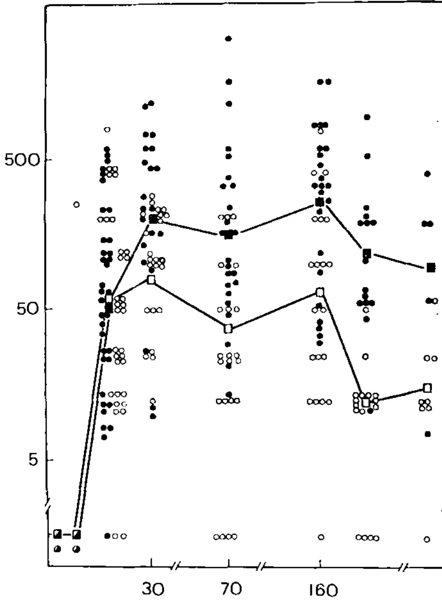


Fig. 6.5. Development of neutralizing (●) and complement-fixing (O) antibodies in the sera of 30 individuals vaccinated with HDCS vaccine after contact with a rabid animal (after Kuwert et al., 1978) The abscissa shows the reciprocal antibody titre, the axis shows days after vaccination.

tone, adsorbed and concentrated using aluminium phosphate. Volunteers were given a series of 5 injections at 1, 3, 7, 14 and 28 days and an early antibody response in volunteers not given immune globulin simultaneously was detected at 7 days, and by 14 days all volunteers had responded (Table 6.6). Minor post-vaccination reactions such as muscle ache were noted in most patients and constitutional reactions noted in 6 volunteers (Table 6.7). The antibody response was similar to that following human diploid vaccine.

6.7.5. Primary hamster kidney cell rabies vaccine

Fangtao et al. (1983) have described clinical experience with a Chinese rabies vaccine produced in primary hamster kidney cells. This may have the considerable advantage of cheapness. The vaccine is immunogenic (Table 6.8) compared to Semple vaccine, even when administered without adjuvant. Side reactions occur (Table 6.9) but are reduced in some respects compared to Semple vaccine. Booster immunizations with the new vaccine produce a satisfactory serological response while post exposure immunization has been successfully employed in 21 patients with severe bites.

TABLE 6.6.
Rapid fluorescent focus inhibition test titres after rhesus diploid rabies vaccine (adsorbed) administration[a] (after Berlin et al., 1983)

	Day of vaccination				
	3	7	14	28	42
HRIG[b]					
Lot A					
Median, titre, IU	0.19	0.20	5.0	11.0	23.0
n	24	24	24	24	23
No HRIG					
Median titre, IU	<0.02	0.04	5.4	18.0	18.0
n	23	23	23	23	21

n is the number of vaccinees. A titre of 0.5 IU is an acceptable response.
[a] Vaccine was given in 1-ml intramuscular doses on days 0, 3, 7, 14, and 28
[b] HRIG indicates human rabies immune globulin, given on day 0

6.7.6. Biosynthesis of rabies glycoproteins in *E. coli*

A modern trend with inactivated virus vaccines (e.g. influenza) has been to separate immunogenic virus glycoproteins and use these proteins as purified vaccines. To some extent this approach can be superceded now by investigations of immunogenic virus proteins produced by genetic engineering techniques in prokaryotes (see Chapter 2). Thus, an alternative for rabies virus subunit vaccine design would be to isolate the appropriate gene and provide it with new regulatory controls in a prokaryote.

As noted above, five viral proteins have been identified from cells infected with

TABLE 6.7.
Local reactions after rhesus diploid cell rabies vaccination (after Berlin et al., 1983)

Day of inoculation	Muscle ache or pain					
	<24 hr		>24 hr		Inflammation	
	Antiserum plus vaccine	Vaccine alone	Antiserum plus vaccine	Vaccine alone	Antiserum plus vaccine	Vaccine alone
0	23/35	10/25	9/35	11/25	4/35	2/25
3	11/35	13/25	6/35	3/25	4/35	1/25
7	12/34	10/24	17/34	10/24	10/34	3/24
14	12/34	7/22	16/34	12/22	12/34	7/22
28	11/31	9/21	6/31	3/21	9/31	3/21

TABLE 6.8.

Titres of neutralizing antibody to rabies virus in 228 healthy volunteers injected with various forms of primary hamster kidney cell rabies vaccine or Semple vaccine (after Fangtao et al., 1983)

Vaccine (*n*)	Potency[b]	Administration of vaccine			Geometric mean (range) reciprocal titre of antibody		
		Days	Dose (ml)	Route	Before immunization	Day[a] 21	Day[a] 45
Plain PHKC (53)	354 800 or 371 510	0–14	2	sc	<5	113 (10–800)	74 (<10–800)
Adjuvant PHKC (45)	660 710	0, 7, 14	2	sc or im	<5	95 (10–1000)	54 (<10–200)
Concentrated PHKC (44)	1 230 000	0, 3, 7, 14	1	im	<10	521 (100–3240)	313 (50–1000)
Concentrated adjuvant PHKC (46)	1 480 000	0, 7, 14	2	im	<10	884 (100–3240)	426 (100–3240)
Semple (40)	371 500	0–14	2	sc	<10	35 (10–1080)	29 (10–1080)

[a] After the first inoculation

[b] The potencies of vaccines were determined according to the method of Habel (data are Habel index values – see Johnson, 1973, Habel, 1973)

TABLE 6.9.
Clinical reactions among healthy volunteers injected with plain primary hamster kidney cell rabies vaccine or Semple vaccine (after Fangtao et al., 1983)

Reaction	No. (%) of patients with reaction	
	PHKC vaccine (n=53)	Semple vaccine (n=40)
Redness at injection site (diameter)		
1.0–2.5 cm	3 (6)	3 (8)
2.5–5.0 cm	25 (47)	31 (79)
>5.0 cm	6 (11)	6 (15)
Induration at injection site	0	40 (100)
Pain and itching at injection site	20 (38)	39 (98)
Fever		
37.1–37.5°C	23 (43)	12 (30)
⩾37.6°C	0	0
Headache or dizziness	3 (6)	3 (8)
Paresthesia	0	1 (3)
Local lymphadenitis	0	2 (5)
Pain in joints	1 (2)	0
Lumbar pain	1 (2)	0
Sore throat	1 (2)	3 (8)
Malaise	0	3 (8)

rabies virus and from purified rabies virions. Of these, the glycoprotein (or G protein) forms the external surface of the virus and is responsible for its immunogenicty. Dietzschold et al. (1978) showed that 9 nanograms of purified glycoprotein was equivalent to 1630 ng of purified whole virus in a mouse protection test even though the glycoprotein constitutes about 40 percent of the rabies virion protein. The amino acid sequences of the glycoproteins of the rabies virus CVS and ERA strains have now been deduced from DNA copies of the respective genes (Anilionis et al., 1981). The homology (about 90 percent at the amino acid level) is in conformity with the closely related antigenic characteristics of the two virus strains. Yelverton et al. (1983) have undertaken the engineering of a rabies subunit vaccine consisting of the glycoprotein gene product from CVS rabies virus strain to test against many strains of infectious rabies virus.

From polyadenylated messenger RNAs (mRNAs) of cells infected with the rabies CVS strain, Yelverton et al. (1983) prepared double-stranded complementary DNA (cDNA) species, introduced single-stranded homopolymeric tails, and annealed them by means of DNA base pairing into the bacterial cloning vehicle pBR322. The heterogeneous hybrid plasmids were then transformed into *E. coli* to form a colony library. From the library a plasmid, pRab91, was identified which selectively hybridized to rabies virus glycoprotein mRNA. Nucleotide sequencing of the cloned

DNA, which consisted of >2000 base pairs, showed that it encoded most of the hydrophobic signal peptide and all of the mature rabies virus glycoprotein, whose NH_2 and COOH-terminus has been established by direct amino acid analysis.

When Yelverton et al. (1983) assayed the insoluble protein fractions from cultures of *E. coli* W3110/pRabdex 1, and *E. coli* W3110/pRabdex2, it was found that the proteins G_{427} and G_{505} were only about 2 to 3 percent as effective per weight as purified authentic glycoprotein in binding antibodies to glycoprotein. To recover more activity from the bacterially synthesized proteins, a rather vigorous solubilization procedure was used. The enriched preparations were resuspended in 7M guanidine-hydrochloride at room temperature followed by dialysis into 7M urea and 5mM Tris, pH 8.0. Under these conditions, the proteins dissolved and their apparent activity increased more than 30-fold as indicated by the ELISA. Freshly solubilized preparations of G_{505} reacted with the antibody about 90 percent as effectively as natural glycoprotein, while the activities of solubilized preparations of G_{427} were no higher than 70 percent those of authentic glycoprotein. The amount of activity recovered suggests that most antigenic determinants present on the authentic glycoprotein are conserved in the bacterial counterpart, G_{505}. It has yet to be discovered whether the incomplete recovery of antigenic activity in the preparations of G_{505} is artefactual or signifies the absence of specific immunogenic domains formed only in the virus-derived, glycosylated molecule.

6.8. Future work on vaccines

As we have seen above, in order to understand antigenic variation of rabies virus, and to develop a logical basis for the selection of new vaccine strains, which currently are derived from a limited number of isolates (Wiktor and Koprowski, 1982), it is necessary to know something of the antigenic structure of the rabies virus glycoprotein as well as biochemical properties of the protein. In a recent report Lafon et al. (1983) described a functional antigenic map of the CVS-11 glycoprotein obtained by a comparative analysis of antigenic variants selected in the presence of monoclonal antibodies. These variants arose with high frequency and could also be isolated in vivo. Neutralization-resistant variant viruses were isolated in vitro at high frequency (10^{-4} to 10^{-5}) in the presence of anti-glycoprotein monoclonal antibody. Analysis of these variants identified at least three functionally independent antigenic sites, based on the grouping of variants that were no longer neutralized by one or more of a panel of 24 monoclonal antibodies. Competition radioimmunoassay suggested that one of these three antigenic sites was topologically distinct, with the other two in close proximity. In addition, it was shown that most (but not all) neutralization-resistant variants failed to bind the relevant monoclonal antibody. Viruses with altered antigenicity were shown to accumulate in virus stocks following several passages in vitro in the absence of antibody. The demon-

stration of selection of antigenic variants by monoclonal antibody in vivo has a number of implications. In view of the danger of an antigenic variant arising as a direct result of treatment, a single monoclonal antibody should not be used in place of immune gamma globulin in the post-exposure treatment of rabies. It may be possible, however, to use mixtures of monoclonal antibodies directed against several antigenic sites, thus reducing the probability of variant selection to an acceptable level. Secondly, it is assumed that immunity following vaccination or infection is directed against all antigenic sites. Lafon et al. (1983) have shown that the frequency of variant selection is dramatically reduced when this is the case. However, some influenza variants (differing in a single antigenic site) are not recognized by human antibody, especially that of children: a restricted anti-influenza clonal repertoire may allow such variants to escape humoral immune mechanisms. Yet another possibility is that the selection pressure is not immunological, and that such non-immunological selection results in viruses showing a concomitant change in antigenic profile.

Thus, the use of monoclonal antibodies as diagnostic tools and also to select antigenic variants of rabies has highlighted antigenic differences between rabies viruses (Wiktor and Koprowski, 1978). Moreover, immunization with one rabies serotype may not provide full protection against challenge with another serotype. Therefore, more attention may have to be paid in the future to a search for a broadly reacting antigenic determinant and, alternatively, the production of vaccines containing several rabies viruses.

Nearly all antirabies vaccines throughout the world are prepared from a virus strain originally isolated by Pasteur in 1882. The antigenic potency of this vaccine is currently evaluated by the challenge of immunized mice with another virus derived from the Pasteur strain (Habel, 1973) although single radial diffusion (SRD) techniques are being developed (Ferguson and Schild, 1982). This vaccine is used for the protection of men and animals against viruses which may, in different parts of the world, represent variants expressing antigens that do not react with virus strains involved in the vaccine evaluation procedure (Crick et al., 1981). Failures in post exposure rabies treatment have been attributed also to either the low potency of the vaccine or to delay in the initiation of treatment. Antigenic differences among strains of rabies have also been suspected as the cause of such failures; however, until now, there has been no way to test this hypothesis. With the availability of monoclonal antibodies, strains of virus can be easily identified and their patterns of reactivity compared with the reactivity of the virus used for vaccine production.

6.9. Principles of rabies control in animals

It is clear from the brief summary above of the epidemiology of the virus that rabies in humans can be prevented by eliminating exposure to rabid animals and also by

prompt local wound treatment and immunization after exposure (see Table 6.10 for summary). In this regard, it should be emphasized that a characteristic of most rabid animals (e.g. foxes) is a change in normal behaviour. Foxes, for example, may lose fear of humans and walk during the daytime among domestic animals, or enter homes. They generally attack when threatened or sometimes without provocation. Rabid badgers behave like rabid foxes. As regards control of domestic animals, ex-

TABLE 6.10.
Specific treatment for rabies (after Christie, 1980)

Nature of exposure	Status of biting animal (irrespective of whether vaccinated or not)		Recommended treatment
	At time of exposure	During observation period of ten days	
I. No lesions; indirect contact	Rabid	—	None
II. Licks:			
(1) unabraded skin	Rabid	—	None
(2) abraded skin, scratches and unabraded or abraded mucosa	(a) healthy	Clinical signs of rabies or proven rabid (laboratory)	Start vaccine[a] at first signs of rabies in the biting animal
	(b) signs suggestive of rabies	Healthy	Start vaccine[a] immediately; stop treatment if animal is normal on fifth day after exposure
	(c) rabid, escaped, killed or unknown	—	Start vaccine[a] immediately
III. Bites:			
(1) mild exposure	(a) healthy	Clinical signs of rabies or proven rabid (laboratory)	Start vaccine[a] at first signs of rabies in the biting animal
	(b) signs suggestive of rabies	Healthy	Start vaccine[a] immediately; stop treatment if animal is normal on fifth day after exposure
	(c) rabid, escaped, killed or unknown	—	Start vaccine[a] immediately
	(d) wild (wolf, fox, bat, etc.)	—	Serum[a] immediately, followed by a course of vaccine

TABLE 6.10. *(continued)*

Nature of exposure	Status of biting animal (irrespective of whether vaccinated or not)		Recommended treatment
	At time of exposure	During observation period of ten days	
(2) severe exposure (multiple, or face, head, finger or neck bites)	(a) healthy	Clinical signs of rabies or proven rabid (laboratory)	Serum[a] immediately; start vaccine[a] at first sign of rabies in the biting animal
	(b) signs suggestive of rabies	Healthy	Serum[a] immediately, followed by vaccine; vaccine may be stopped if animal is normal on fifth day after exposure
	(c) rabid, escaped, killed or unknown		Serum[a] immediately, followed by vaccine
	(d) wild (wolf, jackal, pariah dog, fox, bat, etc.)		Serum[a] immediately, followed by vaccine

[a] Human immunoglobulin 20 IU per kg of body weight is advised. Regardless of any pre-exposure prophylaxis, persons exposed to rabies should receive, for example, two 1.0 ml doses of HDCV vaccine.

perience in the USA has shown that an essential feature is that local authorities should initiate and maintain an effective programme to remove stray and unwanted animals and to ensure vaccination of all dogs and cats. As a result of these procedures confirmed rabies cases in dogs (Fig. 6.6) has decreased dramatically. At present in the USA the incidence of cat rabies is similar to that in dogs, and so immunization of cats is being encouraged. All cats and dogs should be immunized commencing at 3 months of age. It is not considered necessary in the USA to vaccinate livestock, although certain breeding stock in areas where wild life rabies is epizootic can be immunized. Any domestic animal that is bitten and scratched by a bat or by a wild carnivorous mammal that is not available for testing is regarded in the USA as having been exposed to a rabid animal and unvaccinated dogs and cats should be destroyed immediately or quarantined for 6 months. Vaccinated animals should be revaccinated and confined for 90 days.

The control of rabies in wild life is a much more difficult or even impossible task. In the USA continuous trapping or poisoning as a means of rabies control is not recommended. Rather, limited control is maintained in high contact areas such as picnic grounds. Similarly, bats should be eliminated from houses, but more widespread control is not feasible.

Fig. 6.6. Rabid dog (from Tierkel, 1975).

6.10. Chemotherapy of rabies

Since vaccine has been used so successfully to prevent rabies and also can be administered *after* a bite and during the incubation period, there has been little need for antiviral agents. Similarly, only a few experiments have been carried out with interferon. As an example of the experimental studies that have been carried out, Vesicular stomatitis virus and Chandipura virus, both Vesiculoviruses in the Rhabdoviridae family were shown to be inhibited by the nucleoside analogue ribavirin (see Chapter 7 for further details of this molecule), the MICs ranging from 1.5 to 320 μg/ml (Sidwell, 1980). Antiviral activity, similar to the DNA-containing herpes viruses, is somewhat cell dependent, with the least antiviral activity being detected using Vero cells. The presence of a viral RNA polymerase enzyme could be utilized for the selection of new antiviral compounds.

6.11. Summary

The prevention of rabies virus itself has been an early success story from the point of view of vaccination. New human diploid cell and other vaccines, have been developed, although the cheaper duck embryo vaccines will continue to be used in many areas of the world in the near future. Studies of antigenic variation of rabies virions (established with monoclonal antibodies) are of particular interest and show that even with an apparently successful vaccine a close observation has to be kept on its use and possible failures in the field. Chemotherapeutic agents would also be useful in certain circumstances but few investigations are under way at present, partly because of the difficulty in laboratory handling of the virus and partly because of the apparent success of the vaccine. Studies are in progress on the determination of virulence of rabies strains and of the precise composition and sequence of immunogenic virion proteins. This could lead to the development of short peptide vaccines in the future.

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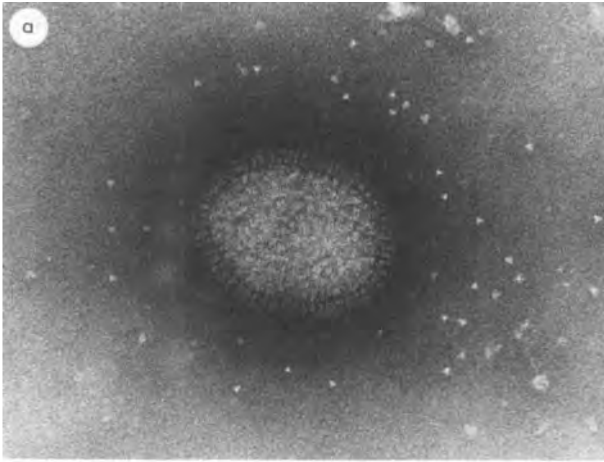
CHAPTER 7

Orthomyxovirus infections

The earth is a unity for influenza A virus in a manner not yet found for probably any other parasite (Hope-Simpson, 1979, Stuart-Harris, 1981, Kilbourne, 1975, Pereira, 1979) and epidemics occur in all inhabited parts of the globe regardless of latitude, longitude, altitude, climate, rainfall, temperature, humidity, race and sex. Influenza A is the classic pandemic virus infection of man (although now not the only pandemic virus, since enterovirus type 70 is essentially similar in epidemiology, see Chapter 4) and influenza B virus also can cause sharp outbreaks, resulting in significant mortality.

7.1 Structure of influenza A virus and protein composition

The influenza A virion is a rather pleomorphic enveloped virus, often taking a 'doughnut' shape when visualized using negative staining techniques (Fig. 7.1). The lipid envelope (which is derived from the host cell in which the virus has replicated) is studded with the two morphologically distinguishable glycoprotein spikes, namely the haemagglutinin (HA) and the neuraminidase (NA). The latter takes the morphological form of a mushroom but with a narrow extended stalk, while the HA is triangular in cross section rather like a Toblerone chocolate bar. Both spikes penetrate through the lipid bilayer via 'tails' of hydrophobic amino acids and contact the underlying layer of matrix (M) protein. The M protein forms a monomolecular layer and probably contributes in a major way to the structural integrity of the virion. Enclosed in the matrix shell is the nucleoprotein and the P1, P2 and P3 complex, closely assocciated with the ss RNA of the virus. The three polymerase pro-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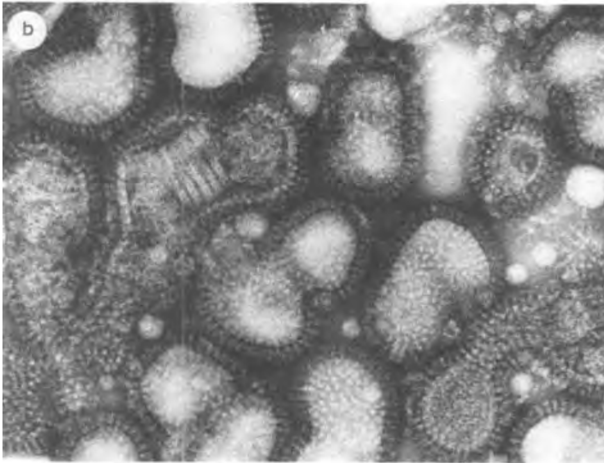
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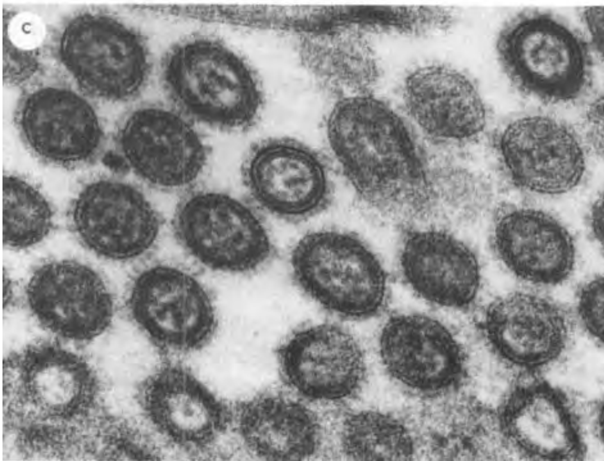
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teins constitute an RNA transcriptase enzyme (Kawakami and Ishihama, 1983, reviewed by McCauley and Mahy, 1983) responsible for initiating RNA replication in the infected cell. The virion RNA exists as 8 separate single stranded fragments which are replicated independently as described below.

We shall examine in some detail the structure and function of some of these structural proteins and RNAs of influenza virus (Table 7.1). More is known about the composition and replication of influenza than probably any other human virus. We can therefore use the influenza virus as a model and safely conclude that future scientific investigations of other viruses will follow a similar pattern.

7.1.1. HAEMAGGLUTININ (HA)

The haemagglutinin (HA) of influenza A virus is a trimer of 224 K M.W. It may be solubilized from the viral membrane by bromelain enzyme digestion, which removes a 5406 M.W. C-terminal hydrophobic (anchoring) peptide from each subunit. The haemagglutinin is a typical integral membrane protein, characterized by a three-domain structure with a large hydrophilic, carbohydrate-containing domain on the external surface of the membrane, a small, uncharged hydrophobic peptide of 24–28 amino acids spanning the virus membrane, and a small, hydrophilic domain (10–55 amino acids) on the internal side of the membrane (reviewed in Webster et al., 1982, Palese and Kingsbury, 1983).

Typical of membrane glycoproteins, the haemagglutinin chain is initially synthesized to include an N-terminal hydrophobic 'signal' peptide, which is subsequently removed as part of the process by which the protein is transported across and anchored into the membrane. Each polypeptide chain of the trimer is glycosylated at seven sites with a total carbohydrate of 13 000 M.W. (19% by weight).

The HA protein is a major determinant of virulence of influenza virus (Bosch et al., 1979) and also contains important antigenic determinants which stimulate the production of neutralizing antibody following infection or immunization. The HA protein has important biological functions of attachment to host cell receptors and, once inside the cell mediates fusion events which result in release of RNA and cell infection. Therefore, the HA represents an important target protein both from the point of view of vaccines and also chemotherapy and hence it is probably the most well studied human virus protein. An important characteristic of the HA is the mosaic characteristic of its antigenic determinants ('antigenic drift', resulting from single or a few amino acid substitutions) and its ability also to undergo major antigenic changes ('antigenic shift'). Amino acid sequence analysis of the HA of different subtypes shows, however, that certain stretches are conserved and this raises

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Fig. 7.1. Electron micrograph of influenza A virions. a, Single virion surrounded by HA spikes (viewed end); b, Partially disrupted particles showing matrix layer and NP-RNA complex; c, Sections of virus showing matrix layer and lipid bilayer. (Courtesy of Dr. D. Hockley.)

the possibility that these could be used as specific targets for new antivirals and vaccines. This is discussed in more detail below when we refer to the work on oligopeptides as inhibitors of virus replication. From the point of view of vaccines, earlier studies in animals showed cross-protection (Schulman and Kilbourne, 1965, Werner, 1966) between subtypes of influenza A virus (but not between influenza A and B viruses) and this was thought to be HA mediated. Therefore shielded antigenic determinants could exist which are recognized more easily in certain animals than in humans. Again this emphasizes the possibility of obtaining immunogens which induce cross-reactive antibody against a wide variety of strains. At present antigenic drift and shift pose major problems for vaccine efficacy.

Detailed studies have now been carried out on the HA of influenza A viruses using X-ray crystallography (Wilson et al., 1981) which together with earlier sequencing data (Porter et al., 1979, Gething et al., 1980) has allowed the construction of accurate 3 dimensional models of the HA and the precise localization of the cell binding site, antigenic determinants (Fig. 7.2) and fusion sequences.

As regards the binding site, the distribution of conserved amino acids on the 3 Å structure reveals a highly conserved region in a surface pocket on the distal end of the molecule that seems suited for a binding to host-cell oligosaccharide. This tentative receptor binding pocket includes conserved residues Tyr 98, His 193, Glu 190, Trp 153 and Leu 194.

The most highly conserved sequence in the haemagglutinin is the amino terminus of HA2, where 1 substitution in the first 11 and only 5 in the first 23 amino acids have been observed. This sequence is associated with the activity by which the virus penetrates a host-cell internal lysosomal membrane to initiate infection. Cleavage of HA into HA1 and HA2 at this point is required for infections penetration and for in vitro membrane fusion (An homologous sequence is present at the cleavage-activation site on the Sendai virus fusion protein, where cleavage is required both for infectivity and membrane fusion activity.) The HA2 N-terminal sequence is strongly nonpolar, the first charged side chain occurring at position 11 (Glu). It is unexpectedly rich in glycines (1, 4, 8 and 13, 16, 20, 23) which could indicate flexibility or the presence of an unusual conformation.

As regards antigenic binding sites, four (A, B, C and D) have been identified (Fig. 7.2) by analyzing antigenic mutants selected in the laboratory under immunological pressure from monoclonal antibodies (Yewdell et al., 1970). Site A is an unusual protruding loop from amino acids 140 to 146 which projects 8 Å from the local molecular surface, and forms the centre of the most obvious antibody-binding site. The haemagglutinin of each antigenically distinct virus of epidemic significance has a mutation in this region. Site B comprises the external residues 187–196 of an α helix, and adjacent residues along the upper edge of a pocket tentatively implicated in virus receptor binding. Two substitutions in the haemagglutinin of the 1972 virus, A/Memphis/103/72: 188, Asn to Asp, and 155, Thr to Tyr; and two more in that of the 1975 virus, A/Victoria/3/75: 189, Gln to Lys and 193, Ser to Asn charac-

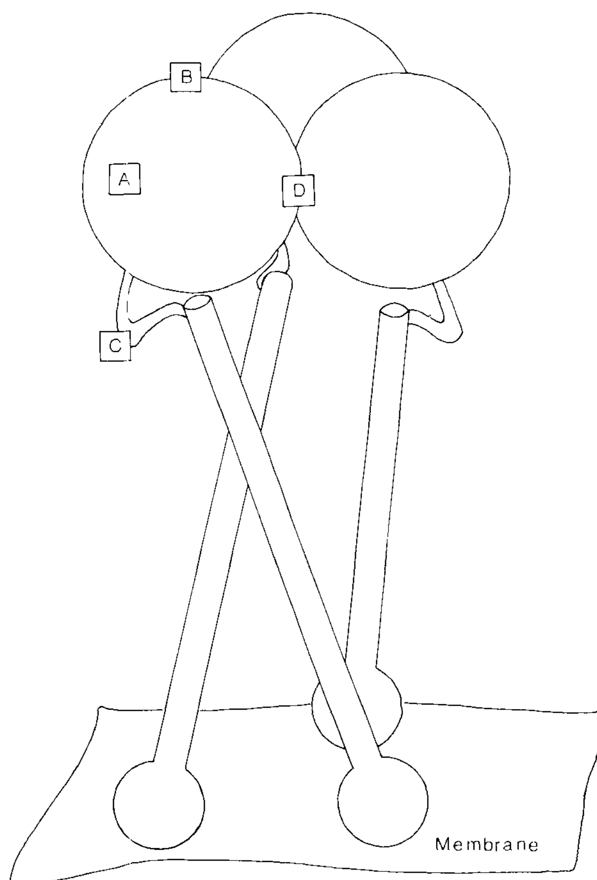


Fig. 7.2. Schematic diagram of the HA trimer of A/Aichi/68 virus. Each trimer contains a stem and a globular domain which contains the variable antigenic determinants A, B, C and D. It is not known at present whether the influenza B HA molecule is identical, but it is assumed to be similar.

terize the site, but the lack of sequence information for this region of the haemagglutinins of several variants leaves the comparison incomplete. Site C is a bulge in the tertiary structure at the disulphide bond between Cys 52 and Cys 277, 60 Å from the distal tip of the molecule and comprises another antibody-binding site (see Fig. 7.2). The haemagglutinins of the viruses from both epidemic periods have substitutions clustered in this region.

In the first three sites noted above, the amino acid substitutions noted as causing antigenic variation are external. Site D departs from this. Several amino acid substitutions in the haemagglutinins of both natural and laboratory-selected antigenic mutants occur in the interface region between subunits in the haemagglutinin trimer. These amino acids may be recognized as a result of a relative movement of the globular regions of HA1 to expose the interface regions. However, it is possible that

the actual antibody binding site is remote from amino acids but affected by the exact fit at the interface, and might be disturbed by the substitutions listed.

7.1.2. THE MATRIX PROTEIN (M)

The complete sequence of RNA segment 7 (coding for the group specific matrix antigen) of two strains of A/PR8/34 (H1N1) and of A/Udorn/72 (H3N2) has been reported as well as partial sequences of a number of other strains (reviewed in Palese and Kingsbury, 1983). Following the first AUG codon in the positive strand RNA, a 252-residue protein, rather hydrophobic and rich in arginine, is encoded. Comparison of the sequences of RNA segment 7 of the H3N2 (Udorn) and H1N1 (PR8) strains shows that the sequences coding for the matrix (M) of these viruses isolated 38 years apart are highly conserved, in keeping with antigenic studies. Comparison of 230 nucleotides of RNA segment 7 from five human H1N1, H2N2 and H3N2 strains isolated over a 43 year period suggests that the same segment 7 was retained throughout the antigenic shifts of HA and NA type (H1N1 to H2N2 to H3N2). In addition, the complete sequences contain a second open reading frame which overlaps the M protein sequence by 68 nucleotides.

Three mRNAs transcribed from RNA segment 7 have been isolated. One (M1 mRNA) consists of an uninterrupted, nearly full-length, copy of RNA segment 7 and is responsible for the production of the M protein. An M2 protein is generated from a spliced product of the M1 mRNA, such that after the nucleotides encoding the N-terminal nine amino acids, nearly 600 nucleotides are spliced out and the reading frame is changed; a protein product corresponding to this has been identified in infected cells. In addition, a third mRNA has been found, which would code only for an 8-residue peptide identical to the C terminus of M1. Such a product has not yet been isolated.

7.1.3. NUCLEOPROTEIN (NP)

The nucleoprotein is one of the group-specific antigens of influenza viruses that distinguishes between influenza A, B and C viruses (Stuart-Harris and Schild, 1979). It probably constitutes the backbone of the helical internal complex that is associated with the RNA segments and the three different polymerase proteins.

The NP gene of A/PR/8/34 virus is 1,565 nucleotides long and is capable of encoding a protein of 498 amino acid residues (M.W. 56 106) rich in arginine. Double immunodiffusion tests showed antigenic differences between the NPs of H1N1 and the H3N2 strains and recent studies with monoclonal antibodies to the NP of A/WSN/33 (H1N1) viruses have shown that antigenic variation occurs in this molecule (Schild et al., 1979). The NP molecule possesses at least three non-overlapping antigenic areas, one area being the same on all strains tested. Monoclonal antibodies to this conserved domain inhibited in vitro transcription of viral RNA, suggest-

ing that this region of the NP is involved in RNA transcription (Van Wyke et al., 1980).

7.1.4. The non-structural proteins

RNA segment 8 codes for at least two non-structural polypeptides, NS1 and NS2, which are translated from separate mRNAs (reviewed by Palese and Kingsbury, 1983, Lamb et al., 1980, Inglis et al., 1979). Mapping and sequence studies have shown that NS1 and NS2 overlap by 70 amino acids that are translated from different reading frames. Polypeptides NS1 and NS2 share 9 amino acids at their N termini, but after this sequence the mRNA for NS2 has a deletion of 423 nucleotides, then rejoins the rest of the mRNA in the +1 reading frame. The function of NS1 or NS2 has not been established. NS1 is made in large amounts and accumulates in the nucleus, whilst NS2 is made late in infection and is found predominantly in the cytoplasm.

Because NS1 and NS2 are internal proteins of infected cells and hence less available to antibodies, they would be expected to show less sequence variation than the surface glycoproteins (HA and NA). Accordingly, comparison of the sequences of the NS genes of fowl plague and the two human influenza strains A/Udorn/72 (H3N2) and A/PR/8/34 shows only 8-11% differences (see Palese and Kingsbury, 1983). An open reading frame potentially coding for an extra polypeptide has been noted in the noncoding, virion RNA of the NS genes of A/PR/8/34, Udorn/72 and FPV, but is not present in the NS gene of duck/Alberta/60/76. No protein corresponding to this extra 'gene' has yet been identified.

7.1.5. Polymerase proteins

The three largest proteins of the virion (P1, P2, P3) with molecular weights of 96 000, 87 000 and 85 000, respectively, are found in association with the nucleoprotein and virion RNA and carry the polymerase activity which transcribes the invading viral RNA. Proteins P1 and P3 are probably required for complementary RNA synhesis and P2 and NP for virion RNA synthesis. The complete nucleotide sequences of two of the three polymerase genes of the A/PR/8/34 strain have been determined, but the extent of variation in the polymerase genes is unknown, although these may play an important part in host range and virulence (Almond, 1977).

Defective interfering (DI) influenza virus particles are generated by high multiplicity passage in permissive cells. These particles are of interest because they facilitate the establishment of persistent infection in cell cultures and could therefore be concerned in latency. They contain new small RNA molecules which are absent from infectious virus and which are generated predominantly by massive internal deletion from the three P genes, although three sequences of one small RNA is a mosaic of several segments from at least two of the polymerase genes.

7.1.6. NEURAMINIDASE (NA)

The NA is the second glycoprotein on the surface of the virion. Several roles have been suggested for the neuraminidase. The enzyme catalyses cleavage of the α–ketosidic linkage between terminal sialic acid and an adjacent sugar residue. This reaction permits transport of the virus through mucin and destroys the haemagglutinin receptor on the host cell, thus allowing elution of progeny virus particles from infected cells. The removal of sialic acid from the carbohydrate moiety of newly synthesized haemagglutinin and neuraminidase is also necessary to prevent self-aggregation of the virus. In general, then, the role of neuraminidase may be to facilitate mobility of the virus both to and from the site of infection.

The protein exhibits both antigenic shift (a major change in amino acid sequence) and antigenic drift (minor changes in amino acid sequence). Two serologically distinct subtypes of neuraminidase are known in human influenza viruses. The N1 subtype was associated with virus isolated between 1933 and 1957, after which time the N2 subtype appeared in 'Asian' influenza. No major change in the structure of the neuraminidase has occurred since, although the haemagglutinin subtype changed from H2 to H3 in 1968 in the Hong Kong pandemic.

The neuraminidase of influenza virus is an integral membrane glycoprotein. It is a tetramer of molecular weight 240 K, reducing to 200 K when solubilized from the virus with pronase. Sequences of neuraminidases from several strains of N1, N2 and B subtypes are known. A hydrophobic N-terminal region serves to anchor the neuraminidase in the membrane. The N2 enzyme subunit contains 469 amino acids, with carbohydrate attached in four places. The crystallizable product of the pronase digestion begins at residues 74 or 77. These so called neuraminidase 'heads' carry the full antigenic and enzymatic capability of the membrane-associated neuraminidase. Varghese et al. (1983) have now described the detailed structure of the NA at 2.9 Å resolution. The NA structure is unusual and is a 4-fold symmetric oligomer of identical polypeptide chains with a box-shaped 'head' connected to the virus membrane by a long slender stalk. For crystallization, the stalk was removed by pronase digestion, liberating the square head which retains full enzymatic and antigenic capabilities. The fold of the polypeptide chain is unique. Each monomer contains six β-sheets; each sheet contains four strands with the topology of a 'W'. Viewed from above the head, each monomer consists of six of the four-stranded sheets arrayed like the petals of a flower but twisted like the blades of a pinwheel. (reviewed by Wiley, 1983):

"Although the three-dimensional structure of the stem is unknown, it may be even more unusual. Amino acids 1–6 are on the cytoplasmic side of the membrane followed by an uncharged and primarily hydrophobic peptide (7–35) which spans the membrane, anchoring the protein to the lipid. The anchor also acts as a signal peptide during membrane translocation but is not removed by signal peptidase. The slender stem (residues 36–73) is unusual in containing 50 per cent of the oligosac-

charides of the molecule (four sites) and in exhibiting absolutely no amino acid sequence homology among NAs from various virus strains – although the remainder of the molecule shows highly significant (> 50 per cent) homology. Thus, both viral membrane glycoproteins, the NA and HA, exhibit novel structural features: the NA, a β-sheet pinwheel and a slender stalk; and the HA, a loop-like topology beginning and ending at the membrane, and a fibrous stem centred on an 80 Å long triple-stranded coiled-coil of α-helices."

The four carbohydrate chains in crystalline NA are distributed two on the top and two on the bottom of the box-shaped head. One oligosaccharide is found at a subunit-subunit interface. Both on the NA and on the HA most of the oligosaccharides are attached on the lateral surfaces of the glycoprotein in positions where the glycoproteins might be expected to contact other proteins embedded in the same membrane.

The catalytic site of the NA has been located by difference Fourier analysis of crystals soaked in sialic acid. The site is surrounded by 14 conserved charged residues and contains three hydrophobic residues, Tyr, Trp and Leu.

By examining the location of amino acid substitutions in a series of 'drifted' field strains and in three antigenic variants selected by growth in monoclonal antisera, Colman and colleagues (1983) have been able to propose the location of antigenic determinants on the NA. The antigenic sites are composed of loops connecting strands of β-sheet. They form a nearly continuous surface that encircles the catalytic site on the top of the NA. Although residues in the active site itself are conserved from strain to strain, the proximity of the variable loops to the site suggests that a significant part of the variable loops could interfere with any antibody contacting the active site cavity (reviewed by Wiley, 1983).

7.2. Early influenza virus–cell interactions

Very little is known in precise terms about the molecular details of interaction between human viruses, including influenza, and cell receptors. However, with the advent of rapid sequencing of virus glycoproteins involved in these interactions our knowledge of sequences involved should increase rapidly. We should recognize the pitfalls of studies of early interactions between viruses and cells, an important one being whether the interaction we observe will lead to infection, or whether we are simply observing non-infectious interaction. Nevertheless more studies are urgently required and data obtained could easily be applied, for example, to the design of specific inhibitors for chemoprophylaxis and also help to understand important problems of antibody-virus interaction and neutralization, virus cell fusion and virus host range. Sialic acids are known to be essential components of the cell surface receptors for influenza, paramyxoviruses, polyoma and encephalomyocarditis virus, but the viruses nevertheless appear to exhibit strict and varied specificities for

the precise sialyloligosaccharide sequences that serve as their receptor determinants. Probably the best characterized virus receptor is glycophorin, a sialo glycoprotein binding site for influenza virus on erythrocytes. Sialic acid containing glycolipids (gangliosides) occur naturally in the plasma membrane of cells and act as receptors of cholera toxin, interferon and some glycoprotein hormones as well as certain viruses. Of course, both gangliosides and glycoprotein may be involved in the adsorption of a single virus because on approaching a cell the virus firstly would interact with glycoprotein before more intimate interaction with the plasma membrane containing gangliosides.

We have referred above to the impossibility of making generalized statements about virus receptors and an excellent example of the reason for this, namely, marked differences in virus cell receptor interaction by even genetically closely related viruses is described by Carroll et al. (1981). These authors describe the different adsorption properties of the RI/5 and RI/5$^+$ viruses with erythrocytes and attribute this to the different sialyloligosaccharide receptor specificities of their HAs. Erythrocytes were treated with *Vibrio cholera* neuraminidase to remove sialic acids and then modified with CMP–NeuAc and three purified sialyltransferases to contain either the NeuAcα2, 3 Gal, NeuAcα2, 6 Gal or NeuAcα2, 6 Gal NAc linkages on cell surface glycoproteins. The HAs of the two viruses RI/5$^-$ and RI/5$^+$ had totally different specificities, binding respectively to NeuAcα2, 3 Gal and NeuAcα2, 6 Gal linkages. A further implication of this study is that the receptor site and functional properties of the HA may change independently of the overall antigenic properties. Two antigenic sites B and D on the influenza HA molecule (Fig. 7.2) are near the receptor site and therefore it is possible that variations in receptor specificity might arise from natural selective pressure placed by host specified sialyloligosaccharide present on secreted or cell surface glycoproteins. Indeed, more recently, we have described the selection of subpopulations of influenza A and B viruses by cultivation of virus in MDCK cells or in embryonated hens eggs (Schild et al., 1983). The latter host system is extremely selective and clones out a population of virions with different antigenic composition, as detected with both monoclonal and polyclonal antibodies, compared to the same parental virus cultivated in MDCK cells. The novel implication is that a non-immune selective mechanism involving host cell receptor sites may operate to select and generate antigenic variants of influenza virus, thus contributing towards antigenic drift and evolution of the virus.

Influenza virus is redistributed on the cell surface soon after infection (Patterson and Oxford, unpublished data) but, in contrast to adenovirus, inhibition of redistribution is not paralleled by a block in virus uptake. These differences may reflect the number of membrane receptors available to the two viruses. If a sufficient number of suitable receptors are within close proximity of a bound influenza virus particle there would be no requirement for active movement or extensive diffusion of receptors. There is now evidence that receptor mediated uptake of enveloped viruses can occur at specialized sites on the plasma membrane known as 'coated pits'.

Ultrastructurally the sites are recognized by thickening on the cytoplasmic side of the membrane which is due to a 170K protein called clathrin. Receptor bound ligands are taken up at coated pits into coated vesicles (Dourmashkin and Tyrrell, 1970, Patterson et al., 1979) and then transported to lysosomes or other intracellular sites (Fig. 7.3). The clathrin coat dissociates soon after pincing off and is recycled back to the plasma membrane.

Until recently membrane fusion as a mechanism of entry and uncoating was thought to be mainly restricted to the paramyxovirus group (see Chapter 8) but now there is evidence that fusion plays a role in the uncoating of many enveloped viruses. What is not clear for many viruses is the *site* of fusion. Whether there is fusion

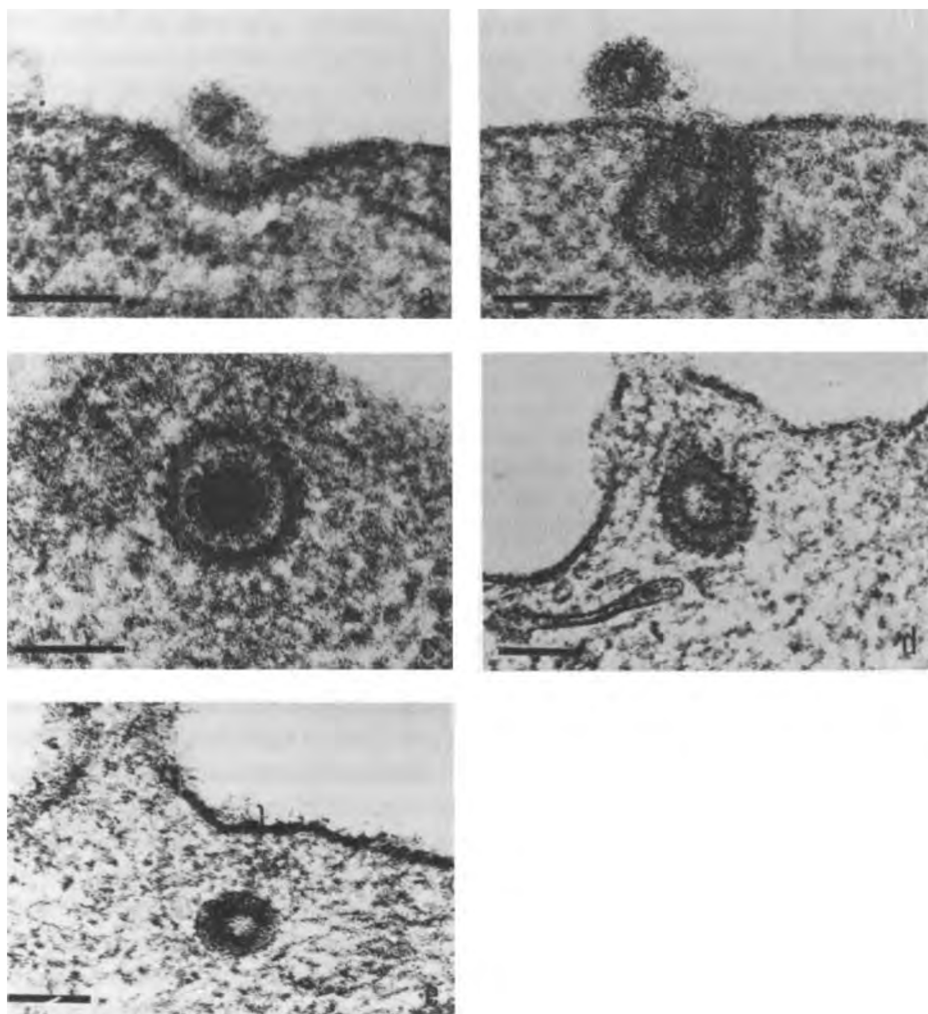


Fig. 7.3. Stages of infection of cell with influenza A virus. (Courtesy of Dr. S. Patterson.)

at the plasma membrane or uptake by pinocytosis into a cytoplasmic vacuole followed by fusion has been disputed for several different virus groups. The finding that the N-terminus of HA2 is highly conserved throughout the different influenza serotypes and is almost identical to the N-terminus of Sendai virus F1 protein has strengthened the fusion argument.

Thus, after uptake into a cytoplasmic vacuole the influenza virus still faces the problem of penetrating a cellular membrane and releasing its nucleic acid in order to initiate infection. In their studies on SFV, Helenius and his colleagues (1980) observed that virions entering via coated pits ended up in lysosomes. In parallel studies SFV was induced to fuse with the plasma membrane of cells by adsorbing cells with virus at 4°C and then briefly exposing the preparations to warm (37°C) medium at pH 5.5. In addition, low pH dependent fusion of virus with liposomes was demonstrated. From this data it was proposed that the low pH environment of the lysosome promotes fusion of the viral and lysosomal membrane, resulting in the release of the viral nucleic acid into the cell cytoplasm. It was claimed that the antiviral action of lysosomotropic agents such as chloroquine, amantadine and NH_4^+ was mediated by an ability to raise lysosomal pH (Ohkuma and Poole, 1978) and block virus infection at an early stage prior to transcription. We shall return to this later in this Chapter.

7.3. Influenza RNA replication

Two types of genome transcript are synthesised during infection, the virus mRNAs which are incomplete transcripts, polyadenylated at their 3′ ends and with additional non viral primer sequences at their 5′ ends, and unpolyadenylated complete transcripts which are considered to be the templates for genome replication (Smith and Hay, 1982). Different mechanisms are responsible for the production of these two classes of cRNAs but surprisingly little is known about the interrelationships between their synthesis and that of vRNA for example. More recent data, however, indicates that vRNA synthesis is regulated throughout infection and this may involve selective transcription of its template. Regulation of vRNA synthesis may be largely responsible for the controlled production of virus mRNAs (Smith and Hay, 1982). The cell nucleus plays an important role in virus replication for influenza is unable to replicate in enucleated cells and replication in nucleated cells is prevented by actinomycin D or mitomycin C treatment before, or early in infection (See Fig. 7.4. for extra details).

In certain respects the initiation of influenza virus RNA transcription resembles the initiation of protein synthesis on cellular messenger RNAs in eukaryotic systems. Affinity labelling and affinity chromatography experiments have demonstrated that a polypeptide of M.W. 24 K, a component of eukaryotic initiation factors 3 and 4B, is involved in cap binding in host cells. More recently it was shown that

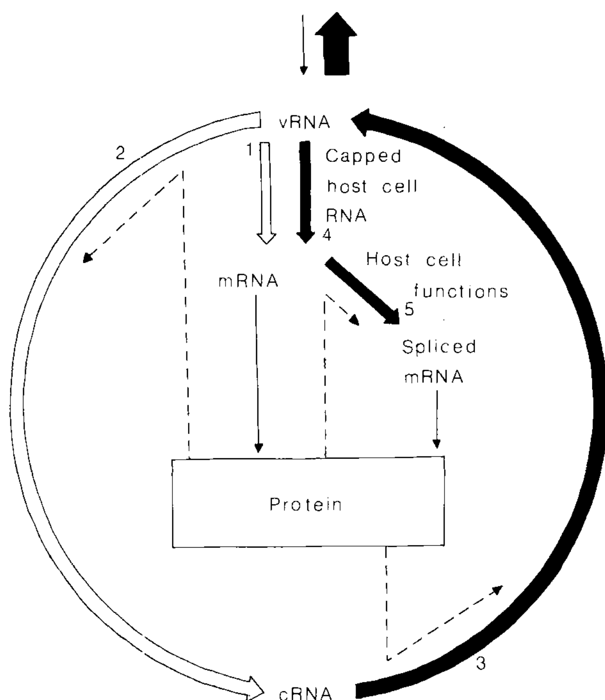


Fig. 7.4. A scheme for the replication of influenza virus genome (after McCauley and Mahy, 1983). ⇒, transcription from input genome RNA; →, transcription from cRNA or newly synthesized vRNA; - - →, necessary interactions. Step 1: primary transcription, which occurs in the absence of protein synthesis but requires host cell transcription. Step 2: cRNA synthesis from input genome vRNA . Step 3: selective and amplified vRNA synthesis directed by cRNA. Both require ongoing protein synthesis. Step 4: transcription of newly synthesized vRNA to form mRNA requires host cell caps. Step 5: modification of mRNAs 7 and 8 by splicing requires protein synthesis and undefined host cell functions.

in the presence of ATP and Mg ions oxidized reovirus mRNA is crosslinked to two proteins of M.W. 28 K and 50 K which may be structurally related to the 24 K polypeptide (Sonenberg, 1981). Studies with monoclonal antibodies indicate that the 24 K cap-binding polypeptide may be contained in even larger protein precursors (Sonenberg et al., 1981). It has also been demonstrated that translation of capped mRNAs can be competitively inhibited by various cap analogues (Both et al., 1976, Roman et al., 1976). Influenza virus has the unique feature to use host cell-coded capped RNAs as primers for the synthesis of its own mRNA (Fig. 7.5). In the priming reaction the cap together with some 10 to 15 nucleotides is cleaved from host RNA by a viral endonuclease and is subsequently used to initiate viral RNA transcription (Krug et al., 1981). As a result a host RNA-derived capped sequence is found to be linked to the 5′-end of the viral mRNA. This cap transfer reaction can also be performed in vitro using a variety of mRNAs such as globin mRNA or reovirus mRNAs as primers. The structural requirements for an RNA

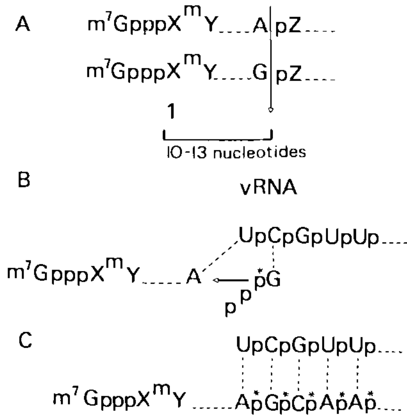


Fig. 7.5. Mechanism for the priming of influenza viral RNA transcription by capped RNAs (after Krug et al., 1981). A, cleavage; B, initiation; C, elongation.

to be active as a primer have been investigated and only RNAs containing a capped 5′-terminus can efficiently initiate viral mRNA synthesis. In addition to natural mRNAs, synthetic polynucleotides such as capped poly (A) and capped poly (AU) can prime viral transcription. Thus, binding of the capped RNA to the viral transcription complex is clearly caused by interactions other than base pairing with the viral template RNA. This leaves the cap structure as the only common signal of recognition of the primer RNA. Therefore it seems likely that some cap recognition site must be present within the polymerase complex and recently the cap binding protein has been established as the virion polymerase protein P2 (Blaas et al., 1982).

In more detail, Krug et al. (1981) identified those bases of a representative primer, β-globin mRNA, that were transferred to the viral RNA transcripts. Using ^{125}I-labelled globin mRNA as primer for in vitro transcription, he found that the predominant sequence at the 5′ end of each viral mRNA segment was identical to the first 13 nucleotides (plus the cap) at the 5′ terminus of β-globin mRNA, which has the sequence: m^{7}Gpppm^{6}1AmC(m)ACUUGCUUUU13GAC....[8]. Because only the C residues were labeled with ^{125}I, these results indicated that possibly the first 12, 13 or 14 5′-terminal bases of β-globin mRNA were transferred to the viral mRNAs (Fig. 7.5). Analysis of the minor ^{125}I-labeled oligonucleotides found in the viral mRNAs indicated that shorter, 5′-terminal fragments of β-globin mRNA (8–11, or 2–3 bases in length) were sometimes transferred and that the transferred pieces were most likely linked to G as the first base incorporated by the transcriptase.

The priming mechanism involves recognition of the 5′-terminal methylated cap structures (m^{7}GpppXm), because only RNAs containing a cap are active as primers. Removal of the m^{7}G of the cap by chemical or enzymatic treatment eliminates all priming activity, and this activity can be restored by enzymatically recapping the RNA. The absence of either methyl group from the cap of an RNA greatly re-

TABLE 7.1.
Influenza virus-coded proteins

Designation	Approximate no. of molecules per virus particle	Molecular weight estimated by:			Remarks
		Gel electrophoresis	Gene sequence	Gene+protein sequence	
P1 polymerase 1		96K			Internal proteins associated with RNA transcriptase activity. Cap binding
P2 polymerase 2	30–60	87K			
P3 polymerase 3		85K			
HA haemagglutinin	500				
HA1				36 074+11 500 (Mem/102/72)	Surface glycoprotein responsible for attachment of virus to cells.
HA2				27 368+1400 (Mem/102/72)	Trimer composed of two polypeptides HA1 and HA2 formed by post-translational cleavage of the primary translation product.
NP nucleoprotein	1000	50–60K	56 106		Internal protein associated with RNA and polymerase proteins, helical arrangement.
NA neuraminidase	100	48–63K	50 087		Surface glycoprotein, with enzyme activity. Tetramer molecule with a 200 000-M_r head.
M1 matrix	3000			27 861	Major virion component surrounding the core, involved in assembly and budding.
M2 matrix		15K	11 000		Coded from the same gene segment as M1 in a second reading frame, a non-structural protein, function unknown.

TABLE 7.1. *(continued)*

Designation	Approximate no. of molecules per virus particle	Molecular weight estimated by:			Remarks
		Gel electrophoresis	Gene sequence	Gene+protein sequence	
NS1 non-structural protein		25K	26 815		Non-structural protein, function unknown.
NS2 non-structural protein		12K	14 216		Non-structural protein coded for in a second reading frame from same gene segment as NS1, function unknown, synthesized late in infection.

For additional references see McGeoch et al., 1976, Skehel, 1972, Lamb et al., 1980, Webster et al., 1982.

duces its priming activity and the absence of both methyl groups completely eliminates activity.

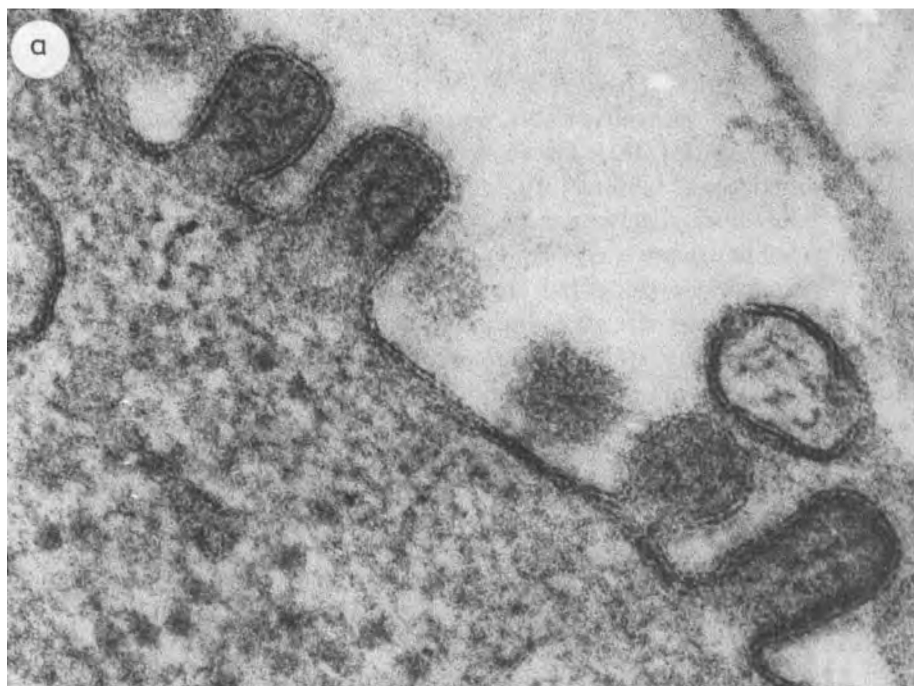
Because similar priming by capped RNAs also apparently occurs in the uninfected cell, the α-amanitin-sensitive (RNA polymerase II) step required for viral RNA transcription in vivo and alluded to above, is explained, i.e. the host RNA polymerase II is required for the synthesis of capped RNA primers. Only those capped cellular RNAs made after infection, and not those pre-existing before infection, serve as primers. One possibility why only newly synthesized capped cellular RNAs serve as primers is that pre-existing, but not newly synthesized, capped RNAs are tied up in ribonucleoprotein structures (including polyribosomes) and cannot be used by the viral transcriptase. If so, the viral transcriptase would be expected to function near the site of synthesis of capped cellular RNAs in the nucleus. Several lines of evidence have suggested that some steps in viral RNA transcription occur in the nucleus, but this has not yet been directly demonstrated. The above sequence of events represents a further target for the development of new inhibitors of influenza virus.

7.4. Later events in influenza virus replication

Much less is known about the later events of influenza virus replication. The virion proteins are synthesized and transported to sites of virus budding. The glycoproteins NA and HA are inserted in the plasma membrane, as detailed above, whilst presumably because of trans-membrane crossing of 'ends' of these glycoproteins, matrix protein aligns itself on the internal side of the plasma membrane. It is assumed that there are specific sites of interaction between NP (containing the RNA and P protein complex) and M, which results in spontaneous formation of complete particles which then bud from the cell surface (Fig. 7.6). Obviously, many questions remain to be answered including the mechanism (if any) for assuring the correct amount of RNA per virion, the triggering process for budding and details of budding itself. It is considered that enzymatic activity of NA ensures release of virions (since inhibition of NA activity results in aggregates of virus at the cell surface) and absence of self aggregation via NA–HA bonding.

7.5. Epidemiology of influenza virus

Influenza viruses are classified into three types – A, B and C – on the basis of their type-specific nucleoprotein and matrix protein antigens although the three viruses also show differences in biological and epidemiological properties. The majority of pandemics so far recorded have been caused by influenza A virus, which is also the type associated with influenza of avian, equine and swine species (Easterday, 1975,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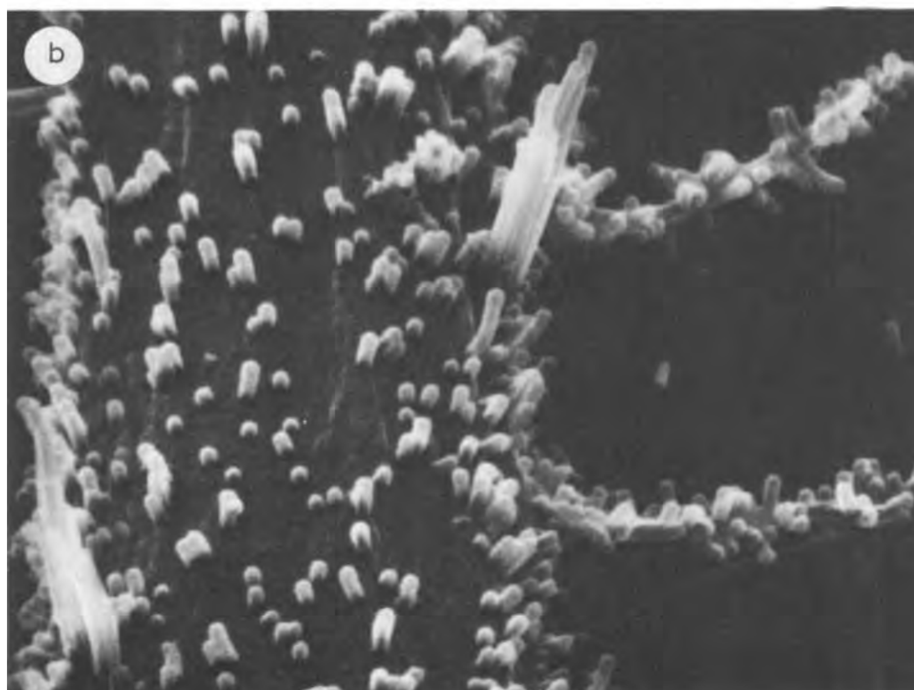
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Laver and Webster, 1979). Type B influenza causes more restricted epidemics and has only been isolated from humans, whereas type C causes even fewer clinical problems in humans, but has also been isolated in pigs (Guo et al., 1983). Type A influenza viruses are further classified into subtypes based on the antigenic characteristics of their surface antigens, haemagglutinin (HA) and neuraminidase (NA). Twelve distinct HA subtypes and nine NA subtypes are now recognized in the nomenclature system for influenza A viruses recommended by the World Health Organization, and a thirteenth HA subtype has recently been identified (Table 7.2). It is implicit in this system that all viruses with common H or N subtype have HA or NA antigens shown to be related by conventional laboratory tests, while such relationships do not occur between subtypes. However, included among viruses of a common subtype designation will be strains showing considerable degrees of antigenic variation ('antigenic drift'). Thus, the human virus A/Bangkok/1/79 (H3N2) contains HA and NA antigens demonstrably related to those of A/Hong Kong/68 (H3N2) virus but showing considerable degrees of difference from them.

Within strains of a single subtype, the differences in deduced amino acid sequence are generally less than 10% but recent extensions of the data have revealed differences in the N-terminal region of HA1 from two strains each of H7 and H10 viruses close to 20%, almost equal to the difference between the amino acid sequences of the two closest subtypes (H2 and H5). Thus, the change from H2 to H3 must involve a more drastic mechanism. Several possibilities could be mentioned to explain this 'antigenic shift': (1) the 'new' virus may have caused an epidemic in man many years previously and have remained hidden ever since. Evidence for this kind of event has been obtained. Thus, the strain of 'Russian flu' (H1N1) which reappeared in Anshan in northern China on 4 May 1977 and subsequently spread to the rest of the world, seems to be identical, in all genes, to the virus which caused an influenza epidemic in 1950. This 'hidden place' could include an animal reservoir, refrigerator or even a persistent or latent infection of humans. (2) Some of the 'new' viruses may be derived from animal or avian viruses. One strain of human influenza has been shown to be such a recombinant (reassortant). The Hong Kong (H3N2) virus contains the NA (and other) genes from an Asian (H2N2) strain of human influenza and an HA which is antigenically related to that of A/Duck/Ukraine/63 (H3N8) and A/equine/2/Miami/63 (H3N8) viruses. The amino acid sequence homology between the HAs of A/Duck/Ukraine/63 and A/Aichi/2/68 viruses (both of subtype H3) is 96%. The donating virus could have been one which contained Hong Kong HA that had persisted from a much earlier human influenza epidemic maintained unchanged in the same way as the Russian 'flu. (3) An animal or bird virus could become infectious for man (Easterday, 1975, Laver and Webster, 1979). This unpre-

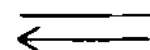


Fig. 7.6. Influenza A viruses budding from infected cells. a, sectioned cell with budding viruses (× 195000) b, scanning electron micrograph of influenza virus budding from human diploid cells (× 26000). (Courtesy of Dr. Hockley.)

TABLE 7.2.
Classification of influenza viruses: proposed grouping of haemagglutinin and neuraminidase antigens

Proposed new designation	Previous subtype designation	Proposed prototype strains
H1	H0	A/PR/8/34
	H1	A/FM/1/47
	Hsw1	A/swine/Wisconsin/15/30
H2	H2	A/Singapore/1/57
H3	H3	A/Hong Kong/1/68
	Heq2	A/Eq/Miami/1/63
	Hav7	A/duck/Ukraine/1/63
H4	Hav4	A/duck/Czech/56
H5	Hav5	A/tern/South Africa/61
H6	Hav6	A/turkey/Mass/3740/65
H7	Heq1	A/equine/Prague/1/56
	Hav1	A/FPV/Dutch/27
H8	Hav8	A/turkey/Ontario/6118/68
H9	Hav9	A/turkey/Wisconsin/66
H10	Hav2	A/chick/Germ/N/49
H11	Hav3	A/duck/England/56
H12	Hav10	A/duck/Alberta/60/76
N1	N1	A/PR/8/34
		A/FM/1/47
		A/swine/Wisconsin/15/30
N2	N2	A/Singapore/1/57
		A/Hong Kong/1/68
N3	Nav2	A/tern/South Africa/61
	Nav3	A/turkey/England/63
N4	Nav4	A/turkey/Ontario/6118/68
N5	Nav5	A/shearwater/E.Aust/72
N6	Nav1	A/duck/England/56
N7	Neq1	A/equine/Prague/1/56
N8	Neq2	A/equine/Miami/1/63
N9	Nav6	A/duck/Memphis/546/74

dictability is one of the major worrying features about influenza, together of course with its demonstrated virulence for humans and explains why so much work is carried out with the virus.

Mortality data from 1200 million people living in 88 of the 156 or so WHO member states (approximately one quarter of the world's population) show that more than two million deaths from acute respiratory disease occur annually (reviewed by Lennette, 1981) and that the majority of acute respiratory disease is attributed to viruses of 5 families. Influenza A and B viruses cause a very significant number

of these deaths. The highest mortality during an influenza epidemic is always noted among certain groups which are identified as 'special risk groups'. We shall see the significance of this group of persons later when we discuss prevention of influenza by vaccines and chemoprophylaxis, because special risk persons constitute the main target groups. Mass vaccination and chemoprophylaxis against influenza is not generally practiced because of the absence of broad spectrum antivirals and the less than 100% efficacy of the current vaccines. Persons in these special risk groups are old people (generally over 65 years of age (see Table 7.3)), babies under 18 months of age, persons with diabetes or chronic heart, kidney and respiratory ailments. As early as 1847, William Farr estimated the impact of influenza in London by estimating excess mortality, viz. he subtracted the number of deaths in an influenza-free winter from the number of deaths in an epidemic winter. Stepwise multiple regression models are now used (Tillett et al., 1980) to obtain these estimates using, in the UK, data from the office Population Censuses and Surveys and generally figures over an 8–11 year period are analyzed. Factors known to influence the 4 weekly returns include seasonal variation and time trends in morbidity and mortality. The results of such an analysis are shown in Table 7.3. The influenza outbreak in 1975–76 was caused by two influenza A (H3N2) variants and also influenza B viruses and 22 250 excess deaths were recorded, most of them due to respiratory causes. This represents the deaths of approximately 1 in 275 of the elderly population in the UK. In the same winter there were 908 000 excess sickness benefit claims, equivalent to an illness in approximately 1 in 20 of those persons insured (viz. 56% of the 16–64 age group). So the total morbidity in the adult population was 1.6 million. This analysis does not include children.

It is widely recognized therefore that influenza A and B viruses form very worth-

TABLE 7.3.
Estimates of excess deaths and sickness benefit claims in England and Wales attributable to influenza (after Tillett et al., 1980)

Winter	Excess total deaths all causes	Excess deaths respiratory causes	Excess deaths 65+ age group all causes	Excess new claims for sickness benefit (×1000)
1975–76	22 250	17 210	19 590	908
1976–77	4060	5790	NA	322
1977–78	5910	6080	NA	364
1978–79	5430	5500	NA	237
Approx. standard error of estimates	5000	2500	4300	225

NA, data not available

while 'targets' for new drugs and vaccines. Although influenza A virus is one of the most studied viruses, particularly from the point of view of molecular biology of virus replication, morphology and antigenic structure, many important questions concerning the biochemical basis of virulence and even the epidemiology of the virus remain unanswered. Specific preventive measures are often undertaken haphazardly and with no very great enthusiasm in many countries.

7.6. Influenza – clinical features

Characteristically the illness begins suddenly (incubation period 2–3 days) with shivering, malaise, headache and aching of limb muscles and a pain in the back (Christie, 1980). The temperature rises rapidly and the afflicted person takes to bed. The main feeling is one of general malaise, pain and discomfort. The temperature ranges from 38.3 to 40.5°C and commonly cheeks may be flushed, eyes infected, throat congested. Fortunately the illness is most often short lived, the temperature drops by day 3 and the patient is out of bed by day 5 or 6 and working on day 10. However, in older patients the recovery period can be quite prolonged and weakness and lassitude marked. In general, mortality figures suggest 1 death in 6000 cases. Young children tend to have a milder disease than older children or adults (reviewed in Stuart-Harris and Schild, 1976). Aspects of diagnosis of influenza are also considered in Fig. 7.10.

Chest complications tend to occur in patients previously suffering from chronic bronchitis or rheumatic, ischaemic or hypertensive heart disease. Pneumonia may result from infection by the virus itself, invasion by staphylococci after virus damage of epithelial cells or invasion by pneumococci. Viral pneumonia presents as an overwhelming infection with profound toxaemia – so much so that the respiratory signs may not be noticed. In staphylococcus pneumonia the clinical signs may be more noticeably respiratory and the person may have partially recovered from influenza when the recovery is interrupted by a sudden onset of dyspnoea. Cyanosis may be featured with signs of shock and collapse of blood pressure. Pneumococcal pneumonia may often be less severe and onset may be delayed until the patient appears to be at the post influenza convalescent stage. Classical signs of lobar consolidation are noted but the response to antibiotics is usually rapid. A rare complication, which is frequently fatal, of influenza B (and influenza A) infection is Reye's syndrome, a fatty degeneration of the viscera and especially the liver.

7.7. Pathogenicity

The main individual aspects of the pathogenicity (viz. virulence) of any virus are mucous surface interaction, entry to host tissues, virus replication, avoidance of

host defences spread, damage to the host, and tissue or organ specificity (reviewed by Sweet and Smith, 1980). The overall 'virulence' of the virus may depend on complex interactions between these varying factors, or, in some cases a single factor will predominate (Mims, 1982, Bosch et al., 1979, Burnet, 1979, Scholtissek et al., 1977, Rott et al., 1976).

Assessments of the degree of virulence of a pandemic and moreover an antigenically variable virus such as influenza is probably impossible, because it is difficult to establish if recorded differences in mortality, for example, between the 1918 influenza pandemic and the 1957 pandemic (estimated 16 times higher mortality in 1918) were caused by an increased virulence of the virus causing the former pandemic or whether the immune status of the population was different, or whether additional factors such as population movements, unusual foci of high population density (because of the world war) contributed. Nevertheless it is quite clear that variations in virulence of influenza viruses do occur in the laboratory.

Following an infection in humans, influenza virus invades the epithelial surfaces of the respiratory tract and many different types of cells can be infected including ciliated, intermediate, basal and goblet cells of the nasal epithelium or more rarely in the case of pneumonia, cells lining the alveoli (reviewed by Sweet and Smith, 1980). Humans may be infected initially by the settling of large particles containing virus in the upper respiratory tract or more fine particles in the lower respiratory tract. It is particularly interesting that, most commonly, influenza is an infection of the *upper* respiratory tract in humans. It can be speculated that in the lung, alveolar macrophages may be particularly efficient at removing and destroying virions. The presence of non-specific inhibitors such as mucoproteins can neutralize influenza virus infectivity by preventing attachment of virus to new susceptible cells. Influenza virus can be carried and hence spread downwards in the respiratory tract by mucociliary action or downward drainage and rarely (in humans) can infect lung tissue and cause death by primary virus pneumonia.

7.8. Inhibitors of influenza virus

Table 7.4 summarizes (although not exhaustively) attempts over the last 3–4 decades to obtain effective inhibitors of influenza A and B virus. Only two compounds (apart from interferon) have confirmed antiviral activity in man, namely amantadine and ribavirin, and therefore particular attention is devoted to these two molecules. Amantadine can be used as an excellent illustration of the problems and frustrations of developing an anti-influenza virus compound (Tables 7.5, 6).

We would like to emphasize two important points at this stage. Firstly, influenza virus may represent a rather unique virus where chemotherapy *and* vaccines may have to be used as a complete strategy for virus control in the field. Secondly, it should be realized that most of the past searches for new influenza inhibitors have

TABLE 7.4.
Inhibitors of influenza virus

Function	Inhibitor	Mode of action
Virion attachment and penetration	Sulphated polysaccharides	Inhibition of virus adsorption
	Amphotericin B methyl ester	Acts on lipid membranes
	Amantadine hydrochloride and derivatives	Inhibition of initiation of transcription by virion transcriptase, or fusion events
RNA transcription	Halogenated ribofuranosyl-benzimidazoles	Unknown
	Ribavirin triphosphate	Selective inhibition of viral RNA polymerase?
	Gliotoxin	Reacts with sulphydryl groups
	Aranotin	
	Selenocystine	
	2-Acetylpyridine-3-thiosemicarbazone (2-APTSC)	High activity against transcriptase enzyme in vitro but not in vivo
	Ca-EDTA and Ca-DTPA liposomes	Act against zinc metalloenzyme by formation of apoenzyme zinc-ligand complex
	Triphenylmethane compounds	Active against RNA transcriptase
Viral protein synthesis and later events of transport of viral proteins	Plant antiviral peptide (PAP)	Elongation of proteins. Effect on ribosomes?
	2-Deoxy-D-glucose	Inhibition of glycosylation
	Glucosamine	
	Tunicamycin	
	Mithramycin	Inhibited HA, NA and M protein protease inhibitors
	Canavanine	Inhibition of RNP formation
	Concanavalin A	Inhibits release of newly formed virions
	FANA	Inhibits virus budding, effective in tissue culture, but not in mice. Neuraminidase inhibitor

See also Oxford, 1977

been carried out using biological screening procedures and more or less random testing of synthesized molecules (Swallow, 1978). Of course, in retrospect, it is not possible to deduce whether the rather depressing lack of success has been due to the particular features of the virus itself, or alternatively to the 'randomness' of the search (Galasso et al., 1979). This question may be answered in the next few years as more groups utilize what appears to be more 'designed' approaches searching for inhibitors of RNA transcriptase, fusion, neuraminidase, mRNA capping etc. Sequence studies of HA, NA and other virus proteins have now provided a vast amount of hard biochemical data on which new more logical screening procedures

TABLE 7.5.
Biological activities of amantadine and recently synthesized analogues

Molecule[a]	Chemical structure	Biological activity
1	amino adamantane (amantadine)	Influenza A virus inhibitor in vitro, in vivo and in man. Effective in Parkinson's disease
2	α-Methyl-1-adamantane methylamine (rimantadine)	Influenza A virus inhibitor in vitro, in vivo and in man (no activity in Parkinson's disease)
3	1-amino-3,5,dimethyl-adamantane (memantine, DMAA)	More potent than amantadine as stimulator of motor activity. Parkinson's drug
4	1 substituted adamantyl hydrazine derivative	Anti mycoplasma and fungal and herpes (HSV) activity
5	N substituted 1 adamantyl carboxamidines	Antiviral activity versus influenza A, vaccinia and herpes virus. The 4 amino derivative had anti polio activity whilst the adamantyl acetamidine inhibited NDV
6	acylated adamantyl thio urea derivatives	In vivo anti influenza A activity (not active versus HSV, SFV or Sendai)
7	alkyl substituted thio ureas	In vitro activity versus HSV, vaccinia and adenovirus
8	amide derivatives: N-(1-adamantyl) cinnamide	In vitro versus influenza, HSV, vaccinia. In vivo versus influenza A
9	propyl carbamate	antifungal
10	4 homotwistane derivatives	Amino and amino ethyl analogues were active against NDV
11	aminospirane	In vitro against influenza A and B viruses. In vivo activity versus influenza A. The dialkyl aminoalkyl derivative inhibited rhinovirus type 14 in vitro
12	2 substituted 1-(aminoalkyl) adamantanes	Some molecules may have anti Parkinson's activity
13	phencyclidine analogues	Anti cholinergic activity
14	bicyclo-octyloxyaniline	Hypo beta lipoproteinaemic agent

[a]See Fig. 7.7 for molecular structure of the compounds.

can be based. An excellent example of such a new approach is the synthesis of short peptides, whose sequence is deduced from that of the known fusion sequence at the N terminus of HA2 (Richardson et al., 1980).

7.9 Amantadine

The cyclic primary amine Amantadine (Symmetrel) was the first, and remains at present the only, inhibitor of influenza A virus replication to be licensed in several

TABLE 7.6.
Brief history of the development of amantadine as an antiviral compound

Event	Reference
1. Influenza A and B viruses shown to be inhibited by amines and ammonium compounds	Eaton et al. (1962)
2. Amantadine selected as an amine with in vivo activity but only against influenza A viruses	Davies et al. (1964) Grunert et al. (1965)
3. Mode of action of amantadine established approximately as inhibiting an early event of virus replication	
4. Clinical efficacy of amantadine described in prophylactic studies in volunteers with H2N2 viruses	Jackson et al. (1963)
5. In vitro and in vivo drug resistant H2N2 viruses isolated	Oxford et al. (1970)
6. Clinical efficacy of therapeutic administration	Hornick et al. (1970)
7. 'Re-emerged' H1N1 viruses and also H3N2 viruses inhibited in vitro and in clinical studies	
8. Aerosol usage of amantadine investigated in experimental and clinical pneumonia	
9. Clinical comparison of amantadine and rimantadine show approximately comparable antiviral activity but rimantadine may be less toxic	Zlydnikov et al. (1981) Dolin et al. (1982)
10. Mode of action of amantadine reinvestigated to include fusion events and lysomotropic activity. Multiple modes of action?	White et al. (1981) Bukrinskaya et al. (1980)
11. General consensus that rimantadine (200 mg/day) is less toxic than amantadine (200 mg/day) although this may relate to different pharmacokinetics. Rimantadine is recommended therefore for prophylaxis and *either* compound for therapy.	Galasso et al. (1984)

Note that amantadine is also used in the treatment of Parkinson's disease (Timberlake and Vance, 1978, Schwab et al., 1972)

countries and to be used as an antiviral in the clinic and general community (reviewed by Oxford and Galbraith, 1980, Hoffman, 1980, Smorodintsev et al., 1970, Tables 7.5 and 7.6). Nevertheless, neither the original compound nor molecular derivatives (Table 7.5) such as rimantadine (Zlydnikov et al., 1981, Indulen and Kalninya, 1980) have gained universal acceptance among clinicians as fully effective compounds to be used prophylactically or therapeutically against influenza virus infections. There is little doubt that the relatively poor usage of amantadine has reduced interest in the search and development of new antivirals against influenza and other respiratory viruses, and it is of some importance to establish if the problems faced by amantadine are unique to this compound or would be similar for other (and hopefully even more effective) antivirals against these respiratory viruses. It could be added that in comparison to the dearth of antivirals against respiratory viruses, compounds acting against herpes virus infections have been developed (acyclovir, and PFA) and may soon be established as the first antivirals to be used on a large scale in the community (see Chapters 12, 13).

7.9.1. Inhibition of influenza A viruses by amantadine – do different influenza A virus subtypes vary in their degree of inhibition by the compound?

Laboratory studies of the inhibition of influenza viruses by amines (including amantadine) and ammonium compounds established quite early that the spectrum of antiviral activity of certain (but not all) of these compounds was somewhat restricted (Fig. 7.7). Thus, although certain simple primary amines such as methylamine inhibited both influenza A and B virus in vitro (Eaton et al., 1962, Oxford and Schild, 1968) amantadine had little or no effect against influenza B viruses and only inhibited influenza A viruses (Tables 7.7, 8). In addition, studies indicated some difference even between influenza A viruses of different subtypes as regards their degree of inhibition by amantadine. Certain viruses, such as A/PR/8/34 (H1N1), were poorly inhibited by the compound, whereas viruses of the H2N2 subtype, for example, were well inhibited (Grunert et al., 1965, Schild and Sutton, 1965). In retrospect this 'increased' resistance might have been caused by the multiple mutations undoubtedly accumulated by A/PR/8/34 virus during extensive laboratory passage.

This early laboratory data which showed an apparent heterogeneity of response of influenza A viruses to inhibition by amantadine gave rise to concern that new epidemic subtypes of influenza A might not be inhibited by amantadine. As a result the compound was only licensed initially in the USA for prophylaxis of influenza A viruses of the H2N2 subtypes, which were demonstrably sensitive to the com-

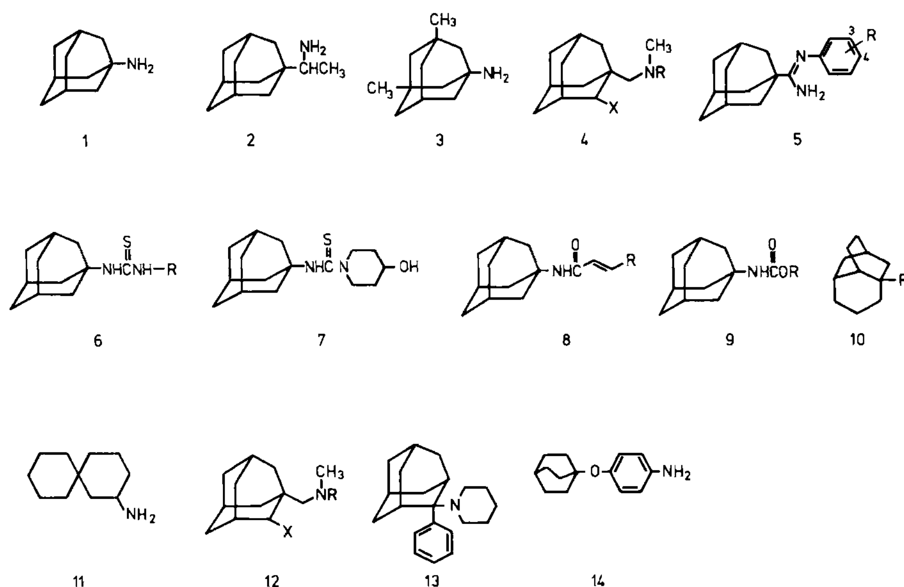


Fig. 7.7. Amantadine molecules tested for antiviral activity (see Table 7.5).

TABLE 7.7.
Inhibition of influenza A and B viruses by amantadine and ammonium ions

Virus	Reduction in virus end point titre ($log_{10}TCID_{50}$/ml)		
	Amantadine (25 μg/ml)	Ammonium acetate (100 μg/ml)	Rimantadine (25 μg/ml)
A/NWS(H1N1)	2.0	1.7	1.5
A/Singapore/1/57(H2N2)	2.5	3.5	Not tested
A/Scotland/49/57(H2N2)	6.0	4.5	5.5
B/England/13/65	0.5	2.6	0.5

MK cell cultures were infected with influenza viruses and incubated in the presence or absence of drugs for 3–4 days. Virus infectivity endpoints were determined by haemadsorption. A reduction of 1.0 $log_{10}TCID_{50}$/ml or 90 per cent inhibition of virus growth is considered significant in this test.

pound. When the new pandemic influenza A/Hong Kong/68 (H3N2) virus appeared, an opportunity was lost to use and test the compound on a large scale during the first wave of the epidemic at a time when no influenza vaccine was available. However, the compound is now licensed in the USA and UK for the prophylaxis and therapy of *any* human influenza A virus.

In summary, therefore, it would appear that most but not all (see below) recent and unpassaged influenza A viruses of the different human subtypes (H1N1), (H2N2) and (H3N2) are well inhibited by amantadine in vitro and in laboratory animal model systems. Furthermore, with the earlier H2N2 virus isolates a good correlation was established in most, but not all studies, between the in vitro inhibitory effect of amantadine and clinical efficacy and therefore it was anticipated that the recent H1N1 and H3N2 viruses would be inhibited in clinical practice. Recent clinical trials have confirmed this optimism (see below).

7.9.2. Amantadine-resistant influenza viruses

Earlier studies (Oxford et al., 1970) showed that amantadine-resistant influenza A viruses could be selected by passage of virus in mice treated with very high (150 mg/kg/day) doses of the compound. Before passage in vitro the influenza A virus was inhibited by 0.3 μg/ml amantadine, whereas after a single passage in the presence of the drug, 6 μg/ml of amantadine was required to inhibit replication of some virus isolates. After six passages in vivo most influenza strains were completely resistant to amantadine and the related drug rimantadine.

A number of laboratories have since investigated the genetic basis of amantadine resistance by producing virus recombinants between amantadine resistant and

TABLE 7.8.
Inhibition of polypeptide synthesis of influenza A and B viruses by amantadine

Virus	% Inhibition of virus induced polypeptide synthesis by 25 μg/ml amantadine		
	NP	NS1	M
Hswine1N1			
A/NJ/8/76	80.0	92.0	80.5
H1N1 subtype			
A/Jap/93NS/78	69.8±15.3	91.7±3.2	99.1±1.3
A/Lackland/AFB/3/78	90.5	96.6	94.0
A/Brazil/11/78	96.5	98.4	ND
A/Fukushima/78	95.0	95.0	ND
H2N2 subtype			
A/Leningrad/549/80	77.4	91.9	86.8
H3N2 subtype			
A/England/641/78	87.4	86.8	86.5
A/England/939/78	83.9	93.8	93.1
A/England/938/78	60.2	87.5	69.0
A/Alaska/78	73.9	88.2	99.2
A/Bangkok/1/79	74.3	90.1	93.6
Influenza B/Singapore/79	0	0	0

Vero cell cultures were infected with 10 EID_{50}/cell of virus and incubated in the presence or absence of amantadine overnight when the cells were pulsed with ^{35}S methionine for 30 min and cell lysates analysed by electrophoresis in polyacrylamide gels. Following autoradiography, the quantities of virus induced polypeptides were estimated by densitometer analysis of autoradiographs and analysis of the tracings using a Kontron MOP digiplan apparatus. Note that polypeptide synthesis of all the influenza A viruses is well inhibited by amantadine but no antiviral effect is detected against a representative influenza B virus.

amantadine susceptible influenza A viruses (Scholtissek and Faulkner, 1979, Lubeck et al., 1978). In this way transfer of drug resistance can be correlated with transfer of a particular gene or group of genes. At present the results from different laboratories are somewhat conflicting, although several groups agree that gene 7, coding for matrix protein, appears to co-segregate with amantadine resistance. The interpretations, however, are complicated to some extent by the observation that using different in vitro techniques the same influenza A virus may appear inhibited or resistant to the drug. In addition, data on determinants of influenza virulence suggest a multi-gene linkage with these biological properties. It is quite possible, however, that several gene products are involved in the mode of action of amanta-

dine and that the product of gene 7 may have 'helper' activity.

At present little extensive field work has been carried out to search for rimantadine or amantadine resistant viruses in contacts or in persons being treated for influenza. This is an important aspect to investigate since it is quite likely that amantadine resistance could spread among field viruses by genetic reassortment. Some influenza H3N2 or H1N1 viruses circulating at present in the community are known to be recombinants (see Chapter 17) containing genes of both virus subtypes and thus intra- or intertypic recombination is probably occurring with a relatively high frequency (Ghendon et al., 1981). Heider et al. (1981) have recently reported two relatively resistant influenza A H3N2 virus isolates in Berlin where rimantadine has not been used as a prophylatic. However, even the amantadine sensitive viruses in this work showed a rather poor dose response to amantadine and therefore the study needs to be extended and confirmed. Our own studies in the UK would suggest that occasional field isolates of H3N2 and H1N1 viruses are partially resistant to amantadine.

7.9.3. Inhibition of virus replication by amantadine — mode of action at the molecular level

Early biological studies demonstrated that amantadine acted at an early stage in influenza A virus infection and later, more detailed studies established the point of action at approximately the late stage of virus uncoating (reviewed by Oxford and Galbraith, 1980). More recently, recognition that the N terminus of the HA2 polypeptide of influenza haemagglutinin has an amino acid sequence similar to that of the fusion sequence of the F protein of Sendai viruses, and also the demonstration of fusion and haemolysis events between influenza viruses and cells at low pH (White et al., 1981) have led to the hypothesis of an important role of fusion during infection of cells with influenza A virus. A possible re-interpretation of the above data on the mode of action of amantadine at present would be that influenza viruses penetrate susceptible cells by viropexis and thus enter the cell cyptoplasm in coated vesicles (Fig. 7.8). Comparable pre-lysosomal cytoplasmic vacuoles have been shown to have a low pH and under these conditions influenza HA-mediated fusion could occur, since a configuration change in the HA could result in the N terminus of HA2, which is normally some distance from either end of the molecule contacting the membrane of the vacuole (Wilson et al., 1981). Fusion of the viral and vacuole membranes would then occur, resulting in release of viral RNA and subsequent transport to the cell nucleus, where initial viral RNA transcription is known to occur. Amantadine and other amines are known to increase the pH of intracytoplasmic vacuoles and so the drug could act by simply increasing the pH to 6.5 or 7.0 when fusion could not occur and viral infection would be blockaded. The hypothesis is most attractive, but some important observations remain contradicting at present. Thus, as we noted above, amantadine resistance is known to be cor-

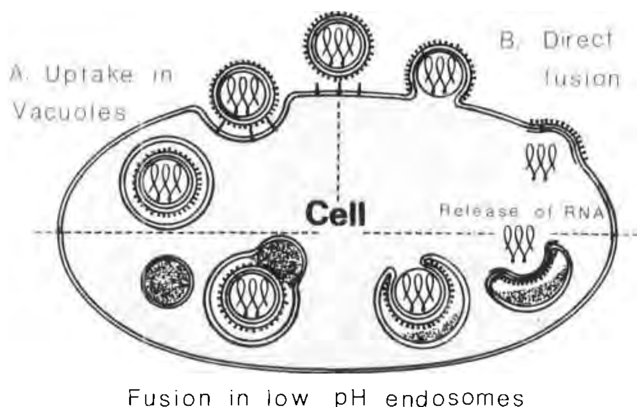
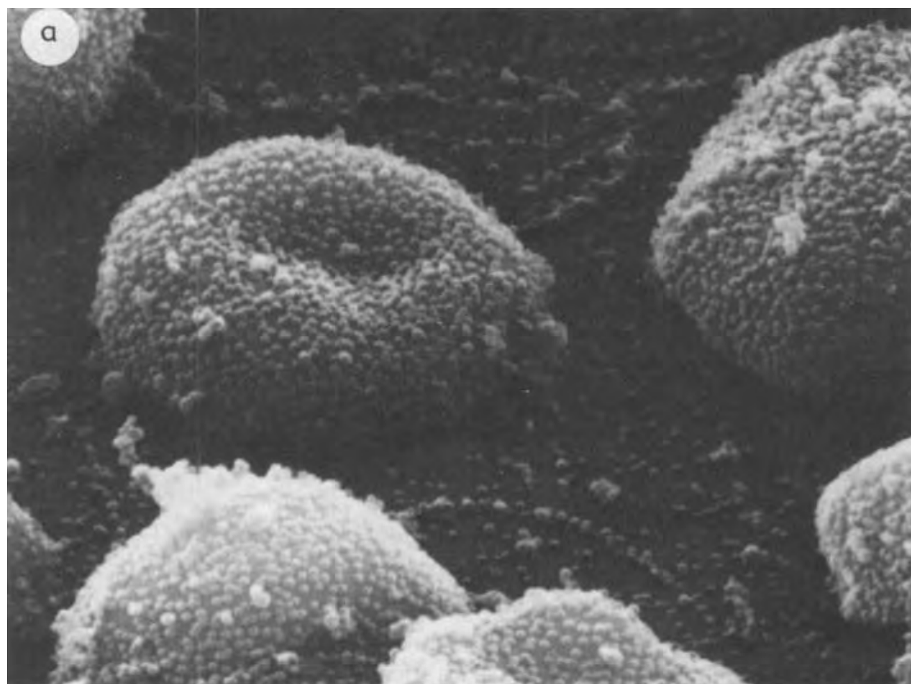


Fig. 7.8. A possible mode of action of amantadine in preventing fusion (drawing courtesy of Dr. D. Hockley).

related with gene 7 (coding for M protein) and not gene 4 (coding for HA). In addition, there is the problem also referred to above, of why some viruses such as influenza B would be resistant to amantadine. Finally, in a recent study Richman et al. (1981) showed that when amantadine-treated cells were washed in compound free medium, though relatively high concentrations of amantadine remained intracellularly, the cells were now *susceptible* to infection. Yet immediately before, with amantadine in the culture medium and equilibrated in the cytoplasm, the cells resisted infection. This implies an antiviral role of amantadine at the superficial external plasma membrane of the cell rather than an intracellular action. Earlier biological experiments with amantadine-treated cells showed that after incubation with trypsin (which removed surface adhering material and presumably amantadine) cells become susceptible to viral infection.

We have investigated the pH optimum of haemolysis (Fig. 7.9) of a number of A and B viruses as part of a separate study on biological characteristics of field isolates of influenza virus. If the pH optimum of fusion as shown by haemolysis for influenza B viruses and drug-resistant A viruses was higher than for amantadine sensitive influenza A viruses, fusion and hence infection would proceed even in the presence of amantadine at pH 6.6, whereas corresponding events would be blocked with most amantadine-sensitive influenza A viruses. Although a small but reproducible difference in pH optimum and pH maximum for haemolysis was noted between an amantadine-resistant virus (maximum pH 6.2) and the parental amantadine-sensitive virus (maximum pH 5.6) this was nevertheless close to the range of pHs shown by other influenza A viruses (Table 7.9). In addition, recombinants such as X-49 and NIB-4, NIB-7 and NIB-8 which are known to have inherited gene 7 and other genes from A/PR8/34 (H1N1) virus, and are thus relatively resistant to amantadine like the A/PR8/34 parent, show a similar pH optimum and maximum for haemolysis to the second parental strains which are all inhibited by amantadine.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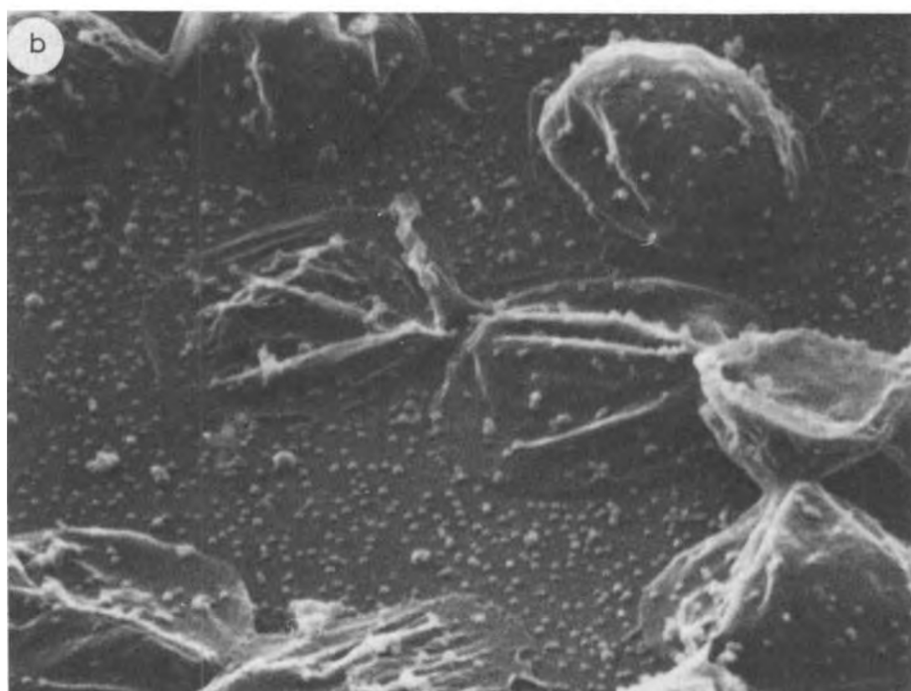

b

TABLE 7.9.
pH optimum and pH maximum of haemolysis for a range of influenza A and B viruses

Viruses		pH maximum	Amantadine resistance
B/HK/73		5.8	+
A/Leningrad/80	(H2N2)	5.2	0
A/Brazil/78	(H1N1)	5.4	0
A/PR/8/34	(H1N1)	5.3	+
A/USSR/77	(H1N1)	5.1	0
A/Bangkok/79	(H3N2)	5.6	0
Recombinants			
NIB-8		5.6	+
NIB-4		5.4	+
NIB-7		5.3	+
Passaged strains			
A/Krashnidor/59	(H2N2)	5.6	0
A/Krashnidor/59R	(H2N2)	>6.2	+

Note that A/Krashnidor/59/R had been derived from the amantadine-susceptible parent by in vitro passage in the presence of rimantadine (M. Indulen, personal communication). Approximately 10 μl of purified virus (10 mg/ml) was adsorbed at neutral pH to 0.1 ml of 10% suspension of erythrocytes. 2 ml of phosphate-citrate buffer was added at pH varying from 5.0 to 6.5 in 0.1 unit steps. After incubation at 37°C for 1 h the lysed cells were deposited by centrifugation and the optical density of the supernatant fluid determined using a Unicam SP 1750 spectrophotometer to estimate the degree of haemolysis compared with RBC incubated in the absence of virus. pH maximum is the higher pH at which significant haemolysis occurred.

Influenza B/HK/73 virus showed a high pH optimum and a high pH maximum of haemolysis, but B/Le/40 was similar to influenza A viruses in its pH profile although neither virus is inhibited by amantadine (Table 7.9). Finally, as anticipated, high concentrations of added amantadine did not inhibit haemolysis of red blood cells by amantadine-sensitive influenza A viruses.

Thus, further work is required to investigate and establish any role for a fusion event, to determine if low pH haemolysis of red blood cells is caused by a fusion process and to look for a correlation between pH optimum for this biological event and amantadine resistance or susceptibility.

←

Fig. 7.9. Low pH induced haemolysis of RBC studied by scanning electron microscopy. a, red blood cells at normal pH with virions adsorbed; b, red blood cells with virus attached at pH 6. – extensive lysis of cells has occurred. (Courtesy of Dr. D. Hockley.)

7.9.4. Possible influence of genetic and phenotypic variation among influenza viruses on amantadine-induced protection

Genetic and phenotypic heterogeneity of viruses may be of considerable practical importance in attempts to control certain virus diseases by chemo- or immunoprophylaxis (see Chapter 17). Thus, certain naturally circulating influenza A viruses may be resistant to antiviral drugs such as amantadine by virtue of mutations in gene 7 or may differ in virulence, antigenic or biological properties and thus may be able to circumvent drug induced protection. Studies of influenza A and B viruses circulating in the community have shown that quite extensive genetic and phenotypic variation occurs. Thus, a number of recently isolated influenza viruses of both H3N2 and H1N1 antigenic subtypes have a temperature sensitive (*ts*) phenotype and differ in virulence for volunteers (Chapter 17). Laboratory studies of artificially induced influenza *ts* mutants have demonstrated clearly that such mutants are attenuated for man and that the shut-off temperature is related to attenuation. The occurrence of non-*ts* and *ts* viruses in nature probably indicates that influenza A viruses of varying virulence are circulating in the community. In our studies, even viruses isolated from the same city varied considerably in the phenotypic *ts* character. Influenza A and B viruses circulating in the community may also differ in respect to the biological property of plaquing in MDCK cells and to the electrophoretic properties of structural and non-structural polypeptides and RNA. Finally, analysis of field isolates of influenza A and B viruses using panels of monoclonal antibodies to virus HA indicates a considerable degree of antigenic heterogeneity even among viruses isolated in rather circumscribed outbreaks in single towns or schools (Chapter 17). It is quite possible that biological techniques currently used to estimate the degree of inhibition of viruses by amantadine are too insensitive and that significant but small differences in drug resistance between viruses may be missed. These differences might, however, have a significant effect on the circulation of viruses in the community.

7.9.5. Pharmacology of amantadine

In summary, it has been established that, following oral administration of amantadine to man or mice, concentrations of amantadine could be attained in the tissues which would be expected to have influenza A virus inhibitory effects (around 1 μg amantadine/g tissue). However, what does *not* appear to be clearly established is the exact intracellular localization of administered amantadine and the correlation with antiviral activity (Richman et al., 1981).

Following single oral doses of amantadine hydrochloride, an average of 86% of the dose was recovered from the urine in five different subjects on nine occasions when urine collections were continued for 4 days or more. The average excretion in 24 hours was 56% of the dose based on 21 determinations in 16 subjects (Table 7.10).

TABLE 7.10.
Blood levels of amantadine in human subjects following single-oral doses of the hydrochloride (after Bleidner et al., 1965)

Time after dose	Blood level (μg/ml) dose		
	2.5 mg/kg	4.0 mg/kg	5.0 mg/kg
0	0	0	0
0.5	0	0.1	0.1
1	0.1	0.3	0.6
2	0.2	0.3	0.6
4	0.3	0.5	0.4
6	0.2	0.3	0.3
8	0.2	0.3	0.5
24	0.1	0.1	

The maximum blood level was generally reached in 1 to 4 hours after an oral dose (Table 7.11). There was no evidence of acetylated or methylated forms of amantadine in any of the human urine samples examined in spite of efforts to demonstrate their presence, and no extraneous peaks have been observed that can be attributed to metabolites of the drug (Bleidner et al., 1965).

Aoki et al. (1979) investigated the disposition of doses of 25, 100 and 150 mg amantadine taken every 12 h for 15 days in 13 healthy young adults. The authors detected a rather slower absorption of the drug compared to previous studies. The average time to peak plasma concentrations was 3–4 h. Almost complete oral bioavailability of amantadine was indicated by the recovery of approximately 80% of a single oral dose. The median ratio of plasma to renal clearance of amantadine approximated unity, which suggested that the compound was not extensively metabolized.

Distribution of an amantadine derivative (1-amino-3,5-dimethyl adamantane)

TABLE 7.11.
Urinary excretion of amantadine in human subjects (after Bleidner et al., 1965)

Time after dose (hr)	Dose range (mg/kg)	Recovery (% of dose)	Recovery range (% of dose)
0–24	2–7	56±13	27–78[a]
0–96	2–4	86± 9	62–93[b]

Human volunteers were given a single oral dose of amantadine and levels of amantadine in the urine were determined at various times thereafter.
[a] 21 determinations in 16 subjects
[b] 9 determinations in 5 subjects

was established in post-mortem tissues of a 77-year-old woman with Parkinson's disease (Wesemann et al., 1980). The patient had been treated with 2×10 mg of the amantadine molecule daily for 53 days. Levels of the compound (μg/g) in the tissues were as follows: kidney (0.18), lung (0.17), spleen (0.1), blood (0.07), cerebellum (0.22). Thus, it is of a particular interest that relatively high levels of the molecule were found in the lung.

Levels of amantadine in tissue specimens were also determined in a 5-month-old girl with influenza A virus pneumonia (Fishaut and Mostow, 1980). 2.5 mg/kg of amantadine each 12 h was administered and tissue specimens obtained $4\frac{1}{2}$ h after the final dose. Serum concentrations ranged from 0.8 to 1.64 μg/ml whilst higher concentrations of amantadine were found in the lung (21.4 μg/ml). This level of compound would significantly inhibit the replication of recent H3N2 and H1N1 influenza A viruses.

In other studies of pharmacology, volunteers were given aerosols of amantadine (Hayden et al., 1980). One hour after aerosol treatments with 1.0 g amantadine per 100 ml of solution in the glass nebulizer, amantadine levels in nasal wash samples (mean 30.3 μg/ml) greatly exceeded blood and nasal wash levels following oral administration.

Little detailed work has been published concerning the pharmacology of many of the derivatives of amantadine which have been synthesized and tested as antivirals. However, the varied biological activities of some of these molecules would suggest that differences in tissue distribution and adsorption might occur. As an example, rimantadine induces fewer CNS side effects in humans than amantadine and this might be related to reduced levels in the CNS since, for example, the compound has no anti-Parkinson's disease activity unlike amantadine itself which is active in this region.

7.9.6. Toxicology of amantadine

A central nervous stimulant effect of amantadine was described (Vernier et al., 1969) in acute and chronic toxicity studies in animals, but only at concentrations around 30 mg/kg orally, which is about 10 times the dosage in man. The predominant signs of central nervous stimulation were increased motor activity, tremors, anorexia, increased sensitivity to environmental stimuli or convulsions in some cases. The stimulant effects have also been described in volunteers given high doses of the compound. At relatively high doses in animals, other effects were transient vasodepressor effects, cardiac arrhythmias, weak ganglionic blocking effect, increase of myocardial contractile force or blocks of phenethylamine vasopressor response (Vernier et al., 1969).

7.9.7. Absence of subtle induced CNS effects of amantadine and rimantadine

Millet et al. (1982) have carried out a controlled comparison of amantadine and rimantadine on CNS effects and included a commonly used antihistamine compound (chlorpheniramine). The study was carried out at the University of California and 52 adult volunteers participated, with a mean age of 25 years. There was no significant difference among treatment groups with respect to age or sex of the subjects. Mild symptoms occurred with approximately equal frequency in all groups and therefore were excluded from further analysis. Table 7.12 shows the frequency and cumulative scores of moderate or severe symptoms, which were most commonly reported within each treatment group. The frequency of reported symptoms was low in the amantadine and placebo groups. Anti-histamine-like side effects, such as drowsiness and dry mouth, were less frequent and severe in the amantadine group than in the chlorpheniramine group. However, moderate to severe inability to concentrate, dizziness, and fatigue were reported more frequently by subjects who received the combination of amantadine and chlorpheniramine. Two subjects who received this combination reported additional symptoms of confusion and distorted depth perception as well as nausea and chills.

There were no significant differences between the group mean scores on the second practice trial and the pretreatment trial of the Critical Tracking Test among all treatment groups (Table 7.13).

The effects of amantadine or rimantadine on higher central nervous system functions, such as memory and attention, become more important when considering its use for prophylaxis of large populations. The data of Millet et al. (1982) shows that

TABLE 7.12.
Frequency and severity of subjective side effects analyzed by treatment group (after Millet et al., 1982)

Symptoms	No of subjects experiencing side effects in group treated with:[a]				
	Amantadine (10)	Amantadine-chlorpheniramine (11)	Chlorpheniramine (11)	Rimantadine (10)	Placebo (10)
Decreased concentration	1 (6)	4 (25)	2 (11)	0 (0)	0 (0)
Dizziness	0 (0)	2 (13)	0 (0)	1 (2)	2 (6)
Headache	0 (0)	1 (3)	1 (2)	1 (2)	0 (0)
Fatigue	0 (0)	2 (11)	1 (6)	2 (4)	0 (0)
Drowsiness	1 (9)	4 (13)	3 (14)	3 (12)	1 (6)
Dry mouth	0 (0)	2 (11)	1 (4)	1 (6)	0 (0)

[a] Numbers in parentheses represent the total symptom score (moderate complaint, 2; severe complaint, 3). Total scores within symptom categories for each treatment group represent the sum of the total daily moderate and severe complaints during the treatment period (4 days).

TABLE 7.13.
Psychomotor test performance in drug and placebo groups (after Millet et al., 1982)

Test	Group mean scores before and during treatment with:[a]				
	Amantadine	Amantadine-chlorpheniramine	Chlorpheniramine	Rimantadine	Placebo
Attentional					
Critical tracking	−0.085 (17.19)	−0.059 (12.68)	−0.039 (13.11)	+0.044 (9.81)	+0.027 (11.83)
Children's checking	+0.060 (1.35)	−0.027 (0.55)	−0.045 (0.77)	+0.800 (1.21)	−1.300 (0.89)
Cognitive					
Grammatical transformation	−1.000 (1.46)	−2.280 (3.65)	−0.166 (3.02)	+3.370 (2.07)	+7.800 (5.18)
Memory					
Memory for designs	+0.500 (0.83)	+0.090 (0.59)	+0.540 (0.59)	+0.400 (0.74)	+0.500 (0.42)
Symbol digit modalities					
Written	+6.400 (2.08)	+10.100 (2.99)	+10.200 (2.39)	+5.400 (2.08)	+6.000 (2.71)
Oral	+3.600 (1.47)	+8.800 (1.96)	+9.640 (2.41)	+8.700 (2.21)	+4.700 (2.13)

[a] Difference among group mean scores before and during treatment. Numbers in parentheses represent the standard error of the mean. −Value means decreased performance; +value means improved performance. In the amantadine, amantadine-chlorpheniramine, chlorpheniramine, rimantadine and placebo groups, there were 6, 7, 8, 8 and 8 subjects, respectively.

neither of these drugs had a significant effect upon performance of tasks which involved attention, cognition, and memory.

The early clinical studies of rimantadine in the USSR (Smorodintsev et al., 1970) and more recently in the USA have apparently indicated that the molecule is less toxic than amantadine (Dolin et al., 1982) and fewer CNS side effects are noted (although these effects are very mild with amantadine and are detected in no more than 10% of patients as described above – see Table 7.14). In a recent study Hayden et al. (1983) found that at similar plasma concentrations amantadine and rimantadine did not differ in the frequency or severity of their side effects. Oral dosing of volunteers with the same concentration (200 mg/day) of amantadine and rimantadine resulted in two fold different plasma levels (4 h after the initial dose of drug) of 300 ± 98 ng amantadine and 140 ± 68 ng rimantadine. Moreover, the plasma drug concentrations correlated significantly with total symptom score (Tables 7.15

TABLE 7.14.
Selected summary of reported side-effects of amantadine and rimantadine

Study	Virus	Dose (mg/day)	No. treated	No. given placebo	No. with side-effects[a]	
					Treated	Placebo
Amantadine						
Togo et al. (1968)	H2N2	200	29	29	0	0
Wingfield et al. (1969)	H3N2	100	23	48	0	0
Galbraith et al. (1969a)	H3N2	200	94	82	2	0
Galbraith et al. (1969b)	H3N2	200	102	100	3	0
Hornick et al. (1970)	H3N2	200	94	103	0	0
Knight et al. (1969)	H3N2	200	13	16	0	0
Togo et al. (1970)	H3N2	200	54	48	0	0
Smorodintsev et al. (1970)	H3N2	100	1313	512	94 (7.1%)	26 (5.2%)
Oker-Blom et al. (1970)	H3N2	200	192	199	— (8.7%)	— (3.4%)
Kitamoto (1971)	H3N2	200	182	173	18 (9.9%)	21 (12.1%)
					9 (4.9%)	9 (5.2%)
					5 (2.7%)	6 (8.5%)
O'Donoghue et al. (1973)	H3N2	200	50	61	0	0
Dolin et al. (1982)	H1N1	200	145	148	23 (15.9%)	7 (4.8%)
Bryson et al. (1980)	—	200	60	49	(33%)	(10%)
Rimantadine						
Zlydnikov et al. (1981)	H3N2	50	1647	1498	53 (3.2%)	32 (2.1%)
Zlydnikov et al. (1981)	H1N1	50	2998	1498	88 (2.9%)	32 (2.1%)
Dolin et al. (1982)	H1N1 and H3N2	200	147	148	10 (6.8%)	7 (4.8%)

[a] Side-effects in treated or placebo groups included insomnia, headache, nausea, vomiting, 'jitteriness' and diarrhoea.

TABLE 7.15.
Plasma concentrations of amantadine and rimantadine (after Hayden et al., 1983)

Drug	Initial dose (mg)	Daily dose (mg)	Plasma level[a] (ng/ml)	
			First	Second
Amantadine	100	200	300± 98	723±366
Rimantadine	100	200	140± 68	442±149
Amantadine	200	300	633±145	1405±437
Rimantadine	200	300	301± 75	913±270

[a]First plasma level was obtained at 4 h after initial dose. Second plasma level was obtained 4 h after ninth drug dose.

and 16). Thus, an important conclusion was that amantadine and rimantadine appeared to differ in their pharmacokinetics, but not in their potential for side effects at comparable plasma concentrations.

7.9.8. Effects of amantadine on pregnancy in animals

In a study by Kyo et al. (1970) amantadine hydrochloride was administered orally in two separate doses of 120 mg/kg (higher dose group) and 40 mg/kg once a day for 6 successive days from the 9th to the 14th day of pregnancy to nullipara rats of Wistar strain at the age of 3–4 months, in order to examine its effects upon the foetus during the final stage of pregnancy and their postnatal growths. The results indicated a slight retardation of increase in the body weight of dams in the higher dose group, but amantadine had no effect on the number of nidations at the end of the final stage of pregnancy. In the higher dose group, however, the mortality

TABLE 7.16.
Relationship between occurrence of moderate or marked side effects in amantadine and rimantadine recipients and plasma drug concentration after 4.5 days of drug administration (after Hayden et al., 1983)

Symptom type	Drug	No. of volunteers with adverse symptoms/total at plasma drug concentration /ng/ml)				
		≤500	501–1000	1001–1500	1501–2000	>2000
CNS	Amantadine	0/5	2/20	8/32	7/19	3/8
	Rimantadine	0/12	2/48	4/17	0/2	0/0
Sleep	Amantadine	0/5	5/20	10/32	5/19	3/8
	Rimantadine	0/12	6/48	2/17	0/2	0/0
GI	Amantadine	0/5	4/20	4/32	4/19	0/8
	Rimantadine	0/12	5/48	2/17	1/2	0/0

rate of the foetus and the drop in body weight of surviving littermates showed a significant difference from those of the control group, although no malformation was observed in the group. Finally observations on the growth of the littermates up to the end of the 6th postnatal week in the spontaneous parturition group indicated that the parturition rate was significantly lower in the higher amantadine dose group than in the control group. Amantadine at the doses tested had no effect on suckling rate, external differentiation, survival rate, auditory senses, motility and development of gonadal functions or skeletal structure.

In contrast, in a study of Lamar et al. (1970), Holtzman rats and New Zealand white rabbits were dosed orally with amantadine (0, 50 and 100 mg/kg) from 5 days prior to mating until day 6 of pregnancy. In rats, but not in rabbits, results of autopsies performed on day 14 of gestation showed significant decreases in the number of implantations and increases in the number of resorptions at 100 mg/kg. Teratology studies were performed in rats (0, 37, 50 and 100 mg/kg) by administering the drug orally on days 7 and 14 of gestation. Autopsy was just before expected parturition. Increases in resorption and decreases in the number of pups per litter were noted at 50 and 100 mg/kg. Gross examination of rat pups at these dose levels revealed no malformations at 37 mg/kg. Malformation at 50 and 100 mg/kg included oedema, malrotated hindlimbs, missing tail, stunting and brachygnatha. Examination of cleared and alizarin-stained skeletal preparations of foetuses revealed cases of absent ribs and absence of the lumbar and sacral portions of the spinal column in the 50 and 100 mg/kg groups. Thus, in rats but not in rabbits, amantadine seems to be embryotoxic and teratogenic. Teratogenicity in rats occurs at 50 mg/kg/day, or about 12 times the usual human dose.

7.9.9. Prophylactic and therapeutic clinical trials with amantadine versus infection with influenza A (H2N2), (H3N2) and (H1N1) viruses: how effective is the compound as an antiviral?

Many but not all organized clinical trials conducted under vigorous double blind, placebo controlled conditions have established a prophylactic and therapeutic effect of amantadine and rimantadine against influenza A viruses (reviewed by Oxford and Galbraith, 1980). In contrast, only a few controlled trials have shown negative effects, but of course these are of considerable interest. There is general agreement in the literature that the protective effect of amantadine (used prophylactically) against influenza A virus would approximate to 70–80% (with a range from 0–100%). To place this figure in perspective, clinical trials with influenza vaccines have, over the same period of time shown similar results with protective effects varying from 0–100% with a mean approaching 70–80%. The death incidence in a moderate influenza outbreak approximates to 1 in 5000 whereas the incidence of death following vaccination is estimated at 1 in 2 million or lower. This yields a maximum benefit risk ratio of 400:1 in favour of immunization. For both amanta-

dine and vaccine with 70% protective efficacy and a 10% attack rate, 14 persons would have to be immunized or given amantadine to prevent influenza in one person. Assuming a 0.1% mortality rate then approximately 14 000 persons would have to be treated or immunized to prevent one death.

With this background, it is of some comparative interest to examine representative clinical trials carried out between 1965 and 1984 and to illustrate exactly how effective amantadine is in preventing or curing influenza A infection. We should then be able to assess whether the compound is active enough to warrant more extensive use in the future or whether amantadine or rimantadine should be considered only as the first anti-influenza compound and whether new compounds of a much higher degree of activity would be required. Alternatively, are expectations of the clinician based on several decades of experience with antibacterials, too high to be realised with antivirals? It might suitably be mentioned here that an important property of an anti-influenza compound, particularly to be used as a prophylactic, would be *not* to completely abort influenza virus infection, but rather to reduce virus replication and abort clinical signs. The patients would thus be able to produce a natural immunological response, so conferring protection against reinfection with the same virus immediately drug prophylaxis ceases.

Following satisfactory safety testing of amantadine in animals, and clearance by the Food and Drug Administration in the USA, human volunteer studies were begun using attenuated strains of influenza A virus as a challenge. We shall consider representative artificial and natural challenge studies of amantadine as a prophylactic and therapeutic agent. (We have not attempted to analyze the data from all clinical trials reported in the literature – rather the review is selective.)

7.9.10. Artificial challenge studies

The advantages of this method, involving as it does the challenge of human subjects with a known virus, are twofold. The first is administrative convenience in that results of such clinical investigation are generated immediately, as compared with the delay involved when waiting for a naturally occurring outbreak, or epidemic of influenza. The second concerns the use of a virus of known virulence to the host and sensitivity to the chemoprophylactic agent. The design can be planned in meticulous detail, including the screening of all volunteers for pre-existing antibody to the challenge virus. It is thus possible to involve groups of volunteers with low or absent antibody to the challenge virus, and to compare the response seen in volunteers who already possess a significant level of antibody to the virus at the time of challenge. A possible objection to this method of investigation of an anti-influenza agent may be its dissimilarity to the infection as it occurs in natural epidemic form and the use of high challenge doses of virus thus presenting an over vigorous testing (Beare and Reed, 1977).

Initially, following the discovery by the Du Pont Company of the antiviral activi-

ty of amantadine, in the United States, clinical studies were confined to America. However, as evidence of prophylactic action in influenza was disseminated, clinical investigations were set up in Europe and the Far East. In general, the dosage used was 100 mg every 12 h, but some studies, notably that by Smorodintsev in 1969, employed 100 mg daily. Clinical studies in the Soviet Union using rimantadine have been reviewed recently (Indulen and Kalninya, 1980, Zlydnikov et al., 1981) and therefore will not be discussed here in detail but summarized in Tables 7.23 – 7.25.

In initial challenge studies, Jackson et al. (1963) selected volunteers from amongst 735 college students who submitted blood specimens for determination of the influenza antibody titre. Two-thirds (497) of the students were considered to have a high serum antibody titre, 1:20 or greater. Subjects for placebo challenge were randomly selected from the high antibody group; 21 subjects served as placebo controls, and 18 were observed for drug toxicity. Among the 735 initial subjects, 199 were in the low antibody group (1:10 or less). These subjects, who were challenged with influenza virus, were placed in groups which received either placebo or 100 mg amantadine. One half of each group was given the capsule as pretreatment, beginning 18 h before virus challenge. The other half received treatment beginning 4 h after challenge. All treatment was continued for the next 6 days. After challenge, illness, virus recovery, exfoliative cytology and serology were studied. Among the 199 volunteers challenged with influenza virus, 89 (45 per cent) had a four-fold or greater rise in antibody in convalescent sera, indicating infection with the challenge virus. This represented an infection rate of 70 per cent among subjects with a pre-challenge antibody titre of 1:10 or less. Volunteers who had a pre-challenge antibody titre of 1:20 had an infection rate of only 22%. Among those with a higher serum level of antibody, only 9% became infected. Among the two placebo groups, 66% and 73% of the subjects with a low antibody titre became infected as judged by a serological rise in titre. Among those with higher antibody, 14% and 26% were infected. Among subjects *pre*-treated with the drug, a considerable reduction in infection was observed in volunteers of either antibody status. Low antibody subjects given amantadine had an infection rate of only 37% which was a statistically significant reduction ($P < 0.01$) compared with the combined groups. No therapeutic effect was detected (see below).

However, an early challenge study, carried out at the Common Cold Research Centre in England, using an attenuated strain of influenza A sensitive to amantadine in in vitro laboratory studies failed to demonstrate prophylactic action of the drug clinically or serologically (Tyrrell et al., 1965). Relatively large doses of egg-adapted virus were used in the volunteers and the numbers involved were small.

Togo et al. (1968) reported a study performed with the cooperation of volunteers at the Maryland House of Correction. Sixty-five men with titres of 1:2 or less of neutralizing antibodies were enrolled and, after full baseline evaluation, were housed in the research ward at the prison. Oral temperature, pulse rate and respiration rates were recorded at four-hourly intervals. Follow-up specimens were collected

at 7, 14, 21 and 28 days post challenge. Seven trials were conducted. In the preliminary potency-testing of the inoculum, seven men were challenged. In the following six drug-evaluation studies, viral challenge was performed on a total of 58 men, volunteers in each group numbering 14, 6, 12, 6, 8 and 12. Amantadine and lactose-containing placebo capsules were administered by double-blind technique from randomly numbered bottles. The drug was given in 100 mg doses twice daily for eight days in the first two studies and for nine days in the following four studies, starting about 26 h prior to the viral challenge. The drug-treated group received a total of 300 mg of amantadine before the virus dose of 64 000 $TCID_{50}$ was given nasopharyngeally. The virulence of the virus inoculum was examined in 7 men who received undiluted virus fluid containing 64 000 $TCID_{50}$. Clinical illness observed was classified according of the severity of signs and symptoms. The following criteria applied:

- 2+ = moderately ill with temperature above 38.3°C and occasional respiratory tract signs.
- 1+ = mild illness, significant symptomatology and temperature 37.8°C.
- ± = questionable illness, no fever, but comprising of pertinent symptoms.
- 0 = no suspected illness.

Subsequent experience with the 18 volunteers enrolled in the drug evaluation study showed similar clinical responses and the induced illness was generally milder than naturally occurring influenza. The prophylactic effectiveness of amantadine in volunteers with experimentally induced influenza A infection was assessed in six separate but consecutive double-blind trials. The most severe illnesses (24) occurred only in the placebo-treated subjects. The occurrence of six instances of 2+ illnesses in the placebo-treated subjects compared with none in the drug-treated group is a statistically significant difference ($P = 0.011$). There was a striking disparity in the overall incidence of clinical illness between the two groups. Febrile illness, with ratings of 1+ or 2+ were observed in thirteen patients in the placebo-treated group, compared with five patients with 1+ febrile illness in the amantadine group, and this is statistically significant.

7.9.11. Prophylactic trials in the community

The first study of amantadine in the family environment was performed by Galbraith et al. (1969a) with the cooperation of family doctors, the majority of whom were members of the epidemic observation unit of the Royal College of General Practitioners. Each doctor, many of whom had experience in the conduct of serologically controlled clinical trials, was asked to include in the study the families of up to five index cases and five contact cases. In the study, 'the family' was defined as all occupants of a household over 2 years of age living in daily contact with each other. The 'index case' was defined as the first person over the age of 2 years to contract clinical influenza in a household, and 'contact cases' were defined as individuals living in the household and having contact with the index case. All index

cases received placebo medication in order not to influence the possible spread of influenza, while the families of index or contact cases received drug or placebo by random allocation. All the members of one family except the index cases, received the same treatment (drug or placebo). The diagnosis of influenza in the index case was made on clinical and epidemiological grounds. At the doctor's first visit, blood was taken from the index case and from as many of the other members of the family as practicable. A second blood sample was taken two or three weeks later, and tests for HI and CF antibody were performed. Daily records were kept of body temperature and the presence of a cough. A cough, accompanied by a rise of temperature to 37.8°C or higher, was taken as criterion for a diagnosis of clinical influenza.

Twenty-two family doctors studied 52 families comprising 208 contacts who were divided between treated and placebo groups. Of the 52 index cases, 35 (67 per cent) showed serological evidence of influenza A infection. In the 35 families in which there was serological evidence of influenza A infection in the index case, two of 55 (3.6%) contacts in the amantadine-treated group developed clinical symptoms of influenza, whilst 12 of 85 (14.1%) contacts in the corresponding placebo group developed an influenza-like illness. The difference in incidence in these two groups was of marginal significance ($P = 0.07$). However, there was no serological evidence of infection with influenza A virus in the two individuals of the amantadine group who developed clinical illness. In contrast, in the placebo group, ten of the 69 contacts from whom paired sera were available developed antibody rises to influenza A (Table 7.17). When serologically confirmed cases of influenza are considered, the difference between the drug and placebo treated groups is significantly different statistically ($P = 0.05$–0.01) The authors also observed that when the proportion of contacts with serological evidence of influenza A infection, irrespective of clinical

TABLE 7.17.
General practice (UK) study: effect of amantadine on incidence of clinical influenza in contacts of index-cases (after Galbraith et al., 1969)

Laboratory evidence of influenza A infection in index case	Treatment	No. of families	Contacts who developed clinical influenza within 10 days of entering the study: All cases			Confirmed serologically		
			No.	%	*P* value	No.	%	*P* value
Present	Amantadine	13	2/55	3.6		0/48	0	
	Placebo	22	12/85	14.1	0.07	10/69	14.5	0.05–0.01
Absent	Amantadine	11	1/45	2.2		0/43	0	
	Placebo	6	3/23	13.0	0.3–0.2	2/21	9.5	0.10

This study examined the prophylactic effect of amantadine by determining the effect of the compound on spread of influenza from an initial index case (which was left untreated) to the rest of the family.

illness, was compared for the placebo and drug-treated groups (Table 7.18), the difference between the groups was highly significant ($P = 0.001$–0.01).

The following winter, the investigators repeated the family study in the face of an epidemic of a new pandemic virus Hong Kong influenza – A/HK/1/68 (H3N2). Seventy-two general practitioners volunteered to take part and 58 families were included, comprising 176 individuals. The only difference in design between this study and the previous year's was that the index case in each family received amantadine or placebo in keeping with the rest of the family. As before, the results of cases suffering from clinical influenza and those with serological proof of influenza A infection, were submitted to statistical analysis. In this instance, the drug and placebo-treated individuals behaved similarly and amantadine failed to protect persons receiving the compound from influenza. The virus itself was equally sensitive to amantadine as the previous year's strain and the authors sought to explain this reversal by considering the initial antibody status of those cases under study. It was seen that during the 1967/68 study, the initial level of HI antibody to the current strain of influenza A was higher (40 per cent with initial HI antibody below 1:12) compared with the 1968/69 study when 90 per cent of the contacts studied possessed initial HI titres below 1:12. This may have been responsible for the difference but the administration of amantadine to half the number of index cases could have influenced the infectivity of these individuals so that a direct comparison of these two trials is not possible.

7.9.12. Therapeutic activity of amantadine

Studies in influenza infected mice indicated unexpectedly, that administration of amantadine could have a significant therapeutic effect. Clinical trials were set up

TABLE 7.18.
General practice (UK) study: effect of amantadine on the incidence of clinical and subclinical influenza A infections (after Galbraith et al., 1969)

Laboratory evidence of influenza A infection in index case	Treatment	Contacts with serological evidence of influenza infection					
		clinical and subclinical infections			Subclinical infections only		
		No.	%	*P* value	No.	%	*P* value
Present	Amantadine	7/48	14.6		7/48	14.6	
	Placebo	27/69	39.1	0.001–0.01	17/69	24.6	0.2
Absent	Amantadine	7/43	16.3		7/43	16.3	
	Placebo	5/21	23.8	0.7	3/21	14.3	0.8

This study examined the prophylactic effect of amantadine by determining the effect of the compound on spread of influenza from an initial index case (which was left untreated) to the rest of the family.

in the USA and the therapeutic effect of amantadine in man was clearly established (Togo et al., 1970).

A study of the therapeutic effect of amantadine amongst patients in the family environment was carried out by general practitioners in the United Kingdom and reported by Galbraith et al. (1971, 1975). Fifty-seven doctors took part and included 203 patients with clinically diagnosed influenza. Amantadine was provided in capsule form (100 mg) or as a syrup (50 mg in 5 ml) and patients received active or placebo medication by random number allocation on a double-blind basis. Adults received 100 mg every 12 h and children aged 10–15 years 100 mg daily, younger children (2–10) a proportional dose of syrup. Medication was started from the time the patient was first seen by the doctor and was continued for 7 days. A blood sample was taken at the doctor's first visit and a second 2–3 weeks later. These were tested for HI antibody with A/HongKong/68 virus and for CF antibody. A fourfold or greater rise in either or both these tests was taken as evidence of influenza A infection. Of the 203 patients entered, only 153 provided results which satisfied the criteria for analysis. Of the 153, 72 received amantadine and 81 placebo. The mean duration of fever is shown in Table 7.19 where the differences between drug and placebo-treated patients were significantly different. When symptomatology excluding fever was considered, no differences were demonstrated between the two groups, but this may have been due to the lack of sensitivity in the method of recording clinical illness.

7.9.13. More recent clinical trials with influenza A (H3N2) and (H1N1) viruses and amantadine or rimantadine

More recently a number of excellent double blind placebo controlled trials have been carried out and the data obtained has confirmed the data from earlier trials with H2N2 and H3N2 viruses. In such a prophylactic trial Dolin et al. (1982) reported the first well controlled comparison in the USA of the prophylactic effects of rimantadine and amantadine in an area where an active influenza surveillance

TABLE 7.19.
Therapeutic trial of amantadine in general practice (UK study) (after Galbraith et al., 1971)

	Duration of temperature (hrs)			*P* value
	Amantadine	Placebo	Difference	
Males	55.1	71.5	16.4	$0.05 > P > 0.02$
Females	37.7	80.6	42.8	<0.01
Both sexes	46.6	75.1	28.5	<0.01

In this double-blind placebo controlled trial, medication with amantadine was initiated within 24 hrs of onset of symptoms and continued for 7 days.

indicated early that an influenza A outbreak had commenced caused by H3N2 (20% of cases) and H1N1 viruses (80% of cases). A total of 450 volunteers enrolled with a mean age of 25.0 ± 0.5 years and with no significant differences in age, race, male: female ratios or level of pre-existing HI antibody. Throat swabs for virus isolation were taken 2 times per week and volunteers were assigned to amantadine, rimantadine or placebo groups. A 100 mg tablet was taken twice daily for 7 days and any symptoms were recorded. Each week the volunteers returned the symptoms diary to the co-ordinating centre and received a further 7 days supply of tablets. If any respiratory illness occurred, volunteers were asked to return at once to the centre, and were examined by a physician. Influenza-like illness was defined as a cough and/or fever greater than 37.7°C and two or more of the following symptoms: sore throat, headache and myalgia. The trial lasted six weeks and a serum sample was obtained at the beginning and again at the end of the study for serological analysis. Significantly more placebo recipients (40.9%) developed influenza-like illness compared to amantadine (8.9%) or rimantadine (14.3%) groups giving a reduction in the rate of illness of 78.2% and 65.0% respectively (Table 7.20). However, as noted in previous trials (reviewed by Oxford and Galbraith, 1980), the rates of laboratory confirmed influenza-like illness were reduced by 85.4% by rimantadine and 91.2% by amantadine suggesting that a proportion of influenza like illness observed during the study was not caused by influenza A virus.

Of a small group of amantadine patients sampled 89% had drug detectable in the urine (52–438 μg/ml) suggesting a good compliance rate. In total 62 volunteers left the study during the 6 weeks because of possible side-effects. The withdrawal

TABLE 7.20.
Effect of rimantadine and amantadine in preventing influenza-like illness and laboratory confirmed influenza among volunteers (after Dolin et al., 1982)

Group	Number	Number with influenza-like illness or influenza	Per cent
Influenza-like illness			
Placebo	132	54	40.9
Rimantadine	133	19	14.3
Amantadine	113	10	8.9
Laboratory confirmed influenza			
Placebo	132	27	20.5
Rimantadine	133	4	3.0
Amantadine	113	2	1.8

Illness was defined as cough and/or fever 37.7°C p.o. and two or more of the following: sore throat, headache, myalgia.
Laboratory confirmed influenza was determined by virus isolation and/or serum antibody rises.

rates were 10.8% for placebo, 9.5% for rimantadine and 22.1% for amantadine and the excess rate in the latter group was mainly caused by CNS effects including insomnia, jitteriness and difficulty in concentrating, although symptoms generally cleared within 48 hours of the cessation of medication. Both amantadine and rimantadine were thus highly effective in preventing illness and/or infection with no statistically significant differences between the efficacy rates of the two compounds. However, amantadine treated persons had significantly more CNS side effects with an excess rate of 9.0% compared to placebo patients. The authors concluded that rimantadine might be the compound of choice for the chemoprophylaxis of influenza A infection in young volunteers, but further trials could be carried out in the elderly and high risk individuals.

Another recent clinical trial has compared the *therapeutic* effect of amantadine versus rimantadine against an H1N1 virus (Van Voris et al., 1981). This trial will also be described in some detail, because it illustrates both clinical and serological criteria which need to be applied in a comprehensive clinical therapeutic study of an anti-influenza compound. The study was carried out in a group of university students naturally infected with A/USSR/77 (H1N1) virus. Clinical criteria were used for the initial diagnosis of influenza and included the presence of a headache, fever, malaise and myalgia of less than 48 hours' duration. This is in accordance with the majority of the previously reported therapeutic studies, where benefit was not noted in persons entering treatment later than 48 hours after the initial symptoms. A complete history was taken on admission to the trial and thereafter clinical examinations were carried out at 4, 48 and 72 hours, 7 days and 3 weeks, and specific signs categorized and graded on a scale of 0 to 3. Oral temperatures were measured by the clinician daily. Nasal washings for virus isolation were taken at the time of admission to the study and at 48 to 72 hours. Acute and convalescent sera were obtained and tested for rising titres of HI antibody to confirm the clinical diagnosis. All students took a tablet (100 mg) each morning and night. After 48 h of therapy, blood was taken to test for levels of drug – this is an important factor to control that students were indeed taking the medication (levels ranged from 0.3 to 0.8 μg/ml, whilst the virus was inhibited by 0.2 μg/ml of drug). Of the 54 volunteers to enter the study, 45 were proven to have been infected with A/USSR/77 (H1N1) virus. This is a rather high rate of clinical diagnosis, since, for example, typical general practitioner trials in the UK normally record a 60% success in influenza diagnosis (Galbraith et al., 1969). When the total symptom scores were calculated for the three groups, an arbitrary 50% level of clinical improvement (compared with pre-treatment) was reached in the amantadine and rimantadine groups at 48 hours. This was not reached until 72 hours by the placebo group ($P < 0.25$). An improvement was noted in the drug treated groups in both respiratory and systemic signs. Mean temperatures for the drug treated groups were significantly lower ($P < 0.01$) than the placebo group. An interesting finding (for students!) was that both at 48 and 72 hours after commencement of the study more students in the drug treated groups

were able to attend class compared to the placebo group. Although the initial titres of the excreted virus were similar in each group, by 48 hours the proportion of students shedding virus was significantly lower in the groups treated with amantadine or rimantadine. All students developed post-infection HI antibody to the infecting virus. Minor CNS side effects were noted in a proportion of students in the amantadine group by day 5, but these appeared to be less important than the relief of influenza symptoms, since students in this group returned to class more rapidly than students in the placebo group. The authors concluded that a therapeutic advantage in favour of amantadine occurred, and that the 24–48 hours benefit appeared to justify the therapeutic use of amantadine or rimantadine especially in comparison with the current usage of non specific drugs such as antibiotics, antihistamines and cough suppressants.

In summary, therefore, amantadine or rimantadine in most clinical trials with influenza A viruses have not resulted in 100% protection against infection or clinical disease. Its prophylactic efficacy would approximate to that of influenza vaccine but with the qualification that amantadine has no effect against influenza B viruses. The compound has been used successfully to prevent the spread of virus in families, hospitals, factories and closed communities such as prisons and antiviral effects are consistent with viruses of the different subtypes H1N1, H2N2 and H3N2. A quite clear therapeutic effect is also obtained resulting in a 1–2 day faster recovery, lower temperatures and fewer clinical symptoms with these viruses, and, perhaps more significantly from the point of view of virus spread in the community, reduced excretion of virus.

7.9.14. Could amantadine be used successfully on a wider scale?

At present health authorities in most countries recommend the routine use of influenza vaccine to prevent influenza infection in persons designated at special risk of mortality, e.g. older persons, diabetics, asthmatics and persons with chronic obstructive heart disease and bronchitis. Little attempt has been made to provide protection for the community at large or to abort an epidemic, in spite of the well documented economic and social disruption and mortality caused by influenza A and B viruses (reviewed by Stuart-Harris and Schild, 1976). Notable exceptions have been the attempt to immunize the population of the USA with A/Swine virus containing vaccine following the outbreak of influenza A/NJ/76 infection in the Fort Dix camp in the USA, and the necessarily (because of their size) uncontrolled trials of live attenuated influenza vaccine in cities in the USSR and China. It could be argued that general prophylactic measures could and should be instituted with more enthusiasm and vigour than at present and that such measures could include prophylaxis in certain groups with amantadine. A consensus meeting in the USA (Elliot, 1979) and also a more recent meeting in Vienna (Galasso et al., 1984) suggested that the following groups should be considered for amantadine prophylaxis. (See also Tables 7.21 and 22.)

1. Unvaccinated children and adults at high risk of serious mortality and mortality because of underlying diseases, which include pulmonary, cardiovascular, metabolic, neuromuscular, or immunodeficiency diseases.
2. Adults whose activities are vital to community function and who have not been vaccinated with an appropriate contemporary influenza vaccine: for example, policemen, firemen, selected hospital personnel. Such persons are in frequent contact with others who may have influenza and should be considered at higher risk of contracting influenza than the general population.
3. Persons in semiclosed institutional environments, especially older persons, who have not received the current influenza vaccine.

The groups for which the panel concluded the benefit-to-risk considerations are less clear include all elderly patients (65 years or older) who have not received influenza vaccine. In addition, the use of amantadine hydrochloride for prophylaxis in hospital patients in the presence of a demonstrated outbreak should take into consideration local and particular risk factors and conditions; for example, the patient who

TABLE 7.21.
Population groups in which amantadine or rimantadine has demonstrated prophylactic and therapeutic activity

Groups	Countries in which placebo controlled trials have shown efficacy	References
1. Families	(UK and USSR)	Galbraith et al., 1969 Zlydnikov et al., 1981
2. Hospitals	(USA and Hungary)	Nafta et al., 1970
3. Schools	(UK and USA, USSR)	Finklea et al., 1967 Smorodintsev et al., 1970
4. Prisons	(USA)	Bloomfield, 1970 Togo et al., 1968 Hornick et al., 1970
5. University campuses	(USA, Finland, USSR)	Jackson et al., 1963 Smorodintsev et al., 1970 Dolin et al., 1982 Oker Blom et al., 1970
6. Open studies (towns)	(USSR)	Zlydnikov et al., 1981
7. Army and Navy barracks and camps	(USSR, USA, Hungary)	Nafta et al., 1970
8. Factories	(Yugoslavia)	Likar, 1970

Note that the summary of countries involved with these trials is not exhaustive. The early licensing and usage of amantadine was subject to two critical reviews by Sabin (1967, 1978). However, the more extensive field use of the two compounds in the last 5 years has provided data to answer many of the earlier questions.

TABLE 7.22.
Population groups in which a more extended use of rimantadine and amantadine, versus influenza A virus, could be suggested

1. Persons (vaccinated or not) presenting with clinical influenza within 48 h of the onset of clinical signs. Future clinical trials may establish if amantadine or rimantadine used therapeutically can reduce mortality in persons at special risk, or reduce the rate of complications such as pneumonia.
2. Adults, such as hospital workers, public transport personnel etc. in the face of an epidemic when insufficient vaccine is available, or when contraindications exist to vaccine.
3. Households contacts of an index case of influenza.
4. Hospital patients and personnel, to prevent hospital spread when patients with influenza A virus infections are admitted. This would mainly be prophylactic use of the compound.
5. Persons in institutions, such as old persons homes and boarding schools. Prophylactic and therapeutic.
6. Unvaccinated adults, who nevertheless have serious underlying disease which place them in a potentially high mortality group following an attack of influenza e.g. persons with pulmonary, cardiac, metabolic or immunological deficiencies. Amantadine or rimantadine could be used prophylactically and persons vaccinated at the same time.
7. Vaccinated adults who are at high risk from an attack of influenza. Rimantadine or amantadine-supplemented protection would be expected to raise the basic 70% protective effect of vaccine alone. Single non reactogenic doses of vaccine against a new pandemic virus subtype would not be expected to give significant protection – two doses of vaccine would be required – particularly with subunit or split virus vaccines. Therefore amantadine could be administered prophylactically during the two–three week period of development of vaccine induced immunity.
8. In the event of the arrival of a new pandemic strain of influenza A virus *all* the above groups and additionally individuals in the general community who would like prophylaxis. (Excluded would be young children and pregnant women because of the limited amount of clinical and toxicological data in these two groups.)

See Galasso et al., 1984, Elliot, 1979.

is to undergo inhalation anaesthesia may be at higher risk of serious complications. Finally, an obvious possibility would be to supplement vaccine induced (partial) protection with amantadine (partial) protection. Only a few studies have investigated the possible additive effects of vaccine and amantadine in this approach and preliminary results have been encouraging (reviewed by Zlydnikov et al., 1981) raising protective efficacies from 32–37% in vaccine or rimantadine groups to 60% in the group given both vaccine and rimantadine.

7.9.15. Differential diagnosis of respiratory infections – a problem for all antivirals with a narrow antiviral spectrum of activity?

Figure 7.10 illustrates the complexity of the clinical diagnosis of influenza A from a number of other viruses causing a rather similar clinical picture. Also influenza

A infection itself in individuals can range from asymptomatic (there probably exists at least a 1:1 ratio of clinical : subclinical cases) through mild cold symptoms, to typical acute influenza and, in the extreme case, virus infection of the lower respiratory tract and pneumonia. A major limitation of amantadine in one respect has been the narrow spectrum of antiviral activity, limited to influenza A virus with only marginal activity (and here limited to in vitro data) against para-influenza viruses, VSV, rubella, arenaviruses and influenza B (reviewed by Oxford and Galbraith, 1980). On the other hand, in influenza A virus epidemic years, the clinical diagnosis rate of influenza by general practitioners can be relatively high, as is shown by the controlled clinical trials in the UK (Galbraith et al., 1969). It is apparent then that any extended use of amantadine must be accompanied and initiated by clear laboratory and virological data and establishment of influenza A virus spread in the community. This has been clearly recommended in the Vienna report

RESPIRATORY VIRUSES AND SYNDROMES

INFLUENZA	Influenza A	Influenza B	Influenza C	Parainfluenza Rhinovirus Enterovirus Adenovirus
COMMON COLD	Rhinovirus	Coronavirus	Influenza Parainfluenza RSV Enterovirus Adenovirus	
SORE THROAT (PHARYNGITIS; TONSILLITIS)	Adenovirus types 3,4,7, 14,21	Enterovirus	Rhinovirus Herpesvirus Parainfluenza RSV Influenza	Haemolytic *Streptococcus*
CROUP	Parainfluenza	RSV	Influenza	Rhinovirus Echovirus Adenovirus
BRONCHITIS	Rhinovirus	Parainfluenza	RSV	Influenza Coronavirus Adenovirus
BRONCHIOLITIS	RSV	Parainfluenza	Rhinovirus Influenza *Mycoplasma pneumoniae* Coronavirus	
PNEUMONIA	Secondary bacterial	*Mycoplasma pneumoniae* (atypical pneumonia)	Influenza Parainfluenza RSV Rhinovirus Coronavirus Adenovirus	

Fig. 7.10. Respiratory viruses and syndromes (after Stuart-Harris and Schild, 1976).

(1984). In the UK, the PHLS and the Royal College of General Practitioners have considerable experience in quickly and accurately estimating outbreaks of influenza A virus in the community. Similar monitoring is carried out in the USA and USSR and therefore in these three countries particularly, prophylactic measures could be initiated with some degree of virological certainty. In the USSR, for example, communities already are encouraged to use rimantadine prophylactically over a period of 3–6 weeks during a city outbreak of influenza A virus. Rimantadine can be purchased from pharmacies without prescription for approximately £1.20 for a three week course, but is also administered free of charge in certain organizations (M. Indulen, Latvian Academy of Sciences, personal communication). In the UK and USA the amantadine has to be prescribed by a medical practitioner and the cost for a 10 day course (in the UK) is approximately £3.50. A parallel situation is occurring with certain of the new anti-herpes compounds, where rapid self-prescription by patients at the first signs of a virus induced lesion is required to show beneficial clinical effects. As mentioned briefly above, certain concepts which have been established about the use of antibacterials may therefore have to be modified for antivirals to include more reliance on a patient's ability to initiate prophylaxis. It must be expected, however, that widespread usage of antivirals may quickly result in the selection and spread of drug resistant variants. Drug resistance is a major clinical problem with antibacterials but has, at the same time, encouraged the search for more effective molecules.

The Vienna report suggesting population groups for amantadine and rimantadine therapy, or prophylaxis using rimantadine, based its conclusions on more recently published data which has established that although rimantadine and amantadine have similar antiviral properties, rimantadine at 200 mg/day has no demonstrable toxic effects and therefore it is the choice for prophylaxis. On the other hand, the very mild side effects of amantadine (less, for example, than many nonspecific compounds used at present to treat the clinical effects of influenza) are not noticeable in persons already suffering from the clinical effects of influenza and so either rimantadine or amantadine can be used for therapy. It should be added that early studies in the USSR established the usefulness of rimantadine and these are summarized in Tables 7.23, 24 and 25.

7.9.16. Therapeutic effects of amantadine aerosols

Hayden et al. (1980) established that aerosols of amantadine in young adults experiencing acute illness caused by influenza A virus had a discernable but modest therapeutic and antiviral effect. (It could be added that aerosol administration has been used more recently with ribavirin and, in particular, against respiratory syncytial virus infections in both adults and children – Chapter 8). In the Hayden trial (1980) the effect of small-particle aerosol therapy with amantadine was assessed in a randomized, double-blind study of 20 patients with naturally acquired influenza A vir-

TABLE 7.23.
Summary of data on prophylactic efficacy of amantadine and rimantadine against infection with influenza (USSR trials) (after Zlydnikov et al., 1981)

Subjects no. in group	Strain of virus	Drug, dosage schedule	Results
Volunteers infected by inoculation	A/Hong Kong/68		
49		Amantadine, 100 mg per day, starting 24 h before virus inoculation, for 7 days	Protection index, 60.2%; fever rate, 30.6%
36		Rimantadine, 100 mg per day, starting 24 h before virus inoculation, for 7 days	Protection index, 87.2%; fever rate, 11.1%
47		Placebo	Fever rate, 80.9%
Volunteers infected by inoculation	A/Hong Kong/68		
109		Amantadine, 100 mg per day, starting 24 h before virus inoculation, for 7 days	Rate of clinical symptoms, 34.8%
90		Rimantadine, 100 mg per day, starting 24 h before virus inoculation, for 7 days	Rate of clinical symptoms, 17.7%
118		Placebo	Rate of clinical symptoms, 81.3%
Seven groups of industrial workers	Influenza epidemic 1975 A/Port Chalmers/1/73		
1881		Rimantadine, 50 mg per day, for 20–27 days	Efficiency index according to clinical diagnosis, 2.04; according to serologic diagnosis, 3.2; and compared with the outer control group, 4.8
1711		Placebo-treated group, with inner and outer control groups	
Industrial workers living in hostels	Influenza epidemic of 1976 (A/Victoria/3/75)		
402		Rimantadine, 50 mg per day, for 15 days	Efficiency index, 1.5; coefficient of efficacy, 32.9% (according to clinical data and serologic diagnosis, 2.4 and 58.3%, respectively)

Subjects no. in group	Strain of virus	Drug, dosage schedule	Results
425		Placebo	
Family contacts	Outbreak of influenza B in 1977		
66		Rimantadine, 50 mg twice per day for adults and 50 mg per day for children 5–15 years of age (the drug was given for 7–10 days)	Morbidity rate, 27%
72		Placebo	Morbidity rate, 72%
Four collectives of adults	Epidemic foci in influenza epidemic 1974–1975 (A/Port Chalmers/ 73) and 1975–1976 (A/Victoria/3/75)		
5935		Rimantadine, 50 mg per day, for 17–25 days	3.3-fold decrease in influenza morbidity in 1975 and 2.6-fold decrease in 1976

us infection. Aerosol treatments of 20 min with either distilled water or with amantadine hydrochloride (1.0 g/100 ml of distilled water) were given three times a day for four days. The amantadine-treated patients experienced a significantly more rapid resolution of clinical signs and symptoms when compared with placebo-treated patients. The resolution of fever was similar for both groups. Aerosol delivery of amantadine did not affect the frequency of viral isolation from upper respiratory tract secretions, but was associated with a trend toward reduced quantity of viral shedding. Serial pulmonary function tests found no important differences between the groups. Amantadine-treated patients experienced a greater frequency of mild local side effects (rhinorrhea, nasal irritation) during aerosol exposures but aerosol treatments did not cause any apparent decline in pulmonary function.

In summary, therefore, the amantadine group of molecules has had a long gestation of two decades but the accumulation of scientific data is now overwhelming in its indication of the clinical usefulness of the compounds against influenza A virus infections. Future studies must monitor closely for development of drug resistant strains, the usefulness of amantadine at lower dosage levels and the usefulness of both amantadine and rimantadine in preventing mortality in the special risk groups.

TABLE 7.24.
Adverse effects of rimantadine administered prophylactically during influenza epidemics (USSR trials) (after Zlydnikov et al., 1981)

Epidemic	Treatment of group, no. of subjects	Dosage	No. (%) of subjects reporting					Total no. (%) reporting adverse effects
			Dyspepsia	Sleep disorders	Headache	Malaise	Other complaints	
A/Victoria/75 (H3N2) 1975–1976	Rimantadine (1647)	50 mg per day for 20–27 days	15 (0.91)	0	14 (0.85)	1 (0.16)	23 (1.39)	53 (3.22)
	Placebo (1498)		2 (0.13)	0	12 (0.80)	4 (0.27)	14 (0.93)	32 (2.14)
A/USSR/77 (H1N1) 1977–1978	Rimantadine (1351)	50 mg per day for 15 days	11 (0.61)	1 (0.07)	9 (0.66)	2 (0.14)	12 (0.88)	35 (2.6)
Total	Rimantadine (2998)		26 (0.86)	1 (0.03)	23 (0.76)	3 (0.1)	35 (1.16)	88 (2.93)
	Placebo (1498)		2 (0.13)	0	12 (0.80)	4 (0.27)	14 (0.93)	32 (2.14)
	P		<0.05	>0.05			>0.05	>0.05

TABLE 7.25.
Summary of data on therapeutic efficacy of rimantadine in patients with influenza (USSR trials) (after Zlydnikov et al., 1981)

Subjects no. in group	Strain of virus	Drug, dosage schedule	Results
Adult outpatients with natural infection	A/Hong Kong/68		
164		Rimantadine, 150 mg per day for 5 days (100 patients treated with rimantadine, 64 with placebo)	Decreased intensity of toxic and catarrhal symptoms, reduction in the duration of clinical symptoms by 2–3 days as compared with control group
Inpatients with natural infections	A/Port Chalmers/1/73, influenza epidemic		
116		Rimantadine, 50 mg 4 times per day for 5 days	Decreased intensity and shorter duration of fever and toxicity; no influence on catarrhal symptoms
39		Rimantadine, 75 mg 3 times per day for 5 days	
43		Placebo	
Outpatients	Influenza epidemics (H3N2) 1973–1975		
240		Rimantadine for 6 days, according to the scheme: days 1–2, 100 mg 3 times per day; days 3–4, 100 mg 2 times per day; days 5–6, 50 mg 2 times per day; 120 patients treated with the drug, 120 with placebo	Shorter periods of toxicity and of catarrhal symptoms, faster clearing of virus as revealed by immunofluorescent technique, reduction (2 times) in the number of complications

7.10. Inhibitory effects of ribavirin

A synthetic nucleoside analogue 1-β-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide (ribavirin, virazole, Fig. 7.11) has been shown to inhibit the multiplication of both DNA- and RNA-containing viruses including influenza viruses (Sidwell et al., 1972, Witkowski et al., 1972, Smith and Kirkpatrick, 1980) (Tables 7.26 and 27). Influenza type A and B virus replication is inhibited in tissue culture cells by ribavirin and

the compound acts at an early stage in the influenza-infected cell, possibly by inhibiting the production of essential nucleotides and hence RNA synthesis. More recently, aerosols of ribavirin have been shown to have a clinical effect against influenza B virus infection in humans and this is considered in more detail below (McClung et al., 1983).

The inhibitory effect of ribavirin on the replication of a number of different influenza A and B viruses was measured in an early study using the egg piece system (Fazekas de St Groth and White, 1958). In these experiments ribavirin was added to egg pieces in WHO plates at a final concentration of 0.04 mM at the time of virus infection and the virus was titrated in ribavirin treated and in control untreated plates and the plates incubated for 3 days at 37°C (Oxford, 1975). The nucleoside analogue at 0.04 mM caused a very significant inhibition (>4.0 log ID_{50}/ml) of the replication of a range of influenza A strains of human, equine, avian and swine origin including A/Duck/England/62 (Hav3Navl), A/Swine/Cambrigde/39 (Hsw/in-e1N1), A/Equine/-Miami/63 (Heg2Neg2) and A/Port Chalmers/1/73 (H3N2) viruses. In addition, influenza B/Hong Kong/8/73 virus was inhibited to a similar degree to the influenza A viruses. The multiplication of fowl plaque virus A/FPV/Dutch/27 (Hav1Neq1) in CEF cells was also inhibited by ribavirin as determined by plaque inhibition tests. The effect of ribavirin against respiratory virus infections in mice is shown in Table 7.27.

7.10.1. Teratogenic effects of ribavirin

Kochhar et al. (1980) investigated the possible teratogenic activity of ribavirin in the mouse model. Pregnant ICR mice were injected at 10th–13th days of gestation

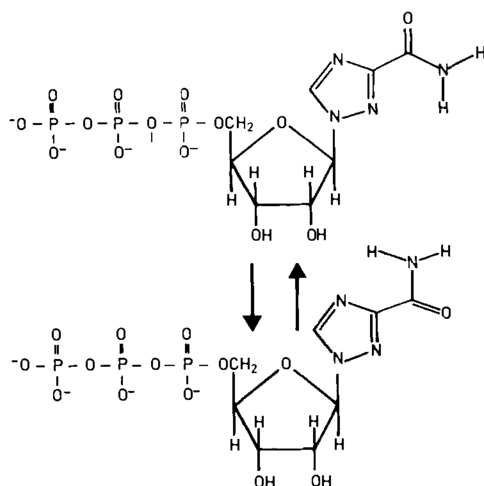


Fig. 7.11. Molecular structure of ribavirin triphosphate.

TABLE 7.26.
In vitro antiviral activity of ribavirin (after Sidwell et al., 1972)

Virus	Cell line	Virus rating
DNA viruses		
HS type 1 (HF)	KB	1.2
HS type 2 (MS)	KB	1.1
PR (RK17C24, derived from Aujeszky)	RK-13	0.0
MCM (Smith)	ME	0.9
Vaccinia (Lederle CA)	KB	1.0
Myxoma (Sanarelli)	RK-13	1.7
AV type 3 (GB)	KB	0.7
RNA viruses		
PI type 3 HA-1 (C243)	KB	0.8
PI type 1 (Sendai)	CE	⩾10
Influenza A_2 (Jap/305)	CE	3.2
Influenza B (Lee)	CE	10
RV type 1A (2060)	KB	0.6
RV type 13 (353)	KB	0.8
RV type 56 (Phillips)	KB	0.7
Coxsackie B_1 (Conn.-5)	KB	0.4
PV type 2 (MEF-1)	KB	0.0
VS (Indiana)	KB	0.7
SF (original)	L-929	0.6

A virus rating of 1.0 or greater indicates a significant effect.
Virus abbreviations: HS, herpes simplex; PR, pseudorabies; MCM, murine cytomegalovirus; AV, adenovirus; PI, parainfluenza; RV, rhinovirus; PV, poliovirus; VS, vesicular stomatitis; SF, Semliki Forest. Cell abbreviations: KB, human carcinoma of the nasopharynx; RK-13, continuous rabbit kidney; ME, primary mouse embryo; CE, primary chick embryo; L-929, mouse fibroblast.

with a single i.p. dose of ribavirin in the range of 10–200 mg/kg. All dosages in excess of 25 mg/kg were teratogenic. The optimal teratogenic dose varied with the stage of development, being higher at advanced stages of development. Depending on the dose and stage of treatment virtually all parts of the skeleton including the craniofacial and limb bones were susceptible to ribavirin. Both the frequency and multiplicity of skeletal defects increased as the dose was raised. The stage dependency of defects in the orofacial bones was markedly apparent. Treatment on day 10.5 resulted in shortened maxilla in all survivors, while treatment on either day 11 or 11.5 resulted in a high frequency of reduction in the length of both upper and lower jaws. Treatment on the 12th day resulted in a very low incidence of effect on the maxilla (4%) but a high frequency (88–100%) of reduction and deformation of the mandible. Ribavirin, both in vivo and in vitro, inhibited embryonic DNA synthesis. The inhibition was transitory and did not seem to be directly related to the embryolethal activity of the drug. Although the role of metabolic inhibition in

TABLE 7.27.
Activity of ribavirin against lethal respiratory virus infections (10 to 20 LD_{50}) in mice (after Sidwell et al., 1972)

Ribavirin (mg/kg per day, p.o.)	Survivors (%)		P	Day of death (mean)		P
	T	C		T	C	
Influenza A (PR8)						
75	90	20	<0.001	20.1	10.2	<0.001
37.5	40	20	>0.3	12.4	10.2	<0.05
Influenza A (Jap/305)						
150	100	30	<0.001	>21.0	10.0	<0.01
75	70	30	<0.001	16.8	10.0	<0.001
Influenza B (Lee)						
30	40	0	<0.05	16.0	8.4	<0.001
15	20	0	>0.3	12.2	8.4	<0.05
Parainfluenza I (Sendai)						
75	90	0	<0.001	19.9	9.8	<0.001
37.5	70	0	<0.001	18.3	9.8	<0.001

Groups of 10 to 20 mice were used in each experiment (T, treated; C, control).

precipitating teratogenesis is not clear, cytotoxic action of ribavirin against proliferating limb bud mesenchymal cells is directly associated with the origin of limb deformities. A review of the toxicology of ribavirin by Hillyard (1980) summarizes data with the compound.

7.10.2. Clinical efficacy of ribavirin versus influenza A and B virus infection

Early clinical trials with ribavirin and influenza gave somewhat conflicting results (Table 7.28). Thus, Salido-Rengell et al. (1977) evaluated ribavirin in a natural outbreak of influenza A virus (H3N2) in a girls' school in Mexico City. Therapy begun very early in the course of illness and was associated with less severe illness and inhibition of viral shedding. Magnussen et al. (1977) also observed a beneficial effect of ribavirin in young adult volunteers experimentally challenged with influenza virus A/Victoria/3/75 (H3N2). The drug-treated group had significantly fewer individuals with severe illness, and an inhibitory effect on viral shedding was also observed. In contrast, Cohen et al. (1976) and Togo and McCracken (1976) were unable to demonstrate a beneficial effect of ribavirin in young adult volunteers experimentally infected with two strains of influenza A virus (H3N2) and one of influenza B virus. Differences in ribavirin dosage regimens and in the severity of illness produced by natural vs. experimental infections probably contributed to the

TABLE 7.28.
Double-blind controlled trials of ribavirin vs. influenza

Investigator	Type study	Virus	Ribavirin dose	Number studied	Effect of ribavirin
Salido-Rengell et al., 1977	Natural infection	A/England/72 (H3N2)	200 mg/day 5 days	61	↓ severity clinical illness ↓ virus isolations ↓ HI antibody response
Cohen et al., 1976	Challenge	A/Maryland/74 (H3N2) + A/Dunedin/73 (H3N2)	600 mg/day 10 days (starting −2 days)	37	None
Togo et al., 1976	Challenge	B/Georgia/74	600 mg/day 10 days (starting −2 days)	30	'Marginal' decreased severity of clinical illness
Magnussen et al., 1977	Challenge	A/Victoria/75 (H3N2)	1000 mg/day 5 days (start +6 hrs)	29	↓ severity clinical illness ↓ virus shedding

inconsistent results of these investigations. Except for mild transient rises in total serum bilirubin levels in approx. 25% of treated subjects, ribavirin appeared to be well tolerated.

More recently Smith et al. (1980) investigated the effect of ribavirin against naturally acquired influenza A (H1N1) virus infection in young adults. A double-blind placebo-controlled trial of ribavirin was conducted in 97 young adult males naturally infected with influenza virus similar to A/Brazil/11/78 (H1N1). Ribavirin was given orally at a dose of 1000 mg/day for five days beginning within 24 or 48 h after onset of clinical influenza. The clinical signs and symptoms of influenza and quantitative viral shedding were the same in ribavirin- and placebo-treated groups. Ribavirin treatment was associated with significantly fewer fourfold or greater rises in antibody to influenza A viral antigen by the complement-fixation test, while rises in haemagglutination-inhibiting antibody titres occurred with equal frequency in both groups. The ribavirin-treated group experienced significant increases in bilirubin and in reticulocyte counts after onset of therapy. This suggested that ribavirin treated subjects had decreased synthesis and/or increased destruction of erythrocytes during treatment but the effect was not of sufficient magnitude to suggest that haemolysis was involved.

7.10.3. Ribavirin aerosol treatment in influenza B infection

Following the successul demonstration of antiviral efficacy of aerosolised ribavirin

in animal models versus influenza A virus and also in volunteers (Knight et al, 1981), McClung et al. (1983) have recently investigated antiviral efficacy versus influenza B virus. This is an interesting approach because few antivirals apart from ribavirin has been shown to be active in animal models versus influenza B, which is a virus which can cause mortality and serious morbidity in some years (see above).

Students coming to a health centre with an oral temperature above 38.3°C and an illness of less than 24 h duration suggestive of influenza were included in the study. Treatment with ribavirin aerosol was commenced within 1 hour of admission and lasted for 16 h continuously. The following morning a regular schedule of 4 hours treatment at 7 am, 2 pm and 7 pm was initiated for 3 days (McClung et al., 1983).

On entry into the study the severity in clinical signs was similar in both groups but by 17 h illness in ribavirin treated patients had improved to a greater extent than in controls (Table 7.29). Similarly, as regards temperature, the maximum temperature was lower in treated patients and remained lower throughout the trial period (Table 7.30). After the first 8 hours a decline in virus titre in nasal washes was detected in the treated group which was significantly different from the control groups during the next 2 days. In conclusion, a more rapid recovery of ribavirin treated patients was noted, and the results were comparable to those obtained previously with influenza A infections (Knight et al., 1981). The estimated dose of ribavirin deposited in the respiratory tract was 55 mg/h. This represents the first report of successful treatment of influenza B infection in humans, since amantadine, for example, is only effective against influenza A viruses.

Amantadine and ribavirin represent the only classes of compounds to have demonstrable effects against influenza A and B viruses in the clinic. Each compound however has its weaknessess: amantadine is only active against influenza A viruses, whilst ribavirin, as a nucleoside analogue (Witkowski et al., 1972) is teratogenic and immunosuppressive in animals (Potter et al., 1976) and hence may have insur-

TABLE 7.29.
Mean maximum daily temperature in ribavirin aerosol treated and control patients infected with influenza B virus (after McClung et al., 1983)

	Temperature, °C			
	Day 0	Day 1	Day 2	Day 3
Treated (n=11)	39.2	38.5	37.8	37.3[a]
Control (n=10)	39.0	39.1	38.3	37.7[a]
t-test, one-tailed	N.S.	0.02	0.028	N.S.

[a] For treated group, n=6.
N.S., not significant.

TABLE 7.30.
Systemic illness in ribavirin aerosol treated and control patients infected with influenza B virus (after McClung et al., 1983)

	Mean symptom scores[a]						
	Day 0	Day 1		Day 2		Day 3	
		am	pm	am	pm	am	pm
Treated (n=11)	2.4	1.2	1.1	1.0	0.64	0.78[b]	0.78[b]
Control (n=10)	2.2	1.9	1.6	1.5	1.2	1.3[b]	1.0[b]
Wilcoxon's rank-sum test, one-tailed	N.S.	0.008	N.S. (0.084)	N.S. (0.096)	0.045	N.S.	N.S.

[a] Range of severity, 0 through 3+
[b] For treated group, n=6, for control group, n=6
N.S., not significant.

mountable problems of toxicity, particularly when used orally as a prophylactic. Work continues to find more effective inhibitors and more recently this has tended to centre around inhibitors of specific virus enzymes, such as the influenza RNA transcriptase and neuraminidase, and synthetic competitive inhibitors of the fusion sequence of influenza HA. Both the former and latter approaches have the theoretical advantages of broad specificity, since the RNA polymerase enzyme is constituted by NP and P polypeptides (shared by virtually all influenza A viruses), whereas the 'fusion' sequence at the N terminus of the HA_2 molecule is also shared by the HA of different subtypes of influenza. It is thought likely then that any inhibitors so developed would inhibit *all* influenza A viruses and, hopefully, influenza B viruses as well. In the next few pages we shall attempt to summarize some of this work and to emphasize approaches which could be exploited in the near future.

7.11. Inhibitors of influenza RNA polymerase

Influenza A and B viruses have RNA-dependent RNA polymerase activity associated with their cores (Chow and Simpson, 1971, Skehel, 1971). This RNA-polymerase activity has been detected in the microsomes and nuclei of influenza-infected cells, and the virus-associated enzyme can transcribe, in vitro, the influenza virus genome, suggesting that the enzyme is required for virus multiplication. Therefore inhibitors of the RNA polymerase enzyme have potential application as chemoprophylactic agents against RNA-containing viruses (Helgstrand and Öberg, 1978, see also Chapter 3). Ho and Walters (1971) described the inhibition of cell-associated RNA-dependent RNA polymerase of influenza A/PR8 (H1N1) virus by selenocys-

tine, and the related compound selenocystamine dihydrochloride inhibits the virus-associated RNA polymerase enzyme of a number of influenza A and B viruses (Oxford and Perrin, 1974). We have described the in vitro inhibition of influenza virus-associated RNA-dependent RNA polymerase by selenocystamine dihydrochloride, bathophenanthroline disodium disulphonate and certain heterocyclic thiosemicarbazones (Fig. 7.12). A property common to these compounds is the ability to chelate soft, heavy, metal ions such as zinc and copper. Conversely, similar types of compounds in which the possibility of chelation was diminished showed significantly (e.g. 3-acetyl-pyridine-thiosemicarbazone) less inhibitory activity against influenza virus RNA-dependent RNA polymerase (Table 7.31). Mass spectrometry and atomic absorption techniques have detected the association of zinc with purified influenza B virus and the hypothesis was advanced that the RNA-dependent RNA polymerase enzyme of influenza virus was a zinc-activated enzyme.

Table 7.31 shows the in vitro inhibition of RNA-dependent RNA polymerase of

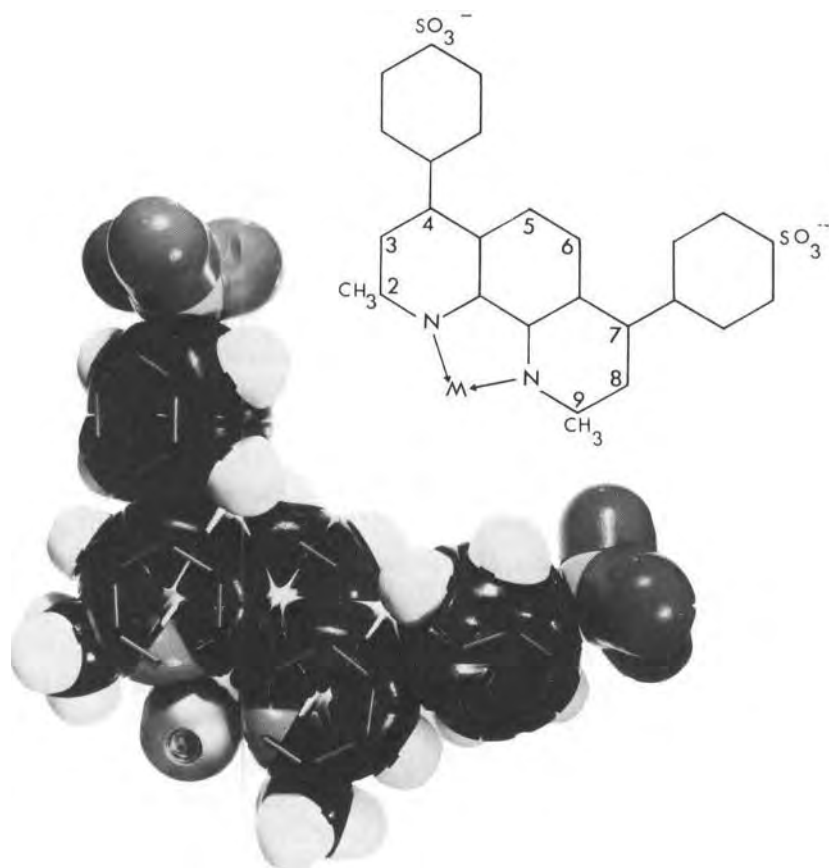


Fig. 7.12. Influenza RNA polymerase inhibitor (1:1 bathocuproine–zinc complex). Normally the zinc would be an integral part of a zinc RNA polymerase metalloenzyme.

TABLE 7.31.
Effect of chelating agents on influenza virus and bacterial RNA and DNA polymerases

	Concentration of compound (mmol) to inhibit incorporation of ^{3}H-UMP or ^{3}H-TMP by 50%			
	A/RI5⁺ RNA polymerase	B/LEE RNA polymerase	*Escherichia coli* RNA polymerase	*Micrococcus lysodeikticus* DNA polymerase
Bathocuproine disodium disulphonate	0.02	0.08	0.08	0.30
2-acetylpyridine thiosemicarbazone	0.003	0.002	0.20	0.20
3-acetylpyridine thiosemicarbazone (control)	1.0	1.0	N.T.	N.T.
Isatin 3-thiosemicarbazone	0.06	0.02	0.20	0.20

N.T., not tested

influenza A and B viruses by selenocystamine dihydrochloride and other molecules. This former compound also inhibited the DNA-dependent RNA polymerase of *E. coli* at a concentration of 0.04 mmol, but had little effect on the DNA-dependent DNA polymerase of *Micrococcus lysodeikticus*. Thus, a certain degree of selectivity was detected, particularly for influenza B/LEE RNA polymerase and *E. coli* RNA polymerase, although the differences between inhibitory concentrations for the latter enzyme and the RNA polymerase of A/RI-5$^+$ were not significant.

If the RNA polymerase of influenza virus is a metallo-enzyme, more active and more selective chelating agents might be designed and tested. The compounds tested at present also inhibit *E. coli* RNA polymerase, which is a zinc metallo-enzyme but any inhibitory effect on mammalian cell polymerases would depend on configuration of RNA polymerase enzyme polypeptides near the zinc binding site and also on the relative stability constants of zinc for the polypeptide ligand and any competing ligand.

7.11.1. Targeting of inhibitors using liposomes

An additional problem is to target the RNA polymerase inhibitors into virus-infected cells. Attempts to do this have been made using liposomes, since certain compounds described above are either relatively insoluble or too highly charged to penetrate the plasma membrane of cells.

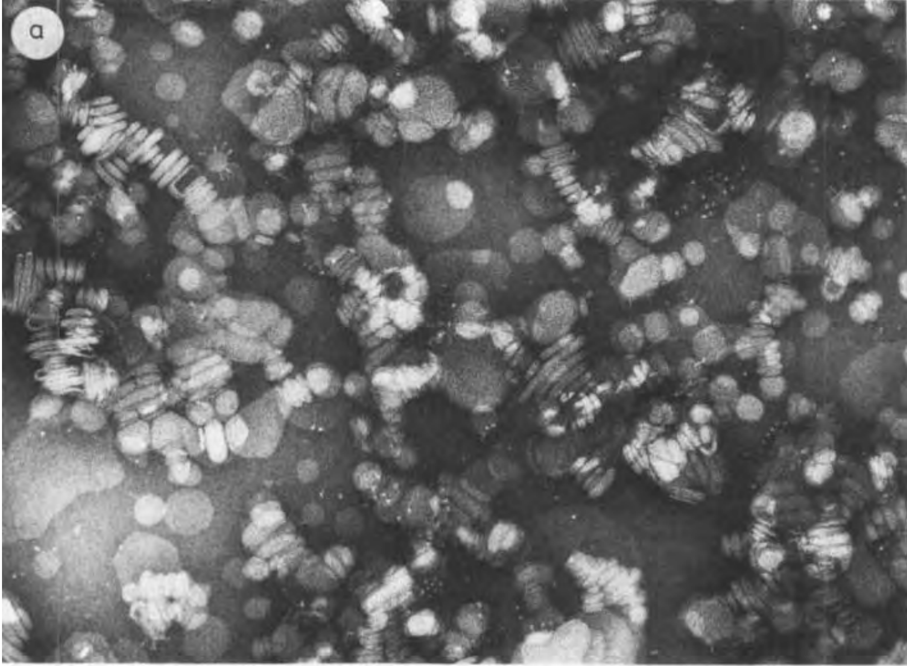
The use of liposome-encapsulated chelating agents for the selective delivery of chelating agents to the interiors of cells of the respiratory tract is an attractive possi-

bility (Perrin, 1977). Liposomes are finely dispersed phospholipid spherules, or vesicles around 1–10 μm in diameter, made up of concentric multiple bilayers that incorporate water and low-molecular-weight solutes in compartments between bimolecular lamellae (Fig. 7.13). Liposomes can be taken up into a cell by pinocytosis or can be engulfed by phagocytes. Once inside a cell, the liposome is broken down by lysosomal lipases and the chelating agent or other drug is liberated. Liposomes may protect drugs from metabolic modification and immunological reaction. The lipid composition may be varied considerably, giving a range of membrane structures, and charged liposomes can be formed by incorporating bases such as stearylamine or anionic species such as diacetyl phosphate or phosphatidic acid.

By using encapsulation in liposomes, the deposition and tissue-retention of highly charged inhibitors may be significantly increased. In model experiments Ca-EDTA or Ca-DPTA were used which are effective chelators of zinc but have a low and defined toxicity in experimental animals and man. The rationale of the experiments was to deplete the zinc levels in cells of the upper respiratory tract and hence to prevent the formation of, or inhibit the function of, the zinc containing influenza RNA transcriptase enzyme. However, in preliminary experiments in influenza virus-infected ferrets and mice, no antiviral activity was detected with the latter compounds, although only very low concentrations have been tested to date. In further experiments we selected the most active thiosemicarbazone (2-APTSC) for further in vivo studies in mice and ferrets. However, we were not able to detect any virus inhibitory effect or any effect of the compound on mouse mortality or mean day of death, or on the clinical signs of influenza in the ferret. Animals were given 5 mg/kg of 2-APTSC at –24, 0, 24, 48, 72 and 96 hours. For the above experiments we used A/Hong Kong/1/68 (H3N2) virus and included either amantadine or ribavirin as control compounds with demonstrable inhibiting effect in the particular model system.

7.12. Alternative animal models for assessment of anti-influenza virus activity

Potter et al. (1981) have described an interesting and relevant infant rat model. In essence when influenza virus replicates in the turbinates of infant rats they become more susceptible to challenge with *Haemophilus influenza*. Initial studies were carried out with amantadine and a polynucleotide (poly CS^4U). In the first study, 9 of 11 infant rats inoculated with influenza A/Victoria/75 virus and later given *H. influenza* developed bacteraemia and 6 of 11 developed meningitis; in contrast, virus-infected rats given amantadine at a concentration of 50 mg/kg by the subcutaneous, interperitoneal or oral route showed a significantly reduced incidence of bacteraemia and amantadine treatment by the intranasal route produced the most significant reduction in bacterial infection. These results were also shown in parallel studies using influenza A/Texas/77 virus. In a second, similar study, using influenza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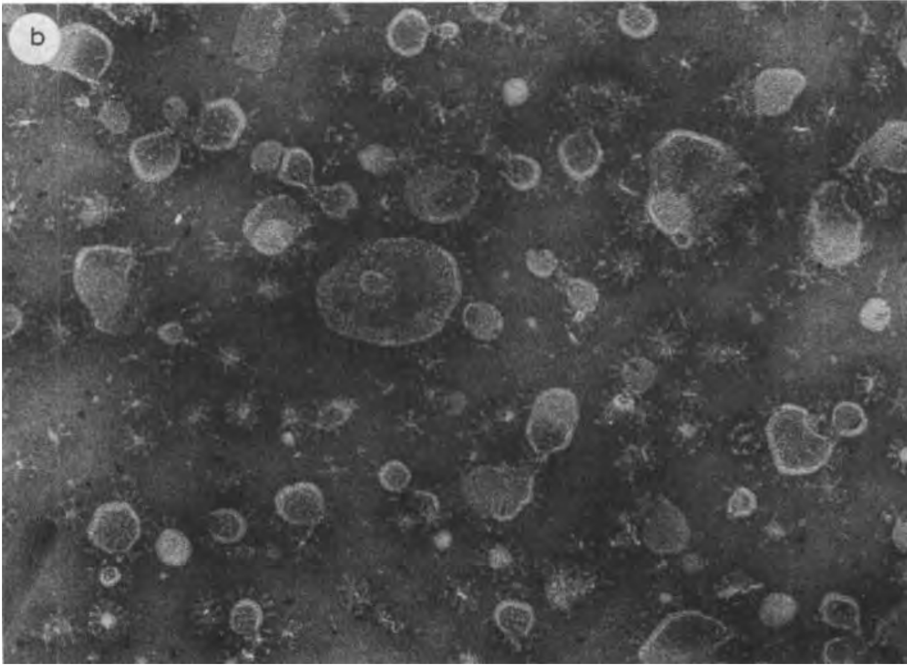
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TABLE 7.32.
Incidence of bacteraemia and meningitis in rats following influenza virus infection and treatment with poly CS[4]U (after Potter et al., 1981)

Virus inoculation	Poly CS[4]U (30 μg/kg×4)[a]	Number of rats with bacterial infection[b]			
		Bacteraemia		Meningitis	
		No.	%	No.	%
A/Texas (10^4 EID_{50})					
+	–	8/11	73	5/11	45
–	–	2/13	15	0/13	—
+	+(i/n)	2/19	10[c]	1/19	5[c]
–	+(i/n)	1/10	10	0/10	—
+	+(i/p)	11/29	38	2/19	10
–	+(i/p)	6/20	30	1/20	5
A/Victoria (10^4 EID_{50})					
+	–	9/9	100	6/9	67
–	–	1/10	10	0/10	—
+	+(i/n)	8/15	53[c]	3/15	20[c]
–	+(i/n)	1/10	10	0/10	—

[a] Compound given i/n or i/p at 0, 6, 26 or 30 h following virus inoculation.
[b] Rats inoculated with 4.5×10^5 cfu of HIb at 48 h following infection with influenza virus; bacteraemia and meningitis assessed at 72 h after H.inf. infection.
[c] Significant protection ($P = <0.05$).

virus A/Victoria/75 and A/Texas/77, poly (CS[4]U) also reduced the incidence of systemic infection (Table 7.32).

7.13. New compounds (and interferon) inhibiting influenza virus

Sodium 5-aminosulfonyl-2, 4-dichlorobenzoate (M12325) was evaluated for antiviral activity in tissue culture and infected mice (Ohnishi et al., 1982). At concentrations ranging from 2.5 to 75.8 μg/ml, the drug inhibited the cytopathic effects of 10 mean tissue culture infective doses of influenza virus A/WSN, A/FM, A/Kumamoto, and B/Great Lakes (Table 7.33). Concentrations up to 150 μg/ml did not inhibit the cyptopathic effects of herpes simplex virus, vaccinia virus, or adenovirus and concentrations up to 3160 μg/ml did not inhibit the growth of MDCK, Vero or HEL cells in culture, and hence a relatively large therapeutic index was estab-

←

Fig. 7.13. Influenza experimental virosome vaccine (Courtesy of Dr. D. Hockley). a, phospholipid vesicles; b, phospholid vesicles with HA attached by sonication ("virosomes").

TABLE 7.33.
Antiinfluenza activity, cytotoxicity, and therapeutic ratio of M12325 in culture (after Ohnishi et al., 1982)

Virus	Cell	MIC (μg/ml)		IC_{50} (μg/ml)		Therapeutic ratio	
		M12325	Amantadine	M12325	Amantadine	M12325	Amantadine
INFV-W	MDCK	2.5 (0.5–5.0)	3.7 (0.5–5.0)	>3160	102±9	>1260	27.6
INFV-W	MDCK	3.1 (0.5–5.0)	32.5 (15–50)	>3160	102±9	>1020	3.13
INFV-F	MDCK	13.3 (5.0–15)	7.8 (1.5–15)	>3160	102±9	>243	13.1
INFV-K	MDCK	3.8 (1.5–5.0)	3.3 (1.5–5.0)	>3160	102±9	>831	30.9
INFV-G	MDCK	7.0 (0.5–15)	32.5 (15–50)	>3160	102±9	>451	3.13

lished. Single oral doses of M12325, ranging from 10 to 300 mg/kg, administered 1 h before and 1 h after challenge, reduced mortality in mice inoculated intranasally with influenza A/WSN virus. Twice daily oral doses for 14 days effected significant reductions in the mortality of mice infected intranasally with influenza A/WSN, A/FM, A/Kumamoto, and B/Great Lakes, and parainfluenza virus, but they were not effective in mice infected with herpes simplex virus. Multiple doses of 10 and 30 mg/kg, administered intraperitoneally, reduced lung consolidation and virus titre whereas M12325 was well tolerated in multiple doses up to 1 g/kg orally.

Finally, in vitro and in vivo studies have clearly demonstrated that influenza A and B virus replication is inhibited by interferon (see also Chapter 3, Table 3.9). Moreover, the few experiments performed to date in controlled studies in volunteers have confirmed this activity (Merigan et al., 1973). Obviously further detailed studies are required with both influenza and other respiratory viruses, because interferon would be expected to have a broad spectrum of antiviral activity.

7.14. Influenza vaccines

Vaccination remains, at present, the main way of preventing epidemic influenza A and B viruses but, at least more recently vaccination has been confined to groups at special risk of mortality from influenza. The earlier widespread use of live attenuated vaccine in the USSR has now declined somewhat and most countries only produce enough vaccine to immunize some 10% of the population. Again, more recently, this vaccine has tended to be inactivated whole virus or subunit vaccine (reviewed by Selby, 1976). These (whole virus) vaccines have been produced for the past 4 decades and with improving technologies of ultra-centrifugation and gel filtration are now relatively pure virus proteins. HA antigen content is well controlled by single radial diffusion techniques (Wood et al., 1977) but it must be admitted

that many problems remain to be solved. Some of these are discussed in more detail below, whilst the reader is also referred to Chapter 2 where some more modern approaches of gene cloning are discussed and to Chapter 17 where some problems of antigenic and genetic variation are outlined (see also Table 7.34). Laboratory studies have established clearly that HA and NA antigens contain the main antigenic determinants of the virus responsible for inducing protective immunity (reviewed in Potter and Oxford, 1979). Passive antibody to HA and NA protects mice against lethal infection with influenza A virus, as well as immunization with HA and NA antigens. Immunization or passive immunity with antibody to M or NP for example has no protective effect (Virelizier et al., 1976, Fazekas de St. Groth and Graham,

TABLE 7.34.
Some currently used influenza vaccines and some experimental approaches

Vaccine	Comments or reference
Inactivated	
subunit	True subunit preparation with HA and NA removed by centrifugation
split virus	Contains all virion protein but lipids are removed and the virus disrupted
whole virion	The most immunogenic vaccine but also produced the most side reactions, particularly in children
Live attenuated	Florent et al., 1977; Florent, 1980
recombinant with host range mutant A/PR/8/34 (H1N1) virus	Occasionally such recombinants may be virulent for humans (Oxford et al., 1978)
ts or *ca* mutants made by recombination	Problems of 'revertants' in young children have yet to be overcome
Experimental vaccines	
virosomes	Liposomes are sonicated with HA and NA to reconstitute a synthetic virus. More immunogenic than HA alone
HA aggregates	A new approach to increase immunogenicity of HA (Morein et al., unpublished data)
Virus grown in human diploid cells	Cell grown virus may exhibit different antigenic determinants (Schild et al., 1983)
HA cloned in mammalian cells or bacteria	Chanock and Murphy, 1979; Chanock, 1982; Heiland and Gething, 1981
Genes from avian 'enteric' influenza viruses – and administration of live vaccine orally	Recombinants may be made with genes of avian duck influenza A viruses (Murphy et al., 1983). Russian workers have administered live vaccine orally.
Oligopeptides of antigenic determinants of 'fusion' sequence	Green et al., 1982; Atassi and Webster, 1982

1954). Although cell mediated immunity (see Chapter 2 and Wells et al., 1979) may aid in the recovery from influenza, nevertheless the most important correlates of protective efficacy are local nasal IgA and serum levels of IgG neutralizing antibody (reviewed in Stuart-Harris and Schild, 1976). Underpinning the mountain of difficulties is the major one of original antigen sin (Francis, 1953), whereby it was observed that the first attack of influenza leaves an indelible immunological memory to the HA of that virus. Subsequent stimulation of the immune system by infection or immunization more often than not leads to an increase in synthesis of antibody to the HA of this first virus, rather than to the virus actually in the current vaccine. It is difficult to imagine how any approach to influenza vaccine could overcome this difficulty and this again emphasizes that a combined approach with antivirals and vaccines may represent the best strategy.

7.14.1. Inactivated influenza virus vaccines

Inactivated influenza A and B virus vaccines are prepared nowadays most often by the purification of recombinant (Kilbourne, 1969; Schulman and Palese, 1978) influenza virions harvested from infected allantoic fluids of embryonated hens' eggs by rate-zonal centrifugation. The virus (which is approximately 98% pure) is then inactivated with formalin or β-propiolactone and standardized by radial immunodiffusion (Wood et al., 1977) or rocket immunoelectrophoresis to contain around 30 μg of protein per human vaccine dose. Using high growth 'recombinant' viruses (Kilbourne, 1969) which contain as many as 6 genes from the high yielding laboratory virus A/PR/8/34 (H1N1) and only 2 genes (coding for HA and NA) from the 'wild' virus, approximately 2–3 doses of virus vaccine may be obtained from one embryonated egg. But it is quite apparent that the application of a considerable technology and time span is required to produce enough vaccine to immunize a population of 200 million persons in the USA, for example (Table 7.35). A period of many weeks must necessarily elapse from the initial outbreak caused by a new

TABLE 7.35.
Use of influenza vaccine in the USA

Year	Total population		Population aged 65 and over	
	Number of persons immunized $\times 10^6$	Percentage of total immunized	Number of persons immunized $\times 10^3$	Percentage of total immunized
1968–69	21	10.7	3506	19.0
1972–73	15	7.7	3209	15.8
1973–74	17	8.3	3638	17.4

Source: US Immunization Survey, 1969, 1973, 1974

TABLE 7.36.
Production of inactivated vaccine for A/Hong Kong epidemic, 1968

Isolation	4 days
Identification, recombination, distribution of strain	8 weeks
From seed strain for first release	10 weeks
To production of first million doses	2 weeks
Total	20 weeks

5 million doses 22 weeks
20 million doses 29 weeks
Source: Murray, R. Bull. WHO 41, 495.
Note: there is little reason to suppose that this period would be significantly shorter today

influenza A virus and the production of vaccine against the antigenic variant (Table 7.36). (In contrast, the efficacy of existing antivirals such as amantadine can be established within days.) Large scale immunization (Table 7.37) has been attempted in the USA in the face of a threatened outbreak of virus caused by A/New Jersey/76 (H1N1), a virus genetically related to the causative virus of the 1918 pandemic which had remained in the pig population in the USA since the pandemic in humans. However, the mass vaccination campaign was halted when a hitherto unrecognized complication of vaccination was observed – Guillain-Barré syndrome, a progressive disorder of the central nervous system (Keenlyside et al., 1980). Nevertheless, the campaign was a considerable technological achievement, with rapid production of vaccine in quantity, and efficient administration on a large scale using jet guns.

The other problems of inactivated influenza vaccine relate to the rather low efficacy (approximately 70% protection), poor longevity of the immune response (1 year), evasion of the induced immune response by antigenic mutants of the virus, and residual toxicity of the vaccine itself. As regards the latter, most adult persons receiving current inactivated vaccines notice only local pain and stinging at the site of inoculation and although this may deter future acceptance of vaccine it is not a serious reaction. However, in children or unprimed adults, whole virus vaccine

TABLE 7.37.
Population groups in which a more extended use of inactivated influenza A and B vaccine could be recommended

1. Persons at special risk of mortality e.g. preexisting pulmonary, cardiac, metabolic and immunological deficiencies and older persons (over 60 years of age).
2. Persons in institutions and boarding schools, where attack rates can be very high.
3. Hospital patients and personnel, public transport personnel, police, factory workers.
4. In the event of a new pandemic of influenza A virus as many of the community who wish to be included in a vaccination campaign.

See also Smith et al., 1976

may induce more serious side reactions, including fever. To circumvent these adverse effects, more recently developed vaccines are 'subunit' preparations containing only the relevant antigens which induce protective immunity – namely the two surface glycoprotein spikes of the virus, haemagglutinin and neuraminidase (reviewed by Tyrrell and Smith, 1979; Webster and Laver, 1966). Such subunit vaccines are less reactogenic but unfortunately they are also less immunogenic in unprimed individuals. Studies are continuing in attempts to improve the immune response to HA subunits. For example, addition of synthetic adjuvants such as muramyl dipeptide (MDP or 6-stearoyl MDP) may increase the B cell response to the HA molecule, at least in animal models.

An exciting new approach has been the incorporation of a synthetic gene transcribed from gene 4 (coding for HA protein) by a reverse transcriptase into an *E.coli* plasmid system (Emtage et al., 1980). The result of this genetic engineering experiment was a bacterium which synthesized influenza HA in amounts detectable by immunoprecipitation (around 2 μg/ml) (see also Chapter 2 and the experiments of Gething and Sambrook, 1982). However, at this stage many obstacles remain. Firstly, the HA constitutes only 2–3% of the total protein synthesized by the bacterium and is not transported to the exterior. Thus, the HA has to be released by lysing the bacteria and purifying it from *E.coli* proteins and endotoxins. Although the HA clearly possesses some antigenic determinants, of prime importance for vaccination is retention of immunogenicity. It is probable that enzymatic degradation occurs in the presence of *E.coli* proteases and thus the HA molecule may be in monomer form, similar to HA removed from virus by the proteolytic enzyme bromelain. In this case the molecule may be poorly immunogenic and may need to be reconstituted onto a virosome, for example, to increase the immune response. On the other hand, the technology of bacterial cultivation on a large scale is well advanced and a single large fermentation batch could produce theoretically 100 mg of HA, enough antigen for 10 000 doses of vaccine at the current formulation.

7.14.2. Synthesis of an antigenic site on influenza HA

As noted above, the N-terminal region of HA2 (the fusion region) is believed to be involved in the initial infection and uncoating of the influenza virus and, from the X-ray structure, the first 10 residues of this region appear to be accessible to antibody (at least at low pH). Because of its involvement in viral infection, antibodies may arise against this region in the course of defence against viral infection. The elegant studies of Atassi and Webster (1983) with synthetic peptides comprising the HA2 region (residues 1–11) of influenza A virus and influenza B virus were carried out to investigate the antigenicity of this region of the HA molecule. Two peptides, comprising the fusion region (residues 1–11 of the HA2 part of HA) of strain A and strain B influenza virus, were synthesized and their abilities to bind rabbit, goat, and human anti-influenza antibodies were determined. In quantitative im-

munoadsorbent titrations, the two peptides bound considerable amounts of antibodies in rabbit and goat antisera against virus or HA of the A or B strain, as well as in several human sera from patients recovering from influenza A. Of the 30 anti-HA monoclonal antibodies, 5 bound completely and 4 bound partially to the peptides. Antibodies were raised in rabbits against the peptides by immunizing with peptide-bovine serum albumin conjugates, or with the free peptides. Anti-peptide antibodies were bound by HA and by the intact virus of the respective strain. However, these antisera failed to exhibit significant virus neutralizing activity. The finding that sera from several individuals after viral infection had large amounts of antibodies directed against the fusion region unequivocally established it to be an important antigenic site on virus HA for humans under conditions of natural influenza infection.

In comparison with the relatively strong immunogenic activity of this location, weak antigenicity has been reported in the region of residues 91–108 and in some of the carbohydrate side chains of HA1. Recently, 20 peptides comprising almost 75% of the HA molecule were synthesized (Green et al., 1982) and, even though most were antigenic when coupled to protein carrier, none of the peptides reacted with anti-HA antibodies, indicating that they did not contain any antigenic sites of HA. The antigenic site described by Atassi and Webster (1983) is not one of the four antigen regions suggested from examination of the X-ray structure of HA (see above). However, it is accessible and is involved in infectivity. Also, this site is not predictable from the empirical approach on the basis of hydrophilicity index. In fact, the fusion region is so hydrophobic that the synthetic peptides were insoluble in aqueous solvents.

Antigenic sites should be expected to reside in accessible surface regions, but not every surface region constitutes an antigenic site and thus, exposure is not a sufficient criterion for immunogenicity. Furthermore, antigenic sites are not necessarily highly hydrophilic regions, and hydrophobic interactions frequently provide major contributions to the binding energy. Also, as seen here and reported for haemoglobin α-chain, major antigenic sites could reside in surface regions that have very low hydrophilicity or are mostly hydrophobic. Such regions are rendered accessible by the three-dimensional and oligomeric characteristics of a particular protein.

In summary, the study is an excellent start but many potential peptides now remain to be investigated. It is also possible that the antibodies induced, although having no conventional neutralizing activity, may still inhibit virus release or infection in a biological system.

7.14.3. Problems and perspectives with inactivated influenza vaccines

Since we shall examine data in some detail of the *ca* live virus approach (see below) it will be of interest to examine some problems with conventional inactivated influenza vaccines. In an excellent study in the Post Office in the UK data was accrued

TABLE 7.38.
Post Office – Telecommunications: Main causes of sickness absence 1974–75

	Days (thousands)		Days/employee	
	Men	Women	Men	Women
Respiratory	514.4	421.4	3.3	5.2
(influenza)	(147.0)	(98.0)	(0.9)	(1.2)
Injuries	258.9	90.9	1.6	1.1
(at work)	(57.2)	(4.7)	(0.4)	(0.1)
Gastrointestinal	182.1	127.5	1.1	1.6
Musculoskeletal	162.9	109.4	1.0	1.3
Cardiovascular	154.6	52.5	0.9	0.6
Psychiatric	58.9	67.6	0.4	0.8
Malignant	8.3	6.5	0.1	0.1
Other	416.3	510.0	2.4	6.3
Unspecified	54.9	48.6	0.3	0.6
All causes	1838.5	1434.4	11.1	17.6

TABLE 7.39.
Subjective reactions in 247 influenza vaccinees (after Smith et al., 1976)

	Type of vaccine			
	Injected	Killed nasal	Attenuated nasal	
			First dose	Second dose
Number vaccinated	89	78	80	75
% with no reactions	3	36	43	67
% with general reaction	63	63	57	33
% with local reaction	92	—	—	—

which answered questions about vaccine efficacy, safety and acceptance by healthy persons working in industry. Firstly the data in Table 7.38 emphasizes the importance of influenza as a cause of sickness absence in this group of workers. Table 7.39 shows data on subjective reactions in some of the employees following inactivated or live influenza vaccines. Most vaccinees experienced some local or even general reactions to vaccination and this was, perhaps, reflected in the acceptance rates for vaccine in successive years which declined (Table 7.40). Of particular interest is the observation that over the 5 year period, days lost from sickness or absence were fewer in the vaccinated group compared with the unvaccinated group, although fluctuations were noted from year to year. Certificated sickness absences were also reduced significantly, both in the influenza and non-influenza period

TABLE 7.40.
Acceptance rate of influenza vaccine in industrial workers (after Smith et al., 1976)

Factory	Nature of factory	1971/2	1972/3	1973/4	1974/5
Post Office					
Telecomms.	Skilled technical	42%	34%	35%	32%
Posts.	Postmen and counter staff	—	36%	28%	23%
A	Light industry and office	40%	26%	22%	19%
B	Light industry and office	42%	27%	20%	14%
C	Office staff	—	32%	26%	27%

Based on 1973/4 total population

TABLE 7.41.
Influenza vaccination study in the Post Office. Days lost from sickness absence/week/100 employees (after Smith et al., 1976)

		1971/72		1972/73		1973/74	
		Vacc.	Unvacc.	Vacc.	Unvacc.	Vacc.	Unvacc.
Excess in influenza period compared with non-influenza period	Telecomms.	4.1	6.4	8.0	6.3	2.3	2.3
	Posts	—	—	3.6	5.1	3.4	4.4
Average over whole study period	Telecomms.	22	22	25	24	21	23
	Posts	—	—	29	31	30	32

TABLE 7.42.
Certificated sickness absence[a] in factory employees, 1972–73 (after Smith et al., 1976)

Category of employees	No. of employees	Average no. of days lost from sickness absence/100 employees/week		
		Influenza period	Non-influenza period	Excess in influenza period
Vaccinated A+B	646	4.6	2.4	2.2
Vaccinated B	524	6.9	2.8	4.1
Non-vaccinated	3280	7.9	3.9	4.0

[a] Excluding absentees in week of vaccination and absences over 9 weeks' duration.

(Tables 7.41, 42) (showing the placebo effect of medical attention on persons reporting ill!). It might be added that a recent analysis of economic savings from vaccinating old persons in the USA has shown a considerable economic benefit (Riddiough et al., 1983).

7.14.4. Live attenuated influenza virus vaccines

An alternative approach is the use of live attenuated influenza vaccines (Beare et al., 1975; Richman and Murphy, 1979). In theory, there should be a greater opportunity for rapid production since up to 1000 doses of live virus vaccine can be recovered from a single embryonated hen's egg. Alternatively, influenza A virus strains can now be cultivated in human diploid cells and this may also have theoretical advantages. (Herrero et al., 1983, Schild et al., 1983). There is also experimental evidence that the immunity resulting from the administration of live attenuated vaccines is broader than that induced by inactivated vaccines and this may help circumvent the perennial problem of antigenic drift (reviewed by Potter and Oxford, 1979).

In early studies two groups of virologists, one in Leningrad (Alexandrova and Smorodintsev, 1965) and one in Ann Arbor (Maassab, 1967) adapted influenza viruses to low temperatures (cold adapted mutants or *ca* mutants) and investigated the potential of these viruses as attenuated virus strains. The viruses will not replicate at 37°C and hence, at least in theory, would not replicate in the lower respiratory tract and would be expected, in view of the accumulated mutations, to show signs of attenuation. Laboratory 'recombinants' or viruses with reassorted genomes between the *ca* mutants and the most recent virulent antigenic variants are made, and assessed for virulence in volunteers. In this way a recombinant can be selected with *ca* genes from one parent and the HA and NA genes from the virulent parent, and such recombinant viruses are apparently attenuated for man.

An alternative and much investigated possibility is to induce a temperature sensitive (*ts*) lesion (or lesions) in a well characterized donor strain of influenza A virus (a 'master' or 'mistress' strain) and then transfer the genes responsible for the *ts* defect, together with genes coding for HA and NA from the current virulent virus, to make laboratory recombinants (Murphy et al., 1976). The rationale is similar to the *ca* approach – viruses which are unable to replicate at 37°C cannot infect cells in the lower respiratory tract of man, and hence would be expected to show reduced virulence properties. However, a complication of some of the earlier studies with *ts* mutants has been some tendency to revert back to a more virulent non *ts* phenotype. But this has been less of a problem with the *ca* mutants which, at present, look more hopeful for future studies.

In a third general approach to live attenuated influenza vaccine viruses, recombinants have been prepared between an old attenuated laboratory virus A/PR/8/34 (H1N1), which is probably a 'host range mutant' since it has been 'adapted' to ferrets, mice and eggs and thus 'de-adapted' to humans, and the recent virulent virus.

Viruses which have HA and NA genes from the virulent virus parent and the remaining genes from the attenuated A/PR/8/34 parent, are investigated in volunteers for attenuation (Beare et al., 1975). It has been shown quite clearly, however, that occasionally such recombinants still retain some virulence for man (Oxford et al., 1978) and such studies have, therefore, to proceed with caution. The present complex epidemiological situation with influenza A virus, with two antigenic subtypes co-circulating (H3N2 and H1N1) introduces a further complication with live vaccines. If two such laboratory attenuated recombinants are administered to man then additional recombination events may occur in naturo leading to 'new' viruses perhaps with more virulent properties (Rott et al., 1979, Scholtissek et al., 1979). However, such processes are probably occurring anyway in nature (Palese and Young, 1982). Two live vaccine viruses administered together may interfere with each other's replication (Potter et al., 1983) and so result in the development of a distorted immune response to a single virus only. Thus, as many problems remain to be solved with influenza vaccines as with the development and use of specific anti-influenza virus inhibitors. The population groups in which more extended use of vaccine could be recommended are similar to those suggested for antivirals (Table 7.37).

An excellent summary of the *ca* virus studies carried out mainly in the USA and USSR has been published (Kendal et al., 1981) and we shall content ourselves here by re-examining some of this summarized data. Typical biological characteristics of these *ca* master or mistress viruses are outlined in Table 7.43, whilst a method of obtaining the *ca* Ann Arbor strain by passage in the laboratory at 35°C is illustrated in Table 7.44. These candidate vaccine strains are tested exhaustively in animal models, where they replicate well in the cooler parts of the upper respiratory tract of the ferret and mouse but not at all at 37°C in the lower respiratory tract. A protocol for phase I testing in volunteers is outlined in Table 7.45, and it is apparent that the candidate virus is tested for immunogenicity and side reactions in

TABLE 7.43.
Replication temperature markers of cold-adapted viruses used as parents in the preparation of recombinant vaccine candidates (after Kendal et al., 1981)

	Replication of cold-adapted parental virus						
	in eggs at (°C)				in CK cells at (°C)		
	25	28	33	39–40	25	33	39
A/Leningrad/9/46 (H1N1)	2.25	8.25	8.25	2.25	N.D.	N.D.	N.D.
A/Leningrad/134/57 (H2N2)	4.75	8.25	8.25	2.25	N.D.	N.D.	N.D.
A/Ann Arbor/6/60 (H2N2)	7.50	N.D.	8.50	1.30	8.30	8.70	<3.0

CK, chick kidney cells
N.D., not determined

TABLE 7.44.
Isolation and passage history of cold-adapted influenza A/Ann Arbor/6/60 mutant used for preparing recombinant vaccine candidates (after Kendal et al., 1981)

Passage level	Conditions
1	Throat swab, incubate at 36°C in CK cells
2	CK_1 isolate, incubate at 36°C in CK cells
3–9	Serial passages, incubate at 33°C in CK cells
10–16	Serial passages, incubate at 30°C in CK cells
17–23	Serial passages, incubate at 25°C in CK cells
24–29	Serial plaque purification, at 25°C in CK cells
30–32	Serial passage at 25°C in specific pathogen-free hens' eggs

TABLE 7.45.
Protocol for phase 1 (safety) testing of live vaccine in healthy young adults in the USA (after Kendal et al., 1981)

A. General procedure
1. Pre-bleed potential vaccine participants.
2. Test pre-study serum by HI, neutralization, or enzyme-linked immunosorbent (ELISA) assay, to identify volunteers who have maximum susceptibility to the vaccine virus.
3. Quarantine all selected volunteers in restricted access area for 2–3 days to monitor for any developing illness.
4. Under blind conditions, administer by drops vaccine or placebo (0.5 ml/nostril) to supine volunteers. Approximately 3/4 of participants receive vaccine, the remainder placebo.
5. Monitor febrile, systemic and respiratory reactions and collect daily nasal wash specimens.
6. After 2 weeks collect final serum specimen. Volunteers depart from restricted access area.

B. Laboratory tests on specimens collected
1. Inoculation of tube cultures of monkey kidney (MK) or Madin–Darby canine kidney (MDCK) cells at the permissive temperature of 34°C with dilutions of nasal wash specimens to titrate post-inoculation replication of virus, and verify cessation of virus shedding. Additional virus isolation studies are undertaken to diagnose possible non-influenza infections in case of illness in participants.
2. Inoculation of the tube cultures of MK or MDCK cells at the non-permissive temperature of 39°C with a low dilution of nasal wash specimens to detect possible revertant (non-*ts*) virus.
3. Titration of virus isolates in primary chicken kidney cells at 25°C, 33°C and 39°C to examine stability of *ca* and *ts* markers.
4. Titration of pre- and post-inoculation sera specimens by HI, and in some cases by neutralization and ELISA procedures, to detect and quantitate humoral antibody responses.
5. In some cases titration of nasal wash specimens by neutralization and ELISA procedures to quantitate local antibody responses.

TABLE 7.46.
Infectiousness, immunogenicity and reactogenicity of cold-adapted H3N2 A/Ann Arbor recombinant vaccines in phase 1 studies with young adult volunteers (after Kendal et al., 1981)

H3N2 recombinants	Dose (log_{10} $TCID_{50}$	No. of volunteers	No. (%) infected[a]	No. (%) infected with	
				HI response	Febrile or systemic illness
CR 18 clone 7	7.5	10	8 (80)	6 (75)	1 (13)
	8.5	12	12 (100)	11 (92)	4 (33)
CR 19 clone 0	6.3	14	13 (93)	13 (93)	0 (0)
	7.5	13	12 (92)	10 (83)	1 (8)
CR 22 clone 1	6.8	9	9 (100)	9 (89)	1 (11)
CR 29 clone 2	7.5	24	18 (75)	16 (89)	0 (0)
CR 31 clone 3	7.7	12	12 (100)	7 (58)	0 (0)
CR 31 clone 10	7.7	17	13 (76)	10 (77)	1 (8)
H1N1 recombinant					
CR 35 clone 2	7.5	25	24 (96)	11 (46)	0 (0)

[a] Determined by virus isolation and antibody response.

a small group of volunteers in quarantined conditions. Samples are collected from the volunteers and examined for biological markers, including the important *ca* property. (A problem with *ts* viruses is that when tested in doubly sero-negative children with no antibody to either HA or NA, non *ts* 'revertant' viruses tend to appear. This has slowed the programme for development of *ts* influenza A viruses in the USA).

Typical results of immunogenicity from published trials with the Ann Arbor and Leningrad vaccine viruses are given in Table 7.46. Both viruses were shown to induce antibody in volunteers, whilst producing no serious side reactions. Most importantly subsequent challenge of these immunized volunteers showed them to be protected against clinical signs of influenza infection. Therefore, studies are progressing with *ca* mutants, but for reasons of safety have to progress slowly. One could ask, for example, whether recombination in nature with a *ca* and wild virus could lead to a more virulent recombinant? Would the *ca* viruses show clinical effects in chronic bronchitics or persons with other pre-existing respiratory diseases? Finally, as alluded to above and in Chapter 2, genetic engineering and biochemical methodology could lead to new vaccine candidates with lesions inserted at specific points in the genome.

7.15. Summary

An overwhelming amount of data has accumulated on the biochemistry, cell biology and epidemiology of influenza, but prospects of control of epidemics in the near future are dim. Meanwhile, a 'holding operation' can be achieved using inactivated vaccine and rimantadine (100 mg/daily) in special risk groups in the population until new more effective vaccines and broad spectrum antivirals (active against influenza A *and* B virus) are developed. Research work is centred now around biotechnology to produce immunogenic peptides and proteins and also more logical searches for antivirals using amino acid sequence data and also virus specific enzymes such as the virion transcriptase as targets.

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CHAPTER 8

Paramyxoviruses

Introduction

The paramyxoviruses are a heterogeneous group of viruses causing a variety of clinical diseases in humans, animals and birds as illustrated in Table 8.1. We shall examine in more detail below the structure and properties of the important human

TABLE 8.1.
Pathogenicity of paramyxoviruses for man and animals (after Kelen and McLeod, 1977)

Virus type	Species pathogenicity under natural conditions[a]								Main clinical syndrome
	H	S	E	Rm	P	C	Rd	A	
PIV-1	+				+		+		ARTD[b]
PIV-2	+					+			ARTD
PIV-3	+	+	+	+			+	+	ARTD
PIV-4	+								ARTD
PIV-5	+	+				+	+		Various
Mumps (MuV)	+								Mumps
Newcastle Disease Virus (NDV)	+							+	Newcastle disease
Measles (MeV)	+	+							Measles
Canine distemper (CDV)						+			Distemper
Rinder pest (RPV)				+					Rinder pest
Pneumonia (mouse) (PVM)							+		ARTD
Respiratory syncytial (RSV)	+	+		+					ARTD

PIV, parainfluenza virus
[a] H, human; S, simian; E, equine; Rm, ruminant; P, porcine; C, carnivore; Rd, rodent; A, avian.
[b] ARTD, acute respiratory tract disease.

TABLE 8.2.
Antigenic composition and reactivity of paramyxoviruses (after Kelen and McLeod, 1977)

Virus types	Core (S) antigen	Surface (V) antigens			
		H	N	HL	CFF
PIV-1–5	+	+	+	+	+
MuV	+	+	+	+	+
NDV	+	+	+	+	+
MeV	+	(+)a	–	(+)a	+
CDV	+	–	–	–	+
RPV	+	–	–	–	+
PVM	+	(+)b	–	(+)b	+
RSV	+	–	–	–	+

a, with erythrocytes of simian origin only; b, with erythrocytes of rodent origin only.

viruses in this group, namely measles, respiratory syncytial virus (RSV), mumps and parainfluenza viruses I–V. In brief, they are all enveloped, negative-stranded, riboviruses of helical symmetry (Fig. 8.1). The genome of the paramyxoviruses is a single negative strand of RNA of molecular weight approximately 5.4×10^6, containing the genes coding for the six known virus specific proteins. The HN (haemagglutinin-neuraminidase) F (fusion) and M (matrix) proteins are associated with the lipid membrane of the typical paramyxovirus. HN and F are glycoproteins (see below) constituting the surface spikes (see Fig. 8.1 for typical paramyxoviruses) and M is a non-glycosylated matrix protein, underlying the viral membrane. The F protein is formed by proteolytic cleavage of a larger precursor glycosylated polypeptide F_0. The three other proteins L, NP and 47K, together with the RNA, form the nucleoprotein core of the virion. The L and 47K proteins may constitute the virus RNA transcriptase enzyme, whose template is the genome RNA, complexed with NP. From the point of view of strategies of antivirals and vaccines, the RNA transcriptase enzyme and the antigenicity (Table 8.2) of the HN and F proteins respectively are important.

In infected cells, the paramyxovirus proteins are synthesized from mRNA species which are complementary to the genome, and which appear to be monocistronic. Two major size classes of mRNA have been detected – a 35S class containing the mRNA species for the L protein, and an 18S class containing at least five mRNA species (for a summary see Ball et al., 1978). The general strategy of replication is that of a typical negative stranded RNA virus (see Chapter 3), although whether precise details will differ between RSV, measles and mumps must await further studies.

8.1. Mumps virus

The catastrophe was dreadful: For the swelled testicles subsided suddenly the next day, the patient was seized with a most frantic delirium, the nervous system was shattered with strong convulsions, and he died raving mad the third day after.

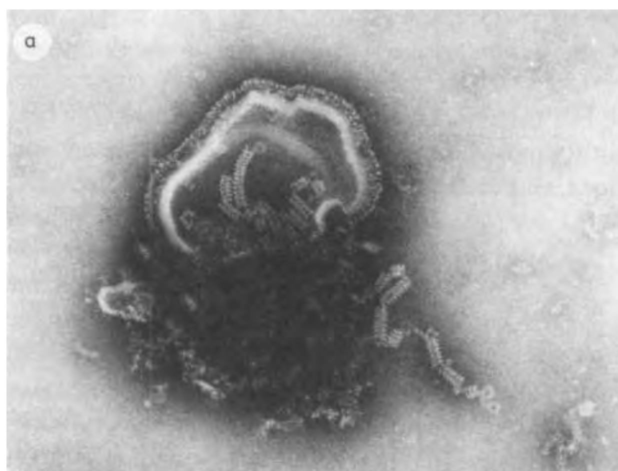
Robert Hamilton.
An Account of a Distemper by the Common People of England Vulgarly Called the Mumps. paper to the
Royal Society of Edinburgh, 1790

The term mumps, probably from an old verb meaning 'to mope', is a good home-spun word for a familiar illness (Christie, 1980). The disease, though, is of great antiquity and was one of the first infections to be recognized and was described by Hyppocrates in the 5th century BC.

8.1.1. THE VIRUS AND MODE OF REPLICATION

The virus itself, like measles, belongs to the paramyxovirus group and is a roughly spherical, enveloped RNA-containing virus, 150–200 nm in diameter (Fig. 8.1). The internal nucleocapsid is surrounded by a lipid envelope (approx. 15 nm in thickness) on which are situated the outer glycoproteins of the virion. The nucleocapsid is a flexible helical structure with a $_{ss}$RNA backbone covered with protein subunits. The RNA approximates to 7×10^6 M.W., and would have coding capacity for at least 8 proteins. A major protein of the virus is the NP and an additional polymerase P protein is thought to be closely linked to the RNA and functioning in an RNA-transcriptase complex. A membrane or M protein is another major constituent of the virion and is assumed to contribute to the structural integrity of the virus, and is located inside the lipid envelope. Two glycoprotein spikes are present, the larger (HN) with HA and NA activities and a smaller glycoprotein F involved in haemolytic and cell fusing activities (Heppertz et al., 1977, Naruse et al., 1981, Orvell, 1978, Herrler and Compans, 1982, Rima et al., 1980). The virus, therefore, has some typical properties of the paramyxovirus group, but compared to other members of the group rather little is known about the detailed biochemistry or molecular biology of the virus.

In a recent study Merz et al. (1983) investigated intracellular mumps virus-specific polypeptide synthesis by pulse- and pulse-chase-labelling with radioactive amino acids and sugars. The major polypeptides seen on SDS-polyacrylamide gels were NP (69 K M.W.), P (45K M.W.) and M (40K M.W.); a non-structural polypeptide (22K M.W.) was also present in infected cell lysates. The HN (74K to 79K M.W.) glycopolypeptide was detected in (^{3}H) glucosamine- and (^{3}H) mannose-labelled in-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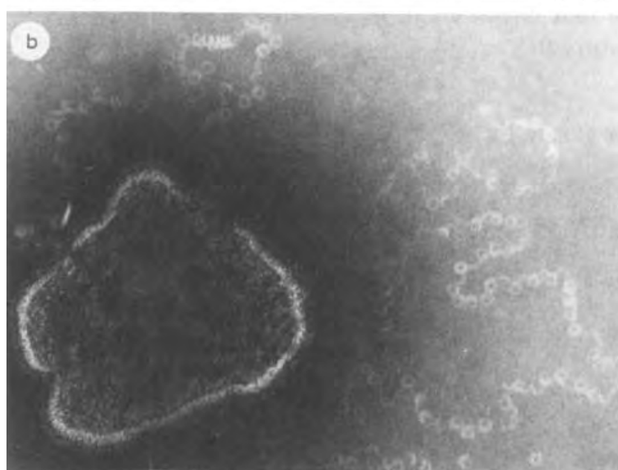
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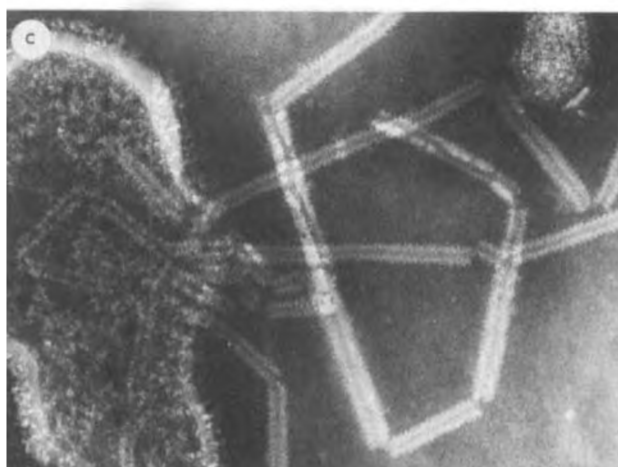
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fected cells. A 65K M.W. species that had incorporated these precursors was seen in pulse-labelled infected cell lysates, and this glycopolypeptide vanished during the chase interval, with the concomitant appearance of two glycopolypeptides (59K M.W. and 14K to 15K M.W.) which represented the F_1 and F_2 subunits of the F glycoprotein. Immunological data confirmed the relatedness of the 65K M.W. glycopolypeptide to the F glycoprotein and identified it as the precursor F_0. The F_0 precursor glycopolypeptide was seen in cells infected with both fusing and non-fusing strains of virus, and F_0 was processed completely to F glycoprotein for all infections.

Thus, mumps virus-infected cells contain seven virus-specified polypeptides, as has been described for other paramyxoviruses. The protein is the most abundant polypeptide in infected cells. Polypeptides corresponding to the nucleocapsid-associated polypeptide P and membrane (M) protein are also present. A polypeptide of M.W. 22K is found in infected cells which does not precipitate with anti-mumps virus antiserum and has a unique limited proteolysis peptide map; this is consistent with it being a non-structural (NS) polypeptide.

8.1.2. Epidemiology and clinical aspects

Mumps is a common disease of childhood, although attacks in adult life are more frequent than of measles or chickenpox for example, suggesting a reduced infectivity of mumps compared with the latter 2 viruses. The virus spreads by aerosol and initially infects the upper respiratory tract. The incubation period varies from 7–23 days, but most commonly is 14–18 days. In experimentally infected children, virus was excreted in saliva for 2–6 days, before clinical signs of parotitis appeared and for 4 days or so after its onset. Approximately half of susceptible persons may never develop clinical signs of mumps infection, although they will commonly secrete virus and so be a source of infection for others. Clinically the disease often begins with malaise, fever and pain near the angle of the jaw. A typical swelling of the face develops within 14–18 hours and often distorts the features considerably. Often both parotid glands are affected, but there may be a delay between the onset in both glands. The patient may have difficulty in opening the mouth and there is often dryness of the throat, presumably because of reduced saliva flow from blocked salivary ducts. The temperature is often high for 2–3 days and the swelling lasts for 3 days but then subsides quickly. The most notorious complication of mumps is orchitis, developing 4–5 days after onset of the parotitis, although it may develop in the absence of preceding parotitis. The first indication is pain in the testicles, rapidly followed by swelling and tenderness. The pain may be very acute. There is often an accompanying sharp general reaction with high temperature and head-

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Fig. 8.1. Electron micrographs of typical paramoxyviruses. a, b, SV5 × 130 500; c, Sendai × 195 000 (courtesy of Dr. D. Hockley.)

ache. Symptoms tend to subside after 3–6 days. It has been estimated that the incidence of orchitis in adult mumps varies from 11.6% to 66% but an average incidence of 1 in 5 is usually accepted by clinicians. Some degree of testicular atrophy is common and may occur in up to half the patients and a further, more serious, complication is sterility. It is estimated that some degree of infertility may be expected in 10% of adult patients suffering from mumps orchitis, although this may be temporary. Christie (1980) believes that the fear of sterility following mumps has been greatly exaggerated.

A more important complication of mumps in children is meningitis (Kilham, 1949, McLean et al., 1961, Russell and Donald, 1958, Donohue et al., 1955) and to a much lesser extent encephalitis. However, the prognosis in aseptic meningitis is extremely good, with few reports of long term sequelae. Mumps encephalitis, on the other hand, can be a severe and sometimes fatal illness (Table 8.3). The essential lesion is demyelination, which occurs in foci distributed throughout the brain or spinal cord. Mumps virus may be found in the cerebrospinal fluid but not the brain tissue itself. Quite often the first indication is a convulsion in a young child or sudden coma in an older child or adult during the convalescent period after mumps, with a sharp rise in temperature. A less dramatic onset may include drowsiness and a change in behaviour. The extent of damage to the CNS varies considerably and some patients may recover, while others may develop paralysis. Mortality is estimated to be around 20%. The overall mortality from mumps is about 1.8 deaths per 10 000 reported cases (Table 8.3).

TABLE 8.3.
Cases, deaths, and case-fatality rates for mumps and mumps encephalitis, United States, 1966 to 1975 (after Hayden et al., 1978)

Year	Mumps			Mumps encephalitis		
	Cases	Deaths	Deaths per 10 000 cases	Cases	Deaths	Deaths per 100 cases
1966	128 295	43	3.4	628	10	1.6
1967	185 691	37	2.0	849	8	0.9
1968	152 209	25	1.6	408	2	0.5
1969	90 918	22	2.4	218	5	2.3
1970	104 953	16	1.5	288	5	1.7
1971	121 924	15	1.2	310	5	1.6
1972	74 215	16	2.2	163	0	0
1973	69 612	12	1.7	214	3	1.4
1974	59 128	6	1.0	149	2	1.3
1975	59 647	8	1.3	166	4	2.4

8.1.3. Prevention of mumps by vaccines

Because of the relatively high incidence of subclinical mumps (Henle et al., 1948) and because patients with parotitis may be infectious even before clinical signs appear, methods of prevention based on isolation of clinical cases or quarantining of contacts are not effective. Therefore work (after the pioneering studies of Enders et al., 1946) commenced in the 1950s to develop both inactivated mumps vaccine and live attenuated vaccine (Buynak and Hilleman, 1966, Ennis and Hopps, 1969, Henle, 1951, Kliachko and Maslennikova, 1957, Stokes et al., 1967). Because of the uncertain nature of the antigenic composition of the virus and because of the poor experience with inactivated RSV and measles vaccines (see pages 361 and 375) early experimental inactivated vaccines (although used quite successfully to immunize young army recruits) were soon superceded by live attenuated vaccines. A further negative aspect of inactivated mumps vaccine was the poor duration of immunity. A live attenuated mumps vaccine was licensed in the USA in 1968 and had been developed from a strain of mumps (Jeryl Lynn, Mumpsvox) initially isolated in fertile hens eggs and which after several passages had been adapted to chick embryo cell cultures (earlier attenuated viruses had been passaged in eggs alone and proved either underattenuated causing mumps in recipients or overattenuated and not provoking antibody in recipients). Originally, mumps vaccine was used in a monovalent form but more recently, it has also been used in the USA in children particularly as a combined vaccine with live measles and rubella viruses (Weibel et al., 1973, 1978, Smorodintsev et al., 1970, Schwarz et al., 1975, Schell et al., 1975, Buynak et al., 1969). Clinical trials over the years have established that the protective efficacy ranged from 70–90% (MMWR Nov. 1982) and there was a good correlation with induction of virus neutralizing antibodies and clinical protection. The vaccine has proved to be safe in usage and no mumps-like syndrome could be attributed to the vaccine virus in controlled clinical trials in 500 or more patients including several hundred potentially susceptible adults. Similar vaccines can also be used in army recruits (Pentinnen et al., 1968). Since licensing of the vaccine in December 1967 in the USA more than 55 million vaccine doses have been used there. Other mumps vaccines have been developed since, particularly in Japan (Isomura et al., 1973, Hosai et al., 1970, Yamanishi et al., 1970).

Since 1968 and the introduction of the vaccine there has been a progressive decline in the number of reported cases of mumps in the USA. Thus, in 1976 a 42% decrease on the average annual total for the years 1971–75 was noted and the incidence of mumps in the USA has now reached an all time low. In 1981 there were only 4729 cases reported, showing a 97% decline from the 185 691 cases in 1967.

It is important that the immunity induced by mumps vaccine should last through adulthood and this feature of the vaccine has now been clearly established. In an early study of longevity of immunity (admittedly in a small number of patients) Weibel et al. (1978, 1980) reported no substantial decline of neutralizing antibody

titre for nearly 12 years. The initial level of neutralizing antibody reached after vaccination was less than that following natural infection but even serum antibody titres of 1:1 were associated with solid immunity to infection with the wild virus.

8.1.4. Development and early clinical trials with a live attenuated mumps vaccine in the USA

An early example of an attenuated mumps vaccine and one still used today (alone or in the form of a triple vaccine) was the Jeryl Lynn strain, named after a child from whom the virus was originally isolated. The virus was attenuated empirically by sequential passage in embryonated hens eggs and in chick embryo cell culture and the first vaccines were prepared using virus grown in the latter cells (Hilleman et al., 1967, 1968). In a large field trial, vaccine was given by subcutaneous injection in a study involving a total of 1337 children. The children were assigned to family or classroom groups or to a classroom-family group if one or more siblings in a family were also entered into a classroom included in the study. The children in classrooms were selected randomly to receive vaccine or to serve as unvaccinated 'sentinels' to enable a study to be made of any (unwanted) spread of vaccine virus. 482 children received vaccine and 855 acted as controls. The age range was from eleven months to eleven years. Any clinical reactions were recorded for 28 days after vaccination. Of the 402 children who were initially seronegative, 395 developed antibody after vaccination (98% seroconversion, Table 8.4). None of 407 contact controls acquired mumps. Surprisingly recipients of vaccine who already had mumps antibody often displayed an increase in antibody titre following vaccination. There were no significant clinical reactions following vaccination. The occurrence of natural mumps in the community during the two years following immunization allowed an assessment of the protective efficacy of the vaccine (Table 8.5). Among families, attack rates were 6.9% in vaccinees and 84.7% in controls (protective efficacy

TABLE 8.4.
Serological response rates in children who received the Jeryl-Lynn strain of live, attenuated mumps-virus vaccine (after Hilleman et al., 1968)

Group of children	Children vaccinated			Children not vaccinated		
	No. in whom antibody developed	Total	Percentage in whom antibody developed	No. in whom antibody developed	Total	Percentage in whom antibody developed
Classroom	224	225	99.6	0	189	0
Family	171	177	96.6	0	218	0
Total	395	402	98.2	0	407	0

TABLE 8.5.
Evaluation of protective efficacy of mumps-virus vaccine among initially susceptible children (after Hilleman et al., 1968)

Interval between vaccination and challenge	Study group	Vaccinated persons at risk			Unvaccinated controls at risk			Level of protective efficacy (%)
		No. of cases	No. at risk	Rate (%)	No. of cases	No. at risk	Rate (%)	
month								
0–10	Family	2	29	6.9	50	59	84.7	91.7
	Classroom	2	114	1.8	49	113	43.4	95.9
11–20	Family	1	14	7.1	22	24	91.7	92.3
	Classroom	1	28	3.6	24	40	60.0	94.1
Total period	All children	5	174	2.9	133	224	59.4	95.1

91.7%). Similarly, in the classroom the comparative rates were 1.8% and 43.4%, giving a vaccine efficacy of 95.9%. In this early study a few children were successfully immunized by jet gun and others with a combined measles–mumps formulation. Finally, the freeze-dried vaccine could be produced in batches of consistent quality, was very stable on storage at 4°C and was also stable after rehydration. A similar vaccine is now used very widely in the USA as a combined triple vaccine with live rubella and measles virus, and long term follow-up studies have indicated persistence of antibody over quite extended periods of time (Weibel et al., 1975, 1970).

8.1.5. A NEW LIVE ATTENUATED MUMPS VACCINE

A trial of a new mumps vaccine was reported recently by Ehrengut et al. (1983) and will be used to illustrate further general features of this type of vaccine (see also Isomura et al., 1973, Rossier et al., 1971, Smorodintsev et al., 1970).

The Urabe Am 9 strain was derived from a mumps virus isolated in primary human embryonic kidney cells from a child named Urabe with typical clinical signs and symptoms of mumps. The wild virus was attenuated by serial passages on several substrates by M. Takahashi, Osaka University, Japan. After isolation, it was passaged once more on human embryo kidney cells (HEK), then once in primary green monkey kidney cells six times in the amniotic membrane of specific-pathogen-free (SPF) hens' eggs, twice in SPF quail embryonic fibroblasts (both were cloning passages) and again four times in eggs. At this stage it was supplied to a vaccine manufacturer where it was passaged five times in the chorioallantoic membrane of SPF embryonated hens' eggs to produce the seed virus for vaccine production in chick embryo fibroblasts.

None of 197 subjects given the vaccine in two clinical trials had local reactions

TABLE 8.6.
The incidence of local and systemic reactions related to the dose of vaccine administered and the serological status of the vaccinees (after Ehrengut et al., 1983)

Study number	Vaccine dose ($TCID_{50}$/dose)	Number of subjects	Average age and range	Serological status		Number with reactions			
						Local		Systemic	
				Neg	Pos	Neg	Pos	Neg	Pos
MUM-018	$10^{4.7}$	34	5.0 (1–9 years)	15	19	0	0	0	0
	$10^{3.7}$	36	4.6 (8 months–7 years)	19	17	0	0	1	1
	$10^{2.9}$	32	5.4 (3–8 years)	15	17	0	0	1	1
MUM-021	$10^{3.7}$	95	5.3 (7 months–8 years)	50	45	0	0	4	7

although a low number developed systemic signs (Table 8.6). Sero conversion was noted in 89–100% of persons in four separate groups. Twenty-seven subjects retested for antibody nearly 3 years later still had EHI antibodies although levels had dropped somewhat. Thus, the Urabe Am 9 mumps vaccine strain in common with other strains used widely (e.g. Jeryl Lynn) is relatively well tolerated and immunogenic, although not completely free from side reactions.

8.1.6. General recommendations for mumps immunization (USA)

It is suggested that susceptible children, adolescents and adults should be vaccinated against mumps, unless vaccination is contraindicated. Mumps vaccine can be of particular value to children approaching puberty and for adolescents and adults, especially males who have not had mumps in childhood. Live mumps vaccine is recommended for all children at any age after 12 months, although it is not recommended for younger infants because of persisting maternal antibodies which would interfere with virus take. Contraindications include pregnancy, since although there is no good evidence of mumps causing congenital abnormalities in humans, nevertheless, the wild virus can infect the foetus and placenta. Mumps vaccine virus has also been isolated from the placenta, although not from the foetus. Since the virus is grown in chick embryo cell culture, persons allergic to egg protein should be vaccinated with extreme caution. Persons with immune deficiency diseases or suppressed immune responses that occur with leukaemia, lymphoma, generalized malignancy or during therapy with corticosteroids, alkylating drugs or radiation should not be vaccinated. This is because the replication of mumps vaccine virus may be potentiated in patients with immune deficiency diseases.

The principle strategy for removing the burden of mumps disease in the USA is through achieving or maintaining high immunization levels and CDC constantly

encourage private physicians and public health clinics to carry out routine vaccination as part of the health care programme. As an example of what can be achieved, the state of Massachusetts initiated a mumps control programme and offered vaccine to 5–14 year olds beginning in 1969 and 1–4 year olds additionally by 1971. A saving of over $300 000 is achieved when the cost of the vaccine programme is compared to the costs of avoided medical care. It seems very likely that the future of mumps vaccine will be directed by the emphasis put on rubella and measles vaccine and combined virus vaccines which, in the USA at least have proven to be efficacious, easy to apply and without serious side effects (Tables 8.7 and 8).

TABLE 8.7.
Occurrence of fever among triple-seronegative children who received combined measles–mumps–rubella vaccine (Moraten measles, Jeryl Lynn mumps, HPV-77 duck rubella) (after Hilleman et al., 1973)

Maximum oral temperature (°F)	Vaccinated children (228): days after vaccination		Unvaccinated children (106): days observed	
	5–12	13–18	5–12	13–18
<99	105 (47%)	140 (64%)	57 (59%)	64 (66%)
99–100.9	86 (39%)	69 (32%)	36 (37%)	25 (26%)
101–102.9	26 (12%)	7 (3%)	3 (3%)	8 (8%)
103–104.9	6 (3%)	2 (1%)	1 (1%)	—
Not taken	5	10	9	9

TABLE 8.8.
Rates of symptoms among vaccinees and unvaccinated controls following measles–mumps–rubella vaccination, University of Lowell, Massachusetts, 1980 (from CDC Measles Surveillance Report, 1982)

Symptom	Percentage of vaccinees	Percentage of controls	*P* value
	n=388	n=175	
Fever	12	8	N.S.
Rash	4	4	N.S.
Sore throat	27	22	N.S.
Cough	23	18	N.S.
Headache	30	20	<0.02
Photophobia	12	14	N.S.
Arthralgia	15	7	<0.01

N.S., not significant

8.2. Respiratory syncytial virus

8.2.1. Respiratory syncytial virus-structure and replication

Electron microscopy shows the virus to be 120 to 200 nm in diameter, with an envelope containing surface projections (Fig. 8.2) (Bloth et al., 1963). Attempts to identify and characterize the proteins of RSV have been hampered by several factors. Loss of infectivity during purification procedures and the fact that 80% of sucrose band-purified material is nonviral make the study of purified viral proteins difficult (Fernie and Gerin, 1980) and may account for some of the discrepancies among

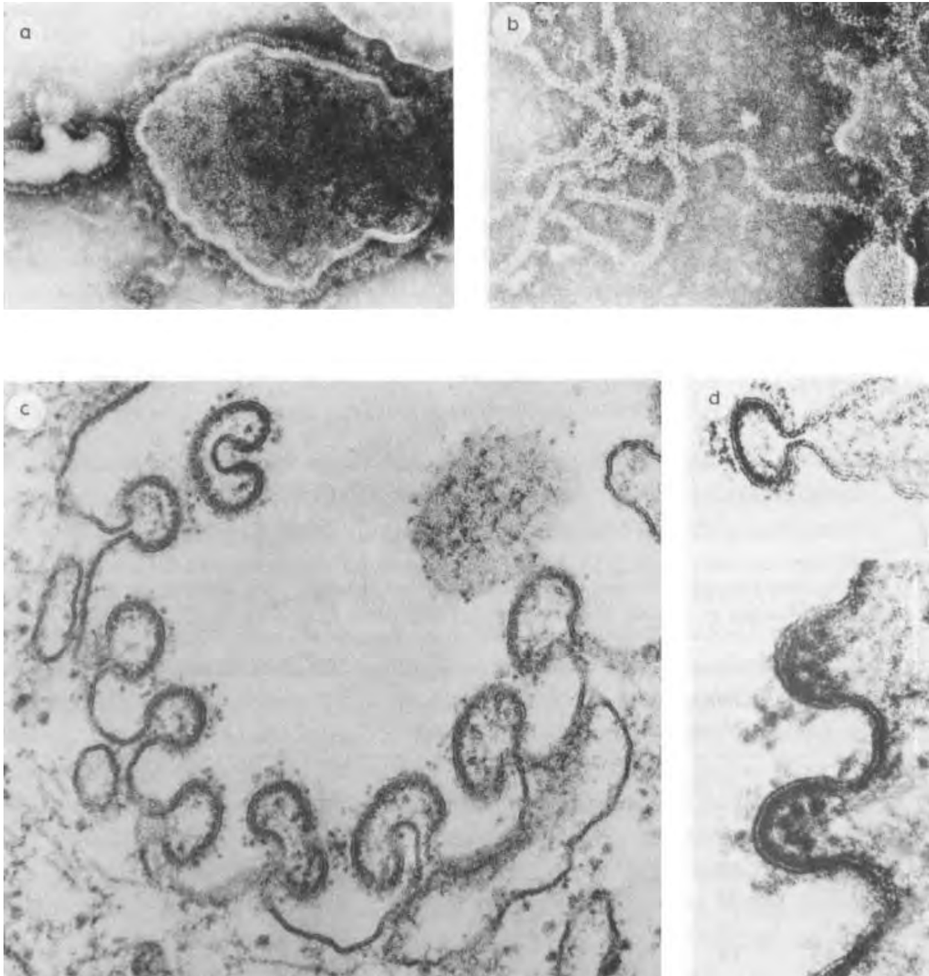


Fig. 8.2. Electron micrographs of respiratory syncytial virus. a, b: negative staining of virions; c, d: sectioned cells with budding RSV (courtesy of Dr. T. Bachi, University of Zurich.)

different laboratories. Between 7 and 10 presumed viral polypeptides ranging in molecular weight from 200 000 (200K) to 10K (Table 8.9 and Fig. 8.3) have been identified by polyacrylamide gel electrophoresis of radiolabeled RSV protein (Fernie and Gerin, 1982, Bernstein and Hruska, 1981). A 90K glycoprotein and a 48K glycoprotein have been identified and suggested as surface glycoproteins based upon their removal by trypsin and detergent treatment of purified RSV. Recently, the 48K glycoprotein has been shown to be connected to a 20K protein by disulfide bonds, forming a 70K protein. The functional designation of the 90K and 70K glycoproteins has not been made. A 41K protein is considered to be the nucleocapsid (N) protein, and a 27K protein is considered to be the matrix protein (Peebles and Levine, 1979).

Although related to paramyxoviruses, RSV does not possess similar biochemical markers such as a haemagglutinin or neuraminidase activity. Like the paramyxoviruses, however, RSV promotes cell-cell fusion, accounting for the characteristic giant cell syncytia formation that is seen when the virus is grown in permissive cell lines. The ability to form syncytia permits the spread of virus by fusion of infected cells to adjacent noninfected cells. It is presumed that a surface protein of the virion is responsible for the fusion process, as has been demonstrated with other paramyxoviruses. In an attempt to define the so-called fusion protein of RSV, Walsh and Hruska (1983) produced six monoclonal antibodies directed against respiratory syncytial virus proteins. Each was characterized by immunoprecipitation and indirect immunofluorescence. One was directed against the nucleocapsid protein, NP 44, two were directed against a 37K protein, two were directed against the 70K envelope protein, VP70 (Table 8.10). Indirect immunofluorescence stain patterns of infected HEp-2 cells defined GP 90 and VP 70 as viral proteins expressed on the cell surface, whereas NP 44 and the 37K protein were detected as intracytoplasmic inclusions. One of the anti-GP 90 antibodies neutralized virus only in the presence

TABLE 8.9.
A revised nomenclature for the polypeptides of RS virus (after Pringle et al., 1981)

New nomenclature	Previous designation		Evidence for virus specificity
	Detroit	Glasgow	
VP200	VP0	VP200	?
GP1	VP1	—	?
VGP48	VP2	VP48	Immunoprecipitation
VPN41	VP3	VP41	Immunoprecipitation
VPP32	VP4	VP32	Mutation
VPM27	VP5	VP27	Immunoprecipitation
VP25	VP6	VP25	Immunoprecipitation
VP10	—	VP10	—

See also Peeples and Levine, 1979; Veba, 1980; Cash et al., 1979; Belshe et al., 1977.

TABLE 8.10.
Monoclonal antibodies to RSV proteins (after Walsh and Hruska, 1983)

Immunoglobulin class	RSV protein specificity (mol wt)	Virus neutralization titre	
		Without complement	With complement
IgG2a	NP 44	<1:4	<1:4
IgG1	VP 37	<1:4	<1:4
IgG1	VP 37	<1:4	<1:4
IgG2b	VP 70	1:512	>1:2.048
IgG2a	GP 90	<1:4	1:2.048
IgG2a	GP 90	<1:4	<1:4

of complement, but did not inhibit cell-cell fusion. The anti-VP 70 antibody neutralized virus without complement and inhibited cell-cell fusion of previously infected HEp-2 cells, thus identifying VP 70 as the fusion protein.

Based on the molecular weight and surface location of GP 90 of RSV, Fernie and Gerin (1980) suggested that it may be analogous to the haemagglutinin of the paramyxoviruses which functions as the viral glycoprotein responsible for adsorption of virus to cells. Although GP 90 of RSV does not possess haemagglutinating or neuraminidase activity, it is reasonable to suspect that it also might function in cell attachment. The inability to neutralize RSV with two anti-GP 90 monoclonal antibodies does not negate this possibility (Walsh and Hruska, 1983). These monoclonal antibodies may be directed toward an epitope on GP 90 distinct from the one responsible for virus-host cell adsorption. That one of the anti-GP 90 antibodies was capable of neutralizing virus in the presence of complement suggests that neutralization is mediated by viral membrane lysis and that antibodies to GP 90 might be important in immunity to infection. Although able to neutralize RSV in the presence of complement, this anti-GP 90 antibody was unable to prevent the spread of infection by cell-cell fusion.

The fusion protein may be involved in the cell penetration step of some enveloped viruses by fusing the viral envelope to the host cell membrane, resulting in delivery of the ribonucleoprotein into the cell cytoplasm. Neutralization of RSV by anti-VP 70 antibody might be mediated by inhibition of virus-cell fusion. The anti-VP 70 monoclonal antibody described by Cote et al. (1981) was able to neutralize virus, but did not inhibit cell-cell fusion. This suggests that either the fusion protein facilitates cell adsorption or that neutralization of virus occurs by a mechanism other than inhibition of cell attachment. It is also possible that virus-cell fusion and cell-cell fusion may be mediated by different regions of the fusion protein.

Reinfection with RSV in the presence of circulating neutralizing antibodies is common in both infants and adults (Hall and Douglas, 1982). However, the protein

specificity of these neutralizing antibodies has not been fully explored. Because of the cell-cell spread of RSV, neutralizing antibody to GP 90 may not be as effective in preventing infection as neutralizing antibody that is directed to the fusion protein and can also limit cell-cell spread of virus. Of course, these studies have particular relevance as regards new RSV vaccines and particularly vaccines containing proteins from cloned genes.

The analysis of the RS virion RNA and the viral-specific messenger mRNAs synthesized in infected cells, has been initiated but is not complete. Seven genetic complementation groups exist, but there is no firm biochemical data describing the number of genes on the viral genome or the number and identification of the gene products specified. Huang and Wentz, 1982 have presented data which suggest that RS virus is a negative-strand RNA virus. One of the characteristics of a negative strand virus is that the virion contains an RNA dependent RNA polymerase that is capable of synthesizing messenger RNA in the presence of inhibitors of protein synthesis. Huang and Wertz (1982, 1983) have established polypeptide coding assignments for six RSV mRNAs by translation in vitro. RNA band 1 is complex, can be separated into at least two components, and codes for three polypeptides of 9.5K, 11K and 14K. RNA2 codes for the 32K polypeptide, RNA3 for the 27K polypeptide, RNA4 for the 41K polypeptide and RNA5 for a 59K polypeptide (which may be a post-translation modified protein).

8.2.2. Clinical aspects

This virus is the most important cause of severe respiratory infections in young infants under the age of 18 months (Chanock et al., 1961, Parrott et al., 1961, Glezen and Denny, 1973, Holzel et al., 1968, Gardner, 1973, Report to the MRC, 1978, Loda et al., 1968, McDonald et al., 1982, Kim et al., 1973) and may also infect (Johnson et al, 1961) and cause exacerbation of bronchitis in adults (Kravetz et al., 1961, Johnson et al., 1961). There has been a close correlation between recovery of RSV and cot deaths. The virus is ubiquitous in both temperate and tropical climates and may cause epidemics of bronchiolitis in infants for several weeks during the winter. Infants tend to shed virus for up to 3 weeks and so may be an important focus of infection even after leaving hospital. RSV can cause a mild upper respiratory tract infection or a severe attack of acute bronchiolitis. Often the attack commences like a common cold but within 24 hours the child may be acutely ill with dyspnoea, being distressed and cyanosed. In other infants the clinical picture may resemble pneumonia (Christie, 1980). The pathology is that of a necrotizing bronchiolitis in which partial blocking of the bronchioles leads to their collapse. Peribronchial infiltration may spread out into the lungs to give widespread interstitial pneumonia. Most adults have detectable levels of neutralizing antibody to RSV but the actual titre of antibody may have no direct relationship with ability to resist infection (reviewed by Jackson and Muldoon, 1975). Young children may have

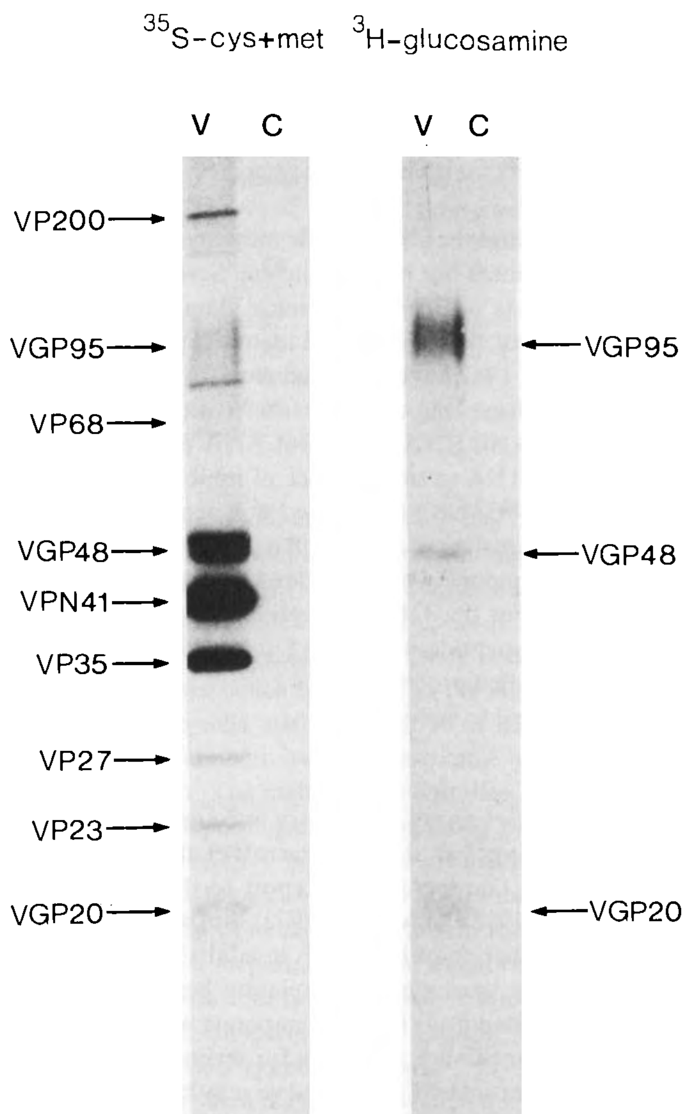


Fig. 8.3. Polypeptides of the Long strain of RSV. Radioimmunoprecipitation analysis and SDS-PAGE of RS virus polypeptides from extracts of infected HEp-2 cells using 1 μl of a human RS virus pneumonia hyperimmune serum. Left: cells labelled with (^{35}S) methionine plus (^{35}S) cysteine; right: cells labelled with (^{3}H) glucosamine. Lanes V, RS virus-infected cells; Lanes C, mock-infected control cells. Bands arrowed represent polypeptides which were consistently revealed during repeat analyses. (after Ward et al., 1983.)

several infections with RSV over a period of only a few years. Rather, resistance to infection appears to correlate more closely with levels of nasal IgA antibody. Repeated natural infections do result in a cumulative acquisition of resistance to illness (Tyeryar, 1983).

8.2.3. Failure of inactivated vaccines against RSV

Soon after the isolation of RSV and delineation of its clinical effects in young children (Chanock et al., 1961) it was realized, that protection against disease would be difficult to achieve by immunization, because moderate levels of serum neutralizing antibody from previous *natural* infection did not protect fully against RSV caused low respiratory tract illness (Kapikian et al., 1969). However, more recent data, summarized by Tyeryar, 1983, indicates that natural infection induces a state of partial immunity to re-infection detectable for at least one year. Also RSV-caused bronchiolitis and pneumonia occurred more often in the first four months of life when maternally transmitted antibody was at high levels. Nevertheless, following preliminary studies of a formalin inactivated RSV vaccine in adults, the vaccine was used to immunize children in a local authority home. The vaccine utilized the Bernett strain of RSV isolated originally in HEK (human embryo kidney) cells and passaged 3 times in HEK cells and 10 times in vervet MK cells. MK cell harvests were clarified by filtration, inactivated with formalin, and safety tested in animals and tissue cultures. The vaccinated and control groups were comparable in age, sex and race and there were no significant side effects following immunization. The vaccine was immunogenic and 96% of children who lacked CF antibody subsequently developed antibody after 1–3 injections. (In the 7 children tested, relatively high levels of neutralizing antibody also developed.) Approximately 9 months later a sharp outbreak of RSV occurred in the home. Virus was recovered from 41% of 146 residents and serological evidence of infection was detected in 92% of 40 seronegative residents. The vaccine not only failed to induce protection but induced an exaggerated and altered clinical response to RSV in the younger vaccinees. Nine of 13 vaccinated children (6–23 months of age) developed pneumonia compared to 4 of 47 non-vaccinated children (Table 8.11). Five of these 13 vaccinated children developed pneumonia serious enough to require hospitalization, whereas none of the 47 non-vaccinated children were hospitalized. This paradoxical effect of vaccination was not observed in Arthur Cottage which housed older children (24–65 months of age), but lack of protection against illness was observed. Kapikian et al. (1969) considered that the failure to produce protection in the older children may have been due to poor induction of locally produced IgA antibody as observed previously with parainfluenza type I virus infections, whereas the *enhanced* clinical effect noted in younger infected children may have resulted from interaction of serum antibody and RSV antigen in the lungs of children who lacked respiratory tract IgA antibody. The authors also concluded that administration of gamma globulin to infants should be approached with caution during periods of RSV prevalence.

Essentially identical results of a paradoxical response to RSV immunization were also reported by Kim et al. (1969). In response to three injections of alum precipitated, 100× concentrated, formalin inactivated RSV vaccine, 43% of infant vaccinees displayed a 4-fold or greater rise in serum neutralizing antibody and 91% displayed

TABLE 8.11.
Incidence of pneumonia and RS virus isolations according to age and vaccine status in residents of Harrison and Arthur Cottages (after Kapikian et al., 1969)

Age (months)	Vaccinated group			Nonvaccinated group		
	No. in group	Pneumonia no.	RS isolated no.	No. in group	Pneumonia no.	RS isolated no.
Harrison						
6–11	2	2 } (69%)[a]	1 } (69%)	7	1 } (9%)	4 } (49%)
12–23	11	7 }	8 }	40	3 }	19 }
24–35	2	0	0	10	0	6
36–58	0	0	0	22	0	3
Total	15	9 (60%)	9 (60%)	79	4 (5%)	32 (41%)
Arthur						
6–11	0	0	0	0	0	0
12–23	2	0	1 (50%)	1	0	0
24–35	9	4 (44%)	3 (33%)	20	6 (30%)	9 (45%)
36–65	11	0	5 (45%)	9	1 (11%)	1 (11%)
Total	22	4 (18%)	9 (41%)	30	7 (23%)	10 (33%)

[a] Percentage significantly different ($P<0.0001$).

a 4-fold or greater rise in serum CF antibody. When RSV became prevalent in the community, the rate of RSV infection in infants who received this vaccine was not different from that in control infants who received parainfluenza vaccines. However, a high proportion of RSV vaccinees required hospitalization at the time of RSV infection, whereas only 5% of such infections among parainfluenza vaccinees (control group) resulted in admission to the hospital (see also Table 8.12). Illnesses among the RSV vaccinees who underwent natural infection included pneumonia, bronchiolitis, and bronchitis, with pneumonia in a majority and rhinitis, pharyngitis and bronchitis in a few. Thus, infants who received this vaccine were not protected against natural infection and also, when they became naturally infected their illness was more severe than that seen in cohorts who received a similar parainfluenza type 1 vaccine. These findings, similarly to those of Kapikian et al. (1969), indicated that vaccine-induced RSV serum antibody alone did not protect against illness and suggested that serum antibody (particularly against certain of the viral proteins) without local respiratory antibody might actually play a part in the production of disease. The highest incidence of serious RSV illness occurring naturally was under six months of age, when maternally derived serum antibody was present. These findings together with those of Kapikian et al. (1969) suggest that RSV illness in infants is, at least in part, an immunologic phenomenon, wherein the virus and serum antibody interact to produce severe illness.

TABLE 8.12.
RS virus infection and illness in groups of infants after receiving 1 or more injections of inactivated respiratory syncytial and parainfluenza vaccines (after Kim et al., 1969)

Vaccine received	No. vaccinees	No. vaccinees with later RS infection as indicated by			No. vaccinees with designated illness at time of RS infection				No. requiring hospitalization at time of RS infection
		Virus recovery	CF antibody rise	Virus recovery and/or CF rise	P	B or B-P	BR-PH	URI	
RS	31	18	20	23	6	13	0	4	18
Para 1	20	7	12	12	2	0	0	10	1
Trivalent parainfluenza	20	7	9	9	0	2	2	5	0
Total parainfluenza	40	14	21	21	2	2	2	15	1

P, pneumonia; B, bronchiolitis; BR-PH, severe bronchitis-pharyngitis; URI, mild rhinitis, pharyngitis and/or bronchitis.

8.2.4. Experimental live attenuated RSV vaccines

Since anti-RSV serum IgG antibody of maternal origin, or antibody induced by inactivated RSV vaccine failed to produce protection against natural disease (Kapikian et al., 1969) efforts commenced to make a cold adapted (*ca*) or *ts* virus, which could be used as a candidate strain for a live vaccine (Gharpure et al., 1969, Belshe et al., 1978). Such a vaccine would presumably induce local IgA antibody in the respiratory tract and so more closely mimic natural infection with RSV. Initial experiments with a *ca* virus (Kim et al., 1971), adapted to 26°C failed to produce a completely attenuated virus because, for example, a child given the virus developed mild lower respiratory tract disease. The same group used the standard mutagenesis technique with 5-fluorouridine (Wright et al., 1970) to produce a *ts* mutant designated RS-A2 ts-1. The mutant, after propagation at 30°C in primary bovine embryo kidney cells did not infect cells at or above 37°C. In the in vivo hamster model the *ts* mutant only infected the upper respiratory tract and, moreover, there was no laboratory evidence of phenotypic reversion, which is the most important problem with *ts* viruses. Initial experiments in adults, who were infected intranasally, indicated that no disease was produced and the vaccinees resisted subsequent challenge with virulent wild type virus (Wright et al., 1971). The next step was to infect infants and children with virus using a coarse spray. The *ts*-1 mutant produced infection in 27 of 32 infants as shown by recovery of virus (Table 8.13) and increases in antibody titre (up to 48-fold) (Table 8.14). Children excreted vaccine virus for a mean of 6.1 days (Kim et al., 1973).

Another candidate live attenuated RSV vaccine strain, *ts*-2, has been found un-

TABLE 8.13.
Evidence of infection in infants and children who reveived the *ts*-1 mutant respiratory syncytial virus (RSV) (after Kim et al., 1973)

Previous RSV infection[a]	No. in group	No. with indicated evidence of infection			
		Virus recovery	Rise in neutralizing antibody		Virus and/or an immunological response
			Serum	Nasal secretion	
No	7	7	7	7	7
Yes	25	20	7	19	25
Total	32	27	14	26	32

[a] Based upon presence or absence of detectable plaque reduction antibody in serum.

TABLE 8.14.
Serum neutralizing antibody response to *ts*-mutant RSV (after Kim et al., 1973)

Previous RSV infection	No. in group	Virus shed		Time (weeks)	Mean serum antibody titre (reciprocal)	Fold antibody rise
		Yes or no	Number			
No	7	Yes	7	0	<20	
				2	117	5.8×
				3	407	26×
				7	683	46×
Yes	25	Yes	20	0	350	
				2	711	2×
				3	841	2.4×
				7	736	2.1×
		No	5	0	518	
				2	525	
				3	477	
				7	537	

suitable for further work because of *over*- attenuation (reviewed by Tyeryar, 1983). When tested in adult volunteers there was only slight evidence of infection and none of the 14 volunteers shed virus, and only 2 had an antibody response. Similarly, only two of seven seronegative children were infected.

In another approach, a live virus vaccine to be administered parenterally has been tested. The vaccine was prepared by passaging a clinical isolate of RSV in WI38 cells. In a randomized, double-blind, placebo-controlled trial encompassing 510 children, 233 received the vaccine and 277 received placebo. Children with low levels of pre-existing neutralizing antibodies did not respond to the vaccine, whereas

seronegative children did produce antibodies, but to titres lower than those observed in children after natural infection. RSV infection occurred in the various groups at similar rates. Of the 112 children for which pre-epidemic/post-epidemic serum pairs were available 48.2% (27 of 56) placebo recipients and 50% (28 of 56) of vaccine recipients had serological evidence of RSV infection. Further, 25% (25 of 100) placebo recipients and 17.3% (19 of 98) vaccinees developed RSV illness, as confirmed by viral isolations. Thus, parenterally administered live RSV vaccine did not protect from subsequent RSV infection or illness and this simply repeats the negative results of earlier trials with killed vaccine documented above. Among the 510 study children there were 23 illnesses requiring hospitalization for respiratory tract diseases during the study. Five of the illnesses were caused by RSV infection, as determined by isolation of virus, fourfold increases in antibody titre, or both. Three RSV illnesses were in the vaccine group, and two were in the placebo group. Fortunately, none of the illnesses was particularly severe, and none was life-threatening. No untoward clinical reactions were noted in most infants (Table 8.15), although in 7 infants a mild non-febrile rhinitis was noted. However, two worrying features of the *ts*-1 vaccine strain were spread to non-vaccinees and laboratory evidence of reversion to wild-type phenotype. Thus, of a total of 139 viruses recovered from vaccinees, 34 showed evidence of genetic alteration from the *ts* phenotype, and 8 viruses completely lost the *ts* property. Although these revertants represent less than 0.01% of virus produced in the respiratory tract at the time of peak virus replication, they nevertheless made the vaccine unsuitable for more widespread use. Experiments with alternative *ts* mutants are still in progress (Richardson et al., 1978).

8.2.5. Application of recombinant DNA technology for RSV

As alluded to above, the poor growth of RSV in tissue culture systems has hampered biochemical work with the virus, and its polypeptide and RNA composition have only recently been described (Huang and Wertz, 1983, Pringle et al., 1981). No satisfactory vaccines have been developed using the conventional biological methods (Hall, 1980) employed so successfully for many other viruses and therefore a remaining possibility is to apply recombinant DNA techniques to isolate gene products and also to analyze the structure and nature of expression of the RSV

TABLE 8.15.
Clinical response to infection with *ts*-1mutant RSV (after Kim et al., 1973)

Previous RSV infection	No. in group	No. infected	No. with indicated response		
			Rhinorrhea	Fever	Otitis media
No	7	7	7	1	1
Yes	25	25	6	0	0

genome. (Venkatesan et al., 1983). Complementary cDNA clones were constructed containing RSV N, P, M and NS gene sequences. (The authors assume that the gene order in RSV is similar to that of paramyxoviruses, namely 3′ NF_0.M P H N L 5′.) In this case Venkatesan et al. (1983) have clones containing sequences adjacent and 3′ to F_0 and the HN equivalent and so it should be possible to obtain cDNAs coding F_0 and HN equivalents in RSV. The cDNA clones could also be transferred to eukaryotic expression vectors. Both the fusion and major surface glycoprotein antigen would be required for any new inactivated vaccine.

8.2.6. Chemoprophylaxis of RSV

Dubovi et al. (1980, 1981) have described the inhibition of RSV induced cell fusion in cells by an aromatic diamidine, bis(5-amidino-2-benzimidazolyl)methane (BABIM) which is active in vitro at 1 μM. The effect was RS virus specific, and did not extend to parainfluenza type III or SV strains, for example. The compound appeared to retard virus penetration and reduced the yields of virus under multiple growth cycle conditions (Table 8.16). It is postulated that 'fusion from within' is inhibited and the compound is known to be a potent reversible inhibitor of trypsin.

8.2.6.1. Inhibition of RSV by ribavirin

The compound inhibited plaque formation by the Long strain of RSV by 50% at concentrations of 3.2 μg/ml in cells (Table 8.17). Similarly 'wild' strains of virus were inhibited in vitro, although they were rather less susceptible than laboratory viruses (Hruska et al., 1980). More than 100-fold reduction in released RSV was detected and the virus induced cytopathic effect was reduced by ribavirin. The compound, therefore, appeared to inhibit RSV to the same extent as influenza A and B viruses (see page 313).

In further experiments Hruska et al. (1982) demonstrated mild in vivo inhibition of RSV replicating in the nasal turbinates and lung tissues of infected cotton rats.

TABLE 8.16.
Effect of BABIM on multiple-cycle yields of RS virus[a] (after Dubovi et al., 1980)

m.o.i.	Yields ($TCID_{50}$/ml)	
	Control	50 μM BABIM
0.1	5×10^7	6.3×10^6
0.01	3.2×10^7	1.2×10^5
0.001	2.8×10^4	1.1×10^4

[a] RS virus at various m.o.i. was adsorbed to Hep-2 monolayers for 2 hr at 36°. Monolayers were rinsed three times and overlaid with MEM containing 2% FBS and 1% DMSO.

TABLE 8.17.
Inhibition of the Long strain of RSV (HEp-2) by ribavirin (after Hruska et al., 1980)

Ribavirin concn (μg/ml)	% Inhibition determined by following assay:				Avg % inhibition
	1	2	3	4	
0	0 (8.45)[a]	0 (1.85)[a]	0 (2.9)[a]	0 (4.2±0.19)[a]	0
1.0	41	32	19		30±11
3.2		62	66	74 (1.15±1.2)	70±20
10.0	99.5	92	93	98.6 (0.06±0.04)	97±3.4
32.0		N.P.D.	N.P.D.	N.P.D.	10
100	N.P.D.				
500	N.P.D.			(*n*=3)	

[a] Titer $\times 10^7$ measured by plaque assay on HEp-2 cells.
N.P.D., no plaques detected.

An antiviral effect was detected when ribavirin was administered by aerosol intranasally and also intraperitoneally (Table 8.18).

8.2.6.2. Ribavirin treatment of experimental RSV infection in adult volunteers

Hall et al. (1983) infected 16 young adults intranasally with RSV (5 $\log_{10}$ $TCID_{50}$) and treated them by ribavirin aerosol commencing on day 3, for 12 hours per day for 3 days (virus shedding and the first clinical signs normally commence on day 3). The aerosol was given during 3 periods in 24 h using a face mask. It was estimated that 12 h of treatment would provide a deposited dose of 660 mg ribavirin per patient.

The average onset of virus shedding of the placebo-treated group on day 4.1 was not significantly different from that of the ribavirin-treated group on day 3.5. The

TABLE 8.18.
Titres of RSV in tissues of cotton rats 4 days postinfection after continuous aerosol treatment with ribavirin (after Hruska et al., 1982)

Treatment	Titre at concentration of ribavirin in reservoir (mg/ml)							
	0 (*n*=6)		1 (*n*=6)		2 (*n*=5)		4 (*n*=10)	
	Lungs	Turbinate	Lungs	Turbinate	Lungs	Turbinate	Lungs	Turbinate
Controls[a]	5.0±0.9	5.4±0.7	5.1±0.7	4.9±0.4	5.3±0.5	5.75±0.3	4.4±0.6	4.35±0.6
Ribavirin	4.5±0.5	5.3±0.3	4.4±0.3	4.0±0.7	4.3±0.5	4.8 ±0.6	3.6±0.1	3.6 ±0.3
P	<0.2	<0.35	<0.1	<0.025	<0.01	<0.01	<0.025	<0.025

[a] Controls were not given aerosol treatment.

peak amount of virus shed on each day varied from 0.7 to 5.45 $\log_{10}$ $TCID_{50}$/ml in the placebo-treated group and from 0.95 to 4.95 $\log_{10}$ $TCID_{50}$/ml in the ribavirin-treated group. If the total amount of virus shed by each subject (i.e., the sum of the average shedding of each day) was compared for the two groups, the average amount of total shedding was greater in the placebo group (1.1×10^4 $TCID_{50}$), compared with the ribavirin-treated subjects (0.6×10^3 $TCID_{50}$). This difference, however, was not statistically significant.

During the first three days of shedding (days 3, 4 and 5 of the study), the proportion of subjects shedding the virus was not significantly different between the two groups. In the latter part of the study, days 6 through 9, however, after the 36 hours of ribavirin treatment, the proportion of infected subjects still shedding the virus was significantly less in the ribavirin-treated group ($\chi^2 = 3.99$, $P < 0.5$).

Twelve of the 13 infected subjects manifested signs or symptoms of upper respiratory tract illness, and one subject in the ribavirin-treated group remained asymptomatic. The frequency of occurrence of minor upper respiratory tract symptoms did not differ significantly between the two groups. The frequency, however, of the more major complaints of systemic symptoms, cough and fever, occurred significantly more often in the placebo-treated group (Table 8.19). Nine (13%) of 70 recorded oral temperatures in the placebo-treated subjects were greater than 37.5°C, compared with one (1.6%) of the recorded temperatures in the ribavirin-treated group ($P < 0.002$). Similarly, the average symptom score for minor symptoms did not significantly differ between the two groups, but the score obtained from systemic symptoms and fever was significantly greater in the placebo-treated group (Table 8.20).

It should be noted that the infection was mild and even so ribavirin only ameliorated systemic symptoms of cough and temperature. However, no toxic signs of the drug were noted suggesting that treatment could be extended.

8.2.6.3. Treatment of infants with RSV with ribavirin

Hall et al. (1983) have also described treatment of infants hospitalized with RSV

TABLE 8.19.
Average symptom score of RSV infected subjects treated with placebo versus subjects treated with ribavirin (after Hall et al., 1983)

	Average score		P^a
	Placebo-treated group (n=7)	Ribavirin-treated group (n=6)	
Minor symptoms	6.2	9.6	N.S.
Systemic symptoms	2.9	0.5	<0.01
Fever	4.4	0.8	<0.01

[a] Wilcoxon's rank test.

TABLE 8.20.
Mean severity score for RSV induced signs or symptoms at start and end of treatment with ribavirin (after Hall et al., 1983)

	Ribavirin group		Placebo group		P value[a] for change in score
	Start	End	Start	End	
Temperature (°C)	37.9	37.2	37.9	37.4	N.S.
Nasal congestion and discharge	1.8	0.6	2.2	1.0	N.S.
Cough	2.3	0.9	1.8	1.6	<0.01
Rales	2.2	0.5	1.6	1.4	<0.01
Wheezing	1.1	0.2	1.3	0.8	N.S.
Retractions	2.2	0.2	1.5	1.0	<0.01
Lethargy	2.3	0.2	2.0	1.2	<0.01

[a] *P* value for unit change in score from start to end of therapy for placebo group versus ribavirin group (Mann–Whitney U test and non-paired *t*-test). N.S. denotes not significant.

infection with an aerosol of ribavirin. The compound was administered continuously, except during a period of 1–3 hours each morning, for a minimum of 3 days giving (in adults) approximately 0.82 mg per kg drug per hour. Thirty-three infants were studied, all with RSV pneumonia. On admission to hospital the severity of illness was the same in placebo and treated groups, but after 1 day of therapy a difference in clinical improvement between the 2 groups in favour of ribavirin was detected. The change in degree of improvement in lower respiratory tract signs, except for wheezing, was significantly greater in the drug treated group, whereas the change in temperature and upper respiratory tract signs was not. By the end of treatment the quantity of virus in nasal washes from the ribavirin group was significantly less (Table 8.21). Moreover, the average number of days of RSV shedding

TABLE 8.21.
Titres of respiratory syncytial virus in nasal-wash isolates (after Hall et al., 1983)

Group (no.)	Titre ($\log_{10}$ $TCID_{50}$/ml)		
	Before treatment	At day 3	At end of treatment
Ribavirin (12)	7		
Mean	2.1[a]	1.2[a]	0.3[b]
Range	0.4–5.7	0–4.2	0–1.7
Placebo (13)			
Mean	3.0	2.1	1.3
Range	0.4–5.9	0–4.2	0–4.2

[a] Not significantly different from value for placebo group.
[b] Significantly different from value for placebo group.

from the start of therapy was 4.3 days in the placebo group and 2.9 days in the ribavirin group. Finally, no toxic side effects of aerosol therapy were detected in these infants.

Since ribavirin therapy is administered by aerosol over long periods it is appropriate at this time only for hospitalized infants. The number of infants requiring hospitalization for RSV infection is nevertheless appreciable. In long-term studies in Washington, D.C., infection with this virus accounted for 23% of all hospital cases of acute respiratory-tract disease, and one of every 100 primary RSV infections resulted in a hospital admission (Parrott et al., 1974). In the UK, the rate of hospitalization for RSV infections is as high as 1 in every 50 infants in the first year of life. Ribavirin therapy may be particularly beneficial for children at risk for severe and often fatal RSV infection, such as infants with congenital heart disease (MacDonald et al., 1982).

8.3. Measles

8.3.1. Measles (morbillus, a diminutive of morbus – a small or childhood disease)

Measles is an Anglo-Saxon word (Middle English: 'maseles') and true to form the disease is still regarded here, as in those earlier times, as an almost normal incident of Anglo-Saxon childhood! This is certainly not so in other countries and, for example, the US may soon rid itself of indigenous measles and may therefore become an island to itself. Other countries with vigorous immunization policies are the USSR, Sweden and the DDR. Measles has also been implicated in at least one neurological disease, resulting from persistent viral infection, namely subacute sclerosing panencephalitis (SSPE) (Hall and Ter Meulen, 1976, Modlin et al., 1977), and also a fatal encephalopathy during immunosuppression (Olding-Stenkvist et al., 1982).

8.3.2. Structure of measles virus and mode of replication

A M.W. of 4.5×10^6 for measles virus genomic RNA has been obtained, which is intermediate between values for other negative strand viruses of 3.8×10^6 for VSV and 5×10^6 for RSV. This is in agreement with the estimated number of virus coded proteins, namely 5 for VSV, 6 for measles and 7 for RSV.

Most studies on virus proteins have been carried out in the last 5 years and as with other paramyxoviruses formidable difficulties have had to be overcome in virus purification. In appearance the virus is a typical paramyxovirus (Fig. 8.4). The virions appear to have six proteins, similar to those of typical paramyxoviruses, namely L, H, P, N, F and M. The precise role of many of these proteins is still

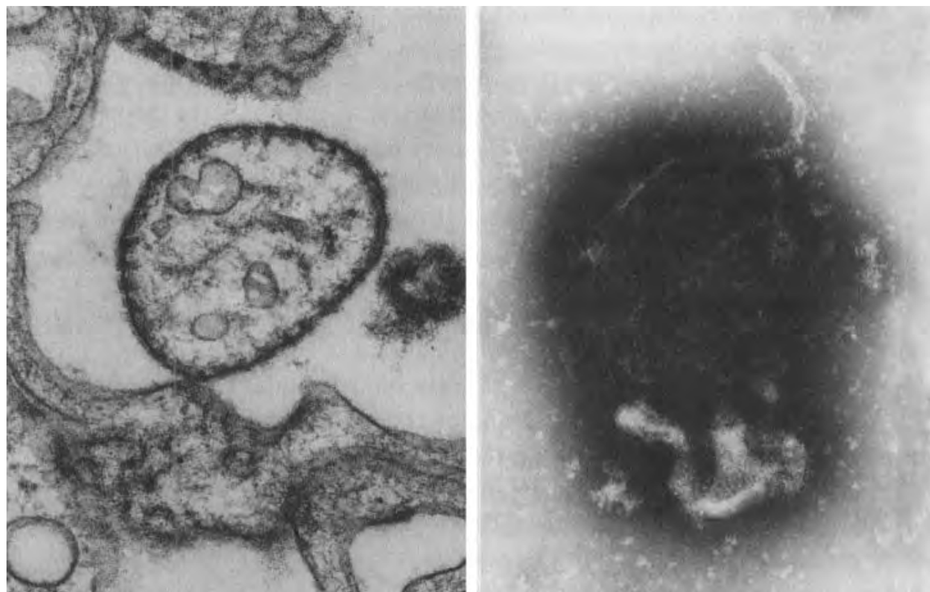


Fig. 8.4. Electron micrographs of measles virus and budding particles. Left, measles-virus budding from surface of infected cell — thin section. × 75 000. Right, measles-disrupted virus showing nucleocapsid and spikes on viral membrane — negative staining. × 130 500. (courtesy of Dr. D. Hockley.)

speculative and even the question as to whether the H and F proteins are present in a single complex spike on the virus is unknown.

Nucleocapsid (N) protein (60K) is directly associated with the RNA genome and is a major protein of all paramyxoviruses in both the virion and the virus infected cell. The electrophoretic mobility of N varies among measles virus strains (Rima, 1983) and some changes could be caused by phosphorylation of the protein. The N protein is a major cross-reacting protein of the morbilliviruses, although monoclonal antibodies may be used to distinguish different members of the group (Birrer et al., 1981a, b).

P protein (formerly called P2) is the second largest structural protein and is designated P because of association with the RNA polymerases. It has a molecular weight of 70K and is phosphorylated (Hall et al., 1980) which may contribute to the observed electrophoretic variations in measles virus strains. The P protein is very susceptible to proteolysis and, for example, in measles virus infected cells a 37K protein derived from P has been described (Rima et al., 1981).

The L protein is the largest protein, is not glycosylated and has a molecular weight between 100 and 200K. It is present in purified virus (Vainionpaa, 1979) and infected cells. The L protein does not appear to be phosphorylated and has been identified as virus specific using monoclonal antibodies (Giraudon and Wild, 1981). The L protein is a very minor component of the virion.

The HA protein (H or G protein) is the major glycoprotein and can be detected in the virion, with a molecular weight of 79–80K. In infected cells and in the virion the H protein is present as 150K–160K dimers. Variation in electrophoretic mobility of H proteins has been observed among strains of measles virus. Monospecific antisera to the H protein of measles virus have neutralizing and HI activity, while some monoclonal antibodies have haemolysis-inhibiting antibody. Antigenic variation among H proteins of measles viruses is also detectable with monoclonal antibodies (Birrer et al., 1981b).

The fusion glycoprotein (F_0) is converted to the biologically active form (F) by proteolysis, and the resulting polypeptides are held together by disulphide bonds. In the morbilliviruses F1 (40K) is not glycosylated. The F2 portion of the F protein is glycosylated and is detected as a 20–25K glycopolypeptide. The proteolytic activation of the F_0 protein appears to depend on the cell line in which the virus replicates. The membrane or matrix protein (M) is the smallest but most abundant protein of the virus with a molecular weight of 36–37K (Mountcastle and Choppin, 1977). The M protein is detectable in measles virus infected cells and under certain salt conditions is associated with the purified nucleocapsid complex. The protein is not glycosylated and can be purified by extraction in 1 M KCl. The M proteins of measles and SSPE viruses can be distinguished by fingerprinting techniques (Hall et al., 1980). The M protein is thought to have a role in virus assembly and budding and may have a function in virus persistence since both the antigenic composition and electrophoretic characteristics of M differs in persistent virus compared to acute infection virus (Ter Meulen and Carter, 1982). Sera from SSPE patients have reduced antibody frequency and titres to the measles virus M protein (Hall et al., 1979b, Machamer et al., 1980) and a number of SSPE strains have been described which produce reduced amounts of M (or no M) in infected cells (Machamer et al., 1981) but other SSPE strains also produce reduced amounts of H or F proteins. It is possible that certain SSPE virus proteins may be rapidly degraded or, alternatively, there may be defects in the transcription or translation of the various RNAs.

Precise definition of the non-structural proteins must await further study but 'candidate' NS proteins are detectable in measles infected cells with molecular weights of 65K, 38K and 18K (reviewed by Rima, 1983).

8.3.3. Clinical disease and epidemiology

Measles virus enters the body and infects via droplet spread, and perhaps also via the conjunctiva. During the incubation period of 8–11 days, virus spreads to the nearest lymph nodes, replicates and is soon detected in the bloodstream, spleen, liver, bone marrow and other organs (Fenner, 1948). Certain symptoms, such as the virus induced lesions or rash (Fig. 8.5) are probably due to inflammatory reactions and to the release of abnormal cell products by virus infected cells. Giant cells containing up to 100 nuclei have been described in the pharynx and tonsils,

skin, respiratory epithelium, lymph nodes and Peyers patches and the brain. The virus is widespread in the skin but disappears quickly. By the time the skin has reacted and the rash is apparent virus content in the internal organs has dropped, and within 1 to 2 days little virus is excreted. The most infectious stage of the disease is for several days before the rash.

There is little doubt that measles is one of the most ubiquitous of human viruses, with a world wide distribution, and can cause disease in any climate or environment (Assaad, 1983, Hethcote, 1983). In countries with a high standard of living, epidemics occur in cycles every 2–3 years, attacking predominantly 3–5 year-olds in a relatively mild way (Table 8.22). In third world countries, on the other hand, measles has a high incidence in the under 2 year olds and is a severe disease with unusual clinical features such as blindness and a high fatality rate (Table 8.23, Morley, 1969, Smith and Foster, 1970, Wakeham, 1978). The state of nutrition may affect the virus epidemiology and malnutrition may reduce the immune response (Chen et al., 1980, Dossetor and Whittle, 1975). The measles then accentuates the effects of malnutrition and diarrhoea, for example, may persist after measles, leading further to protein loss. Death rates of 42%, 17% and 7% have been recorded in Afghanistan, for example, in children aged 0–4, 5–9 and 10–14 years respectively. Certain of the clinical features of measles in third world countries today resemble descriptions of measles in Europe 100 years ago (Christie, 1980, Heymann et al., 1983).

Complications of measles in children in Europe are croup (4%), otitis media, eye complications and central nervous system changes (0.4%). Croup is often a complication of the prodromal phase and nearly every child with a sharp attack of measles has symptoms of bronchitis and X-ray signs of pneumonitis because of virus inva-

TABLE 8.22.
Peak age of incidence for measles

Country	Age (years)
Nigeria (urban)	1–2
Kenya	1–2
Ghana, Zambia, Rhodesia	2–3
Cameroon (urban)	<1
Indonesia	1–4
India	2–4
India (rural)	1–2
Guatemala	1–2
United Kingdom	5–9
United States	
(prevaccination)	4–5
(postvaccination)	10–14
Denmark	6

TABLE 8.23.
Case fatality rates for measles from community studies around the world

Country	Year	Case fatality rate (%)
India (Punjab)	1959	1–2
Chile	1960	4
The Gambia	1961	14
Guatemala	1963	4.5
Nigeria	1963	7
Nigeria (war areas)	1968	15
India (Madras)	1969	1.5
India (Aurangabad)	1972	2.2
Kenya	1976	6.5
Bangladesh	1976	3.7
Ghana	1978	3
Indonesia	1978	25.5
United States (prevaccination)	1961	<0.02
United States (vaccine era)	1975	0.01
United States (epidemic on Indian reservation)	1974	4
United Kingdom	1963	0.2

sion of the respiratory tract (Miller, 1964, Christie, 1980). In underdeveloped countries bacterial pneumonia is a common and often fatal complication of measles including pneumococci, haemolytic streptococci and *H. influenza*. Virus pneumonia caused by measles itself has been reported in children in North Africa and the fatality rate may approach 50%.

Otitis media used to be a common complication of measles but nowadays is much reduced, possibly because of a slow change in the disease itself rather than the use of antibiotics. Convulsions are commonest when the rash is appearing and often are classical febrile convulsion. The incidence of measles encephalitis approximates to 1 in 1000 cases but drowsiness and irritability are very common in measles and so the actual incidence may be much higher. The mortality in patients with coma is around 30–40%. Sub-acute sclerosing panencephalitis (SSPE) occurs at a rate of approximately 1 per 100 000 measles cases.

8.3.4. Prevention by vaccination

Since patients are most infective in the prodromal period and to isolate them when the rash is out serves little purpose, we must orientate particularly towards vaccines or specific antivirals to prevent measles as an epidemic disease. Immunoglobulin given intramuscularly (250 mg to infants, 500 mg to over 1 year olds) to contacts within 6 days or so of exposure to measles either prevents or modifies the disease. Immunoglobulin prophylaxis is considered to be a valuable measure in persons at

special risk and who have not been vaccinated but, of course , could not be considered for use at present on a wider scale.

8.3.5. Early studies with inactivated and under-attenuated live measles vaccines: evidence of side-reactions

Since some of the first produced live attenuated measles vaccines (e.g. 'Enders-Edmonston', isolated from a boy of that name and passaged 24 times in kidney cells, 28 times in primary amnion cells and 6 times in chick embryos) produced unacceptable side reactions in children (Enders et al., 1960), it was initially recommended that either the vaccine be given with gamma globulin or that killed measles vaccine be administered first, followed after several months by a booster of live attenuated virus (MRC report, 1965). This practice is now discontinued because of the development of more suitable attenuated viruses (e.g. Schwartz strain, produced by 77 additional passages of Edmonston virus in chick embryo fibroblast cells at 32°C (Krugman, 1983)) and also because of untoward side reactions noted in some children given the above combination. This again emphasizes the caution with which vaccination trials have to proceed, particularly with viruses where the virus structure is unknown.

In this regard, McNair-Scott and Bonanno (1967) described febrile (104°–105°F) illness in 16 patients who were given the inactivated measles vaccine course followed by a challenge with live measles virus. Redness and swelling at the site of injection of live virus vaccine was noted and blisters developed in 4 and cysts in 3 patients (Table 8.24). The clinical appearance was that of an Arthus type immunological reaction in a sensitised person. Moreover, Norrby (1966) described the occurrence of severe pneumonia after infection with natural measles virus in 5 of 125 children previously immunized with killed measles vaccine, Rauh and Schmidt (1965) described 54 patients infected with natural measles two years after immunization with killed vaccine of whom 9 showed an atypical infection characterized by urticarial,

TABLE 8.24.
Reactions at inoculation site of live measles virus after 2 doses of killed measles virus vaccine more than three months previously (after McNair Scott and Bonanno, 1967)

Type of reaction	No. of children	Stated day of onset	No. of children in whom day of onset was known
Fever	16	1st	2
Local swelling and erythema	13	3rd	1
Local swelling and blisters	4	4th	3
Local swelling and cysts	3	5th	1
Generalized rash	2	6th	3

Average stated duration, before therapy, 3–7 days in 5 patients.

vesicular and petechial rashes, swollen hands and feet, pneumonia (4 cases) and erythema multiforme.

In a further report Fulginiti et al. (1967) described atypical measles illness in 10 children who had received inactivated measles vaccine 5–6 years earlier (Table 8.25). The illness was characterized by a 2–3 day prodrome of fever and pain, followed by a maculo-papular rash with petechial vesicular and urticarial components. Peripheral oedema and pneumonia occurred in almost all the children. All the children were hospitalized and 9 were judged as seriously ill (in fact Rocky Mountain spotted fever was misdiagnosed in one case). The authors concluded that these cases represented only a fraction of the total in the community and recommended that the inactivated measles vaccine should be discontinued.

Nowadays only live 'further' attenuated measles vaccines (MRC report, 1977, Miller, 1982, Krugman, 1983) are used and these are highly effective vaccines (Table 8.26). The vaccine is given currently to children no earlier than 12 months (because of maternal antibodies) and preferably at 15 months of age. Vaccine is not administered to children with acute leukaemia or under immunosuppression (Mitus et al., 1962, Aicardi et al., 1977).

In a detailed follow up study of 5000 children given a single dose of live attenuated measles vaccine (Schwartz strain) when 10 months to 2 years of age, a high level of protection was noted (MRC report, 1977, Table 8.26). Moreover this immunity lasted over a 10 year period (Table 8.27). Throughout the trial when measles occurred in vaccinated children more of the cases were mild than in unvaccinated

TABLE 8.25.
Clinical findings in patients with atypical measles who had been immunized previously with inactivated measles vaccine (after Fulginiti et al., 1967)

Case no.	Age (years)	Sex	Temperature		Rash[b]	Oedema	Pneumonia[c]
			Maximum °F °C	Duration (days)			
1	6	F	105 (40.6)	7	M, V, P	+	RML
2	6	M	103 (39.4)	7	M, P	+	?
3[a]	7	F	104 (40.0)	7	M, V, P	+	B+E
4	7	F	105 (40.6)	7	M, P	+	RLL+E
5	6	M	104 (40.0)	5	M, P	0	B
6[a]	6	F	104 (40.0)	5	M, V, P	+	RML
7	8	F	105 (40.6)	4	M	0	RML
8	6	M	105 (40.6)	6	M, P	0	B+E
9	7	F	103 (39.4)	4	M, V	+	RLL
10	8	F	105 (40.6)	5	M	+	RLL

[a] Siblings.
[b] M signifies maculopapular; V, vesicular; P, petechial.
[c] RML signifies middle lobe of the right; RLL, lower lobe of the right; B, bilateral; E, effusion.

TABLE 8.26.
Confirmed cases of measles in four periods according to vaccine group (after MRC report, 1977)

Period	Group	No. of cases	Doctor's assessment		
			Mild No. (%)	Moderate No. (%)	Severe No. (%)
1964/65 (9 months)	Killed/live vaccine	174	133 (76)	37 (21)	4 (2)
	Live vaccine	183	137 (75)	42 (23)	4 (2)
	Unvaccinated	1993	938 (47)	969 (49)	86 (4)
October 1965– September 1969	Killed/live vaccine	327	245 (75)	77 (24)	5 (2)
	Live vaccine	169	126 (75)	39 (23)	4 (2)
	Unvaccinated	1501	631 (42)	781 (52)	89 (6)
October 1969– September 1972	Killed/live vaccine	119	85 (71)	32 (27)	2 (2)
	Live vaccine	42	28 (67)	14 (33)	0 (0)
	Unvaccinated	203	74 (36)	124 (61)	5 (2)
October 1972– September 1976	Killed/live vaccine	38	20 (51)	17 (45)	1 (3)
	Live vaccine	32	18 (56)	14 (44)	0 (0)
	Unvaccinated	47	16 (34)	30 (64)	1 (2)

children (Table 8.26). For example, no severe cases of atypical measles as noted in the USA after multiple doses of inactivated measles vaccine (Fulginiti et al., 1967) were noted. The persistence of clinical protection was also confirmed by the serological investigations which showed that only one child in the sample investigated (Table 8.27) had no detectable antibody after 10 years.

Common reactions associated with the 'more attenuated' measles vaccine strains used now (Norrby, 1978) are fever and rash occurring in 5–30% of recipients, generally during the second week after immunization. Febrile convulsions are rare. In a recently published study in the UK involving 10035 children vaccinated with the Schwartz vaccine virus, 20% had mild symptoms, 8% moderate symptoms and 1% severe symptoms in the two weeks after vaccination. In all, 5% of children were seen by the doctor for possible serious side effects and 12 (0.1%) were admitted to hospi-

TABLE 8.27.
H.I. antibody levels after 10 years according to vaccine group (after MRC report, 1977)

Vaccine group	No. of children tested	No. and percentage according to H.I. titre[a]							
		<4 No. (%)	4 No. (%)	8 No. (%)	16 No. (%)	32 No. (%)	64 No. (%)	128 No. (%)	256 and over No. (%)
Killed/live vaccine	47	2 (4)	1 (2)	1 (2)	3 (6)	19 (40)	9 (19)	9 (19)	3 (6)
Live vaccine	71	1 (1)	1 (1)	2 (3)	16 (23)	17 (24)	19 (27)	12 (17)	3 (4)
Unvaccinated	37	12 (32)	— (0)	— (0)	3 (8)	5 (14)	7 (19)	7 (19)	3 (8)

[a] A titre of 16 is equivalent to 1 international unit.

tal, nine with convulsions. All of the latter group were febrile. The incidence of febrile convulsions during this two week period was three times that expected for children aged 10–18 months. We should remember that convulsions due to natural measles at this age are 7–10 times more frequent than noted above during vaccination (Miller, 1982). Furthermore, intensive surveillance following the use of 90×10^6 doses of live measles vaccine in the USA has established an encephalitis incidence as low as 1 per million doses of vaccine, as compared to an incidence of 1 per 1000 due to natural measles in a comparable situation.

The Centre for Disease Control in the USA has established a system to monitor adverse effects of measles vaccines and all illnesses occurring within 4 weeks of vaccination in the immunization projects have to be reported (Table 8.28). Unfortunately reports from the private sector in the USA are much less reliable and hence a complete assessment of risk is not possible. However, during 1979–80 there were 19 reported cases of neurological events (excluding cases of febrile seizures) within 30 days of immunization with live measles virus, and one patient died. Three of the cases had a diagnosis of encephalitis or encephalopathy including Reye syndrome and the persons were less than 10 years of age (approximating to 0.64 cases per 10^6 doses of vaccine). Of course it is possible that some of the cases were only

TABLE 8.28.
Serious adverse events within 30 days after receipt of measles-containing vaccines (after CDC Measles Surveillance Report, 1982)

Types of adverse event	Number of adverse events by vaccine							
	MMR[a]		MR[b]		Measles		Total	
	'79	'80	'79	'80	'79	'80	'79	'80
Allergic reactions	12	11	1	1	2	4	15	16
Anaphylaxis	2	1	0	0	0	0	2	1
Arthritis and/or arthralgia	7	8	2	2	0	3	9	13
Convulsions–febrile	22	30	2	0	1	0	25	30
Convulsions–non-febrile	3	1	2	0	1	1	6	2
Encephalitis and/or encephalopathy	1	1	1	0	1	0	3	1
Reye syndrome	0	0	1	0	1	0	2	0
Guillain-Barré syndrome (GBS)	2	1	1	0	0	0	3	1
Paralysis–non-GBS	0	1	0	0	1	0	1	1
Sudden infant death syndrome	0	0	1	0	0	0	1	0
Deaths from all causes	1	2	1	0	2	0	4	2

[a] Measles-mumps-rubella vaccine.
[b] Measles-rubella vaccine

coincidentally related to measles vaccination and may have been caused by other agents.

8.3.6. Global eradication of measles

From a technical point of view both measles and polio could follow smallpox and be eradicated world-wide by mass immunization campaigns. We have noted above the serious morbidity and also mortality caused by measles. Indeed, estimates of 900 000 deaths annually in underdeveloped countries caused by measles are accepted as reasonably accurate. Hinman (1982) has compared and contrasted measles and smallpox as regards several factors which are relevant to worldwide eradication (Table 8.29). Note that measles shares six of the favourable characteristics with smallpox. Three important differing factors are that smallpox was of lower infectivity than measles and so did not spread so rapidly through susceptible populations. therefore smallpox was sometimes eradicated from areas where overall immunization rates were low. In fact, a containment policy was used whereby once a smallpox case was discovered all contacts in the immediate area were vaccinated (see Chapter 15). In contrast, we know that measles may still be transmitted in communities with immunization levels of > 90%, but on the other hand, it is not clear if measles can *persist* indefinitely in these conditions of strong immunity pressure. A crucial remaining factor which could strongly influence such a campaign is the amount of international support for a global immunization campaign against measles. The answer here may be ambiguous and is not helped by countries where immunization rates are low (Table 8.30).

A satisfactory result in the USA, USSR or DDR however, would most likely stimulate international interest and already, another country, Canada, is considering a national programme of measles eradication. It is very worthwhile then to analyze the experience in one of these countries (USA) which hopefully will be repeated

TABLE 8.29.
Factors affecting eradication of smallpox and measles

Factor	Smallpox	Measles
Animal reservoir	No	No
Long-term carrier state	No	No
Obvious illness	Yes	Yes
Immunity from disease	Lifelong	Lifelong
Immunity from vaccine	Long-term	Long-term
Effectiveness of vaccine	High	High
Stability of vaccine	Stable	Labile
Evidence of immunity	Visible	Not visible
Infectivity	Moderate	High
Universal vaccination	Not essential	Probably essential

TABLE 8.30.
Current status of measles vaccination campaigns

Countries with high rate of measles vaccinations	Countries with low or very low rates of measles vaccination
USA (99% reduction in cases)	UK
Canada	West Germany, Scandinavia
Mexico	France
Costa Rica	Belgium
Czechoslovakia	Holland
Albania	Italy
Yugoslavia	Greece
USSR	Central and South American and African countries other than those detailed opposite
Japan	
China (in certain target provinces)	
Cuba	
Brazil (certain areas)	

Note that most vaccines are prepared in chick embryo cell cultures except for the Yugoslavian vaccine which is prepared in human diploid cells, the Iranian AIK-C strain prepared in MRC-5 human diploid cells and the USSR Leningrad-16 vaccine prepared in quail embryo cells. Many virus strains are used, although most of these were derived from the Edmonston B strain of virus. The virus is most often adminstered i.m. but earlier experiments in the USSR using intranasal administration have been reevaluated by Sabin et al. (1983) with good (preliminary) results. (see also Leon de Coto, 1983, Rissi, 1983, Assaad, 1983, Rey et al., 1983.)

in other countries. We shall also briefly review current progress of vaccination campaigns in the USSR and Sweden.

8.3.7. Eradication of measles campaign in the USA

More than 90% immunization has been achieved in DDR, Alaska and USA, but since more recent data is available from the latter country it will be used as an example.

In 1978 a goal was established to eliminate indigenous measles from the USA by October 1st 1982 and the major elements in the strategy were high immunization levels, effective surveillance and aggressive outbreak controls. Obviously this precise goal has not yet been achieved but it may very well be reached this year. Since live measles vaccine was licensed in 1963, approximately 130 million doses have been used in the USA, including 19 million doses of Edmonston B vaccine and 111 million doses of 'more attenuated' virus strains. Since the early seventies, combined measles, mumps and rubella vaccines have been used and in 1980, for example, 75% of measles vaccine used was in the combined formulation. (Similarly, in the USSR between 1967 and 1972, 30 million doses of measles vaccine were used). The general setting or background of this measles eradication campaign in the USA involved the 'Childhood Immunization Initiative' which had two goals:

1. ensuring that at least 90% of children in the USA received vaccine against preventable diseases of childhood such as measles, rubella, mumps, polio, diphtheria, tetanus and pertussis, since even in 1976 it was estimated that as many as 20 million children under 15 years lacked protection against some of these agents in the USA.
2. developing a permanent system to maintain this level of protection. A key factor in this campaign was identifying unvaccinated school children, and the medical records of 28 million school children were reviewed. To ensure that a high percentage of the population would continue to be protected, immunization requirements were enforced strictly as a condition for entering or attending school. By July 1979 all 50 states in the USA had laws requiring that children should have *proof* of immunity before entering school for the first time. In addition, 30 states required such proof for students at all levels from kindergarten to high school. Vigorous enforcement of school immunization laws seem to be the single most important factor in the immunization campaign, and students who do not provide documentary evidence of immunity to measles are excluded from school. In fact, experience has shown that in the successive school years 1978 – 1982, 93%, 94%, 96% and 96% of children respectively had been vaccinated against measles.

A constant pressure has had to be exerted to maintain the immunization programme as was shown in 1970–1971 when, because of the introduction of rubella vaccine, interest was diverted and measles cases increased from approximately 22 000 cases to 75 000 cases reported (Table 8.31). With increased public support, the figure was again forced down to 22 000 cases in 1974. However, it should be stressed that there is not universal agreement about the meaning behind these rises and falls. Sabin (1981), for example, attributes the periodic rise and fall in the number of reported measles cases from 1971 to 1977 to a build-up of susceptible persons, and not to a rise and fall in vaccinations. He suggests that if mass vaccination is carried out in a *short time* then the transmission of measles would be broken.

During the period 1960–81 reported measles deaths declined very significantly in the USA, from around 400 to less than 10 at present. Similarly, the number of reported cases of measles encephalitis has declined from around 330 in 1962 to around 3 at present. Since SSPE is considered to be a rare complication of natural measles, it was of prime importance to monitor cases which might have been caused by vaccine virus. However, since 1960 there has been a marked decline in reported SSPE cases in the USA. The estimated risk of SSPE following wild measles is much higher (6–22 cases per million measles cases) than the risk of SSPE following measles vaccine virus (0.48–1.13 per million measles vaccinations). In the UK, in contrast, 100 000 children a year suffer from measles, and 20 die.

8.3.8. Duration of vaccine immunity and vaccine failures

Immunity following administration of live measles vaccine appears to be long lasting, and in any case up to 10–14 years (Fig. 8.6). Even persons in whom antibody

TABLE 8.31.
Reported measles morbidity and mortality, United States, 1960–81 (from CDC Measles Surveillance Report, 1982)

Year	Cases	Cases per 100 000 population	Deaths	Deaths per million population	Deaths per 1000 cases
1960	441 703	245.4	380	2.11	0.860
1961	423 919	231.7	434	2.37	1.023
1962	481 530	259.2	408	2.20	0.847
1963	385 156	204.3	364	1.93	0.945
1964	458 083	239.7	421	2.20	0.919
1965	261 904	135.3	276	1.43	1.054
1966	204 136	104.4	261	1.33	1.279
1967	62 705	31.8	81	0.41	1.292
1968	22 231	11.1	24	0.12	1.080
1969	25 826	12.8	41	0.20	1.588
1970	47 351	23.2	89	0.44	1.880
1971	75 290	36.5	90	0.44	1.195
1972	32 275	15.5	24	0.12	0.744
1973	26 690	12.7	23	0.11	0.862
1974	22 094	10.5	20	0.10	0.905
1975	24 374	11.4	20	0.09	0.821
1976	41 126	19.2	12	0.06	0.292
1977	57 345	26.5	15	0.07	0.262
1978	26 871	12.3	11	0.05	0.409
1979	13 597	6.2	—	—	—
1980	13 506	6.0	6	—	—
1981	3032	1.3	2	—	—

Note that live attenuated measles vaccine was introduced in 1963.

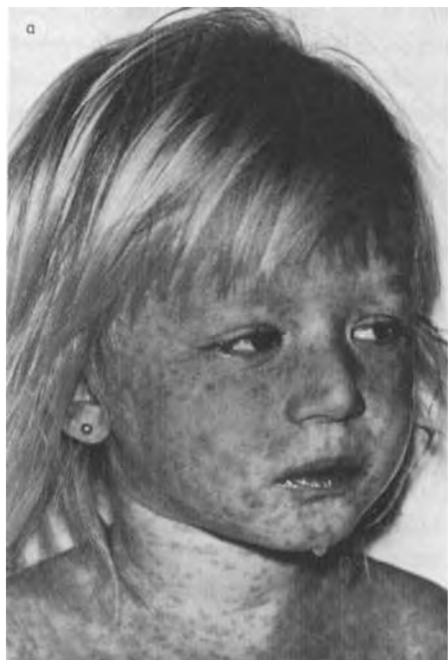
is no longer detectable may still have immunological memory and so may be immune.

Vaccine failures result when patients do not serologically convert (primary failure) and when immunity is subsequently lost following a successful initial seroconversion (secondary failure). The primary failure rate in the USA is less than 5% and is attributed to impotent vaccine because of improper storage or handling or to interference with vaccine virus replication by pre-existing measles antibody. Secondary failure following live measles vaccine has not been demonstrated conclusively.

Measles importations are geographically widespread in the USA and a rising proportion of imported cases has occurred among US citizens returning from overseas travel. Between 1980–81, 209 cases of measles were reported to have been imported, representing 1.3% of the total measles cases during that period.

⟶

Fig. 8.5. Clinical appearance of measles. a, quite severe rash and respiratory symptoms; b, milder measles case. (courtesy of the late Dr. W.C. Marshall, Great Ormond Street Hospital.)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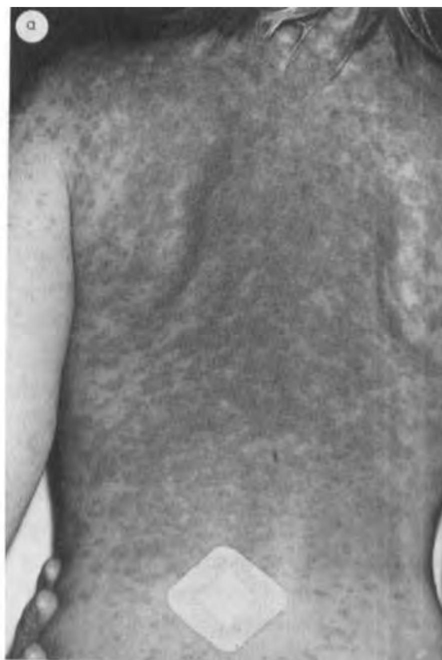
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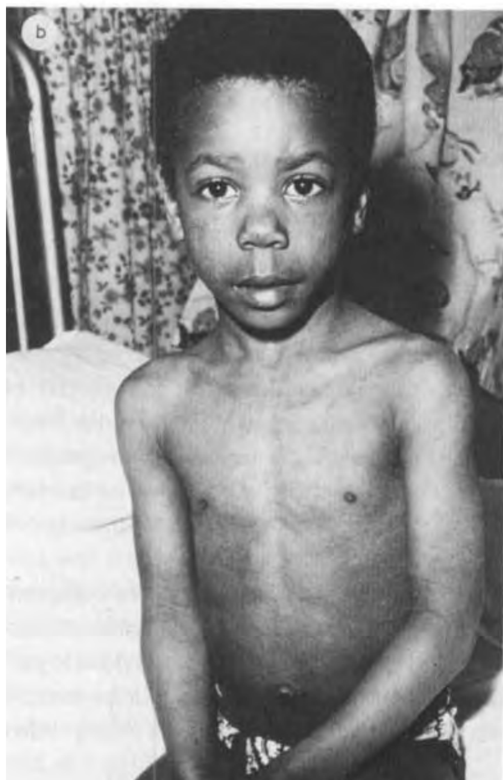
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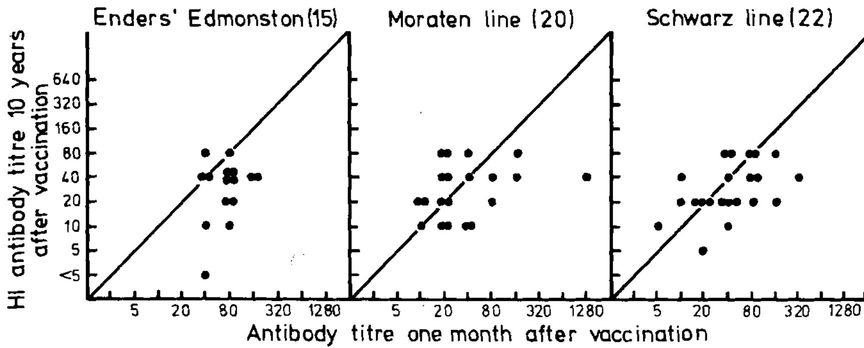

Fig. 8.6. Retention of measles HI antibody 10 years after vaccination (after Weibel et al., 1980.)

TABLE 8.32.
Age distribution of measles cases, deaths, and death-to-case ratios (United States) (from CDC Measles Surveillance Report, 1982)

Age group (years)	1973–1975			1976–1978			Percent change in death-to-case ratio
	Estimated cases	Deaths	Death-to-case ratio	Estimated cases	Deaths	Death-to-case ratio	
<1	3919	13	3.32	4170	3	0.72	−78.3
1–4	14041	21	1.50	14425	6	0.42	−72.0
5–9	22896	10	0.44	33267	3	0.09	−79.5
10–14	22542	11	0.49	43785	13	0.30	−38.8
15–19	7762	4	0.52	25474	3	0.12	−76.9
20+	1998	4	2.00	4222	10	2.37	+18.5
Total	73158	63	0.86	125343	38	0.30	−65.1

The increasing proportion of measles cases in young adults in recent years (Table 8.32) has resulted in the initiation of vaccine programmes for adults, including military camps. Fortunately young adults do not appear to have an increased risk of serious illness from measles vaccine virus. Measles incidence in the armed forces in the US has dropped sharply as a result of a measles immunization programme initiated in 1980. Vaccination certificates are required for attendance at military schools and day-care centres, and all teachers have to be vaccinated or have proof of immunity.

Epidemiologists agree that complete eradication of indigenous measles will eventually have to be achieved through herd immunity and this might be achieved successfully by routinely giving a second dose of measles vaccine to all individuals particularly in cities or areas where there are many immigrants. Although the current nationwide vaccination rates are high in the USA (and the envy of many other

countries, and with accrued social and medical benefits) nevertheless they are not always above levels estimated to be necessary for herd immunity. When local herd immunity is achieved the vaccination programme still has to be continued indefinitely to prevent outbreaks of imported cases.

8.3.9. Mass measles vaccination in the USSR

Mass vaccination against measles virus was started in the USSR in 1967, although some republics had started as early as 1963–64 (Burgasov et al., 1973). The attenuated measles virus Leningrad-16 has been used in this campaign (Peradze and Smorodintsev, 1983). In the years before vaccination, a high morbidity due to measles was noted in the USSR, with an average incidence of 827 cases per 100 000 population. As the scale of vaccination increased a steady decline in morbidity followed and as early as 1969 measles incidence had dropped 4-fold. Serological analysis showed that virus induced immunity persisted for at least 7 years. Thus, of 4793 children's sera analyzed over a period of 7 years at the end of the first year 88.2% of children had antibody and in subsequent years only a small proportion of children (2.5–6.4%) appeared to have lost antibodies. Actual antibody titres remained relatively constant over this period, however. A further point noted was that although no antibodies were detected in the blood of some vaccinated children, nevertheless they were resistant to actual infection. The authors estimated that during the 5 years of vaccination the USSR had saved more than $244 million dollars and the saving in the use of measles gamma globulin alone repaid all the expenses connected with the cost of the vaccine and its administration.

8.3.10. Measles eradication campaign in Sweden

General vaccination with a combined measles, mumps and rubella vaccine was introduced in Sweden in 1982 (see Christenson et al., 1983). The new immunization schedule comprises *two* vaccine injections, given at 18 months and 12 years of age, respectively (see Chapter 2). Vaccinating at 18 months only, was abandoned because of the risk of increasing numbers of people remaining unprotected among those left unvaccinated, or who had failed to seroconvert after the first inoculation. This has indeed occurred in the USA (see above). The aim of the new strategy is rapid elimination of all three diseases. A controlled field study was carried out in 150 children aged 18 months using two different batches of the vaccine. Seroconversion was seen in 99% against measles. Fever and rash as side effects of the vaccines were recorded five to 12 days after vaccination. Moderate fever (38.5–39.4°C) was observed in 22 children, high fever (⩾39.5°C) in 33, and rash in 35. Even with high fever most of the children were not particularly ill, or fretful. Preliminary results obtained by follow up of routinely vaccinated schoolchildren aged 12 indicated a considerably lower rate of fever and rash during the postvaccination period, occurring in 3–10% of cases only.

8.3.11. Combined live virus vaccines (mumps, rubella, measles)

The least costly way to deliver virus vaccines to the patient is in combination (Recommendation MMWR, 1972). For this approach to be successful there must be no interference between the viruses, the immunity for each virus should persist to the same extent as it would following administration of the virus alone (Weibel et al., 1972) and clinical reactions must be low or nonexistent. Combinations of live vaccines have been developed and used, particularly in the USA and USSR but are not used so extensively in the UK or the rest of Europe at present (Buynak et al., 1969, Stokes et al., 1971, Villarejos et al., 1971, Smorodintsev et al., 1970, Schiff, 1980, Sabin, 1981, Weibel et al., 1971). The serological conversion rate to four different combined virus vaccine was 94–99% (Hilleman et al., 1973) and, moreover, the quantity of antibody induced is comparable to the viruses given alone. The virus used is of great importance, since when a less attenuated measles vaccine was combined with the mumps virus, a lowered response to mumps was detected (Hilleman et al., 1973). Perhaps not unexpectedly, most of the fevers noted as side reactions were caused by the measles component.

8.3.12. Control of measles in third world countries

Two host factors seem of primary importance in explaining why measles is so severe in developing countries as compared with the disease in the United States and Europe: first, children experience the infection at a younger age; second, they are usually more poorly nourished (Walsh, 1983). As noted in Table 8.22, the peak age of incidence of the disease in urban areas in developing countries is between one and three years, while in the United Kingdom and Europe it is five years and older, and in the United States it is 10–14 years. In general, infants and toddlers are more likely to experience complications and to die from measles than are older children. The death-to-case ratio for infants less than one year of age is many times greater than it is for older children.

The other factor leading to high mortality from measles in developing areas is malnutrition (Walsh, 1983). Severely malnourished children suffer more complications and are more likely to die than their better-nourished peers. During the Nigerian Civil War, observations made at a time of severe famine revealed that $\leqslant 15\%$ of the children infected with measles died, and 50% of those in a medical centre for treatment of kwashiorkor died (Smith and Foster, 1970). In India, among children hospitalized with measles 89% of those poorly nourished had more than one complication (e.g., pneumonia, otitis, stomatitis, diarrhoea) while only 20% of the well-nourished had more than one complication (Chen et al., 1980).

Measles in malnourished children differs markedly from the disease as usually seen in the United States and Europe. The rash becomes dark red-purple and exfoliates extensively, exposing large areas of epithelial surface to bacterial invasion. Se-

vere changes occur in the mouth, tracheal-bronchial tree, and intestine; extreme soreness of the mouth (stomatitis) prevents the child from eating or drinking; bronchopneumonia, persistent diarrhoea and protein-losing enteropathy then ensue (Axton, 1975). Xerophthalmia that results in blindness occurs in areas of the world where diets are deficient in vitamin A. In these areas, more than half of the incidence of blindness may be attributable to measles (Walsh, 1983). Viral excretion persists much longer in poorly nourished patients (12 days, rather than three days as in those well-nourished). The persistence of virus in lymphocytes and intestinal cells suggests that there is a delay in production of lymphocytes that are competent to destroy these cells. As a result, the virus multiplies for a longer period, more cells are infected, and the disease is more severe. In addition, the depressed cellular immune system increases the patient's susceptibility to secondary infection (Dossetor et al., 1977). Bronchopneumonia and diarrhoea are the most common causes of death from measles.

The heat lability of the measles vaccine greatly complicates efforts to distribute it in tropical climates. The freeze-dried vaccine has to be held at 2–8°C until minutes before injection and unless this temperature range is maintained, the vaccine rapidly loses its potency. However, in the last few years, new stabilizers have been developed that retard the heat inactivation (Heymann et al., 1979). In the presence of these stabilizers, titres of the attenuated virus remain adequate (10^3 median tissue culture-infective doses per dose) for up to three weeks at 37°C and up to six months at 20–25°C in the laboratory (McAleer et al., 1980). In field trials, $>85\%$ of children nine months of age or older seroconverted after receiving vaccine that was stored at 23–25°C for seven to eight days (Heymann et al., 1979).

The final problem in providing effective immunization for children in developing countries is reaching them during the brief interval between the loss of maternal antibodies and the acquisition of natural disease. As soon as maternal antibodies wane, the infants begin to acquire the disease. Ideally, each child should be vaccinated as soon as transplacental immunity fades and as soon as his/her immune system can adequately respond to the vaccine. At six months of age, at least 20% of children will have maternal antibodies, and in these infants, the vaccine virus will not multiply and no protection will be achieved. After immunization, only 60%–80% will seroconvert and develop protective immunity. However, in developing countries, 15%–20% of children may already have had measles if immunization is delayed until the age of nine months (Walsh, 1983, Medical Research Centre, Nairobi, 1982).

Measles vaccination is part of the World Health Organization Expanded Programme on Immunization. Vaccines against diphtheria, pertussis, tetanus, poliomyelitis, and other infections can be administered simultaneously with measles vaccine once a reliable and comprehensive delivery system is available. Prophylaxis for malaria and oral rehydration as well as health education are a few of the other measures that could easily be combined with measles immunization efforts (Walsh,

1983). At present rates of immunization of children against measles in South-east Asia are very low (0.15% of children immunized) compared to 63% in Europe. Recent observations in the United Republic of Cameroon support the WHO recommendations of one dose measles vaccination in tropical Africa at 9 months minimum age. Measles control activities in the capital city began in 1966 and mass vaccination campaigns were held every 2 years among children aged 6–36 months. However, in 1973 despite a sample survey showing that 78% of children in the city had been vaccinated there were over 4000 cases of measles among the under 12-months-old children. It was deduced that most of the vaccine given had been ineffective because of pre-existing immunity from the mother and from the use of impotent vaccine because of storage problems. Between 1975–79 new storage facilities were introduced and also the new WHO policy of vaccination at 9 months. By 1979 a 44% decrease in reported measles attack rates among children under 9 months of age was reported (Heymann et al., 1983).

8.3.13. Measles immunization using aerosols

Early experiments in the USSR (Terskikh et al., 1971) and Japan (Okuno et al., 1965) indicated that administration of aerosols of live measles vaccines could result in sero-conversion, even under circumstances of partial immunity which prevented sero-conversion by the same vaccine injected intramuscularly. Sabin et al. (1983) have adapted a simple aerosol apparatus that would be suitable for use in third world countries by large numbers of non-professional personnel.

Inhalation of undiluted, aerosolized measles vaccine was immunogenic in 100% of 4- to 6-month-old and older children with and without residual maternal antibody when the human diploid cell (HDC) vaccine containing the Ikic (Edmonston-Zagreb) strain and 1% human albumin was used. Prevaccination, residual, placentally-transmitted, plaque-neutralizing antibody that can prevent an immune response after subcutaneous injection of measles vaccine, did not prevent an immune response after inhalation of aerosolized vaccine. There were no immediate clinical reactions in the 160 children who inhaled the aerosolized vaccines, and no significant subsequent reactions among the 96 children who were successfully immunized. There were no contact infections.

This compares very favourably, for example, with a 1974–1981 study in Nairobi, Kenya, which showed measles vaccine failures in 85%, 65% and 48% of infants vaccinated subcutaneously at 4, 5 and 6 months of age, respectively (MRC, 1982). A study in South America showed failure of seroconversion in 42%, 31%, and 18% of infants 6, 7, and 8 months of age, respectively, after subcutaneous injection of measles vaccine. In the Sabin study (1983), it was found that inhalation of the aerosolized HDC vaccine, containing the Ikic (Edmonston-Zagreb) plaque-purified strain of measles virus (Ikic et al., 1968), further attenuated in HDC, and 1% human albumin, immunized all of 39 infants 4 to 6 months of age and all of 21 children

12 to 24 months of age without reference to the amount of maternal antibody measured before vaccination. Sero-conversion was evident at six weeks after vaccination in all but four infants, 4 and 5 months of age, with residual maternal antibody titres of 130 to 512, in whom sero-conversion became evident only in the blood obtained six months after vaccination. However, a rather worrying observation was that another measles vaccine (but cultivated in CEF cells) did not achieve 100% sero-conversion when given by aerosol, even although it contained ten times more virus (but no added protein). Further studies are required to determine whether the human diploid vaccine was intrinsically more immunogenic or whether the 1% human albumin stabilized the virus during the aerosol infection.

8.3.14. CHEMOPROPHYLAXIS AND THERAPY OF MEASLES INFECTIONS

In general it can be stated that the practical and economic success of the measles vaccine (Table 8.33) has reduced the need for effective prophylactic agents. Nevertheless such compounds, if cheaply produced, could be useful in third world countries or in developed countries in special groups of children at high risk but who have not been immunized. Measles virus is inhibited in vitro by the nucleoside analogue ribavirin (Table 8.34) and two small uncontrolled clinical trials have shown the compound administered orally at 10 mg/kg for 7 days at the rash stage to reduce the severity of symptoms (Fernandez, 1980).

Early studies of certain carbobenzoxy tripeptides (carbobenzoxy-D-phe-L-phe-nitro-L-arg) by Norrby (1971) showed that 1 μg/ml was sufficient to inhibit penetration of measles virus into new cells. Cell fusion and haemolytic properties of the virus were also inhibited but no effect was noted on virus adsorption. One hundredfold higher concentrations showed inhibition effects on mumps virus, but no anti-

TABLE 8.33.
Summary of health and resource benefits due to vaccination against measles, United States, 1963–1981 (from CDC Measles Surveillance Report, 1982)

	Number
Cases averted	48420000
Lives saved	4840
Cases of mental retardation averted	16100
Additional years of normal and productive life by preventing premature death and retardation	1439000
School days saved	159309000
Physician visits saved	24880000
Hospital days saved	2762000
Net benefits achieved	$4448000000

(See Chapter 2 for a more extensive discussion of economic benefits of vaccination.)

TABLE 8.34.
Inhibition of measles virus by ribavirin in BS-C-1 cells (after Sidwell, 1980)

Ribavirin concentration (μg/ml)	CPE inhibition (%)	Cytotoxicity
1000	100	+
320	100	+
100	100	−
32	94	−
10	54	−
3.2	50	−
1.0	34	−
0.32	29	−
0.1	26	−
0.032	21	−
0.01	20	−
0.0032	15	−
0.001	0	−

viral effects were detected against Sendai or RSV. The possible biochemical basis of the antiviral action was not appreciated at the time but re-evaluation of the general approach by Choppin et al. (1983) has highlighted what could be an interesting point of action of antivirals. Choppin et al. (1983) noted the similarity in N-terminal sequences of the F2 polypeptide between paramyxoviruses and many of the short peptides which had been shown earlier to be active virus inhibitors in vitro. Therefore, it was proposed that the fusion activity of F protein might be inhibited by oligopeptides which mimicked this region of the protein. It was found that oligopeptides with the appropriate amino acid sequence were highly effective specific inhibitors of virus infectivity at the level of virus penetration and of virus induced fusion and haemolysis (i.e. activities that reflect the membrane fusing activity of the F protein). Oligopeptides were found which inhibited SV5, Sendai, canine distemper virus as well as measles (Table 8.35). However, measles virus was much more inhibited than the other viruses.

From such studies, several conclusions have been drawn. In general terms it was noted that the oligopeptides with the correct amino acid sequence were specific inhibitors, and the longer the peptide, the more active they were. The most effective peptide tested was a heptapeptide with the sequence of the Sendai virus F_1 N-terminus and a 50% effective concentration of 0.02 μM. A carbobenzoxy (Z) group on the N-terminal amino acid increased activity as compared to the same oligopeptide with an unblocked N-terminal residue. Other hydrophobic additions also increased inhibitory activity, such as a dansyl (DNS) or a t-butyloxicarbonyl (t-BOC) group. The steric configuration of the N-terminal phenylalanine also significantly affected activity, e.g., Z-D-Phe-L-Gly was more active than Z-L-Phe-L-Phe-Gly and finally

TABLE 8.35.
Inhibition by oligopeptides of plaque formation by paramyxoviruses and myxoviruses (after Choppin et al., 1983)

Virus	Peptide	50% effective concentration (μM)
Measles	Z-D-Phe-L-Phe-Gly-D-Ala-D-Val-D-Ile-Gly	0.02
	Z-D-Phe-L-Phe-Gly	0.20
	Z-D-Phe-L-Phe-L-(NO_2)Arg	0.20
	Z-D-Phe-L-Phe-Gly(chloromethylketone)	0.20
	Z-D-Phe-L-Phe-L-Tyr	0.20
	Z-D-Phe-L-Phe-L-(Azido-Phe)	0.28
	DNS-D-Phe-L-Phe-Gly	0.34
	Z-D-Phe-L-(pBr)Phe-Gly	0.52
	t-BOC-D-Phe-L-Phe-Gly	2.0
	Z-D-Phe-L-Tyr-Gly	9.3
	Z-D-Phe-D-Phe-Gly	10
	Z-D-Phe-L-Phe-Gly(methyl ester)	20
	Z-D-Phe-L-(Benzyl)Tyr-Gly	20
	Z-L-Phe-L-Phe-Gly	23
	-D-Phe-L-Phe-Gly-D-Ala-D-Val-D-Ile-Gly	130
	Ac-D-Phe-L-Phe-Gly	20
	Z-Gly-L-Phe-L-Phe-Gly	870
Sendai	Z-D-Phe-L-Phe-Gly	320
	Z-D-Phe-L-Phe-L-(NO_2)Arg	540
	Z-Gly-L-Phe-L-Phe-Gly	>1000
SV5	Z-D-Phe-L-Phe-Gly	320
	Z-D-Phe-L-Phe-L-(NO_2)Arg	500
Measles mutant R93	Z-D-Phe-L-Phe-Gly	20
	Z-D-Phe-L-Phe-L-(NO_2)Arg	>1000
Canine distemper	Z-D-Phe-L-Phe-Gly	1.50
	Z-D-Phe-L-Phe-L-(NO_2)Arg	>1000
Influenza A (WSN)	Z-Gly-L-Leu-L-Phe-Gly	20
	Z-Gly-D-Phe-L-Phe-Gly	23
	Z-Gly-L-Phe-L-Phe-Gly	53
	Z-D-Phe-L-Phe-Gly	290

Z denotes a carbobenzoxy group; DNS, a dansyl group; t-BOC, a tertiary butyloxy group; and Ac, an acetyl group.

esterification of the C-terminal amino acid decreased activity. The effects of the substitutions at the termini of the oligopeptides may be related to the positioning of the inhibitor at the site of action. Thus, the carbobenzoxy or dansyl groups add hydrophobicity to the N-terminus, whereas esterification of the C-terminus decreases the polarity of the peptide, and such changes could affect the orientation of the peptide. The peptide may also be protected from proteolytic activity.

The specificity of the oligopeptide inhibitors and the importance of the correct

TABLE 8.36.
Inhibition of wild type and mutant measles viruses by oligopeptides (after Choppin et al., 1983)

Peptide	50% effective concentration (μM)	
	wt	mutant
Z-D-Phe-L-(NO_2)Arg	0.2	>1000
Z-D-Phe-L-Phe-Gly	0.2	21

amino acid sequence was demonstrated by the selection of a mutant of measles virus that was resistant to the action of a tripeptide (Z-D-Phe-L-Phe-L-(No_2)Arg) by repeated passage of the virus in the presence of this peptide. Although the mutant became resistant to this peptide, it remained relatively sensitive to Z-D-Phe-L-Phe-Gly, which differed only in the third amino acid. (Table 8.36).

The oligopeptides did not appear to have significant toxic effects on the cells used in these studies and cells survived for days with no detectable cytopathic changes, and multiplied normally in the presence of the peptides. Preliminary experiments also suggest the oligopeptides are not highly toxic in mice. However, a problem with the compounds is their relative insolubility and this factor makes precise quantitation of antiviral effects difficult. To investigate their site of action, oligopeptides have been synthesized with radioactive or fluorescent (dansyl) labels to monitor their site of binding. Radioactively-labelled oligopeptides were added to purified virus, to mock infected cells, or to infected cells, and after washing, specific binding was calculated. Such studies suggested that the oligopeptides bind to the cell and not to the virus.

8.3.15. Summary

Attenuated measles vaccines have been developed empirically by selection of 'host range' mutants, and are widely and successfully used throughout the world. Using the vaccine, some countries may soon eliminate measles as an endogenous virus but continued problems are anticipated, particularly in adults with viruses re-introduced by visitors from abroad. Basic studies on new antivirals are continuing (particularly with oligopeptides) but antiviral compounds are unlikely to have extended use in the clinic, except perhaps in tropical areas where the disease may be life threatening. However, a vaccination programme in these areas is preferable, and is an urgent need.

8.4. Parainfluenza viruses

8.4.1. Parainfluenza viruses types I–IV

These respiratory viruses are worldwide in their distribution and affect all age groups (Hamre et al., 1961, McLean et al., 1961, Parrott et al., 1959, Kapikian et al., 1960, Smorodintsev, 1962, Chanock et al., 1958, 1959, Andrewes et al., 1959, Bloom et al., 1961, Kelen and McLeod, 1977, Tyrrell et al., 1959, Birkum-Peterson, 1958). A feature of the epidemiology of these viruses is that re-infections are common, although they may often be subclinical. These subclinical infections maintain a huge and active reservoir of infective virus. The viruses are transmitted by airborne droplets and particularly rapid spread is noted in institutionalized children. It has been estimated that parainfluenza viruses constitute up to one third of all respiratory tract infections of humans and 40% of respiratory infection in preschool children and infants.

8.4.2. Clinical aspects

The incubation period varies from 2–4 days in children and 3–6 days in adults. In pre-school children, particularly, a severe respiratory infection often develops and types I and II are most often associated with laryngo tracheobronchitis (croup), whereas types III and IV cause infection of the lower respiratory tract such as bronchitis and pneumonia. When the lower respiratory tract is involved bronchospasms are a predominant clinical sign.

In adults hoarseness is often the main symptom and, in general, all four virus types cause a range of respiratory illness.

In a recent study in Chapel Hill (USA) involving a study of 7000 cases of lower respiratory tract infection in a pediatric practice 18%, 4% and 15% of virus isolates were parainfluenza I, II and III respectively (summarized by Tyeryar, 1983). Of children from whom parainfluenza virus type I and II was isolated, almost 60% had croup. Parainfluenza type III was closely associated with tracheo bronchitis. The risk for hospitalization for illnesses associated with parainfluenza type III was much less than for RSV, and the risk for infection with the former virus during the first four months of life was inversely related to the level of neutralizing antibody measured in the cord serum at birth. Interestingly, patterns of antibody to haemagglutinin and to neuramininidase correlated with antibody patterns by viral neutralization assays. The ability of the assay to detect rises of antibody levels to fusion (F) protein resulted in serological recognition of infection not discernible by antibody responses to HN. Primary infections produced greater antibody rises to HN than to F protein, but peak titres to HN were characteristically delayed up to 10 months.

Secondary infections with parainfluenza virus type III infrequently provided any further immunological responses to the HN protein. The data suggested that a sec-

ondary infection was important for stimulation of antibody to F protein and, in some cases, the rise in level of antibody to F protein was the only marker of secondary infection.

8.4.3. Virus structure and replication

These viruses are typical paramyxoviruses and have a replicative strategy similar to that already described for negative strand viruses such as measles (q.v.).

8.4.4. Prevention of parainfluenza virus infections using vaccines

It might be an obvious deduction from the above comments on natural re-infection in children with parainfluenza viruses that effective vaccines would be difficult to produce. Indeed, earlier studies of experimental vaccines showed that immunized children, when later encountering a wild virulent parainfluenza virus, could still have a severe infection. This again indicates the absence of knowledge about important antigenic determinants on the virus and, in particular, the absence of data on the function of the fusion (F) glycoprotein of these viruses. As with inactivated measles and RSV vaccines, with experimental parainfluenza vaccines the inactivation process during vaccine production very probably inactivated the F protein, and so although some neutralizing antibodies were produced nevertheless, in the absence of antibodies to F protein, immunized children were subsequently not protected against infection.

Fulginiti et al. (1967a, b, 1969) described a vaccine trial in children with parainfluenza virus types I, II and III. The vaccine was prepared by formalin treatment of virus harvested from the amniotic cavity of embryonated hens' eggs. 537 children were immunized and ranged in age from 6 months to 6 years. The antibody response to immunization is summarized in Table 8.37 and in general was satisfactory since most seronegative children developed HI or neutralizing antibodies. Parainfluenza virus infection occurred in the community in the months following immunization and so the protective efficacy of the vaccine was assessed, but no evidence of protection could be established. Indeed, attack rates for subsequent parainfluenza illness were rather higher in the vaccinated group than the control groups (Table 8.38).

An innovative approach to a parainfluenza type III vaccine has been to isolate F and HN glycoproteins in their native antigenic forms and to assemble them into a multivalent structure (reviewed by Tyeryar, 1983). The virus is disrupted with the non-ionic detergent octylglucoside, the glycoproteins separated by ultracentrifugation and the detergent dialysed off, when the glycoproteins reassemble.

TABLE 8.37.
Summary of fourfold or greater parainfluenza HI rise in parainfluenza vaccinees (TPV) compared to controls[a] (after Fulginiti et al., 1969)

Pre-vaccine III antibody status	≥4× antibody rise 30 days post 3rd dose of vaccine		
	Para 1 No./Total (%)	Para 2 No./Total (%)	Para 3 No./Total (%)
Seronegative (<1:8)			
TPV	47/47 (100)	38/43 (88)	10/11 (91)
Control[a]	4/35 (11)	11/27 (40)	0/6 (0)
Seropositive (≥1:8)			
TPV	10/34 (29)	12/38 (32)	20/70 (29)
Control[a]	0/18 (0)	2/26 (8)	5/47 (11)
Totals			
TPV	57/81 (70)	40/81 (40)	30/81 (37)
Control[a]	4/53 (7.5)	13/53 (25)	5/53 (9)

[a] Controls are RS virus vaccine recipients.

TABLE 8.38.
Attack rates for hospitalized illness due to parainfluenza viruses (after Fulginiti et al., 1969)

Vaccine group	No. with para illness	No. with croup	No. virus isolated	No. ≥4× rise anti-body	Attack rate for para illness per 100 at risk
TPV	6	7	P-3 0/1	1/1	1.1 (6/567)
			P-2 1/3	3/3	
			P-1 2/2	2/2	
Control (RSV)	2	1	P-3 0/1	1/1	0.43 (2/464)
			P-1 0/1	1/1	
Control (non-vaccinee)	3	2	P-3 1/1	1/1	0.3 (3/1001)
			P-2 0/1	1/1	
			P-1 1/1	1/1	

TPV, trivalent parainfluenza vaccine; P, parainfluenza type.

8.4.5. CHEMOPROPHYLAXIS OF PARAINFLUENZA VIRUSES

Amantadine was shown in earlier studies (Davies et al., 1964) to have some mild inhibitory effect against RSV, parainfluenza types II and III and Sendai virus, although this was not equivalent to its marked inhibitory effects against influenza (Chapter 7).

Thirty adult volunteers were given 100 mg capsules of amantadine and placebo

twice daily for 12 days. On the fourth day the men were challenged intranasally with $10^{4.5}$ $TCCD_{50}$ of parainfluenza virus (Smith et al., 1967). A mild upper respiratory tract infection developed in 17 of the 30 men and no difference in the number developing illness was detected between the two groups. Also no difference in severity or duration of illness or virus isolation rate was detected. Finally, the serum antibody titres were not different in the two groups and the authors concluded that the compound was not active, with the reservation that a large virus challenge dose was used. Nevertheless, the concentrations of drug detected in the nasal wash (approx. 0.3 μg/ml allowing for dilution) would not be high enough to inhibit replication of parainfluenza viruses (based on in vitro data), although they would be high enough to inhibit replication of influenza A viruses.

In vitro, parainfluenza viruses are inhibited by certain benzimidazole compounds (Bucknall, 1967) a biguanide also has some virus inhibitory effect in cell cultures (Tobita, 1968), and ribavirin inhibits Sendai virus in vivo (page 315).

More extensive studies for new antivirals against parainfluenza viruses are urgently required and could probably be initiated now with good prospects for success.

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CHAPTER 9

Infections caused by rubella, reoviridae, retro, Norwalk and coronaviruses

A number of RNA-containing viruses are brought together in this Chapter rather as 'leftovers' either because they fit uneasily into the existing classifying schemes or because we felt, like rubella, they deserve more attention than being submerged into the (non-arbovirus) Togavirus group! They form a fascinating chapter of infective agents both for the clinician and the virologist.

9.1. Rubella (German measles) infections

Although rubella is a common disease of childhood it is rather benign and probably would not have been considered even worthy of thought for vaccine had it not been for the dramatic discovery by Gregg (1941) in Australia of the teratogenic effect of the virus. In a classic retrospective study, initiated by his observation of an unusual number of cases of congenital cataract in Sydney, he linked this observation with an outbreak of rubella which had occurred 9 months previously. The case histories of 78 infants with congenital cataracts showed that 68 of their mothers had suffered from rubella in the first trimester of pregnancy. Later prospective studies worldwide, established that a syndrome of defects resulted from this intrauterine infection (congenital rubella syndrome) but that a triad of defects were most commonly detected in the infants: cataracts, cardiac deformities (causing cyanosis) and deafness. The so-called 'expanded rubella syndrome' (established by precise virological diagnosis in the mid-1960s) includes, additionally, low birth weight and failure to thrive, purpura, anaemia, brain changes, jaundice, dental defects and neurological manifestations (Table 9.1). Congenital rubella, therefore, often presents

TABLE 9.1.
Frequently encountered signs of congenital rubella

Ophthalmic defects
- Cataracts
- Glaucoma
- Retinal cloudiness

Central nervous system defects
- Sensorineural deafness
- Speech defects
- Mental retardation
- Microcephaly
- Cerebral calcification

Cardiovascular system
- Persistent patent ductus arteriosus
- Intraventricular septal defect

Haemopoietic system
- Anaemia
- Leucopenia
- Thrombocytopenic purpura
- Persistent lymphadenopathy

Bony system
- Osseous malformation metaphyses of long bones

Miscellaneous
- Intrauterine and postnatal growth retardation
- Recurrent infections

For additional details see: Avery et al., 1965, Bellani et al., 1965, Cooper et al., 1965, Erickson, 1944, Hambridge et al., 1966, Horstmann et al., 1965, Keir, 1965, Lundström and Boström, 1958, Lundström et al., 1962, Manson et al., 1960, Menser et al., 1967.

as a complex clinical picture. If the virus infects the foetus during the process of organogenesis, in the first 3–4 months of pregnancy, then it is extremely likely that the infant will be born with gross defects. Nevertheless, some infants escape developmental defects but show other signs of infection, such as low birth weight or hepatosplenomegaly. The virus often persists in foetal tissues throughout gestation and can be recovered from the infant after birth (Menser et al., 1967, Monif et al., 1965). In the latter group, virus can be easily isolated from the throat, csf, urine and faeces and liver for up to 3 months after birth. These rubella syndrome children can, therefore, be an important infectious source for pregnant women. Prospective studies have established that when a mother is infected in the first 4 weeks of pregnancy a minimal figure of 60% of the infants have congenital defects, but other authorities would suggest a higher figure, approaching 90%. The incidence of congenital defects (principally deafness) resulting from intrauterine infection in the first 4 months of pregnancy has been estimated at between 1.5 and 50% (reviewed by Banatvala and Best, 1984). It should also be remembered that rubella infection of the

mother may result in spontaneous abortion in up to 20% of cases, usually when the infection occurs in the first eight weeks.

9.1.1. The virus and mode of replication

Rubella virus is a positive-stranded RNA virus, 40–70 nm in diameter with a lipoprotein envelope. It has been classified as a non-arthropod-borne togavirus and has been placed by itself in the genus Rubivirus. No antigenic relationship has been shown, for example, between rubella and more than 200 alpha and flaviviruses, and no cross reactions are detected with pestiviruses or equine arteritis virus (Brinton, 1980). The nucleocapsid is 30–40 nm in diameter and contains a single molecule of RNA which is infectious when extracted under appropriate conditions. The viral envelope is acquired by budding from the host cell. The pleomorphic character of the virus particle is presumably due to the non-rigid character of the lipid envelope; elliptical and oblong virus particles and particles bearing finger-like protrusions have been described (Fig. 9.1). The envelope bears poorly defined 5–6 nm surface spikes, presumably composed of viral glycoproteins, which carry the pH-dependent HA activity. At least four (see below) major polypeptides have been described, three glycoproteins associated with the envelope (E1, E2a and E2b), and a non-glycosylated core protein (C) which may represent viral structural proteins, precursor polypeptides, or host proteins which have remained associated with the purified virus (Oker-Blom et al., 1983, Alstyne et al., 1981, summarized in Table 9.2). The three glycoproteins have approximate molecular weights of 58K, 47K, and 42K respectively, whilst the molecular weight of the C protein is 37K. This would imply that the structural genes should have a coding capacity of 116K. Subgenomic 24S RNA species have been detected in virus infected cells (Oker-Blom et al., 1983) and, thus, rubella may be similar in this regard to the alphaviruses where a 26S mRNA encodes the 130K precursor of the structural proteins. It is quite likely that only three genes are required to code for the virus proteins, since E2a and E2b are very similar in their tryptic maps.

9.1.2. Clinical rubella in children and young adults

The attack is usually mild and is characterized by a 3 day rash, a few swollen and perhaps slightly tender lymph nodes, a slight temperature rise and some scarcely noticeable malaise. Christie (1980) summarizes it as a trifling ailment causing less inconvenience to the patient than the common cold. In as many cases again no rash is apparent at all and a subclinical infection proceeds silently. Regardless of the severity of rubella in the adult, the foetus can be infected, resulting in congenital infection. The incubation period for rubella is 14–16 days. The rash first appears on the face around the mouth and behind the ears. On the trunk, spots are at first discrete, about the size of a pin head. If the rash progresses then it spreads rapidly to cover

the arms and legs as well. Another tendency with a rubella rash is for it to come and go in an hour or so. Persons are most infectious for a few days before the rash appears and transmission is by virus aerosol and inhalation to the upper respiratory tract.

The most common complication of rubella in the adult is arthritis and arthralgia

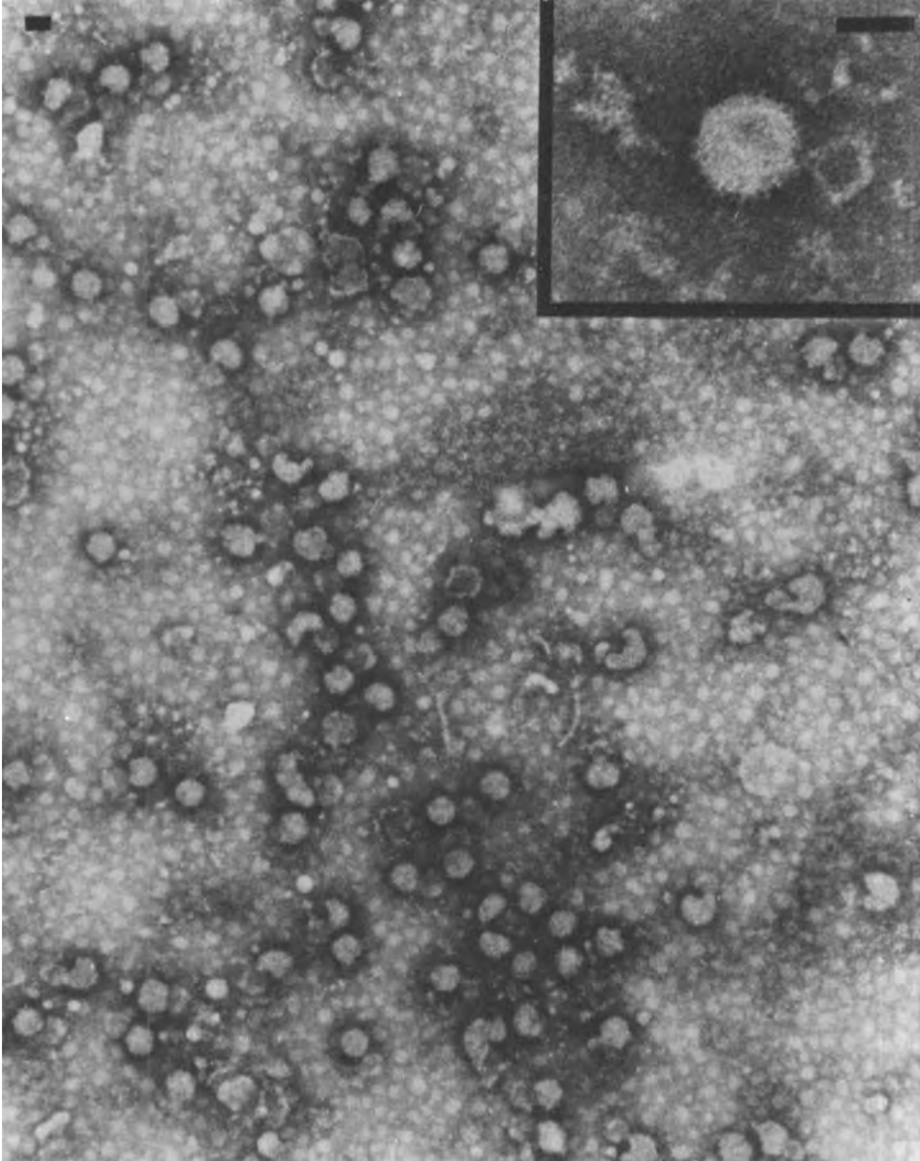

Fig. 9.1. Electron micrograph of rubella virus (kindly supplied by Dr I Chrystie, St Thomas Hospital).

TABLE 9.2.
Biochemical properties of rubella virus (after Banatvala and Best, 1984)

Virus Particle:	Diameter	40–70 nm			
	Buoyant Density	in sucrose	1.16–1.19 g/ml		
		in $CsCl_2$	1.20–1.23 g/ml		
	Sedimentation coefficient	240S (range 240–350S)			
Nucleocapsid:	Diameter	30–40 nm			
	Symmetry	Icosahedral			
	Sedimentation coefficient	150S			
	M.W.	$2.6–4.0 \times 10^6$			
Nucleic acid:		Single strand of RNA			
	Buoyant Density	1.634 g/ml			
	Sedimentation coefficient	38–40S			
	M.W.	$3.2–3.5 \times 10^6$			
Length of surface glycoprotein spikes		5–6 nm			
Chemical composition of virions		RNA	2.4%	Lipid	18.8%
		Proteins	74.8%	Carbohydrates	4 %
Major polypeptides:	E1, E2a, E2b	Envelope 55–62 and $46–50 \times 10^3$			
	C	Nucleo-capsid	31–35	$\times 10^3$	
Thermal stability:	4°C	Stable for 7 days			
	37°C	Inactivated at 0.1–0.4 $\log_{10}$ $TCID_{50}$/ml per hour			
	56°C	Inactivated at 1.5–3.5 $\log_{10}$ $TCID_{50}$/ml per hour			
pH Sensitivity:		Stable at pH 6.0–8.1			
		Unstable at more acid and alkaline pH			
UV Sensitivity:		Inactivated within 40 seconds			
	1350 W/cm^2	Inactivated at 7.0 $\log_{10}TCID_{50}$/0.1 ml per hour			
Photosensitivity:		Labile $K=0.07\ min^{-1}$ in PBS			

(in up to 1/5th of patients) which may persist for some weeks. Most commonly, the smaller joints are involved. Central nervous system complications are rare following rubella, and encephalitis incidence rates of approximately 1 in 5000 are generally assumed. The onset of encephalitis is usually sudden, within a day or two of the rash, and symptoms may vary from headache and drowsiness to convulsions and coma.

9.1.3. Prevention of rubella using vaccines

The important aspect with rubella is to prevent the congenital infection and this seemed an achievable goal following the introduction of several live attenuated vaccines in the mid-1960s. At this time, in the USA alone, an estimated 12.5×10^6 cases of rubella occurred with 20000 malformed infants and 11000 instances of foetal

wastage (reviewed by Hinman et al., 1983, Fig. 9.2). However, nearly two decades later the enthusiastic and high expectancies of those early days (Meyer et al., 1969, Weibel et al., 1969) have not been achieved, at least in many countries (reviewed by Banatvala, 1977, Clarke et al., 1979, 1980, 1983, Grenstein and Greares, 1982). This is not a reflection of the lack of efficacy of the vaccine, but rather a criticism of some national health authorities and general somnolence, and, to some extent, scientific disagreement about how best to use the vaccine.

Following the isolation and cultivation of rubella virus in tissue culture in the early 1960s (Parkman et al., 1962, Weller and Neva, 1962, McCarthy et al., 1963) rapid progress was made in developing a number of simple attenuated vaccines. For example, virus was passaged 77 times in vervet monkey kidney cells (HVP-77 vaccine) (Meyer et al., 1966, 1968, Parkman et al., 1966) and 5 times in duck embryo

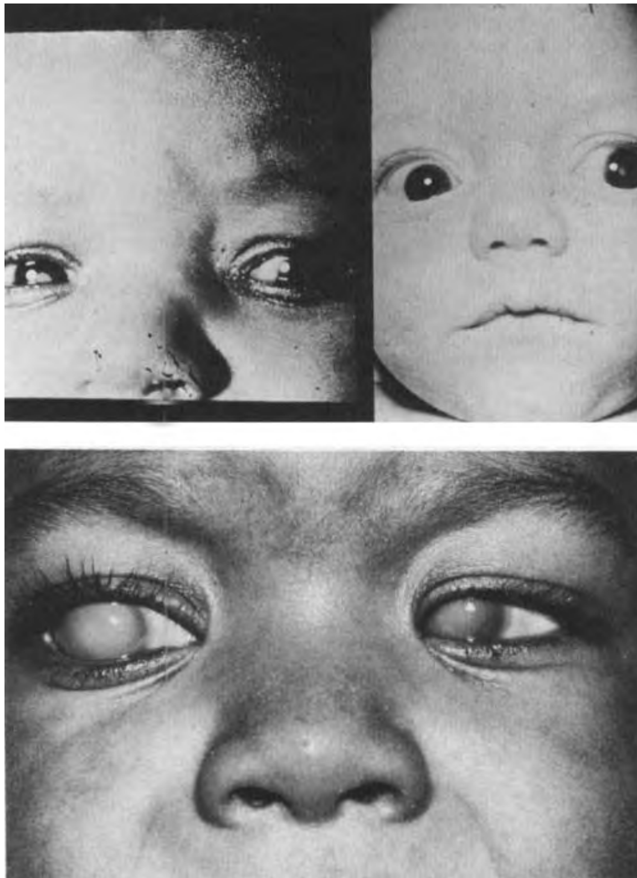

Fig. 9.2. Clinical aspects of rubella and rubella syndrome – cataracts in children with congenital rubella syndrome (courtesy of the late Dr. W. Marshall, Great Ormond Street Hospital for Sick Children.)

fibroblasts (HPV-77, DE5) and was found to be attenuated successfully for children and adults (Buynack et al., 1968, Weibel et al., 1968). Another strain of virus was passaged 51 times in primary rabbit kidney cells (Cendehill vaccine, Huygelen and Peetermans, 1967, Zygraich et al., 1971), whilst a third virus was passaged 4 times in HEK and 25 times in human diploid WI-38 cells (RA 27/3, Plotkin et al., 1973). Finally, and more recently, a Japanese vaccine virus was passaged in vervet MK cells (Table 9.3) 7 times and 20 times in primary guinea pig kidney cells (Best et al., 1974). All these vaccines when administered produced seroconversion in up to 95% of susceptible persons, with only limited side effects. Nevertheless, further experience indicated that the HPV-77 DK12 vaccine produced unacceptable side effects of arthralgia and arthritis and so it was withdrawn in the USA, for example. Later the HPV 77 DE5 vaccine was also withdrawn and replaced by the RA27/3 vaccine (Meruvax 2). Side effects including arthritis and arthralgia are usually mild (Best et al., 1974).

Early studies showed that the vaccine viruses were not transmitted to potentially susceptible sentinel volunteers (Halstead and Diwan, 1971), which was an essential requirement, because it was not known at first whether the vaccine viruses were teratogenic or not.

Immunity induced is long lived (Hoshino et al., 1982, Fig. 9.3) while experience over the last 15 years has shown the rubella vaccine viruses to have no teratogenic activity, although they have been isolated from foetal tissue and from infants whose mothers had been inadvertently vaccinated during pregnancy (Modlin et al., 1976). After experience accumulated with the different rubella vaccines it has become clearer that side effects are more associated with some, and the longevity of immune response induced might also differ. In short, it would now appear that the RA27/3 rubella vaccine strain has more positive features than some of the other viruses, and

TABLE 9.3.
Characteristics of some commonly used attenuated rubella vaccines

Vaccine	Origin of virus	Passage history for attenuation (Nos. of passages)
HPV77	Army recruit with rubella (1961)	Vervet monkey kidney (77)
HPV77, DE5	Army recruit with rubella (1961)	Vervet monkey kidney (77); duck embryo (5)
Cendehill	Urine from a case of postnatally acquired rubella (1963)	Vervet monkey kidney (3); primary rabbit kidney (51)
RA27/3	Kidney of rubella-infected fetus (1964)	Human embryonic kidney (4); WI-38 fibroblasts (17–25)
To-336	Pharyngeal secretion of child with postnatally acquired rubella Toyama, Japan (1967)	Vervet monkey kidney (7); primary guinea-pig kidney (20); primary rabbit kidney (3)
QEF (MEQ_{11})	Throat washing from patient in Osaka (1966) = Matsuura strain	Vervet monkey kidney (14); Chick amnion (65); Japanese quail embryo fibroblast cells (11)

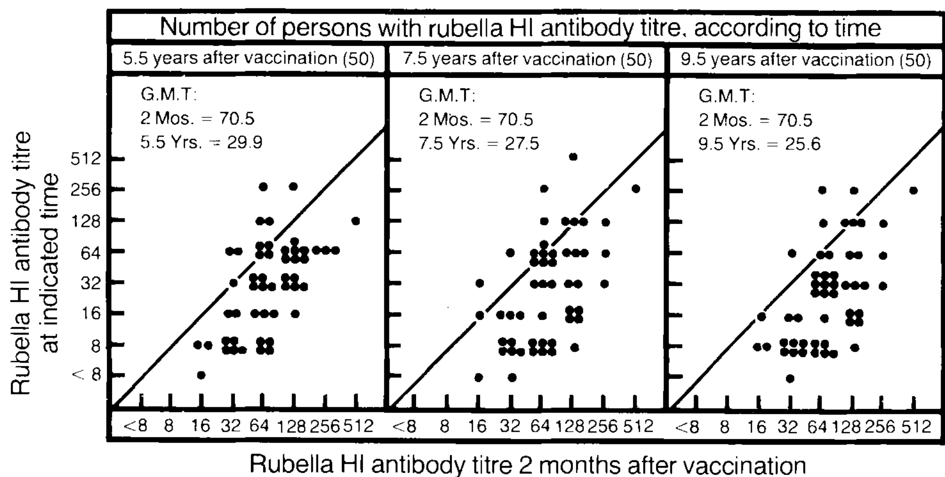

Fig. 9.3. Vaccine induced immunity to rubella is long lived. (after Weibel et al., 1975.)

is currently used on a rather wide scale. Immunity induced by RA27/3 more closely resembles the immune response to naturally acquired infection. This particular virus was first isolated in 1964 from an explant of a surgically aborted foetus by direct inoculation into WI-38 human diploid fibroblasts (reviewed by Plotkin et al., 1973). The virus was attenuated by low temperature passage and cloning in WI-38 cells, and was used for immunization between the 27th and 30th passage in these cells. A dose is 10^4 $TCID_{50}$ and can be administered intranasally or by s.c. injection (the latter technique is now favoured). In eight comparative studies performed in different countries, the mean HI antibody titre after immunization by either route was 2.4-fold higher with RA27/3 vaccine compared to Cendehill and HPV-77 vaccines (Plotkin et al., 1973). Similarly, higher levels of neutralizing antibody were detected following RA27/3 than Cendehill vaccine (Table 9.4) and higher levels of CF antibody than HPV-77 DE vaccine (Table 9.5). Nasal antibody (rubella specific IgA) is regularly detected after intranasal RA27/3 vaccine and frequently detected after s.c. injection with the same vaccine. In contrast, although specific IgA antibody is detected after Cendehill and HPV-77 DE5 vaccines, for example, it does not persist so well (Best et al., 1979) (Table 9.6). It should be noted here that there is no evidence of transmissibility from persons infected intranasally with RA27/3 vaccine.

An important aspect of rubella vaccines is whether recipients could become reinfected at later dates with wild rubella virus, which could still produce teratogenic effects. In early studies of persons receiving HPV-77 or Cendehill vaccines, and who were exposed to wild virus in nature or artificially, vaccines had re-infection rates ranging from 40–100% (Table 9.7). With RA27/3 vaccinees, re-infection rates of 3–10% were observed, which is close to figures obtained following natural infection. In a more recent study Best (personal communication) challenged a number of per-

TABLE 9.4.
Neutralizing antibody responses to 3 rubella vaccines (after Plotkin et al., 1973)

Investigator	Geometric mean neutralizing titres 6 to 8 weeks postimmunization		
	Cendehill	RA27/3 Subcutaneously	RA27/3 Intranasally
Plotkin (unpublished)	5 (6)[a]	15 (7)	13 (7)
Tobin	12 (28)	18 (29)	–
Fogel	6[b]/25	–	39[b]/43

[a] Number in parentheses is number of subjects tested.
[b] Number positive at 1 : 4 screening dilution per number tested.

TABLE 9.5.
Complement-fixation antibody responses to rubella vaccines (after Plotkin et al., 1973)

Vaccine	Percent serocon-verting[a]	Geometric mean titre	Geometric mean titre of seroconverters[b]
HPV-77-DE	73	3.3	4.2 (16)
RA27/3 subcutaneously	100	6.0	6.0 (27)
RA27/3 intranasally	91	6.8	8.0 (22)

[a] Eight weeks immunization.
[b] Number in parentheses is number of seroconverters.

sons who had been vaccinated some 10 years earlier and viraemia was detected in 1 of 19 vaccinees who had antibody levels below 15 international units. Therefore, it is not yet known whether adequate levels of antibody *will* persist in all vaccinees for up to 30 years after vaccination, for example.

In summary, we have a number of successful rubella vaccines, developed empirically without any knowledge of genetics of the virus or the molecular basis of virulence or attenuation. Perhaps because of this absence of precise virological knowledge it has taken 10 years of field trials to establish that the various vaccine strains are not identical and vary, for example, in their ability to produce a long lived immune response and in side effects. The RA27/3 vaccine strain would appear to be a very successful one. It will be of particular interest, though, to analyze the genome of these different viruses and hence to determine, for example, how many mutations

TABLE 9.6.
Intranasal antibody after rubella vaccination (after Plotkin et al., 1973)

Investigator	Vaccine	No. positive/total
Plotkin	RA27/3-IN	5/6
	RA27/3-IN	9/15
	RA27/d-SC	4/9
	Cendehill	0/6
Bellanti	RA27/3-IN	8/8
Schiff-Ogra	Ra27/3-IN	8/10
	RA27/3-SC	4/10
Ogra	RA27/3-IN	13/15
	RA27/3-SC	2/5
	HPV-77-DK	0/30
Total	RA27/3-IN	43/54
	RA27/3-SC	10/24
	Others	0/36

TABLE 9.7.
Reinfection rates after artificial challenge of RA27/3 vaccines with intranasally given rubella virus

Investigator	Route of immunization	
	Subcutaneous	Intranasal
Plotkin (earlier data)	1/8	1/7
	0/7	–
	0/9	–
Liebhaber	1/10	1/9
Naficy	–	2/11
Ogra (unpublished data)	8/20	2/13
Smith, Kline and French (unpublished data)	–	1/10
Total	10/54 (18.5%)	7/50 (14%)

are required to change a rubella strain from a virulent one to an attenuated one. This could lead to the artificial 'construction' of a new generation of attenuated vaccines (see Chapter 2).

9.1.4. Side reactions and contraindications to rubella vaccination

Rubella vaccines are generally well tolerated, but lymphadenopathy, rash, arthralgia or arthritis may occur some two to four weeks after vaccination, although such reactions are usually less severe than those following naturally acquired disease (reviewed by Banatvala and Best, 1984, Best et al., 1974). In a study of 142 seronegative volunteers given Cendehill, HPV-77, DE5, RA27/3 or To-336 (Japanese) vaccines, joint symptoms were detected in up to 42% of vaccinees (Best et al., 1974) and persisted for up to eight days (Tables 9.8, 9).

9.1.5. Strategy for the prevention of rubella syndrome

When the vaccines were licensed in 1969, two strategies for use were discussed, but with a single aim, namely to prevent congenital rubella syndrome (RS). Widespread vaccination of adolescent girls and women would prevent RS, but would not influence virus transmission in the community. On the other hand, routine vaccination of children (boys and girls) early in life would increase herd immunity and interrupt transmission of the disease. The USA adopted the latter policy whilst the UK

TABLE 9.8.
Reactions occurring after vaccination in seroconverted vaccinees and controls (after Best et al., 1974)

	Cendehill	HPV77.DE-5	RA27/3	To-336	Controls
No. of subjects	35	31	36	34	39
Rubelliform rash	3 (8.6%)	1 (3.2%)	9 (25%)	0	1 (2.6%)
Lymphadenopathy	11 (31.4%)	17 (54.8%)	16 (44.4%)	13 (38.2%)	14 (35.9%)
Joint symptoms	8 (22.9%)	12 (38.7%)	15 (41.7%)	6 (17.6%)	0

TABLE 9.9.
Joint symptoms experienced by seroconverted vaccines (after Best et al., 1974)

	Cendehill	HPV77.DE-5	RA27/3	To-336	Total
No. seroconverted:	35	31	36	34	136
Joint symptoms	8 (22.9%)	12 (38.7%)	15 (41.7%)	6 (17.6%)	41 (30.1%)
Arthralgia only	7 (20%)	4 (12.9%)	9 (25%)	2 (5.9%)	22 (16.2%)
Arthritis	1 2.9%)	8 (25.8%)	6 (16.7%)	4 (11.8%)	19 (14.0%)
Multiple joint involvement (three joints or more)	4 (11.4%)	10 (32.3%)	8 (22.2%)	4 (11.8%)	26 (19.1%)
Symptoms lasting seven days or more	0	1 (3.2%)	2 (5.6%)	2 (5.9%)	5 (3.7%)

adopted the former policy. Recent epidemiological modelling studies have shown both countries to have taken the correct decision, because when the fractions of the susceptibles vaccinated is less than 80% it is better to let natural rubella spread (e.g. U.K.), whereas in countries where high rates of immunization are likely to be achieved (e.g. USA) then a policy of eradication can be followed with vaccine given to both boys and girls (Hinman et al., 1983, Anderson and May, 1983). In fact the latter assessment is perhaps rather diplomatic because a more severe critic would pronounce that, whilst the USA policy has at least prevented extensive outbreaks and reduced RS, the UK policy has, up to the present time, done neither.

A summary of the USA experience (Schiff, 1980) has shown the following:

1. the incidence of RS has decreased significantly (Fig. 9.4);
2. the vaccine viruses are not teratogenic;
3. the vaccine of choice may be RA 27/3;
4. herd immunity has not stopped outbreaks, and outbreaks are occurring in unvaccinated groups e.g. young adults;
5. 20–35% of 18–25 year old females remain susceptible.

A much more vigorous campaign is therefore required even in the USA to immunize the susceptible women of child bearing age, and also to raise immunization levels in school children.

Sabin (1981) again takes a rather individual viewpoint by querying whether, in fact, the small number of RS cases justifies a large scale immunization programme. In any case he speculates that a mass immunization campaign over a *short* period of time would be necessary to break the chain of virus transmission.

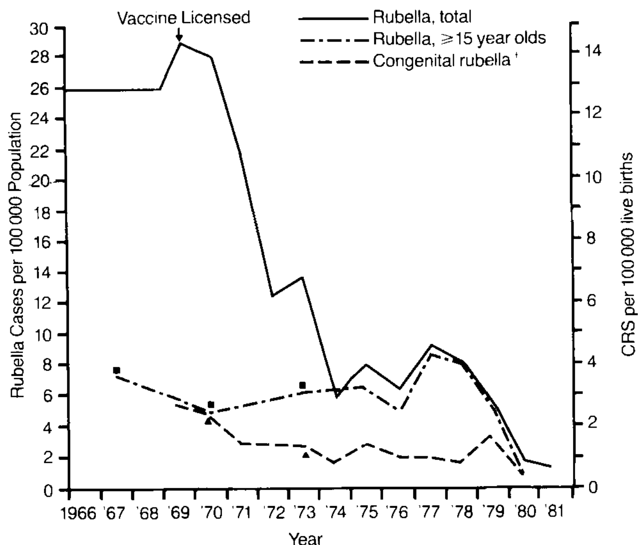

Fig. 9.4. Incidence of reported rubella cases and congenital rubella cases (U.S.A. 1966–81.)

Similarly to measles, since rubella vaccine is a live virus vaccine, it should not be given to patients whose immunity is deficient, whether as a result of disease (e.g. malignancy) or of treatment with corticosteroids, radiotherapy or cytotoxic drugs. Since rubella vaccine may be transmitted transplacentally, pregnancy is a contraindication and should be avoided for three months after vaccination. Examination of the products of conception of rubella-susceptible women vaccinated inadvertently during pregnancy has shown that rubella virus may be recovered from the placenta, kidney and bone marrow. Furthermore, histopathological studies have shown that changes in the placenta, decidua and foetal eye are similar to those occurring in naturally acquired infection. However, clinical and serological follow-up studies carried out on women in the USA, who elected to go to term after inadvertent rubella vaccination in early pregnancy, have shown that none of 277 infants delivered of mothers known to be rubella susceptible and given rubella vaccine within a period ranging from 3 months before to 3 months after conception had major malformations. Many of these infants have been followed up since, and though about 30% have serological evidence of intrauterine infection, none had defects compatible with congenital rubella. The risk of rubella-induced major malformations among infants delivered of susceptible mothers was calculated (based on a 95% confidence limit) as 3.3% – a figure similar to the risk in normal pregnancies (Table 9.10). The United States Immunisation Practices Advisory Committee still recommends that pregnant women should *not* be given rubella vaccine, but now states that inadvertent vaccination should no longer be a reason to recommend termination of pregnancy routinely.

TABLE 9.10.
Consequences of rubella vaccination during pregnancy, US 1969–1982 (MMWR, 32, 1983). Pregnancy outcome in susceptibles going to term

Vaccine	No.	To term	No. vaccinated between one week before to four weeks after conception / No. with date of conception known	Evidence of infection[a]	Abnormalities[b]
HPV77-DE5/ Cendehill	149	94	33/87 (38%)	8/194[d]	0[c]
RA27/3	111	81	28/81 (35%)	1/83	0
A11	260	175	61/168 (36%)	9/277 (3%)	0

[a] IgM present, IgG persisting beyond 6 months or isolation of rubella virus.
[b] Compatible with congenital rubella.
[c] Now aged 2–7 years.
[d] 149 infants whose mothers were susceptible at the time of vaccination and 45 whose mothers were of unknown status.

9.1.6. SEROLOGICAL SURVEYS OF RUBELLA VACCINE INDUCED IMMUNITY

The results of the serological surveys in the UK indicate that the rubella vaccination programme has had an impact on the serological status of the *young adult females* in the study population. It is clear from fig. 9.5 that of women eligible for vaccination at school the proportions who were seronegative were much lower than those for men of the same age, and for older women. The low frequency of seronegative subjects among women born after 1956 was found consistently throughout the studies and relates chronologically to the expected effect of vaccination (Clarke et al., 1983).

In the first 4 years of a blood-donor study the seronegative proportion of women born before 1956 was consistently higher than that for men of the same age (Fig. 9.6). However, in 1980 the percentage who were seronegative of women born before 1951 was lower than that for men of the same age and for women born between 1951 and 1956. A possible explanation for this finding is that this age group included mothers who had been vaccinated at postnatal clinics after the birth of a child. Some indication of the persistence of rubella antibodies after vaccination can be obtained from the results. Women born in 1957 and 1958 would by 1976, the first year of the study, have been vaccinated up to 6 years previously, and by 1980 up to 10 years previously. If it is assumed that the differences in proportions of seronegative subjects between men and women born after 1956 were due to rubella vaccination, then the consequence of a rapid fall in the antibody due to vaccination would be that the proportion of seronegative women would tend to revert to that of men of the same age. There is no evidence that this occurred. Thus, it appears that over this 5-year period the antibody levels persisted from up to 6–10 years after vaccination. Other evidence has indicated that antibody can persist in the majority of vaccinees up to 16 years after vaccination (O'Shea et al., 1982).

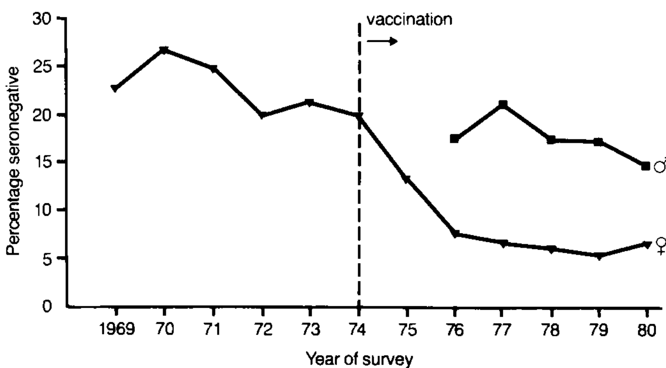

Fig. 9.5. Percentage of students found to be seronegative for rubella antibody in surveys carried out at Nottingham University. Dotted vertical line = earliest year in which student intake would have included females eligible for vaccination at school. (after Clarke et al., 1983.)

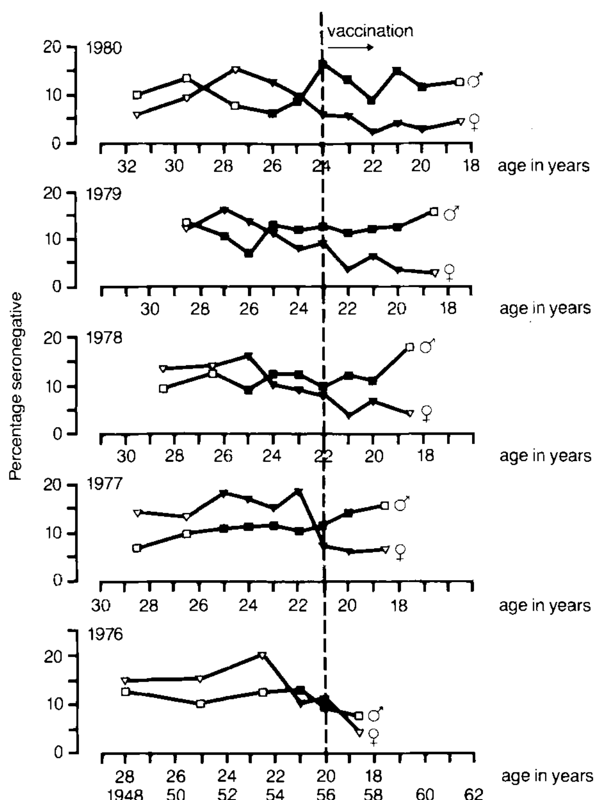

Fig. 9.6. Percentages of young adult blood donors found to be seronegative for rubella antibody in five annual surveys carried out from 1976 to 1980. Dotted vertical line = age group of females offered vaccine at school (i.e., those born from 1956 onwards). Age is based on year of birth. Points to the left of the dotted vertical line represent age groups of females who, because they were born before 1956 were not offered vaccine, points to the right of the dotted line represent age groups of females who were offered vaccination. Open symbols mean that data have been pooled for individuals with consecutive years of birth. (after Clarke et al., 1983.)

Hambling (1980) conducted an 8-year study from 1971 to 1978 in the UK, in which 150 000 serum samples from women of child-bearing age were tested for rubella antibody; he found that the seronegative proportion among women who had been eligible for vaccination at school was lower than that among older women. Other studies have reported similar trends, (Hutchinson et al., 1982) but two studies (Clubb et al., 1981 and Gilmour et al., 1982) failed to find any effect attributable to vaccination; the numbers of serum samples tested in the latter studies were much lower than in the other studies.

Surveys have shown that the frequencies of seronegative schoolchildren under 11 years and of seronegative young adult males in the UK are similar to that reported

before the introduction of vaccine. This finding would be expected because of the selective nature of the vaccination programme. These individuals constitute a susceptible group who could contribute to the spread of rubella in the community. Their influence on the incidence of congenital rubella may be discernible in the future when experience in the UK is compared with that in the USA, where both sexes are vaccinated in infancy (Hinman et al., 1983).

In a survey in 1977, (Peckham et al., 1977) 71% of 16-year old girls were reported to have received rubella vaccine, although uptake varied widely in different parts of the UK. After the rubella vaccination campaign in 1979, the uptake of vaccine increased to 84% in 1980 but it fell short of the 90% aimed at by the campaign. However, extensive local campaigns have achieved uptakes in excess of 90%. Further efforts are obviously needed to increase the uptake of rubella vaccine.

9.1.7. Combined measles–rubella immunization

Serological surveys in certain adolescent and young adult populations have indicated that susceptibility levels to rubella are approximately 15 to 20 percent (see above), not appreciably different from those reported in prevaccine years. Furthermore, adolescents and young adults now account for a high proportion of all reported measles (see Chapter 8) and rubella cases, particularly in the USA, and have the greatest risk of disease. These facts are illustrated by the measles and rubella outbreaks reported in secondary schools, colleges, military installations, and places of employment in the USA. This pattern of disease transmission has led to increased efforts to vaccinate older susceptible persons. Ideally, those in need of vaccination would be identified through a sensitive, specific, readily available, and inexpensive screening technique that provides immediate information. An obvious approach is a history of previous measles and rubella infection or vaccination; however, previous reports have indicated that such information obtained from prospective vaccinees or their parents may not be an accurate predictor of susceptibility. Preblud et al. (1982) in a recent study evaluated the sensitivity and specificity of *histories* of past infection or vaccination and determined the costs and effectiveness of three alternative strategies for vaccinating persons susceptible to measles, rubella, or both: (1) vaccinating *all* persons regardless of past history; (2) serologically screening all persons and vaccinating only those who were susceptible; (3) vaccinating all persons who do not have physician-documented proof of proper vaccination, past infection (measles only), or serological immunity.

These authors found that with few exceptions, any category of history response was associated with a measles susceptibility rate of approximately 5% or less. Maternal histories were statistically no more specific than cadet histories ($P > 0.06$), and identified at most only two thirds of susceptible persons. While both cadet and maternal histories for either infection or vaccination were sensitive (identifying 90% to 95% of all immune subjects), a history of neither was very nonspecific (identify-

ing at most 28% of susceptible persons). Positive histories for previous rubella infection and vaccination were associated with lower susceptibility rates than were negative histories ($P < 0.01$). However, *no* history successfully identified all persons susceptible to rubella. While cadet and maternal histories of rubella vaccination were more specific than other histories ($P < 0.01$), negative histories of vaccinations still failed to identify approximately 10% of the subjects susceptible to rubella. Comparison of discordant responses confirmed that maternal histories were generally no more reliable than cadet histories!

The costs and effectiveness of the three alternative vaccination strategies in a model cohort of 1000 subjects were investigated. Vaccination of *all* persons is the least expensive strategy (both in total cost and cost per susceptible person protected), unless records are available for 75% or more of the population in question, in which case vaccination after record review is less expensive. However, vaccination after record review is associated with a lower proportion of protected susceptible persons compared with the other two strategies, when 75% or more of the potential vaccinees have records (approximately 80% protected vs. 90%). These findings are not altered even if one assumes that records are only 80% accurate or that they are 100% accurate. In general, while there is little difference in the total cost of administering either measles or rubella vaccine alone or both in combination, the cost per susceptible person protected indicates that a combined measles and rubella vaccination programme is highly economical. A combined programme provides a 10% to 15% reduction in cost compared with a rubella-only vaccination programme (with the exception of the serological screening/vaccination strategy, in which case the combined programme costs 4.5% more) and approximately a fourfold to fivefold reduction compared with a measles-only vaccination programme.

9.1.8. Prevention of rubella using immune globulin

Normal immune globulin may prolong the incubation period considerably but since inapparent infection is accompanied by viraemia, foetal damage is not prevented (Forrest and Honeyman, 1973, Polakoff, 1983). However, Peckham (1974) showed that infants of mothers given normal human immune globulin who experienced subclinical rubella in early pregnancy were less likely to be infected in utero than those infants whose mothers were not given immune globulin. It is therefore possible that the administration of normal human globulin reduces the level of foetal infection, damage, or both.

High-titred rubella immune globulin has been used experimentally to determine whether infection induced by rubella vaccine can be prevented (Urquhart et al., 1978). The results were encouraging in that 8 out of 20 volunteers (40%) given high-titre immune globulin and rubella vaccine simultaneously failed to sero-convert, and the remaining 12 exhibited delayed antibody responses compared with volunteers given vaccine alone. This preparation, which is in short supply, is available

via the Scottish Blood Transfusion Service, but has not been properly evaluated in the field. It may be recommended for the few susceptible pregnant women who come into contact with clinical rubella and for whom therapeutic abortion is unacceptable.

9.1.9. Chemoprophylaxis and rubella

Because of the success of the live attenuated rubella vaccines very few studies have been carried out of specific inhibition of rubella virus replication. Early in vitro and in vivo work was initiated in the 1960s with amantadine and other amines but was soon discontinued because of the rather low antiviral efficacy of the molecules investigated. Thus, Oxford and Schild (1965) described the inhibitory effects of amantadine on microplaque formation by several strains of rubella virus (Fig. 9.7 and Table 9.11). Amantadine at 100 μg/ml exerted some toxic effects in the line of rabbit kidney cells used (RK-13), whereas 20 μg/ml amantadine was required to inhibit microplaque formation by 90% – the therapeutic index therefore was low, particularly compared to the in vitro activity against strains of influenza A virus (see Chapter 7). Amantadine was also active as an antiviral in organ cultures of ferret trachea infected with rubella virus strains (Figs. 9.8 and 9.9) but no antiviral effect was noted in prophylactic experiments in laboratory animals (Table 9.11) infected intranasally with rubella virus and given relatively high dosages of the compound (50–100 mg/kg).

Three infants with congenital rubella syndrome were given human leukocyte (al-

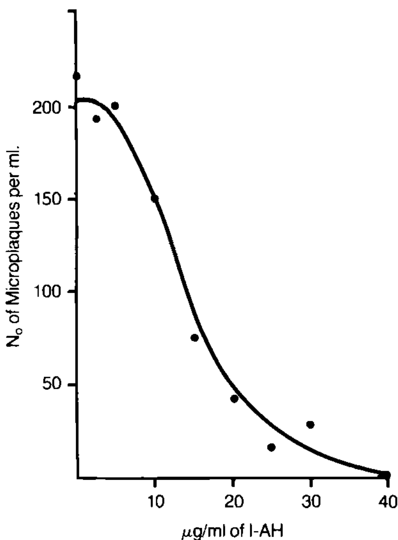

Fig. 9.7. Dose response curve for amantadine and rubella virus.

TABLE 9.11.
Comparison of antirubella virus activity of aminoadamantane (amantadine) in different test systems (after Oxford and Schild, 1965)

Animal species	Inhibition of rubella virus multiplication[a] in:		
	Cell cultures (25 μg/ml aminoadamantane)	Organ cultures (25 μg/ml aminoadamantane)	Experimental animals (50–100 mg/kg aminoadamantane)
Rabbit	0.8–1.0[b]	0.5–1.0 (lung)	0
Ferret	not tested	0.5–1.0 (trachea)	0
Hamster	not tested	0.5–1.5 (lung)	0

[a] $\log_{10}$ $TCID_{50}$/ml reduction of virus titre in cultures treated with aminoadamantane compared to control cultures.
[b] RK-13 cells, continuous rabbit kidney.

pha) interferon at doses of 2×10^5 to 7×10^5 U/kg per day for 10 days, by Arvin et al. (1982). A transient decrease in pharyngeal virus excretion was observed with treatment. No significant side effects were associated with the administration of human leukocyte interferon to these infants (Table 9.12) but no beneficial effect was noted.

9.1.10. Summary

A number of live, attenuated rubella vaccines have been developed empirically and tested over the last two decades, and although all are efficacious, nevertheless, differences in reactivity and immunogenicity have been detected. The rubella vaccine designated RA27/3 (human diploid cell) is now used widely. There appears little or no need for a chemotherapeutic approach or new vaccines, but rather a more intensified vaccination campaign is required so that rubella epidemics and rubella syndrome can be prevented. Much remains to be discovered about genetic and antigenic variation among rubella field viruses, virus structure and biochemistry.

9.2. Reoviridae infections

The family *reoviridae* (the name reo arose from *re*spiratory, *e*nteric, *o*rphan) consists of reovirus, orbivirus and rotavirus as shown in Table 9.13. Viruses from this family seem to infect most humans and vertebrates, and antibodies have been found in all investigated human populations.

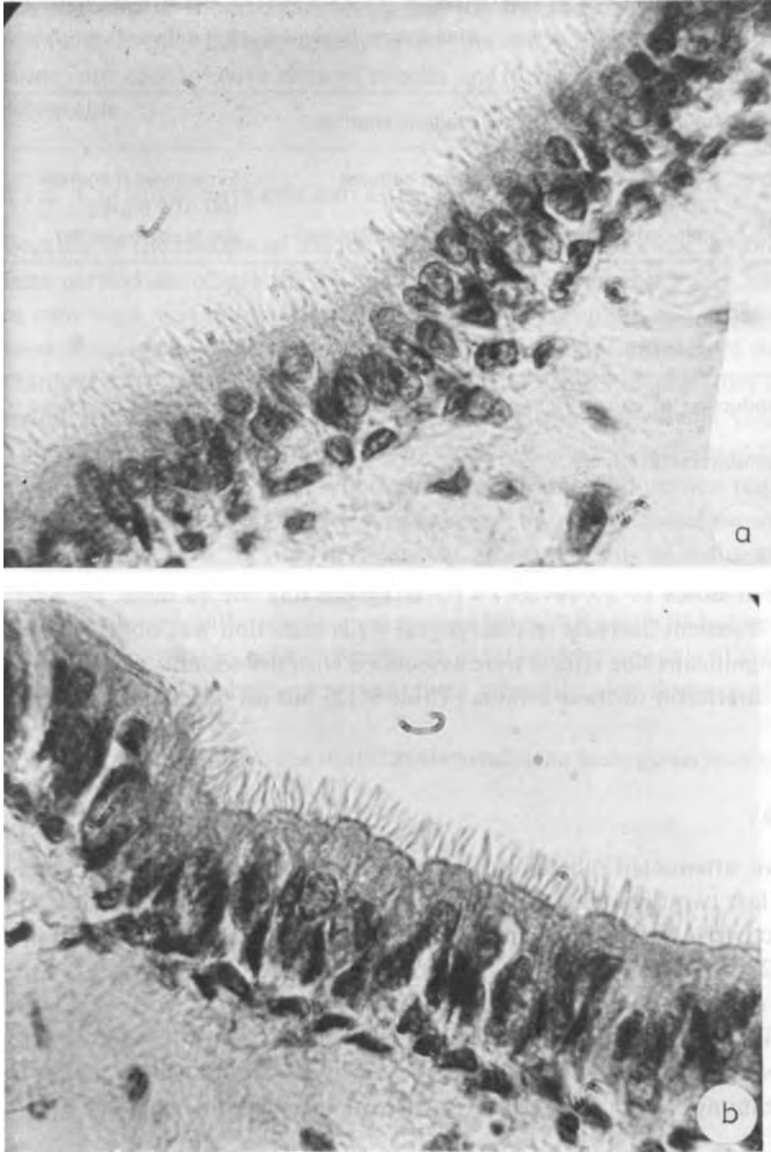

Fig. 9.8. Multiplication of rubella virus in organ cultures of ferret trachea. a, normal trachea; b, rubella virus infected trachea. No overt signs of infection can be seen, but infective virus is released into the medium (see Fig. 9.9.).

9.2.1. THE VIRUSES

The virus particles are 60–80 nm in diameter and contain double-stranded RNA, in reoviruses 10 segments, divided in 3 size classes, with a total molecular weight of 15×10^6, in orbiviruses 10 segments with a total molecular weight of 12×10^6 and

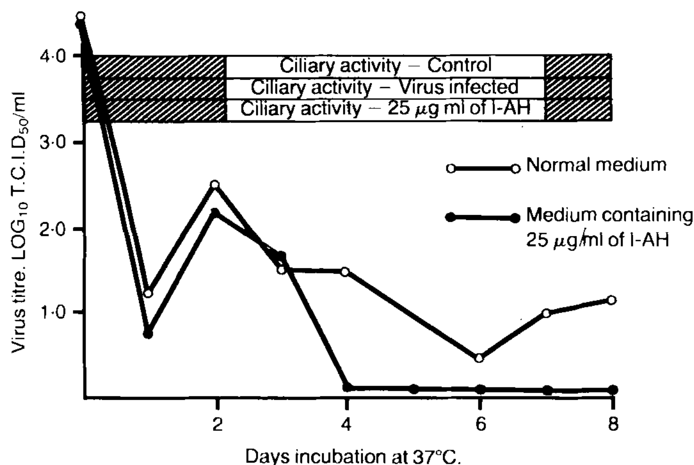

Fig. 9.9. Ferret trachea organ cultures infected with rubella in the presence and absence of amantadine (I-AH).

TABLE 9.12.
Clinical findings in infants with congenital rubella treated with interferon (after Arvin et al., 1982)

Infant	Birth wt (g)	Gestational age (wks)	Status at treatment		Current status	
			Age (mos)	Clinical problems	Age (mos)	Clinical problems
A	1680	34	3	Microophthalmia, cataract, pulmonic stenosis	48	Deafness, impaired vision, delayed growth and development
B	2340	42	5	Cataract, chronic pneumonia, failure to thrive	24	Deafness, chronic pneumonia, delayed growth and development
C	2320	40	3	Microophthalmia, cataract, probable ventricular septal defect	12	Deafness, impaired vision, delayed growth and development

in rotaviruses 11 segments with a total molecular weight of 10×10^6. Fig. 9.10 shows the morphology of the human rotavirus. The particles, which lack an envelope, have icosahedral symmetry, and are made up of two distinct capsid shells. The outer shell can be removed by chymotrypsin treatment to reveal a core. This core contains an RNA dependent RNA polymerase coded by the viral genome and a poly A polymerase activity. The virions also contain 5′-terminal cap-forming enzymes (Yamakawa et al., 1982).

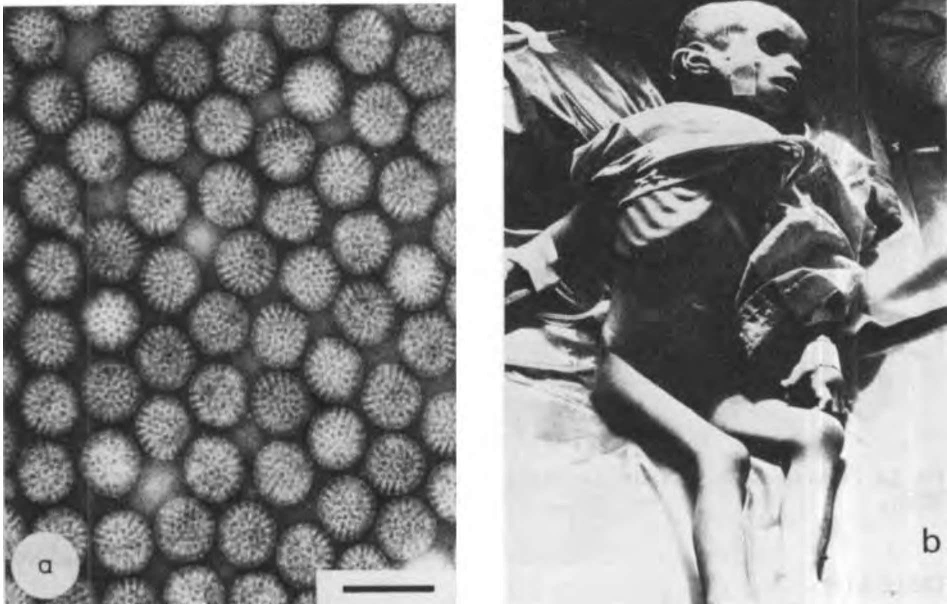

Fig. 9.10. a, Human rotavirus. EM picture of human stool isolate. The bar is 100 nm. (Courtesy of Dr. L. Svensson.). b, Child in a Bangladesh hospital suffering from chronic malnutrition and acute diarrhoeal disease. Development of virus vaccines and also satisfactory nutrition are both urgently required. (From 'World Health', a WHO publication.)

9.2.2. Replication and molecular biology

The virus particles are bound to receptors on the cell, transported into the cytoplasm and uncoated within lysosomes. After removal of the outer capsid by proteolytic enzymes, the core-associated RNA transcriptase enzyme transcribes mRNA from the RNA genome in the virus core. The virus core also contains the viral enzymes necessary for the formation of caps on the viral mRNA (Yamakawa et al., 1982). The cap formation involves the following viral reactions:

1. transcriptase
   pppG + pppC → pppGpC + PPi
2. nucleotide phosphorylase
   pppGpC → ppGpC + Pi
3. guanylyltransferase
   pppG + ppGpC ⇄ GpppGpC + PPi
4. methyltransferase 1
   GpppGpC + Ado Met → m^7GpppGpC + Ado Hey
5. methyltransferase 2
   m^7GpppGpGpC + Ado Met → m^7GpppG^{m}pC + Ado Hey

The viral mRNA does not contain any poly-A sequence at the 3′-terminal.

Reovirus proteins are synthesized and cleaved in the cytoplasm as indicated in Figure 9.11. Synthesis of minus strands to form new double stranded RNA is also carried out by viral RNA polymerase in the cytoplasm using replicative complexes consisting of one of each of the ten or eleven plus stranded RNA molecules (for a review see Joklik, 1980). This detailed knowledge of the replicative events has been established using reovirus and it is not entirely clear if orbi- and rotaviruses differ from this pattern in any respect. However, there seems to be several viral enzymes in the reoviridae family suitable as targets for antiviral drugs.

9.2.3. Clinical aspects

Table 9.13 lists the main syndromes caused by reo-, orbi- and rotaviruses. Reovirus infections in humans seem to result in very mild, or no symptoms. In contrast, rota-

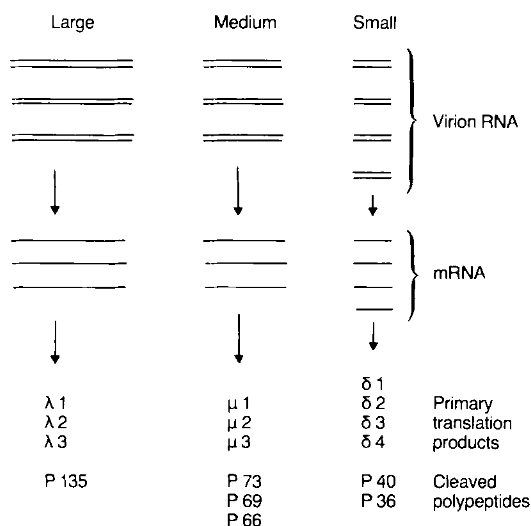

Fig. 9.11. Transcription and translation of reovirus RNA.

TABLE 9.13.
Reoviridae affecting humans

Virus	Disease
Reovirus 1, 2 and 3	Mild fever, diarrhoea and respiratory disease
Orbivirus, Colorado tick fever, and some other tick-borne viruses	Mild fevers
Rotavirus, 4 serotypes, each comprised of sub-groups	Diarrhoea, mainly in infants and young children. Can be severe and is common

viruses are the most common cause of acute gastroenteritis in infants and children, being responsible for about half the cases (Davidson et al., 1975). The symptoms include diarrhoea, fever, abdominal pain and vomiting, resulting in dehydration. This can be severe, especially in third world countries with inadequate medical care, and the result can be a very considerable mortality (see Chapter 1 and Fig. 9.10b). It has been estimated that 3–5 billion cases and 5–18 million childhood deaths occur each year due to rotaviruses in Asia, Africa and South America, making it the single largest cause of disease and death in these areas of the world (Elliot, 1976, Kapikian et al., 1980). Gastroenteritis caused by rotavirus is also found in adults and can be severe in elderly patients (Halvorsrud and Örstavik, 1980).

The orbiviruses, such as Colorado tick fever, cause a mild human disease with fever.

9.2.4. Epidemiology

The faecal-oral route seems to be the main way of transmission of these viruses and the incubation period is 2–4 days. This incubation period has also been found in volunteers challenged orally with rotavirus (Kapikian et al, 1983). In most cases rotavirus infections occur in infants and young children as shown in Table 9.14. A seasonal variation in the prevalence, with the highest figures during the cold season, has been reported in several studies (Middleton, 1982).

High frequencies of antibodies against rotavirus in several populations show that this is a very common infection. About 90% of children at the age of two have antibodies both to type 1 and type 2 human rotavirus (Wyatt et al., 1978) and about 70% of the adult population in industrialized countries have antibodies to rotaviruses (Gust et al., 1977, Middleton et al., 1976). Using a sensitive ELISA method to determine the presence of antibodies to rotaviruses the figures seem to approach 100% in different populations (Ghose et al., 1978, Yolken et al., 1978). The distribution of rotavirus types and subgroups is presently not clear (see Chapter 17 for a discussion of molecular epidemiology).

TABLE 9.14.
Age range of 326 consecutive rotavirus patients in Toronto (after Middleton, 1982)

Patients (No.)	Age groups (%)					
	0–6 months	6–12 months	12–18 months	18–24 months	2–3 yrs	>3 yrs
Patients admitted with enteritis (257)	21.4	40.1	18	5	8.2	7.3
Nosocomial enteritis patients (69)	56	34	6	1	3	–
Total patients (326)	29	39	15	4	7	6

9.2.5. Vaccination and immunotherapy

Different approaches to immunization against rotavirus gastroenteritis have been discussed and the reader is referred to these excellent reviews (Kapikian et al., 1980, McLean and Holmes, 1980, Saulsbury et al., 1980, Chanock, 1981, Kapikian et al., 1981, Wyatt et al., 1981, Kapikian et al., 1983). There are two pieces of information that are important in the context of rotavirus immunization and that we should emphasize here. One is the number of different strains and their antigenic diversity and the other the relative importance of antibodies in serum and in the intestinal fluid. The results of Kapikian et al. (1983) indicate that the presence of serum antibodies could prevent a symptomatic infection by challenge virus. The presence of local neutralizing antibodies in the intestinal fluid has been shown by Davidson et al. (1983) and could also be of importance.

The possibility of using an attenuated live vaccine seems promising and a human type 2 rotavirus strain has been serially propagated in gnotobiotic piglets to yield an attenuated strain WA, which is presently being tested for safety and antigenicity in volunteers (Edelman, 1982, See Chapter 2). Using a live attenuated bovine rotavirus vaccine Vesikari et al. (1984) have recently achieved an 88% protection rate in children during a rotavirus epidemic. The development of a vaccine by hybrid DNA technology or using immunogenic small polypeptides will certainly be investigated against rotavirus in the future.

Passive immunization through antibodies in milk given to infants should also be considered, and is an important argument in favour of breast feeding. Passive immunization has also been used in immunodeficient patients with chronic rotavirus infections (Saulsbury et al., 1980).

When considering the present status of immunotherapy and prophylaxis against rotavirus it should be kept in mind that these viruses have been known only for a short period of time. Vaccination against reo- or orbiviruses seems not to be particularly important in comparison to the very great need for vaccination against rotaviruses, especially in underdeveloped countries.

9.2.6. Chemotherapy

No clinically effective chemotherapy of infections caused by *reoviridae* has been reported. Restoration of electrolyte balance and replacement of fluids by the oral or parenteral route is important in cases of rotavirus infections resulting in diarrhoea.

Ribavirin will, in cell culture, inhibit the growth of rotavirus (Chang and Heel, 1981) but was not active in rotavirus infection in mice (Schoub and Prozesky, 1977). Similar results using ribavirin and some other compounds in cell culture were reported by Smee et al. (1982) and the results are shown in Table 9.15. Although some antiviral effects were shown, none of the compounds or ribavirin mono- and triphosphate had any effect on the rotavirus RNA polymerase in a cell-free assay. Also, these compounds showed very little activity on a murine rotavirus infection in mice.

TABLE 9.15.
Effect of antiviral substances on infectious simian rotavirus yields in MA-104 cells (after Smee et al., 1982)

Drug. conc. μg/ml	Virus yield ($log_{10}CCID_{50}$/0.1 ml)			
	Ribavirin	3-DG	3-DU	(S)-DHPA
10	5.5	4.6	5.5	5.7
100	5.0	2.3	5.5	3.7
1000	3.3	1.7	4.5	2.7

Untreated samples had yields of 5.5–5.7. 3-DG, 3-deazaguanine; 3-DU, 3-deazauridine; (S)-DHPA, 9-(S)-(2,3-dihydroxypropyl)adenine

TABLE 9.16.
Retroviridae

Subfamily	Human viruses
RNA tumour virus group (Oncovirinae)	Human T-cell leukaemia/lymphoma virus (HTLV)
Foamy virus group (Spumavirinae)	Human foamy virus? (not causing tumours)
Meadi/visna virus group (Lentivirinae)	–

The presence of several viral enzymes should make it feasible to synthezise inhibitors and useful animal models are available for rotavirus infections, and so this could be a fertile area for investigation in the future.

9.3. Retrovirus infections

The impact of retroviruses on human diseases is far from clear but they are involved in several animal tumours and probably in some human malignancies as well as implicated in AIDS.

9.3.1. The viruses

The retroviruses can be divided into three main groups as shown in Table 9.16. The unequivocal identification of a human retrovirus (HTLV, see later) is recent although this group of viruses has been studied for many years. The retroviruses contain a dimeric single-stranded viral RNA and a reverse transcriptase enzyme. The enveloped virus particles are 80–100 nm in diameter and have an internal icosahedral capsid containing RNA with a monomeric M.W. of 3×10^6. The viral pro-

teins are glycosylated and the envelope contains lipids derived from the plasma membrane. The virus particles contain both type-specific and group-specific antigens.

9.3.2. Replication and molecular biology

Retrovirus replication starts with an interaction between an envelope glycoprotein and a specific receptor on the cell-surface. After uncoating, the virion RNA is transcribed into DNA by a reverse transcriptase enzyme. Transfer RNA functions as a primer in this process. A linear DNA is formed containing terminal repeats and the dsDNA can circularize. The retroviral DNA integrates into cellular DNA in a way similar to the integration of transposons. Integration seems to be required for virus replication and the integrated viral DNA is transcribed by cellular RNA polymerase II into virion RNA and mRNA. The viral genome contains genes for the group antigen (gag), the reverse transcriptase (pol), the envelope glycoprotein (env) and can also have onc and sarc genes. The integrated viral DNA can enter the germ line and be transmitted as a provirus in a vertical manner.

9.3.3. Clinical aspects

The retroviruses have been associated in animals with leukaemias, lymphomas, sarcomas and carcinomas. In humans a T-cell leukaemia/lymphoma virus (HTLV) has recently been isolated from patients with cutaneous T-cell lymphomas (Poiesz et al., 1980, Poiesz et al., 1981, Miyoshi et al., 1981, Popovic et al., 1982, Reitz et al., 1983, Sarin et al., 1983). The integration of the HTLV genome at the same site in cells from 3 out of 13 patients with mature T-cell leukaemia-lymphoma indicates that integration next to specific cellular genes is important in neoplastic transformations (Hahn et al., 1983). This disease has an aggressive course with poor prognosis and has both skin manifestations and visceral involvement with hypercalcaemia, hepatosplenomegaly and lymphadenopathy. This virus-associated malignancy shows a geographic clustering to parts of the USA, the Caribbean and Japan (Blattner et al., 1983) and the presence of disease is clearly correlated to areas were HTLV infection is prevalent.

No chemotherapy or vaccination against HTLV is presently available but the possible association of HTLV with some cases of AIDS (acquired immune deficiency syndrome) will certainly arouse considerable additional interest (Essex et al., 1983, Gelman et al., 1983, Gallo et al., 1983, Barré-Sinoussi et al., 1983) in prevention and treatment of infections and malignancies caused by HTLV. Several viral enzymes and especially the reverse-transcriptase could be suitable targets for inhibitors. The activity of foscarnet against retroviruses (See Öberg, 1983) gives one example of a drug which should be evaluated in this context.

9.4. Norwalk virus infections

After an outbreak of gastroenteritis (winter vomiting disease) among students and teachers in an elementary school in Norwalk, Ohio, stool filtrates from secondary cases were able subsequently to induce disease in volunteers. By the use of immune electron microscopy (IEM) Kapikian et al (1972) identified the Norwalk agent as 27 nm virus particles. Since then several similar infectious agents have been described.

The classification of the Norwalk group of viruses is uncertain and it has been regarded either as a parvo- or as a calcivirus. The present information seems to favour the latter classification and it is, therefore, discussed in this 'mixed bag' chapter on RNA viruses. The Norwalk group of viruses has recently been the subject of an excellent review by Kapikian et al. (1982).

9.4.1. The viruses

The Norwalk-like agents have a diameter of 25–27 nm, a density of 1.37–1.41 g/cm^3 in CsCl, possess one major capsid polypeptide and have a morphology by electron microscopy similar to that of picorna-, parvo- or calcivirus. A problem with the identification has been that the Norwalk viruses have been impossible to grow in cell culture. An overview of the possible agents included in the Norwalk virus group is given in Table 9.17. This group of agents has been found by EM and IEM in

TABLE 9.17.
Characteristics of Norwalk virus and related agents associated with acute epidemic, nonbacterial gastroenteritis in humans (after Kapikian et al., 1982)

Agent		Induces illness in		
	Size, nm	Humans	Animals	Antigenic relationship
Norwalk	27×32	Yes	No	Distinct
Hawaii	26×29	Yes	No	Distinct
Montgomery County	27×32	Yes	No	Related to Norwalk agent
Ditchling	25–26	Not tested	No	Ditchling and W agents related to each other but not to Norwalk or Hawaii agents
W	25–26	Yes	Not tested	
Cockle	25–26	Not tested	Not tested	Distinct from Norwalk and Hawaii agents
Paramotta	23–26	Not tested	Not tested	Distinct from Norwalk agent
Colorado	27–32	Yes	Not tested	Distinct from Norwalk, Hawaii and Marin county agents
Marin County	27	?	No	Distinct from Norwalk, Hawaii and Colorado agents

None of the viruses has been grown in cell culture.

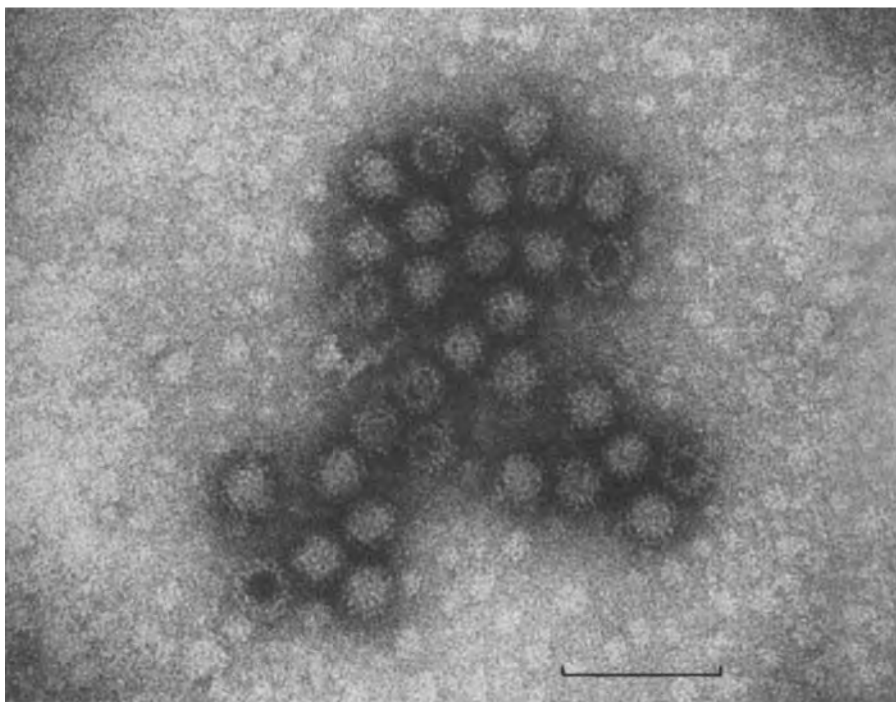

Fig. 9.12. Norwalk-like virus. EM picture from human stool isolate. The bar on the micrograph represents 100 nm. (courtesy of Dr. E.O. Caul.)

stools (Fig. 9.12). Calciviruses, to which group Norwalk virus might belong, contain infectious single-stranded RNA, M.W. $2.6–2.8 \times 10^6$, have a density of 1.36–1.39 g/cm^3 in CsCl, a diameter of 35–39 nm and possess one major capsid polypeptide.

9.4.2. Clinical aspects

The disease induced by Norwalk virus is a mild, self-limiting, gastroenteritis with vomiting and diarrhoea, lasting for 1–2 days. From studies on volunteers the incubation time has been found to be 48 hours and the virus shedding in stools was maximal during the acute illness (Thornhill et al., 1975). The acute infection results in histopathological lesions in the jejunum and a broadening of the villi in the small intestine.

9.4.3. Epidemiology

In healthy adults the prevalence of antibodies against Norwalk virus is between 50 and 90% in different parts of the world (Greenberg et al., 1979). Antibodies are gra-

dually acquired over a period of years in industrialized countries while in developing countries a high prevalence of antibodies is found in children. The acquisition of antibodies against Norwalk virus takes place later than against rotavirus (Black et al., 1982). Contaminated water seems often to be responsible for spreading the virus (Taylor et al., 1981, Baron et al., 1982, Kaplan et al., 1982). Only a short term immunity has been observed in volunteers after a challenge with Norwalk virus although this immunity prevented illness after a second challenge with virus 6–14 weeks later. However, the immunity after one challenge did not prevent illness when the rechallenge was delayed 27–42 months (Parrino et al., 1977). There seems to be no clear correlation between antibody level and development of clinical symptoms after challenge with Norwalk virus (Blacklow et al., 1979, Greenberg et al., 1981). Further studies on the mechanism of immunity and the possible influence of genetic factors are required before immunoprophylaxis can be discussed. No specific antiviral treatment of the disease is presently known.

9.5. Conclusions about reoviridae, retrovirus and Norwalk virus infections

The high incidence of acute gastroenteritis caused by rotaviruses calls for prophylactic and therapeutic measures. Although no vaccine is presently available it seems

TABLE 9.18.
Coronaviruses (see also Wege et al., 1982)

Virus	Host	Disease
Infectious bronchitis virus (IBV)	Chicken	Respiratory disease, nephritis, gonaditis
Murine hepatitis virus (MHV)	Mouse	Hepatitis, encephalomyelitis, enteritis, vasculitis
Bovine coronavirus (BCV)	Cattle	Enteritis
Human coronavirus (HCV)	Man	Respiratory disease
Transmissible gastroenteritis virus (TGEV)	Pig	Enteritis
Haemagglutinating encephalomyelitis virus (HEV)	Pig	Vomiting and wasting disease encephalomyelitis
Probable virus member		
Canine coronavirus (CCV)	Dog	Enteritis
Feline infectious peritonitis virus (FIPV)	Cat	Peritonitis, granulomatous inflammations in many organs
Possible virus member		
Rat coronavirus, sialodacryoadenitis virus (RCV, SDAV)	Rat	Respiratory disease, adenitis
Turkey coronavirus (TCV)	Turkey	Enteritis
Porcine epidemic diarrhoea virus (PEDV)	Pig	Enteritis

likely that vaccines will be developed in the next few years. There are also several rotavirus enzymes useful as targets for antiviral drugs. However, no antiviral drugs have shown therapeutic effects against rotavirus infections.

The newly discovered human retrovirus (HTLV) has not yet been investigated in such detail as to predict the usefulness of vaccine or antiviral drugs. Several compounds are known to inhibit other retrovirus enzymes but the implication of this for chemotherapy of HTLV infection is unknown at present.

The possibility and need for vaccination or chemotherapy against Norwalk virus and related agents is unclear.

9.6. Coronaviruses

Coronaviruses are a group of eleven pleomorphic, positive stranded, RNA-containing enveloped viruses (reviewed by Siddell et al., 1983, Tyrrell et al., 1968, Robb and Bond, 1979) infecting humans, animals and birds (Tables 9.18, 19). Human strains mainly infect the respiratory tract and are confined, normally, to the ciliary epithelium of the trachea, nasal mucosa and alveolar cells of the lungs.

9.6.1. Virus structure and replication

Coronavirions are pleomorphic, 60 to 220 nm in diameter and have club-shaped surface projections about 20 nm in length (Fig. 9.13). In thin sections the virion envelope may be visualized as inner and outer shells separated by a translucent space. The internal ribonucleoprotein (RNP) component of coronavirions has been visualized as a long strand of 1 to 2 nm diameter, or as a helical RNP condensed into coiled structures of varying diameter, normally 10 to 20 nm.

The coronavirion nucleocapsid contains a non-glycosylated protein of 50K to 60K M.W. This protein is phosphorylated and purified Murine Hepatitis Virus (MHV) virions have been shown to contain a protein kinase activity. Coronavirions contain two major envelope proteins. The matrix protein is a transmembrane glyco-

TABLE 9.19.
Antigenic relationships of coronaviruses

	Group 1	Group 2
Mammalian	HCV 229E and other isolates	HCV OC43 and other isolates
	TGEV (1 serotype)	MHV (many serotypes)
	CCV (1 serotype)	RCV (SDAV) (1 serotype)
	FIPV (1 serotype)	BCV (1 serotype)
		HEV (1 serotype)
Avian	IBV (at least 8 serotypes)	TCV (1 serotype)

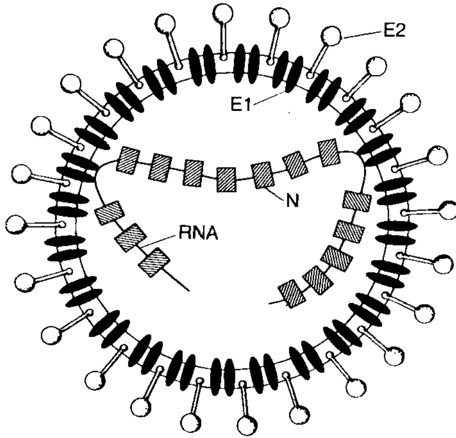

Fig. 9.13. A model of coronavirion structure. Genome (RNA), nucleocapsid protein (N), matrix protein (E1) and peplomer protein (E2) are shown. (after Siddell et al., 1983.)

protein of 20K to 35K. The glycosylated region of the protein is exterior to the virion envelope and in many cases matrix proteins with different degrees of glycosylation are incorporated into virions. The second corona virion envelope protein, which constitutes the surface peplomer, is responsible for eliciting neutralizing antibodies during infection and is thus vital for incorporation into any vaccine (Schmidt and Kenny, 1981). In many cases different molecular weight forms (80K to 200K) of the protein are incorporated into virions. The protein is acylated and complex and mannose-rich carbohydrate side-chains are N-glycosydically linked to the polypeptide. Virions grown in cells treated with tunicamycin lack the peplomer protein and are unable to attach to cells or initiate infection. There are indications that for some coronaviruses proteolytic processing of the peplomer protein occurs during morphogenesis and may be involved in activating functions such as virus-induced cell fusion (Collins et al., 1982).

The coronavirus genome is a linear molecule of single-stranded RNA, which is polyadenylated and infectious. The genome RNA has a M.W. of 5×10^6 to 7×10^6, corresponding to about 15000 to 20000 nucleotides (Fig. 9.14). T_1-resistant oligonucleotide fingerprinting of genome RNA and intracellular viral mRNA confirms the positive polarity of the genome and indicates that it does not have extensive sequence reiteration (Macnaughton and Madge, 1978, Siddell et al., 1983).

As regards early events of virus-cell interaction, Patterson and Macnaughton (1981) have shown that virions initially attach over the whole cell surface but are then rapidly redistributed away from the cell periphery by an energy-requiring process. The reason for this redistribution is unknown. Krzytyniak and Dupuy (1981) have shown that MHV3 uptake into cells is rapid and temperature-dependent. Uptake is not related to the phagocytic capacity of the cells and may, therefore, involve a mechanism such as receptor-mediated endocytosis, as has been reported for other

virus-cell systems (Helenius et al., 1980, and see Chapter 7 for example). The essential features of virus genome replication are: (i) the expression of coronavirus information in the cell is mediated through multiple subgenomic mRNAs, which form a 3′ co-terminal nested set; (ii) as far as is known, each mRNA directs the translation of only one protein; (iii) the size of the translation product for each RNA corresponds approximately to the coding potential of the 5′ sequences which are absent from the next smallest RNA. Although it has not been proven, these features and the inability of ribosomes to initiate translation at internal sites on eukaryotic mRNA suggest that only the 5′ sequences of each mRNA (depicted as genes A, B, C, etc. in Fig. 9.14) are translated into protein. This strategy has many parallels with the strategies of other positive-stranded RNA viruses (see Chapter 4). Also, the coronavirus strategy appears to be a flexible one, allowing for the control of viral protein synthesis at the levels of both transcription and translation. (For additional details see Stern and Kennedy, 1980).

In general, the smallest RNA, RNA7, encodes the intracellular nucleocapsid polypeptide (60K). The next smallest, RNA6, encodes the matrix protein polypeptide (23K) in vitro, or its glycosylated counterpart (25K) in oocytes, and the third major intracellular RNA, RNA3, encodes the peplomer protein core (120K) in vitro or the co-translationally glycosylated peplomer precursor (150K) in oocytes. The translation products of two further MHV RNAs, RNA2 and RNA4/5, have been identified as corresponding to 30K and 14K to 17K intracellular viral polypeptides, respectively. Finally, morphogenic studies on the maturation of coronaviruses have revealed that virus assembly is restricted to the cytoplasm, where progeny virions are formed by a budding process from membranes of the rough endoplasmic reticulum. The virions acquire their lipid envelope from the cells, excluding host cell proteins in the process, and are subsequently transported through and accumulate in the Golgi complex and smooth walled vesicles. There is an absence of budding from the plasmalemma (Beesley and Hitchcock, 1982, Ducatelle et al., 1981, Holmes et

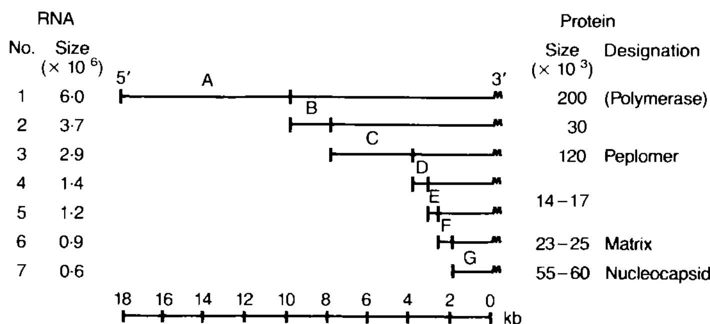

Fig. 9.14. The structure and expression of the murine hepatitis (coronavirus) genome. (after Sidell et al., 1983.)

al., 1981, Massalski et al., 1981) but the precise mechanism of virus release, as with many other viruses, has not been elucidated to date.

9.6.2. Prevention of human disease

Very little work has been carried out to date with *human* coronaviruses, either from the point of view of vaccine development or specific antivirals. Both approaches may be usefully investigated in the future. Genetic cloning may be particularly useful for development of inactivated vaccines, since the virus itself would be difficult to replicate and purify in large quantities for conventional vaccines.

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CHAPTER 10

Parvo, papova and adenovirus infections

10.1. Parvoviridae

10.1.1. The virus

The *Parvoviridae* is a family composed of the smallest, diameter 18–27 nm, animal DNA viruses. It is divided in three genera called parvovirus, densovirus (infecting insects) and adeno-associated virus (AAV). The genome is a single stranded, infectious DNA of M.W. $1.5–2.5 \times 10^6$ surrounded by probably 32 capsomers composed of three different polypeptides. The virus particles have no envelope and are resistant to heat and to solvents like ether and chloroform, and are generally stable at pH 3–9. Parvoviridae can be subdivided into autonomous and defective viruses. AAV are defective viruses requiring adenovirus as a helper for viral multiplication and have been isolated from adenovirus preparations (Hoggan et al., 1966). Defective virus seems to contain complementary DNA strands in different virus particles, while autonomous virus only contains the DNA strands complementary to virus mRNA. The properties of parvoviridae have been reviewed by Tattersall and Ward (1978).

10.1.2. Molecular biology and replication

The multiplication of *Parvoviridae* requires either that the cells are in S-phase (autonomous parvovirus) or the presence of a helper DNA virus (defective parvovirus). Adenovirus and, to some extent, herpes simplex virus can function as helpers. The dependence on S-phase or helper virus indicates that the small parvovirus genome

is heavily dependent on cellular functions for its replication. After adsorption, which is pH-dependent, virus is transported to the cell nucleus for replication. Virus DNA replicates in the nucleus and has palindromic ends allowing hair-pin structures to be formed. These hairpin structures serve as primers for the initiation of replication and are later cleaved by an endonuclease. About 90% of the genome is transcribed into RNA, which is cleaved and spliced giving several mRNA species with partly identical sequences. AAV gives rise to virion polypeptides with M.W. of 91K, 72K and 60K. The AAV infected cell also contains polypeptides of M.W. 25K and 16K. The combined M.W. of the polypeptides exceeds the non-overlapping coding capacity of the genome and this is explained both by the fact that the smaller polypeptides are cleavage products of larger polypeptides and that there are overlapping reading frames in the genome both for defective and nondefective parvoviruses.

A DNA dependent DNA polymerase has been reported to be present in one parvovirus (Kilham rat virus) although a later study failed to find this enzyme activity. Finally, a structural protein has been implicated as necessary for viral DNA synthesis, but no enzymatic functions have been conclusively ascribed to Parvoviridae. Ward and Tattersall (1978) have reviewed the replication of parvoviruses, and this paper should be consulted for further details.

10.1.3. Clinical aspects

Members of the Parvoviridae family cause severe diseases in a number of animal species. A common feature of these infections is a destruction of rapidly proliferating cell populations resulting in foetal and neonatal abnormalities (Kilham and Margolis, 1975, Margolis and Kilham, 1975). In man, antibodies to AAV-2 and 3 have been found in 30% of a child population. AAV-2 has been isolated as frequently as adenovirus from cases of pharyngoconjunctival fever in small epidemics but no particular clinical syndrome has been ascribed to AAV. Serological methods using haemagglutination are available for the identification of different AAV types.

A parvovirus-like virus has been associated with aplastic crisis in children with sickle cell disease and also inhibits haematopoietic colony formation in vitro (Mortimer et al., 1983). A parvovirus has also been implicated in erythema infectiosum (fifth disease) (Anderson et al., 1983). Antibodies to this virus are present in 30–45% of healthy adults. It seems likely that the implications of this virus and other parvoviruses in human disease are underestimated at present. Their teratogenic potential in humans is unknown. Table 10.1 lists parvoviruses isolated from humans.

10.1.4. Vaccines

No vaccine against any member of the Parvoviridae family is available for human use. Vaccines for use in animals have been described, e.g. against feline panleucopenia virus (cat distemper virus).

TABLE 10.1.
Human parvoviridae

Virus	Source	Reference
AAV-2	Ad 12 stock	Hoggan et al. (1966)
AAV-3	Ad 7 stock	Hoggan et al. (1966)
H-T virus ?	Human placenta	Toolan (1968)
H-B virus ?	Human placenta	Toolan (1968)
Aplastic crisis	Sickle cell disease patients	Mortimer (1983)

10.1.5. Antiviral agents

No antiviral agent against Parvoviridae has been described to date. The dependence of cellular or helper virus DNA replication makes it possible that nonspecific DNA polymerase inhibitors might inhibit the virus. The absence of viral enzymes limits the possibility of finding selective antiviral drugs against parvovirus multiplication to viral functions such as adsorption and assembly.

10.2. Papovaviridae

10.2.1. The virus

The family Papovaviridae can be divided into the two genera papillomavirus and polyomavirus. The virus particles (Fig. 10.1) have a diameter of 45–55 nm, do not have an envelope and contain a double-stranded supercoiled circular infectious DNA with a M.W. of $3–5 \times 10^6$. The virus morphology is icosahedral and negative staining reveals 72 capsomeres, composed of three structural proteins. The virus particle also contains cellular histones bound to viral DNA. The infectivity of the virus particle is ether-resistant, acid-resistant and thermostable. Table 10.2 lists papillomaviruses infecting humans and causing different types of warts. The polyomaviruses found in humans are listed in Table 10.3 and constitute SV40 (Simian virus 40), BK virus (human urine) and JC virus (human glia cells). The SV40 virus has been a contaminant in millions of polio vaccine doses but this has not resulted in any clinical illness (See Chapter 4).

10.2.2. Molecular biology and replication

Normally, infection of permissive cells by papovaviruses leads to a lytic infection, while an abortive infection is seen in non-permissive cells. Cell transformation can also be a result of infection. After adsorption and penetration into the permissive cell, viral DNA is dissociated from the capsid and transported together with its bas-

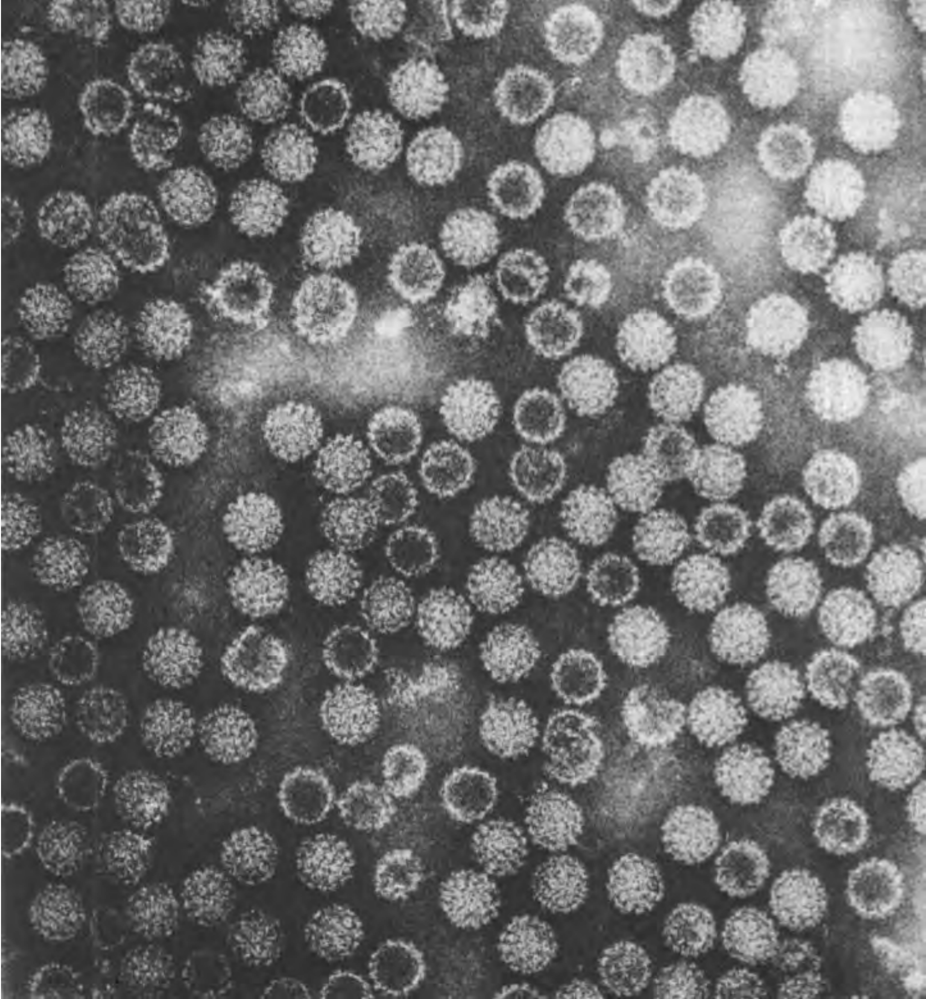

Fig. 10.1. Papilloma virus. The papilloma viruses have similar structures and this EM picture shows purified European elk papilloma virus with a diameter of 55 nm in negative staining. (Courtesy of Dr. J. Moreno-López.)

ic proteins to the nucleus. The circular covalently closed viral DNA is transcribed in the nucleus as exemplified in Fig. 10.2 for SV40, which is the best known of the papovaviruses. A similar pattern has been delineated for polyoma virus. The positions on the genome are related to the cleavage point for the restriction enzyme EcoRI. The two early mRNAs coding for t and T (tumor antigens) are transcribed from the negative strand and contain a common sequence. Two other early proteins are also found, namely U and TST. The early mRNA is formed by splicing as well as the late mRNAs, coding for the structural proteins VP 1, VP 2, and VP 3. It

TABLE 10.2.
Human papillomavirus (HPV)

Syndrome	Virus
Common wart	HPV-1 (plantar wart)
(Verruca vulgaris)	HPV-2 (hand wart)
	HPV-4 (mosaic wart)
	HPV-7 (butcher wart)
Flat wart	HPV-3 (juvenile flat wart)
(Verruca plana)	
Epidermodysplasia	HPV-3, HPV-5, HPV-8
(Verruciformis)	
Genital wart	HPV-6
(*Condyloma acuminatum*)	HPV-11
Laryngeal papilloma	HPV-11
Cervical carcinoma	HPV-6, HPV-11, HPV-16
Epithelial hyperplasia	HPV-13

After Zur Hausen et al., 1981, Gissmann et al., 1983, Pfister et al., 1983, and Dürst et al., 1983.

TABLE 10.3.
Human polyoma viruses

Virus	Source
JC virus	Progressive multifocal leukoencephalopathy (Padgett and Walker, 1973)
BK virus	Urine from transplant patients (Wright et al., 1976)
SV40 ?	Normally monkeys, but has been isolated from progressive multifocal leukoencephalopathy (Weiner et al., 1972) and meningiomas (Weiss et al., 1976) in man

should be noted that this transcription occurs on the plus strand of the genome and that a sequence at position 94–96 has an overlapping code reading in two different phases. The late proteins are synthesized when viral DNA synthesis starts at 15 h after infection. Cellular DNA synthesis is stimulated during the early phase of the infection.

A cellular DNA polymerase is used for SV40 replication and the T antigen initiates replication at position 67 (ori) and replication proceeds in two directions. Continuous protein synthesis is required for viral replication. Assembly of viral DNA and proteins takes place in the nucleus and leads to lysis of the cell. Integration of viral DNA into cellular DNA can result in transformation.

Several cellular enzymes are involved in the replication of the papova viruses, whilst a protease and a protein kinase have been implicated as viral enzymes (Friedmann, 1976, Smith et al., 1979). An increased arginase activity has been observed in warts but there is no indication that this is due to a viral enzyme.

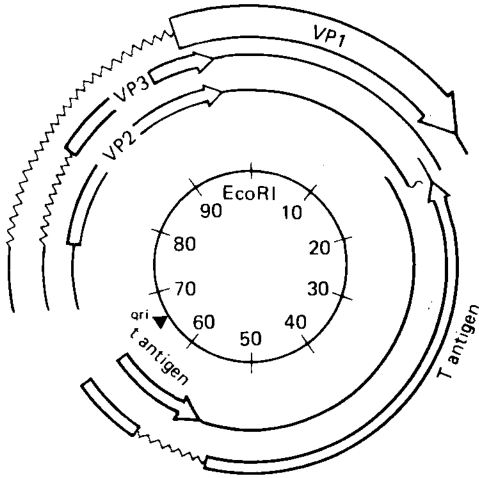

Fig. 10.2. The SV40 genome and the gene products. The origin of replication is denoted ori. Early genes are transcribed into t and T antigens. Late genes are transcribed from the other DNA strand and after splicing the mRNAs are translated to the capsid proteins VP 1-3. The genes for VP 2 and VP 3 overlap and the gene for VP 1 partly overlapping VP 3 is read in another phase.

10.2.3. Clinical aspects

Progressive multifocal leukoencephalopathy (PML)

This is a rare subacute demyelinating disease, which has a world-wide distribution, the patients being mostly middle aged. In most cases the patients have an underlying immunosuppressive disease such as Hodgkin's disease. The disease starts with paralysis, mental deterioration and sensory abnormalities and the patients' condition deteriorates further and death is common in 3–6 months. Papovavirus or viral DNA has been recovered in tissues from PML patients. In most cases the virus isolated has been JC virus, but in a few cases SV40 has been isolated. BK virus has not yet been associated with PML (see Johnson, 1982). It is not unlikely that human papovaviruses are involved in the aetiology of some tumours.

Acute respiratory tract disease

A mild respiratory illness has been recorded coinciding with the appearance of antibodies against BK virus (Goudsmit et al., 1982).

Warts

Different types of human papilloma virus (HPV) induce the formation of several types of warts (Figs. 10.3, 4, 5) which are benign tumours of skin and mucous membranes (Table 10.2). The infection occurs through minor abrasions in the skin and

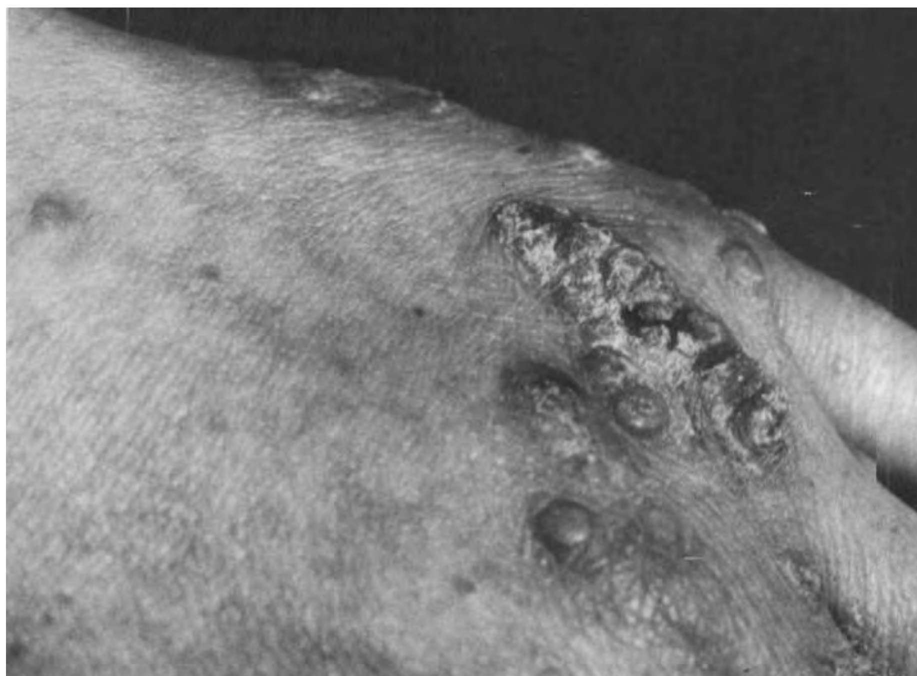

Fig. 10.3. Common hand warts. (Courtesy of Dr. J. Wallin.)

the incubation period is 6 weeks to 8 months. A proliferation of cells in the epidermis or mucosa occurs and virions are found in basophilic inclusions. Large vacuolated cells are found in the stratum granulosum. The warts can persist foı months and the rate of regression seems to be dependent on both humoral and cel mediated immunity. There is possibly a connection between papilloma virus anc genital cancer (Zur Hausen et al., 1981).

10.2.4. Epidemiology

Antibodies to BK and JC viruses are very common and about 70% of all humar sera are positive for these viruses (Gardner, 1973, Padgett and Walker, 1973). BK virus infections were present in 8% of patients with upper respiratory disease anc has been isolated in a high frequency from children with recurrent attacks of acut(respiratory disease (Goudsmit et al., 1982). Reactivation of BK virus in immunosuppressed patients with antibodies prior to virus excretion indicate that the viru! could exist in a persistent state. Recent studies have shown nonintegrated JC viru! DNA to be present in kidney tissue (Dörries and ter Meulen, 1983) and in severa organs of patients with progressive multifocal leucoencephalopathy (Grinnell et al.

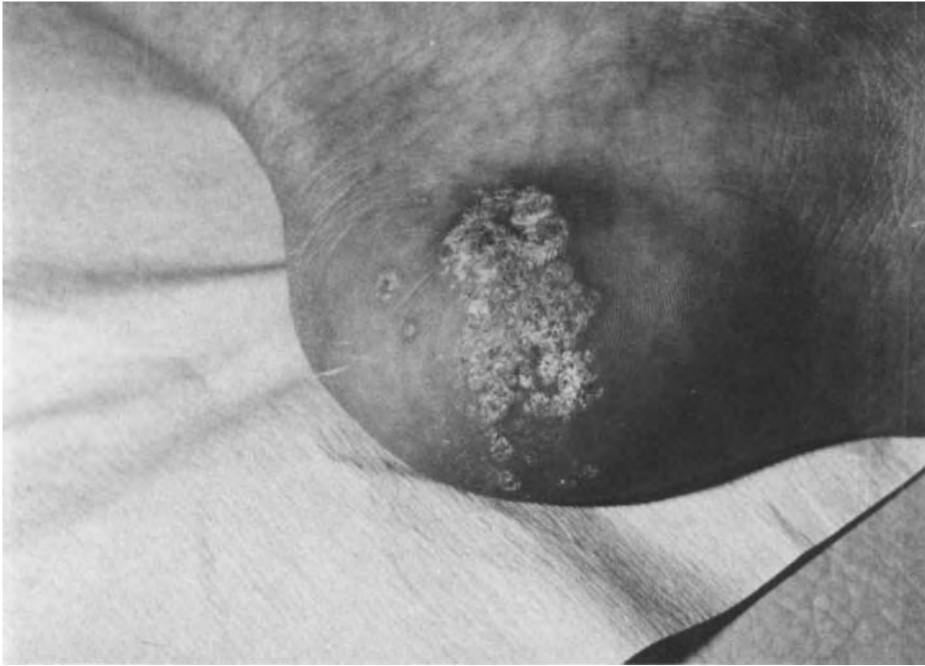

Fig. 10.4. Common foot warts. (Courtesy of Dr. J. Wallin.)

1983). BK virus DNA has also been shown to persist at a high frequency in kidneys of healthy adults (Chesters et al., 1983).

Common warts (Figs. 10.3 and 4) occur in 7–10% of the population (Sanders and Stretcher, 1976) and are common in school children. Genital warts (condylomata acuminata, Fig. 10.5) are common and comprise 6.1% of all reported cases of venereally transmitted diseases in British statistics (Sexually transmitted disease surveillance, 1978). Laryngeal papilloma is a childhood disease and is possibly caused by a perinatal infection from mothers with genital warts (Quick et al., 1978, Gissmann et al., 1983). The human papillomaviruses HPV-6, HPV-11 and HPV-16 have been associated with cervical carcinomas and HPV-13 with focal epithelial hyperplasia (Pfister et al., 1983, Dürst et al., 1983).

10.2.5. Vaccination

Vaccination with autologous wart homogenates has been claimed to be efficacious in patients with different types of warts (Nel and Fourie, 1973, Brandt et al., 1980, Abcarian and Sharon, 1982) but as for many other modes of wart therapy well controlled studies are lacking and no conclusions can be drawn. Several of the vaccines used have been very crude preparations and were not free of DNA. The use of wart

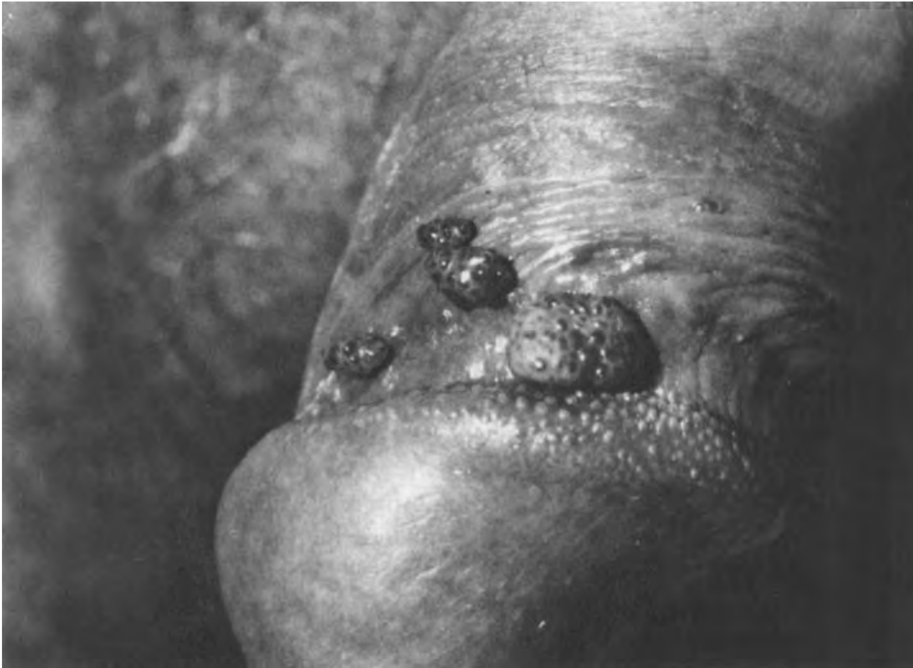

Fig. 10.5. Genital warts (condyloma.) (Courtesy of Dr. J. Wallin.)

virus as a vaccine should preferably not be continued due to the oncogenic potential and a subunit vaccine could be tried in placebo-controlled studies to clarify the possible protective effect.

10.2.6. Interferon and immunomodulators

Leukocyte interferon has been given to children with laryngeal papillomatosis and the results from two studies indicate that the treatment has therapeutic benefits (Haglund et al., 1981, Goepfert et al., 1982) but dosing, the type of interferon used and combination with different types of surgery remains to be optimized and will be facilitated by the increased availability of interferon.

Placebo-controlled trials using levamisole (Schou and Helin, 1977) or transfer factor (Stevens et al., 1975) have failed to show statistically significant effects. Dinitrochlorobenzene has been used as a topical medication to induce sensitization, but the claimed efficacy (Goihman-Yahr et al., 1978, Russo et al., 1975, Eriksen, 1980, Sanders and Smith, 1981) needs to be evaluated in double-blind placebo-controlled trials and the mutagenic potential of dinitrochlorobenzene should be kept in mind.

10.2.7. CHEMOTHERAPY

There is no selective antiviral drug against any papova or papilloma virus. The development of such a drug will be difficult because of the absence of suitable viral enzymes or other viral targets and the absence of cell cultures for papilloma virus growth. However, a vast number of medications have been used for centuries against warts. The psychological component in these older established treatments seems to prevail still in today's treatments. In most cases the therapy involves surgical excision, cryotherapy, trichloroacetic acid, salicylic acid or podophyllin (Bunney, 1977, Sanders and Stretcher, 1976, Rees, 1980, Tring, 1981, von Krogh, 1981) but there is not sufficient data from double-blind placebo-controlled or comparative studies to evaluate the relative merits of the different treatments. The most commonly used medication for genital warts, podophyllin, in a large double-blind study showed that only 22% of the patients were free of warts in three months and there was no difference in the therapeutic efficacy of 10% and 25% podophyllin (Simmons, 1981). It has been suggested that a more frequent use (twice daily for 3 days) of 0.5% podophyllin in ethanol should be the treatment of choice (von Krogh, 1981) but comparative double-blind trials will be necessary to establish this. Salicylic acid paint seems to be the first choice against warts on dry skin. Neither of these treatments are specifically aimed at any viral function. The viral protease and protein kinase have not been utilized as targets for antiviral drugs. Phosphonoacetic acid was effective on rabbit papilloma but this could have been due to non-specific skin toxicity (Friedman-Kien et al., 1976). Phosphonoacetic acid has not been tested on human warts.

10.3. Adenoviridae

10.3.1. THE VIRUS

Adenovirus was first isolated from adenoid tissues (tonsils) and grown in cell culture by Rowe et al. (1953). More than thirty immunologically distinct types of adenoviruses cause infections in humans. The structure and the proteins forming an adenovirus particle are shown in Fig. 10.6. The viral genome is linear double-stranded DNA with a molecular weight of $20–23 \times 10^6$. The base composition (GC-content) differs considerably between different strains.

10.3.2. MOLECULAR BIOLOGY AND REPLICATION

Both strands of the viral DNA are transcribed leading to a sequential formation of early and late mRNA coding for early and late proteins as shown in Fig. 10.7. The primary transcripts are spliced and polyadenylated to form functional mRNA's in several steps as outlined in Fig. 10.8.

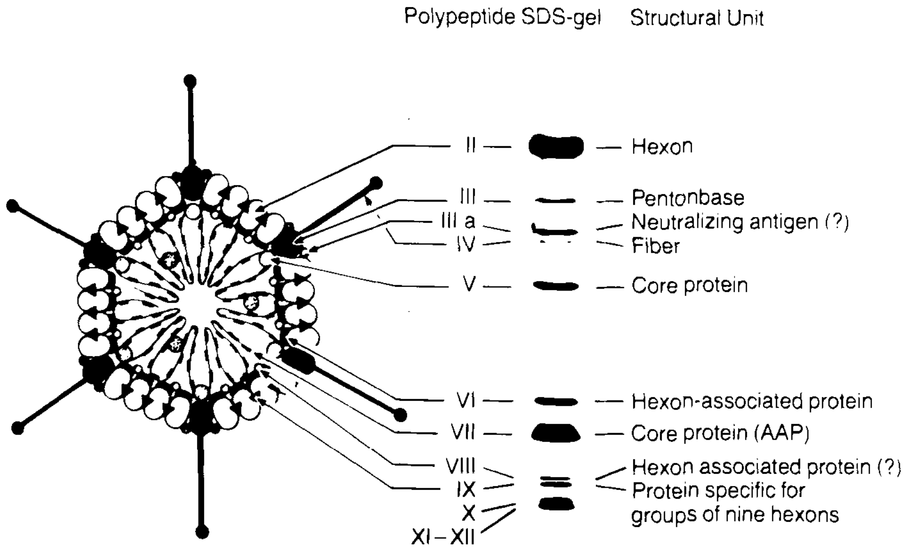

Fig. 10.6. Structure of adenovirus type 2. (Courtesy of Dr. U. Pettersson.)

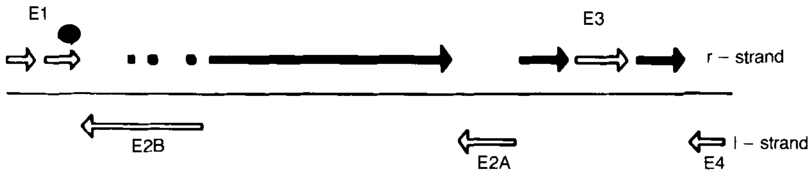

Fig. 10.7. Topography of the adenovirus genome. E1-4 denotes where early proteins are coded and the black areas denote the genes for late proteins. (after Pettersson, 1981.)

The DNA is terminally inverted and redundant resulting in the possibility of the single-stranded DNA forming circles. The replication of the linear DNA takes place using a viral DNA polymerase and viral proteins participate as shown in Fig. 10.9. One strand in the viral DNA is displaced by a newly synthesized strand. The displaced single strand forms a circle with a panhandle structure, which functions as the starting point for the formation of a complementary strand. In this way the synthesis of the two strands occurs independently. The total replication for adenovirus takes about 20 hours in cell culture. A large excess of viral proteins is formed in the infected cell.

The molecular biology of adenovirus transformation has recently been reviewed by Pettersson and Akusjärvi (1983). Adenoviruses have an oncogenic potential and can induce tumours in animals but have not been shown to be associated with any human tumour.

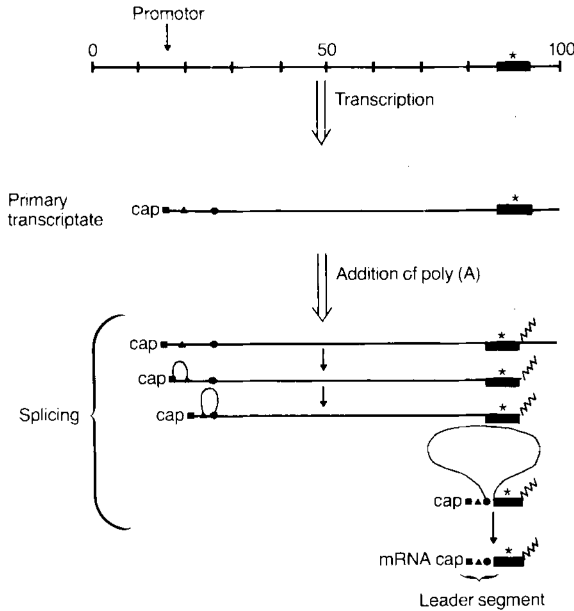

Fig. 10.8. Synthesis of late adenovirus mRNA. The black bar denotes the sequences of the viral DNA coding for the viral protein. A cap structure and a leader segment on one end and a poly A tail (ʌʌʌ) on the other end of the translated part of the mRNA is the result of the processing. (after Pettersson, 1981.)

10.3.3. Clinical aspects

Most children are infected with adenoviruses early in life but probably fewer than 50% of these infections result in disease. The frequency of symptoms appears to be dependent on the type of adenovirus and type 2 for example seems to result in a low percentage of symptomatic infections (Brandt et al., 1969). The symptoms in small children are often a stuffy nose and cough whereas in older children pharyngitis is common. Infections caused by adenovirus type 7 can be severe and even fatal in small children and in older children may cause high fever, pneumonia and abdominal symptoms (Wadell et al., 1980).

Acute respiratory disease in military recruits is caused especially by adenovirus types 4 and 7 and this is characterized by fever, pharyngitis, pneumonia and malaise lasting for about 10 days. Epidemic keratoconjunctivitis is often caused by adenovirus types 8 and 19, but can also be due to other strains such as type 37 (Keenlyside et al., 1983), and has been called 'shipyard eye'. The symptoms are summarized in Table 10.4. Enteric types of adenovirus can be an important cause of acute gastrointestinal disease (Yolken et al., 1982). The association of different adenovirus serotypes with clinical symptoms is shown in Table 10.5.

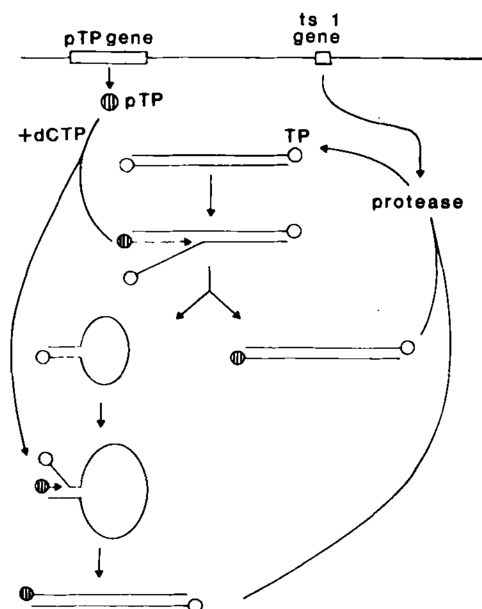

Fig. 10.9. A model for replication of adenovirus DNA. The precursor for the terminal protein (pTP) forms an initiation complex with dCTP. A new DNA strand is formed by strand-displacement. The displaced single strand forms a circular structure with a pan handle identical to the ends of the original dsDNA. This structure is recognized by a new initiation complex and the second strand is formed by complementary strand synthesis. A protease, presumably viral, cleaves the precursor protein into a 55 K protein linked to the DNA. (after Pettersson and Akusjärvi, 1983.)

10.3.4. Epidemiology

A fecal-oral transmission is of importance in young children (Fox et al., 1969). During an acute infection, virus is excreted from the respiratory tract and later an excretion by the faecal route can occur, at least intermittently, for more than a year. Adenovirus is frequently (more than 50% of 86 samples) found in surgically removed tonsils (Evans, 1958). About 7% of infants with respiratory disease were infected with adenovirus, mainly of types 1, 2, 3, 5, 6 and 7 (Brandt et al., 1969).

In military recruits epidemics peak 3–6 weeks after the onset of training and can reach morbidity rates as high as 6–17 percent per week in the USA (Dingle and Langmuir, 1968) and a respiratory spread is thought to be the main route of transmission. A spread of adenovirus in swimming-pools or small lakes can result in epidemics of pharyngoconjunctival fever. Epidemic keratoconjunctivitis seems often to be the result of insufficiently sterilized ophthalmological instruments.

The widespread nature of adenovirus infections is indicated by the presence of neutralizing antibodies in frequencies of more than 50% of the population, as illustrated in Fig. 10.10.

TABLE 10.4.
Occurrence of signs and symptoms in 192 persons with epidemic adenovirus type 8 keratoconjunctivitis in a county in Georgia, October 27, 1977–January 13, 1978 (after D'Angelo et al., 1981)

Sign or symptom	% of total
Redness in one or both eyes	98.4
Discharge from eye	90.6
Pain in eye	70.3
Change in vision	70.3
Sensitivity to light	68.2
Swollen glands	38.5
Headache	26.0
Fever	14.1
Sore throat	12.0
Myalgia	12.0
Cough	7.3
Nausea and/or vomiting	5.7
Diarrhoea	3.1

TABLE 10.5.
Classification of human adenoviruses and association with clinical symptoms (after Sambrook et al., 1980)

Class	Representative serotypes	Haemagglutination group	% G+C in DNA	Oncogenicity in rodents	Target tissue	Epidemiology
A	12, 18, 31	IV	48%	high	gastrointestinal tract infection	cryptic gastrointestinal
B	3, 7, 11, 21	I	51%	weak	pharynx, lungs (upper and lower respiratory tract), haemorrhagic cystitis (lower urinary tract), conjunctivitis (eye)	acute epidemic infection
C	1, 2, 5, 6	III	58%	nil	pharynx (upper respiratory tract)	latent throat infection; cryptic gastrointestinal infection
D	8, 9, 19	II	58%	nil	keratoconjunctivitis (eye)	acute epidemic infection
E	4	III	58%	nil	upper respiratory tract	
F	EA			nil	gastrointestinal tract	enteritis-associated enteric infection

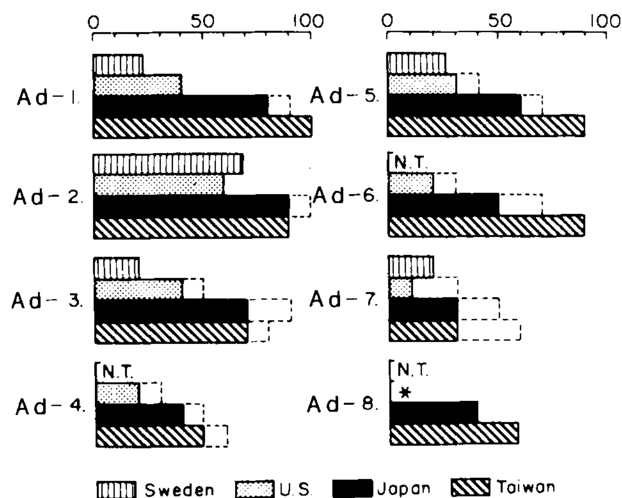

Fig. 10.10. Neutralizing antibodies against adenovirus types 1–8. The bar indicates the percent of antibody in children and the broken lines the percent in adults. N.T., not tested; *, no adenovirus antibodies. (after Foy and Grayston, 1976.)

TABLE 10.6.
Response of previously vaccinated and unvaccinated volunteers to nasopharyngeal challenge with live adenovirus type 5 (after Couch et al., 1973)

Vaccine group	No. of men	Antibody titre, mean ($\log_2$)	Illness, no. of men
Saline	19	0	10
Hexon	15	2.3	3
Fiber	8	4.4	0

The volunteers were vaccinated with saline, 100+100 μg Ad-5 hexon or 200+100 μg Ad-5 fiber.

10.3.5. Vaccination

A trivalent formalin inactivated vaccine (types 3, 4 and 7) was prepared earlier in monkey kidney cells. This was, however, subsequently withdrawn from use because the monkey cells also contained SV40 virus. Live attenuated vaccines against adenovirus types 4 and 7 have been prepared in human diploid cells. These vaccines induce virus neutralizing antibodies and have been used orally in military personnel in the USA. Types 4 and 7 vaccine can be given simultaneously (Top et al., 1971). An experimental subunit vaccine made from adenovirus type 5 hexon and fibre antigens has been used in man and resulted in resistance to challenge with adenovirus type 5 as shown in Table 10.6.

10.3.6. CHEMOTHERAPY

The presence of specific viral enzymes suitable for chemotherapy against adenoviruses offers the possibility of finding a selective inhibitory agent. The compounds so far described as having an inhibitory effect on adenovirus multiplication in cell culture seem to be nonspecific. The use of steroids in keratoconjunctivitis might be beneficial but requires a diagnosis excluding HSV. Idoxuridine has been used for keratoconjunctivitis but might even result in aggravated symptoms. AraA might have a limited effect if used early (Pavan-Langston and Dohlman, 1972).

10.4. Summary

The parvoviruses have not been shown to cause extensive enough disease in man to warrant vaccine development. Vaccines against warts are of uncertain value and the oncogenic potential of DNA containing vaccine should be kept in mind. Inactivated and attenuated adenovirus vaccines have been prepared and have prophylactic effect but are not widely used. The absence of suitable viral enzymes coded by parvo and papova viruses makes specific chemotherapy against these viruses difficult and no drug has been shown to have significant therapeutic activity. Selective antiviral drugs against adenovirus have not been described.

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CHAPTER 11

Herpesviruses, herpes vaccines and antiherpes drugs

11.1. Introduction

The name 'herpes' derives from the Greek and means to creep. Herpesviruses are ubiquitous, infecting most vertebrates but also invertebrates and even fungi. They cause a number of diseases and are rather notorious for their ability to form latent and recurrent infections. Some herpesviruses have also been linked to different malignancies. The family Herpesviridae has been divided into three subfamilies containing the following five human herpesviruses: alphaherpesvirinae (herpes simplex type 1 and 2 and varicella-zoster virus), betaherpesvirinae (cytomegalovirus) and gammaherpesvirinae (Epstein-Barr virus) (Matthews, 1982).

A large number of treatments have been investigated for therapeutic intervention of established herpes disease, some have a true therapeutic activity but many show only placebo effects. Attempts to influence the latent stage of the disease have not been successful so far. The role of vaccines in established latent herpes infections is not clear, but vaccines might be of use for preventing or modifying some herpes diseases. Vaccines will also be discussed in separate chapters for each disease entity. A short description of the herpesvirus replication strategy, immunology and mechanism of action of some antiherpes agents will be presented in this chapter.

Historical background

Herpes diseases have been long recognized and were even described in early Greek medicine as "spreading, creeping cutaneous lesions." Labial herpes was described by a Roman physician, Herodotus (AD 100), as herpetic eruptions appearing around the mouth during a fever and has, naturally, also been observed by Shake-

speare (Romeo and Juliet, Act I, scene IV), but oddly enough not in the Bible. The French physician Astruc (1664) is credited with the first description of genital herpes in men and women. More recently(!) a serological difference between the virus causing genital herpes and the virus causing labial herpes was found first by Huang Zhi-Shang (1957) who in a little observed dissertation presented evidence for serological differences between labial and genital herpes virus and later by Shubladze and Huang (1959), Schneweis (1962) and Plummer (1964) and this resulted in the description of herpes simplex type 1 (labial herpes) and herpes simplex type 2 (genital herpes) and a general, but less than accurate, description of 'above' and 'below' the waist herpes (types 1 and 2 respectively).

Varicella-zoster was for a long time not differentiated from smallpox. It was von Bokay in Hungary, in 1888 who observed that susceptible children acquired varicella after contact with zoster patients (von Bokay, 1908). The reactivation of a latent varicella virus, usually in older persons, to give a zoster infection was first suggested by Garland (1943), and shown in epidemiological studies by Hope-Simpson (1965).

In the 1920s it became clear that the large inclusions in cells from some children, dying of what was considered to be parasitic diseases, were similar to intranuclear inclusions in cells from herpes lesions (Goodpasture and Talbot, 1921). Rowe et al. (1956), Smith (1956) and Weller et al. (1957) independently reported the isolation of the virus causing cytomegalo inclusion disease, and this virus was named cytomegalovirus.

The last of the five human herpesviruses to be characterized was Epstein-Barr virus. The first clear description of infectious mononucleosis was given by Sprunt and Evans (1920) and the aetiological agent for this disease was, in an early experiment by Wising (1942), transmitted by blood to a volunteer. Several attempts to reproduce this experiment failed, but today we know that this was most likely due to antibodies in the volunteers. Transmission of the virus by the more delightful way of kissing was suggested by Hoagland (1955) but the aetiological agent was still unknown. In a pioneering study Burkitt (1958) described a malignant lymphoma in children in East Africa and four years later he suggested a viral aetiology (Burkitt, 1962). Finally, growth of Burkitt lymphoma cells in culture led to the observation by Epstein, Achnong and Barr (1964) of herpesvirus-like particles in the cultured cells. The correlation between Epstein-Barr virus and mononucleosis was accidentally found by Henle et al. (1968) when a technician working with Epstein-Barr virus developed mononucleosis! This person previously lacked antibodies to Epstein-Barr virus, but was found to develop antibodies to this virus during the illness.

11.2. The viruses

The Herpesviridae family is composed of large DNA viruses containing linear dou-

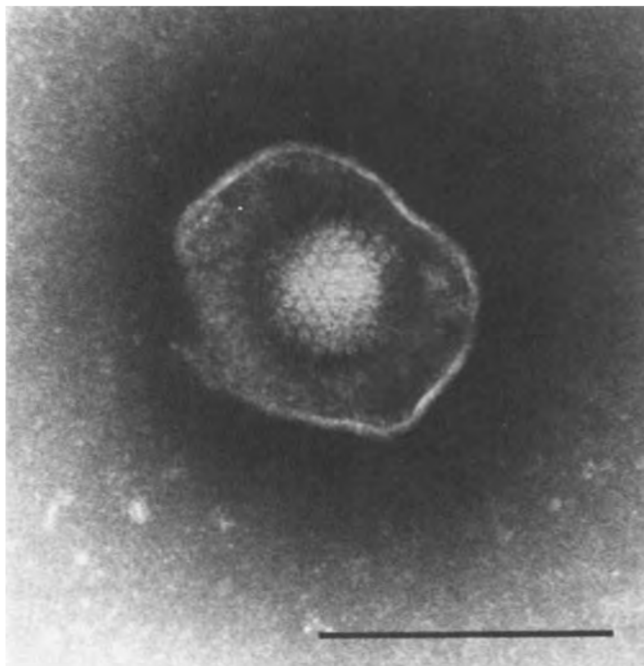


Fig. 11.1 Herpes simplex virus type 1. The bar is 200 nm. (courtesy of Dr L. Svensson.)

ble-stranded DNA with a molecular weight of $80–120 \times 10^6$. The size of the herpes-virus particle is 150–200 nm as shown in Fig. 11.1. It consists of an icosahedral capsid formed from 162 capsomeres and surrounded by a tegument, and outside that a lipoprotein envelope. The five human herpesviruses herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella-zoster virus (VZV), Epstein Barr virus (EBV) and cytomegalovirus (CMV) are listed in Table 11.1 together with their new systematic names and some characteristic properties. Herpesviruses such as B virus infecting monkeys can also infect man with often fatal results.

All herpesviruses are sensitive to extreme pH, heat and ether. A homology in DNA sequences (about 50%) is shown between HSV-1 and HSV-2, but not between other human herpesviruses. This is reflected in some antigenic similarity between HSV-1 and HSV-2, which, perhaps, can be of importance for the design of vaccines.

11.3. Molecular biology and replication

After adsorption to a receptor on the cell membrane the virus particle is taken up by pinocytosis and/or fusion with the cell membrane. The envelope is removed and the nucleocapsid moves to the cell nucleus. With the use of cellular DNA dependent

TABLE 11.1.
Properties of human herpesviruses

Properties	Virus				
	Herpes simplex virus type 1	Herpes simplex virus type 2	Varicella Zoster virus	Epstein-Barr virus	Cytomegalo virus
Abbreviations	HSV-1	HSV-2	VZV	EBV	CMV
Systematic names	Human herpes-virus 1	Human herpes-virus 2	Human herpes-virus 3	Human herpes-virus 4	Human herpes-virus 5
	α Herpesvirus 1	α Herpesvirus 2	α Herpesvirus 3	γ Herpesvirus 4	β Herpesvirus 5
DNA size	96×10^6	99×10^6	100×10^6	110×10^6	150×10^6
GC content	67%	68%	46%	46%	56%
Latent infection	Ganglia	Ganglia	Ganglia	B-lymphocytes?	B-lymphocytes?
Clinical disease	Herpes keratitis	Genital herpes	Chicken pox	Mononucleosis	Mononucleosis
	Labial herpes		Zoster		Hepatitis
	Encephalitis				Neonatal infections

Data from Andrewes et al. (1978) and Matthews (1982).

RNA polymerase the viral genome directs the synthesis of large nuclear RNA. This RNA is capped, cleaved, polyadenylated and transported to the cytoplasm for use as mRNA in the translation to viral proteins. The sequence of events is shown for HSV in Fig. 11.2. Three classes of mRNA have been identified, immediate early (α), early (β) and late (γ). These give rise to proteins of three corresponding classes. A feed-back mechanism has been found whereby early proteins shut off the formation of immediate early, and late proteins the formation of early proteins. As indicated in Fig. 11.3 some proteins belonging to the different classes have been identified and located on the viral genome. The viral genome induces the formation of more than 100 polypeptides. Viral enzymes required for replication are among the first proteins to be found. Among these are the viral DNA polymerase and in the case of HSV-1, HSV-2 and VZV, a viral thymidine kinase (TK).

The viral DNA is replicated by viral DNA polymerase in the nucleus and then as immature nucleocapsids is transported through the nuclear membrane, acquiring an envelope. The formation of nuclear inclusion bodies is typical for herpesvirus infected cells. Virus particles, containing about 50 polypeptides, can be released to adjacent cells without cell lysis or, alternatively, the infected cells can lyse. This is often preceded by a typical rounding-up of the infected cells (cytopathic effect).

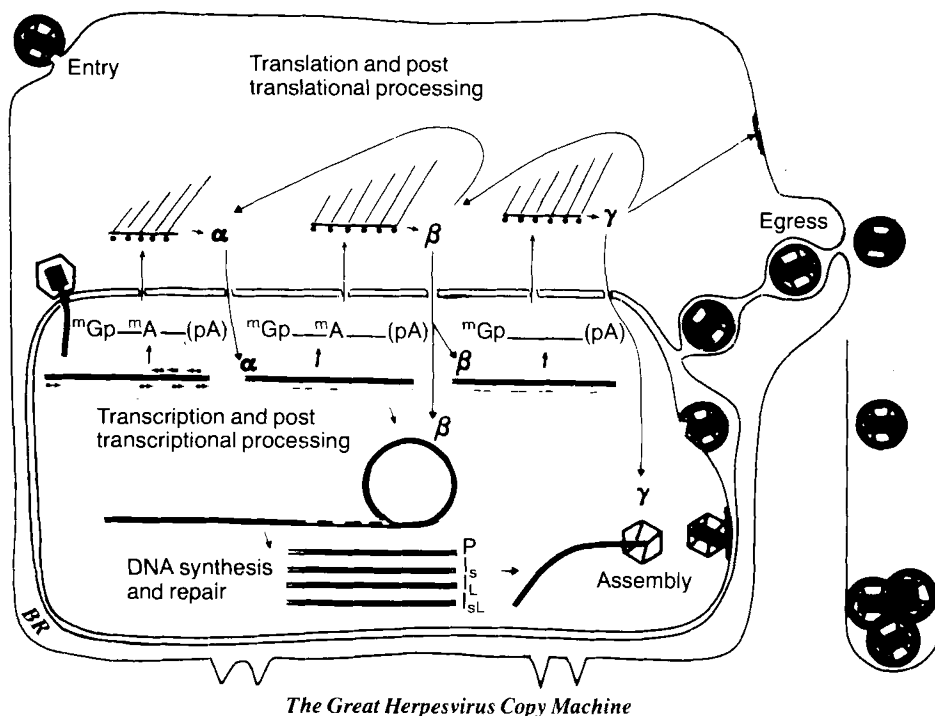


Fig. 11.2. Replication of herpesvirus. Drawn and supplied through the courtesy of Dr B. Roizman.

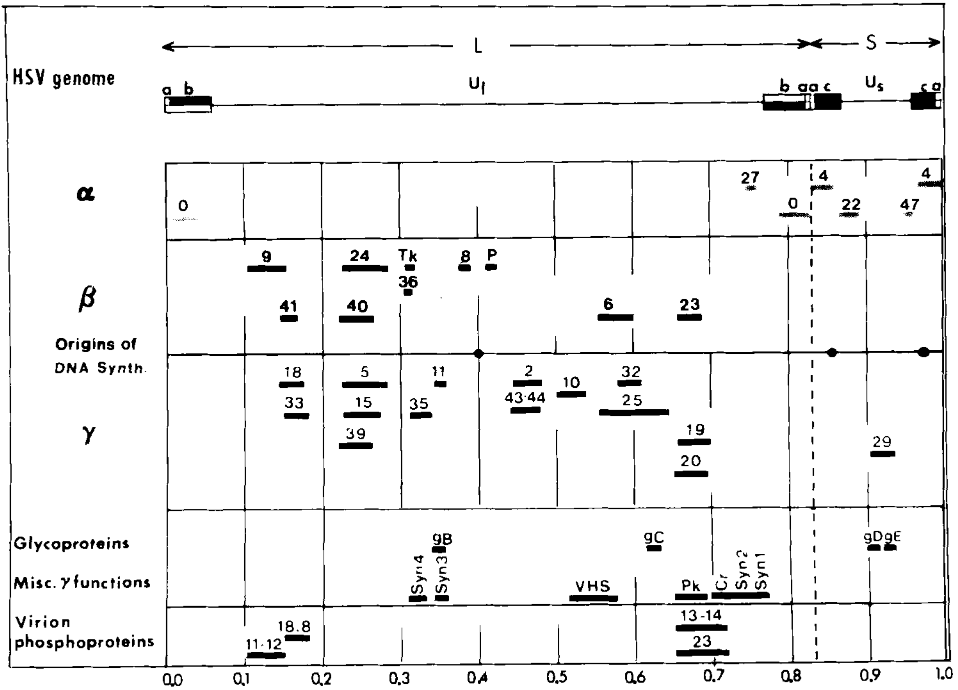


Fig. 11.3. Location of viral genes in the herpes simplex virus genome. (courtesy of Dr B. Roizman.)

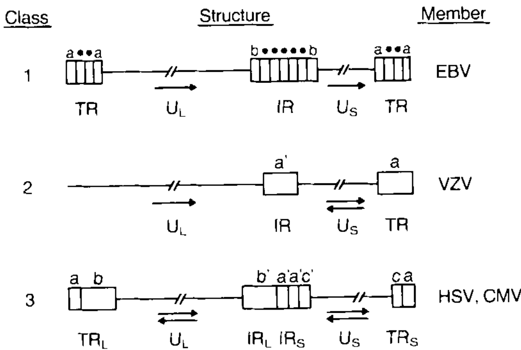


Fig. 11.4. Herpesvirus genomes. U_S and U_L denote the short and long unique DNA sequences respectively. Tandem repeated sequences are indicated by small continuous rectangles. An inverted sequence complementary to sequence a, b or c is indicated as a′, b′ or c′. The arrows indicate the orientation found for the unique sequences. The figure is adopted from Ben–Porat (1981).

The genomes of the five human herpesviruses are shown schematically in Fig. 11.4. The indicated orientations of U_L and U_s (long and short unique DNA sequences) seem not to influence the infectivity of the virus particles. A model for the replication of the viral DNA is outlined in Fig. 11.2. It has been estimated that

copying of the entire genome takes 20–40 min. The viral DNA is in itself infectious.

The enzymes induced by the herpesvirus genomes are listed in Table 11.2 but even more viral enzymes are likely to be found in the future. The viral enzymes are of especial importance as potential targets for antiviral drugs. The herpesvirus DNA polymerases are somewhat similar in their properties to cellular DNA polymerase α but nevertheless sufficiently different to allow a selective inhibition by drugs. The DNase activity observed seems to be closely related to the DNA polymerase and might be part of a replication complex. Herpesvirus thymidine kinase is a key enzyme, phosphorylating nucleoside analogues, with structural similarities to thymidine, to nucleoside-5′-monophosphates. The viral thymidine kinase is less restricted in its structural requirement than cellular cytoplasmic thymidine kinase and this difference is the basis for selectivity of phosphorylating nucleoside analogues such as ACG, BVDU, DHBG and FIAC. The cellular mitochondrial thymidine kinase has an ability to phosphorylate some nucleoside analogues which are not phosphorylated by the cellular cytoplasmic thymidine kinase. Protein kinases are induced by herpesviruses but the role of these enzymes for virus replication is unknown. However, several viral proteins are phosphorylated. 5-p-Fluorosulfonylbenzoyladenosine has been reported to inhibit the protein kinase activity of Tupaia herpesvirus (Flügel and Darai, 1982).

The herpesvirus ribonucleotide reductase differs from the cellular ribonucleotide reductase, and it ought to be possible to find compounds capable of selectively inhibiting the enzyme or of being activated by it. The importance of the ribonucleotide reductase for viral replication or latency is at present unclear but some recent data indicate that this enzyme is involved in cell transformation (Huszar and Bacchetti, 1983).

An increased protease activity in herpesvirus transformed cells could be of importance in tumorigenicity, and protease inhibitors such as leupeptin can inhibit the fibrinolytic capacity of HSV-2 transformed hamster cells (Adelman et al., 1982).

The viral DNA polymerase is an obligatory enzyme for virus replication and mu-

TABLE 11.2.
Enzymes induced by human herpesviruses

Virus	Ribonucleotide reductase	Protein kinase	TK	DNA pol	DNase
HSV-1	+	+	+	+	+
HSV-2	+	?	+	+	+
VZV	?	?	+	+	+
EBV	+	+	–	+	+
CMV	?	?	–	+	+

These can be useful 'targets' for the design of new antiviral drugs.

tants in this enzyme are often lethal. In contrast, the need for the viral thymidine kinase is less clear and HSV mutants in which this enzyme is absent, grow well in cell culture, utilizing cellular thymidine kinase. The viral thymidine kinase could, however, be required during formation of a latent infection and/or reactivation in ganglia (Field and Wildy, 1978, Field and Darby, 1980). A further indication of this is that only the herpesviruses forming latent ganglionic infections (HSV-1, HSV-2 and VZV) induce thymidine kinases while EBV and CMV, probably forming latent infections in other cells, do not seem to possess this enzyme.

11.4. Latency

All five human herpesviruses have the capacity to form latent infections which later can be reactivated once or repeatedly, causing disease. The cells carrying latent CMV and EBV could be lymphocytes. The latency of HSV-1 and 2 in sensory ganglia is well established and it is probable that VZV also has a latent phase in sensory ganglia. However, in animal models it is not excluded that HSV can form a latent infection in the footpads of mice as well as in the ganglia (Al-Saadi et al., 1983 and reviewed by Klein, 1982). In HSV latency in man it is not clear how the genome is preserved in the ganglia but in mice with latent HSV infections it seems likely that the viral DNA undergoes some modification such as integration or formation of circles or large concentrated DNA (Rock and Fraser, 1983). Human ganglia have been found to contain HSV mRNA but it is unclear if this is synthesized during latency or activated during preparation of the ganglia. In a study by Galloway et al. (1982) RNA transcripts from the 30% left-handed part of HSV-2 were found in human paravertebral ganglia. Less RNA was found hybridizing to other sequences of the L-part of the viral DNA and no RNA complementary to the S-part. It also seems clear that a latent HSV infection does not prevent the latent infection by a second strain of HSV. The viral enzymes required for HSV to form and maintain latency are not known. Herpesvirus thymidine kinase negative mutants seem to have a decreased capacity to be recovered from ganglia from infected mice (see above).

Reactivation in humans can be induced by several stimuli such as fever, stress, menstruation, sunburn (labial herpes) and immunosuppression. In the latter case reactivation of more than one type of herpesvirus can occur. It is not clear if these stimuli trigger HSV replication in the ganglia, and if this virus is transported to the skin or if there is a continuous flow to the skin of virus which normally is inactivated by defence mechanisms but which, in the situation of a decreased resistance, gives rise to lesions. It is, furthermore, not clear if HSV in a recurrent lesion travels back to a ganglion and establishes a latent state. This latter question is of importance in the context of resistance development (see Chapter 7).

No antiviral drugs have had any effect on established latent herpes infections and it is at present unclear which viral functions should be attacked by drugs in the la-

tent state. Also, antibodies do not seem to prevent latency to herpesviruses, since persons with latent and recurrent infections have antibodies, and furthermore have a cell-mediated immunity.

11.5. Transformation

Although the subject is outside the scope of this book it should be mentioned that herpesviruses are known to induce tumours in animals. It is likely that HSV-2 in some way is involved in the formation of cervical tumours and that EBV is an aetiological agent in Burkitts lymphoma and nasopharyngeal carcinoma. In the case of HSV-2, no specific part of the genome seems to be consistently associated with cervical tumours, and possibly a viral protein is involved. A hit-and-run mechanism for the transformation has recently been suggested for HSV (Galloway and McDougall, 1983). On the other hand, Robinson and O'Callaghan (1983) found specific DNA sequence in several transformed cells using equine herpesvirus. In any case, inhibitors of herpesvirus enzymes seem not to be directly useful as therapeutic agents against herpesvirus induced tumours. An interesting hypothesis for transformation has been presented by Huszar and Baccetti (1983), who suggest that the HSV-2 ribonucleotide reductase causes imbalance in the deoxyribonucleotide pools in infected cells. This imbalance might result in mutations due to base substitutions, altered proof reading or error-prone repair. This idea is in agreement with a hit-and-run model since the viral genome need not be present in the transformed cell.

11.6. Herpesvirus vaccines

Herpesviruses are extremely complex with virions composed of up to 50 structural proteins and with additional non-structural proteins synthesized intracellularly. The immunology of the virus is incompletely understood, although it is realised that cell mediated immunity (but to which protein?) may be more important than serum antibody in immunity to infection and, moreover, the virus life cycle is complicated by the presence of a latent phase in neurones. The immunology of HSV infections has been reviewed by Kirchner (1982). Moreover, if a vaccine were to be developed who would be target groups? It is unlikely that vaccination would have any effect on recurrent virus infection in persons already latently infected. A further complication derives from the possible association between HSV-2 and cervical cancer, and this would limit development of *live* attenuated herpes simplex vaccines. In spite of these difficulties several experimental vaccines are under development including HSV-2, cytomegalovirus and varicella zoster. Certainly, new vaccines could be developed now to take advantage of recent developments in technology such as genetic engineering and the use of monoclonal antibodies to identify important antigenic determinants of the virus (see Chapter 2).

Animal studies using human herpesviruses to investigate vaccines and immunological functions could be of questionable relevance to the situation in humans where the latent state requires that virus can escape the immunological defences and, furthermore, give rise to disease in individuals with both humoral and cell-mediated immunity. In contrast, a guinea pig inoculated with HSV on its back will form vesicles only after the first inoculation but not if it is reinoculated.

11.7. Structure-activity relationships for inhibitors with different mechanisms of action

Inhibitors of, or compounds activated by, viral enzymes are of two principal types, competitive or un/non-competitive. The action of a competitive inhibitor is dependent on compounds competing with the inhibitor at the active site of an enzyme. The un- and non-competitive inhibitors are not influenced by the concentration of compounds interacting with the inhibited enzyme. The same relations are true when a compound does not inhibit, but is modified by an enzyme. The structure-activity relations for two types of herpesvirus inhibitors acting in two different ways will be discussed below. The two types of inhibitors are illustrated in Fig. 11.5.

Pyrophosphate analogues

A large number of pyrophosphate analogues have been investigated as inhibitors of RNA and DNA polymerases and Table 11.3 shows some examples of structures inhibiting DNA polymerases. The inhibition of these enzymes are un- or non-competitive with respect both to nucleoside triphosphates and DNA template, as illustrated for foscarnet in Fig. 11.6. Active compounds are found within a rather narrow range of structures. The un- or non-competitive type of inhibition will result in a reproducible inhibition of virus multiplication in cell cultures of different age or condition since varying nucleoside or nucleotide concentrations will not affect the inhibition of virus multiplication.

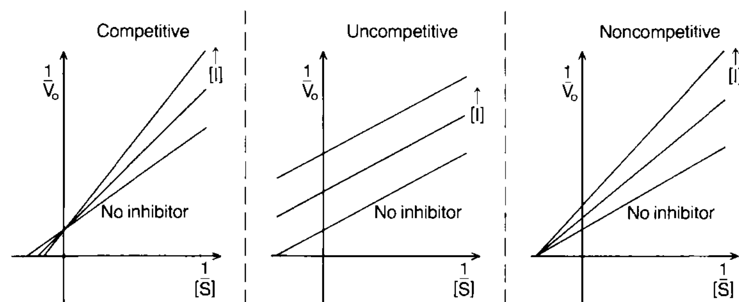


Fig. 11.5. Double reciprocal plots showing the patterns of competitive, uncompetitive and noncompetitive inhibition of enzymes. V_0 is the velocity of the reaction, S the substrate concentration and I the inhibitor concentration.

TABLE 11.3.
Polymerase inhibition by pyrophosphate analogues

Compound	Concentration (μM) giving 50% inhibition						
	HSV-1 DNA pol	HSV-2 DNA pol	HCMV DNA pol	Hepatitis B DNA pol	AMV DNA pol	Influenza RNA pol	Cellular DNA polα
$HO\text{-}C(=O)\text{-}C(=O)\text{-}OH$	115	400	130	>500	>500	>500	>500
$HO\text{-}P(=O)(OH)\text{-}P(=O)\text{-}OH$	150	12	150	97% at 500	25	>500	250
$HO\text{-}P(=O)(OH)\text{-}C(=O)\text{-}OH$ (PFA) Foscarnet	0.3	0.5	0.3	20	8	30	40
$HO\text{-}P(=O)(OH)\text{-}CH_2\text{-}P(=O)(OH)\text{-}OH$	>500	—	>500	>500	>500	>500	>500
$HO\text{-}P(=O)(OH)\text{-}C(=O)\text{-}P(=O)(OH)\text{-}OH$	10	27	6	500	200	280	100
$HO\text{-}P(=O)(OH)\text{-}CH_2\text{-}C(=O)\text{-}OH$ (PAA)	0.5	0.7	0.4	>500	>500	300	35
$HO\text{-}P(=O)(OH)\text{-}CH(CH_3)\text{-}C(=O)\text{-}OH$	15	550	18	>500	>500	>500	>500
$HO\text{-}P(=O)(OH)\text{-}CH(C_9H_{19})\text{-}C(=O)\text{-}OH$	10	5	1.5	—	—	>500	160
$HO\text{-}P(=O)(OH)\text{-}CH(OH)\text{-}C(=O)\text{-}OH$	2	3	3	>500	>500	>500	100
$HO\text{-}P(=O)(OH)\text{-}CH_2CH_2\text{-}C(=O)\text{-}OH$	>5000	—	—	>500	>500	>500	>500

Courtesy of Dr B. Eriksson.
HSV, herpes simplex virus; HCMV, human cytomegalovirus; AMV, avian myeloblastosis virus.

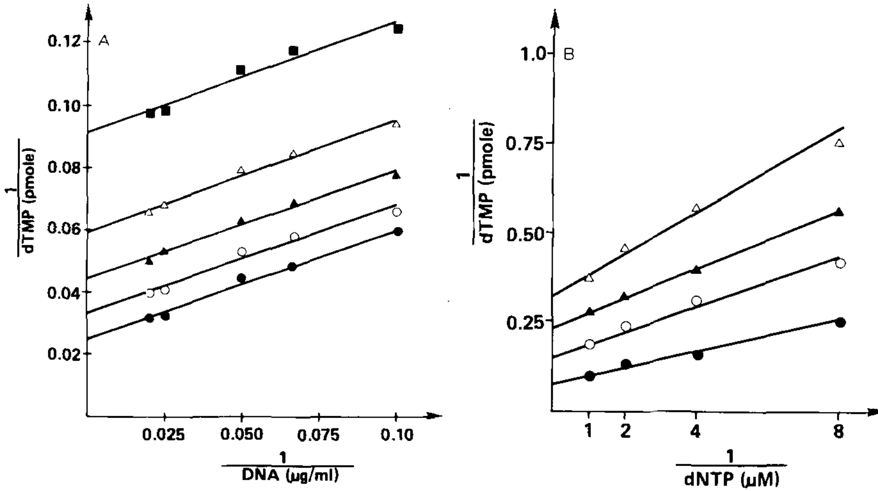
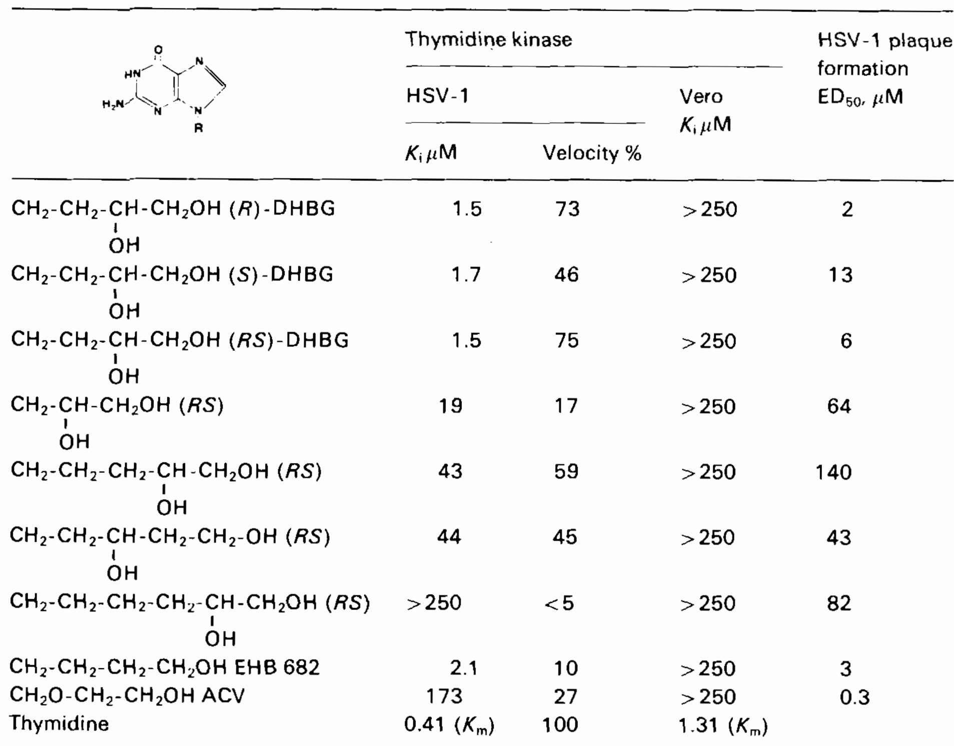


Fig. 11.6. Inhibition of HSV-1 DNA polymerase by foscarnet at varied template (A) and substrate (B) concentration. The concentrations of foscarnet were: 0 μM (●), 0.25 μM (○), 0.50 μM (▲), 1 μM (△). From Eriksson et al. (1980).

Thymidine analogues

A vast number of nucleoside analogues have been evaluated as inhibitors of herpesviruses and this is still a very active research area. Structures with similarities to thymidine can often be phosphorylated by HSV-1, 2 and VZV thymidine kinase but not by cellular thymidine kinase. This is the first step of action of most of the present herpesvirus-inhibitors. Table 11.4 shows some guanine derivatives, some of which can be phosphorylated by HSV-1 thymidine kinase and also in some instances after further phosphorylation can act as selective virus inhibitors. The phosphorylation by viral thymidine kinase is a reaction where the nucleoside analogue has to compete with thymidine, and this is shown in Fig. 11.7. The affinity to the kinases are given by K_i values, a low value represents a high affinity to the enzyme. An important property of a compound acting in this way is that it should be able to compete efficiently with thymidine in order to have a good in vivo activity against herpesvirus multiplication. The importance of this can be illustrated practically by the decreased inhibition shown when thymidine is added to a cell culture where HSV-1 multiplication is inhibited by a nucleoside analogue. Three examples are given in Fig. 11.8 and Table 11.5 where the reversion by thymidine of inhibition by acyclovir, BVDU and DHBG are shown to be competitive processes. In the case of BVDU, the reversion of inhibition could also take place by an increased dThdTP concentration competing with BVDU-TP but not with ACG-TP or DHBG-TP. Table 11.4 also shows that several structures, which are not particularly thymidine-like, can be phosphorylated by this enzyme. The importance of different types of inhibition and affinity to enzymes is also discussed in Chapter 3. The consequences

TABLE 11.4.
Phosphorylation and inhibition of HSV-1 plaque formation of some acyclic guanosine analogues



R	Thymidine kinase			HSV-1 plaque formation
	HSV-1		Vero	ED_{50}, μM
	K_i μM	Velocity %	K_i μM	
CH_2-CH_2-$CH(OH)$-CH_2OH (*R*)-DHBG	1.5	73	>250	2
CH_2-CH_2-$CH(OH)$-CH_2OH (*S*)-DHBG	1.7	46	>250	13
CH_2-CH_2-$CH(OH)$-CH_2OH (*RS*)-DHBG	1.5	75	>250	6
CH_2-$CH(OH)$-CH_2OH (*RS*)	19	17	>250	64
CH_2-CH_2-CH_2-$CH(OH)$-CH_2OH (*RS*)	43	59	>250	140
CH_2-CH_2-$CH(OH)$-CH_2-CH_2-OH (*RS*)	44	45	>250	43
CH_2-CH_2-CH_2-CH_2-$CH(OH)$-CH_2OH (*RS*)	>250	<5	>250	82
CH_2-CH_2-CH_2-CH_2OH EHB 682	2.1	10	>250	3
CH_2O-CH_2-CH_2OH ACV	173	27	>250	0.3
Thymidine	0.41 (K_m)	100	1.31 (K_m)	

Courtesy of Drs. A. Larsson and N.G. Johansson.

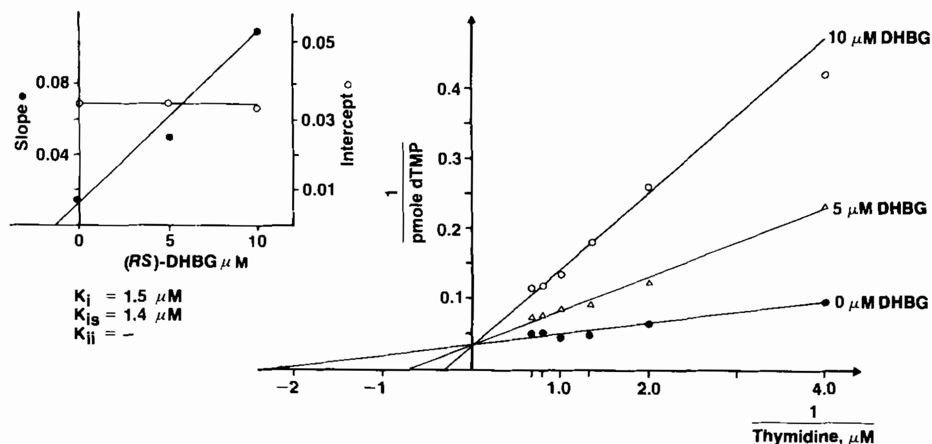


Fig. 11.7. Inhibition of thymidine phosphorylation by (RS)-DHBG. Thymidine was used as the varied substrate in this reaction with purified HSV-1 TK and (RS)-DHBG as inhibitor. From Larsson et al. (1983b).

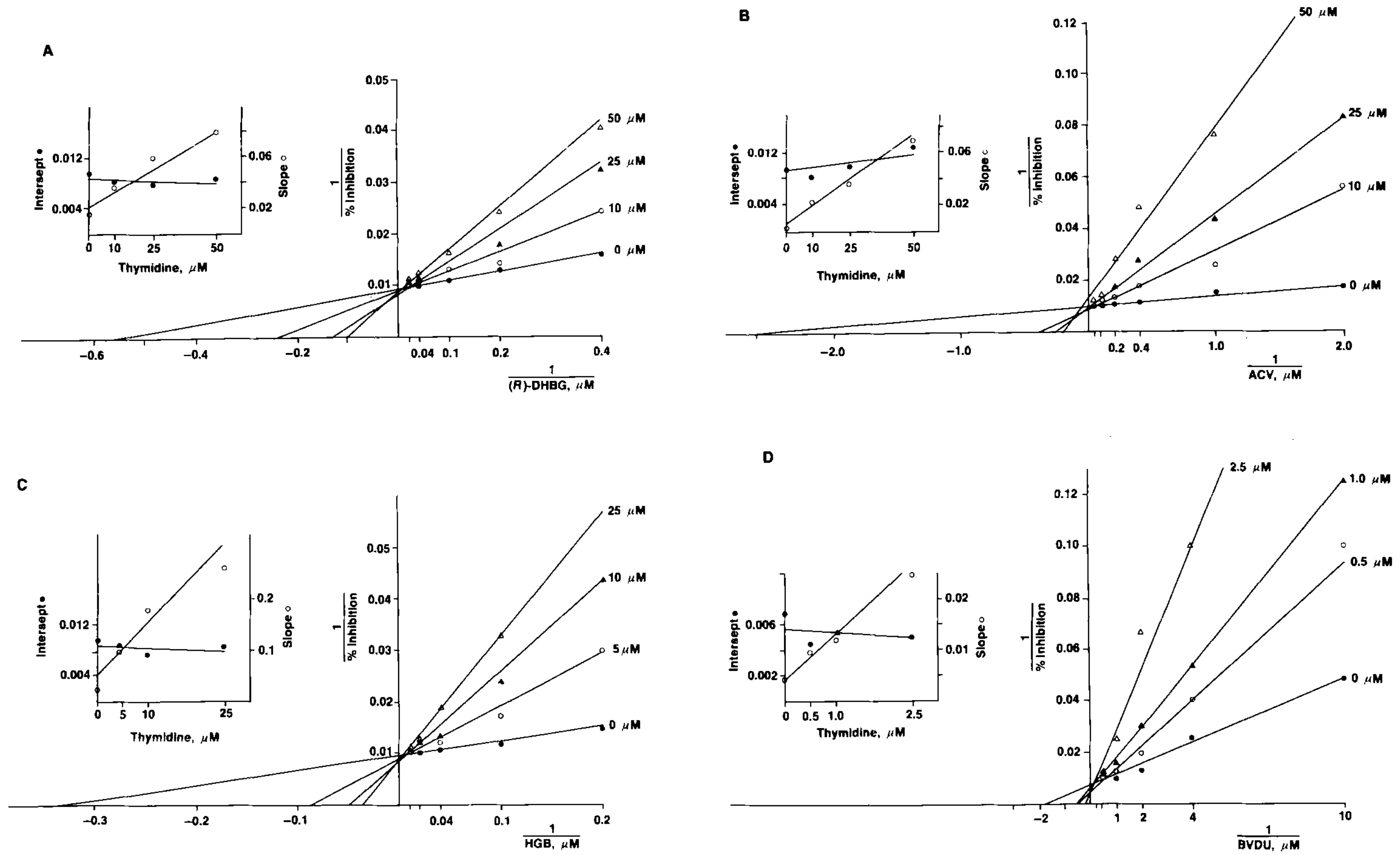


Fig. 11.8. Inhibition of HSV-1 plaque formation by antiherpes drugs, kinetic patterns of reversal by thymidine. Antiherpes drugs were used as the varied substrates and the inhibition of HSV-1 plaque formation was measured at different thymidine concentrations. From Larsson et al. (1983a).

TABLE 11.5.
Reversion of inhibition of HSV-1 multiplication in cell culture (after Larsson et al., 1983a)

μM dThd added	Concentration of drug causing 50% inhibition				
	ACV	(*R*)-DHBG	HBG	BVDU	PFA
0	0.5	1.8	2.8	0.2	48
5	1.8	2.4	6.5	2.0	—
10	2.3	2.8	9.9	2.9	—
25	4.4	3.9	18	7.2	42
50	6.7	6.3	40	13	42
100	14	11	81	22	45
Ratio $\frac{0\ \mu M\ dThd}{100\ \mu M\ dThd}$	28	6	29	110	1

These experiments may have immediate relevance to action of antivirals on HSV lesions in the skin if local dThd concentrations are high.

of the different modes of action and a possible sensitivity to high concentrations of thymidine in skin is illustrated in Table 11.6, which shows foscarnet to be the most active compound on guinea pig skin, although it is not the most active in cell cultures where the thymidine concentration is low.

11.8. Antiherpes drugs and modes of action

The most important antiherpes drugs with present or possible clinical use are listed alphabetically below and, where known, the mechanisms of drug action are described. Their INN names are given first, when available, then the other names and finally the manufacturer. The structural formulae of these compounds are shown in Fig. 11.9. Several reviews discussing mechanism of action have been published and these should be consulted for further details (Becker, 1976, Müller, 1979, Swallow, 1978, Shannon and Schabel, 1980, Becker and Hadar, 1980, Shugar, 1981, Gordon et al., 1981, De Clercq, 1982 and Öberg, 1983a). An accurate discussion of the relative toxicity of the drugs is not possible due to the absence of published data and several compounds such as ribavirin, araT, araC, tromantadine (Viru-Merz), 2-deoxy-D-glucose are excluded from our list due to toxicity, lack of effect on herpes infections in controlled studies, or too limited information to hand.

One group of anti-herpes drugs, containing nucleoside analogues, requires the action of a viral thymidine kinase (TK) as indicated in Fig. 11.10. CMV and EBV do not seem to induce viral TKs and are thus rather insensitive to thymidine analogues requiring this viral enzyme. Since the nucleoside analogues after phosphorylation to monophospates have to be phosphorylated in several competitive steps to

TABLE 11.6.
Effect of antiviral compounds on cutaneous herpes virus infection in guinea pigs

Substance	Vehicle	Cumulative score		Time to healing	
		Mean	S.D.	Mean	S.D.
10% Vidarabine	DMSO	22.9	2.2	11.6	0.5
10% Ara-C	DMSO	15.8	3.2	10.1	1.4
10% Idoxuridine	DSMO	20.9	2.6	10.6	1.1
10% Ribavirin	DMSO	20.8	2.1	10.7	0.8
1% PAA	Water	8.4	2.7	7.0	2.0
2% Foscarnet	Water	5.8	1.7	5.2	1.0
2% Acyclovir	DMSO	13.9	3.0	9.8	0.4
5% Acyclovir	PEG	21.3	2.7	10.3	0.8
Untreated		22.6	2.5	10.9	0.8

Treatment started 48 h post inoculation with HSV-1 and continued for 3 days with 2 daily topical applications of 30μl. DMSO is dimethyl sulfoxide and PEG is polyethylene glycol 200. From Alenius and Öberg (1978), Alenius et al. (1978) and Alenius et al. (1982).

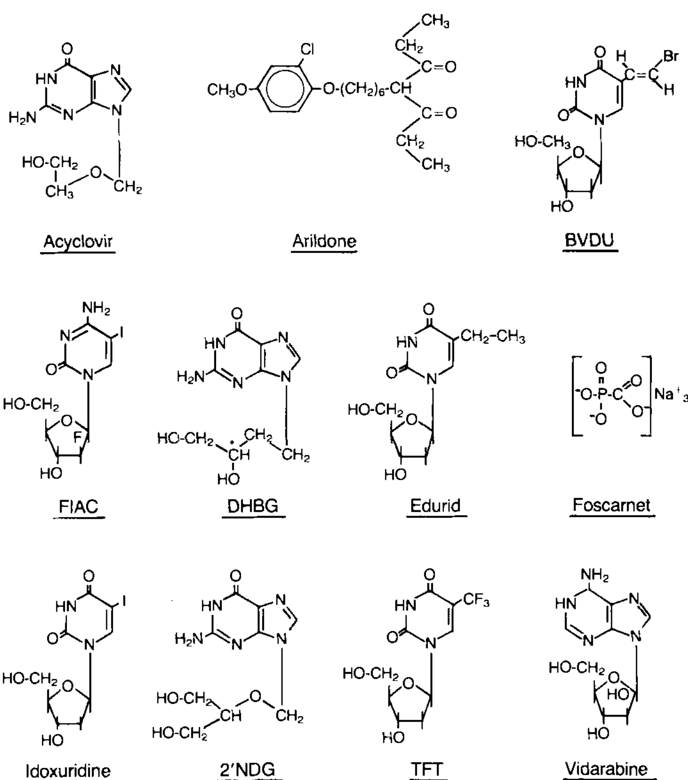


Fig. 11.9. Structures of antiherpes drugs.

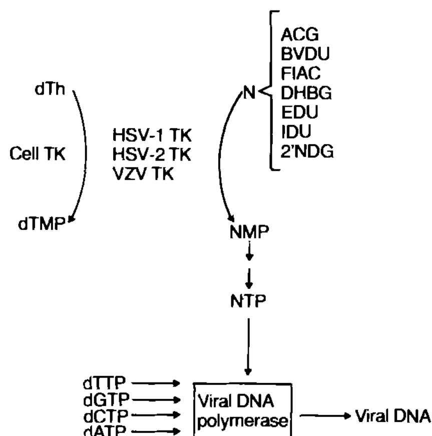


Fig. 11.10. Mechanism of action for antiherpes drugs requiring viral thymidine kinase.

triphosphates, which act as inhibitors in competitive reactions, the final effect of an inhibitor will depend on the concentrations of several cellular metabolites. This has not been sufficiently considered in the past and to predict clinical efficacy an evaluation of this type of anti-herpes drug should be carried out under conditions where the influence of competitive metabolites can be determined (Larsson et al., 1983a).

Acyclovir (ACV, 9-(2-hydroxyethoxymethyl)guanine, acycloguanosine, ACG, Zovirax® – Burroughs-Wellcome). The mechanism of action is outlined in Fig. 11.10 and in more detail in Fig. 11.11. This acyclic guanosine derivative is phosphorylated by HSV-1, HSV-2 and VZV thymidine kinases, but much less so by cellular thymidine kinase, to its monophosphate (Elion et al., 1977, Fyfe et al., 1978, Fyfe and Biron, 1979, Cheng et al., 1981b). The monophosphate is further phosphorylated by cellular GMP kinase (Miller and Miller, 1980) and by several enzymes (phosphoglucerate kinase, GDP kinase and other) to a triphosphate (Miller and Miller, 1982). The affinity of acyclovir to HSV-1 thymidine kinase is low ($K_i = 173 \mu M$) and the presence of thymidine ($K_m = 0.4\ \mu M$) will decrease the phosphorylation of acyclovir in a competitive fashion (Larsson et al., 1983a). Acyclovir triphosphate acts as a chain terminator in DNA synthesis (Furman et al., 1980, Derse et al., 1981) and competes in the polymerase reaction with GTP (Elion et al., 1977). The inhibition by chain termination is selective for herpesvirus DNA polymerase and could partly be due to a high binding affinity of the viral DNA polymerase to DNA chains terminated with acyclovir monophosphate (Derse et al., 1981).

Because of its requirement for a viral thymidine kinase, acyclovir is an efficient inhibitor of HSV-1, HSV-2 and VZV but active only at considerably higher drug concentrations against CMV and EBV. The properties of this interesting compound have recently been reviewed by Rodgers and Fowle (1983).

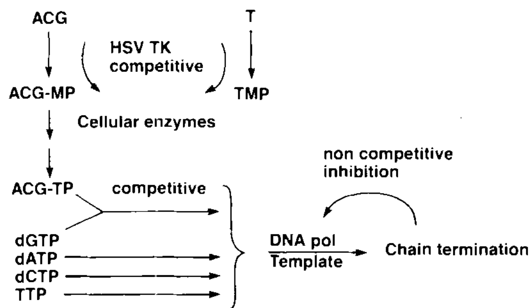


Fig. 11.11. Mechanism of action of acyclovir (ACG).

Arildone (4-[6-(2-Chloro-4-methoxyphenoxy)hexyl]-3,5-heptanedione) – Sterling-Winthrop). The mechanism of action of this compound is not clear, but it seems to act at the uncoating of virus particles (Kurth et al., 1979, McSharry et al., 1979, Schrom et al., 1982). Interestingly, in cell culture, arildone has been active not only against HSV-1 and HSV-2 but also against RNA viruses (Diana et al., 1977, Kim et al., 1980 and see Chapter 4).

[E]-5-(2-Bromovinyl)-2′-deoxyuridine (BVDU, BrvdUrd, bromovinyldeoxyuridine – Searle). This thymidine analogue is selectively phosphorylated by HSV-1 and VZV thymidine kinases, and to a lesser extent by HSV-2 thymidine kinase, but not by cytoplasmic thymidine kinase (Cheng et al., 1981a). As shown in Figs 11.8 and 11.10 the phosphorylation of BVDU is a competitive reaction, being affected by the thymidine concentration. Further phosphorylation to a triphosphate is probably mediated by cellular enzymes, as noted above for acyclovir. BVDU triphosphate shows some inhibition of viral DNA polymerase (Allaudeen et al., 1981, Ruth and Cheng, 1981) but is also incorporated, probably internally, into viral and cellular DNA in the infected cell (Larsson and Öberg, 1982). This seems to result in an altered stability for the viral DNA (Mancini et al., 1983). A certain selectivity for inhibition of HSV-1 DNA synthesis has been observed in the infected cell (Larsson and Öberg, 1981, Larsson and Öberg, 1982). The incorporation and inhibition of DNA polymerase activity is a competitive reaction influenced by the dTTP concentration (Allaudeen et al., 1981, Ruth and Cheng, 1981). The antiviral spectrum in cell cultures corresponds to the phosphorylation by viral thymidine kinases e.g. a very high inhibitory activity against VZV, a high activity against HSV-1 and a low activity against HSV-2 (De Clercq et al., 1979).

1-(2′-Deoxy-2′-fluoro-β-D-arabinosyl)-5-iodocytosine (FIAC, Bristol-Myers). This compound is also selectively activated by herpesvirus thymidine kinase (Cheng et al., 1981b) as indicated in Fig. 11.8. The detailed mechanism is less well worked out than for acyclovir and BVDU but the triphosphate of FIAC has a selective effect on herpesvirus DNA polymerase as shown both with isolated enzymes (Ruth and Cheng, 1981) and as indicated from cell culture experiments with HSV-1 (Lars-

son and Öberg, 1981 and Larsson and Öberg, 1982) FIAC is also metabolized to compounds, especially 2′-fluoro-5-methyl-araU (FMAU), showing antiherpes activity, probably by the same mechanism as the parent compound. The metabolism of FIAC and the inhibition of herpesvirus DNA polymerase by a variety of triphosphates related to FIAC is shown in Fig. 11.12.

9-(3,4-Dihydroxybutyl)guanine (DHBG – Astra). This compound exists in two isomeric forms, R and L, of which the R(+) form has been most active. The mechanism of action seems to be very similar to that of acyclovir (Larsson et al., 1983b) as shown in Fig. 11.11. However, the affinity for HSV-1 thymidine kinase is high ($K_i = 1.4\ \mu M$) and this leads to a small influence as regards the competitive action of thymidine as shown in Fig. 11.8 and Table 11.5. A selective inhibition of HSV-1 DNA synthesis in cell culture has been observed (Larsson et al., 1983b). In cell culture DHBG is active against HSV-1, HSV-2 and VZV but not against CMV, in accordance with its proposed mechanism of action.

5-Ethyl-2′-deoxyuridine (EDU, Edurid – Robugen). This compound is first phosphorylated by viral thymidine kinase as outlined in Fig. 11.10 (Gauri and Walter, 1973; Cheng et al., 1979). The selectivity seems to be less than that of acyclovir and BVDU and the possible further phosphorylation to a triphosphate is speculative. A certain selectivity for inhibition of HSV-1 DNA synthesis has been observed in cell culture, but the detailed mechanism of action remains to be determined. Mutants of HSV-1 which do not have thymidine kinase are not inhibited.

Foscarnet (Trisodium phosphonoformate, phosphonoformic acid, PFA, Foscavir – Astra). As outlined in Fig. 11.13 this interesting compound is a direct non-competitive inhibitor of herpesvirus DNA polymerases at concentrations not affecting cellular DNA polymerases (Helgstrand et al., 1978). Foscarnet is probably bound to the site of the viral polymerases where pyrophosphate is split off during the polymerization of nucleoside triphosphates (Eriksson and Öberg, 1979). The selectivity for inhibition of viral DNA polymerase has been shown both with purified enzymes and in cell cultures. All five human herpesviruses are inhibited by foscarnet. (For reviews see Helgstrand et al., 1980, Öberg, 1983b and Eriksson and Öberg, 1984).

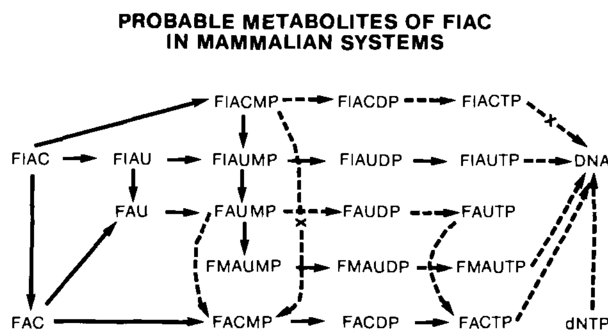
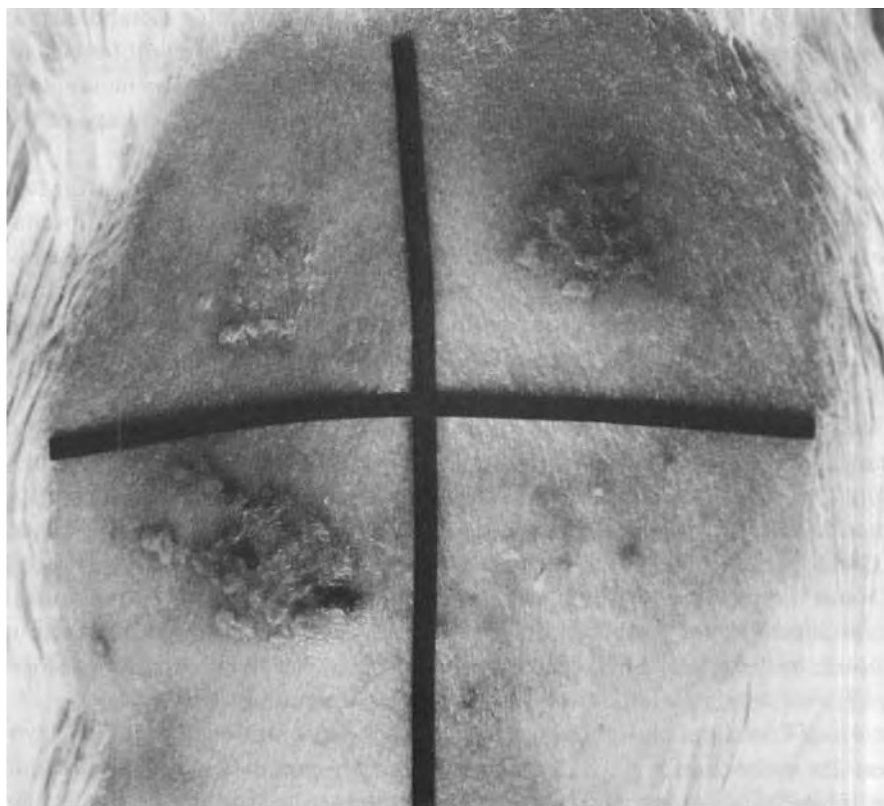
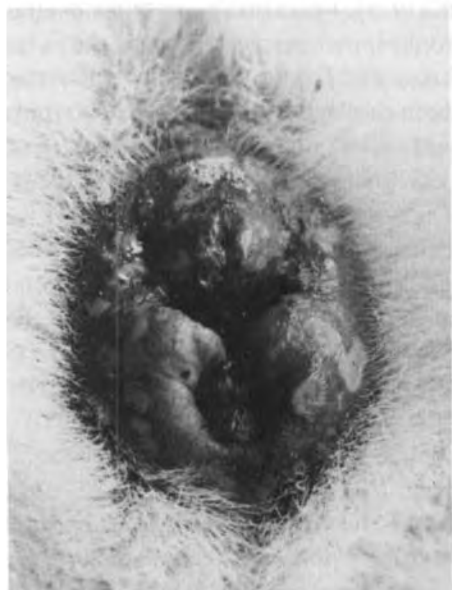


Fig. 11.12. Probable metabolites of FIAC in mammalian cells. (courtesy of Dr R. Schinazi.)





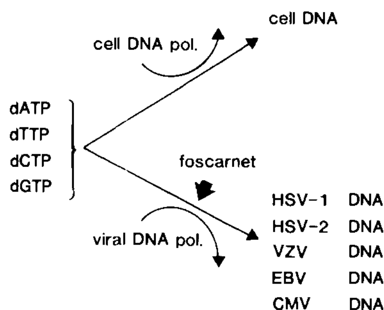


Fig. 11.13. Mechanism of action of foscarnet.

Experiments with HSV-1 mutants (Alenius, 1980) showed that the same mechanism of action operates during inhibition of HSV-1 in infected animals. Fig. 11.14 shows the therapeutic effects of foscarnet on cutaneous and genital herpes in guinea pigs.

5-Iodo-2'deoxyuridine (Idoxuridine, IDU, Dendrid®, Herpid®, Kerecid®, Stoxil®, – SKF, Alcon). Although IDU can be phosphorylated by cellular thymidine kinase, herpesvirus mutants lacking thymidine kinase are resistant to IDU, indicating the requirement of the viral kinase as shown in Fig. 11.8. The reaction is competitive with thymidine and IDU-monophosphate is incorporated into both cellular and viral DNA (Prusoff and Ward, 1976). Little selectivity seems to exist at the polymerase level and part of the antiherpes activity is mediated by miscoding and subsequent synthesis of wrong polypeptides because of the incorporation of IDU into DNA. (This was one of the first antivirals to be studied and is now a little 'dated' and superceded by more active compounds.) Other mechanisms of action such as inhibition of herpesvirus ribonucleotide reductase by IDU diphosphate could also be of importance (Müller, 1979).

2'-Nor-2'deoxyguanosine (9-[(2-Hydroxy-1-hydroxymethyl)ethoxy)-methyl]guanine, DHPG, 2'NDG, Biolf 62 – unclear patent situation, involving Syntex, Burroughs-Wellcome and MSD). The mechanism of action (Fig. 11.10) against HSV-1 seems to be the same as for acyclovir and DHBG (Ashton et al., 1982, Smith et al. 1982a). 2'NDG has a higher affinity than acyclovir for HSV-1 thymidine kinase and a higher rate of phosphorylation. Phosphorylation of 2'NDG monophosphate to the diphosphate by cellular GMP kinase is also more efficient than the cor-

←

Fig. 11.14. Therapeutic effect of foscarnet on cutaneous and genital herpes infections in guinea pigs. Above, guinea pig infected with HSV-1 on four sites on the back. One day after inoculation two sites were treated with placebo cream twice daily for four days and two sites were treated with 3% foscarnet cream in the same way. Five days after inoculation the foscarnet treated areas had healed while the placebo treated show large vesicles. Below, genital HSV-2 infection in female guinea pigs. The right animal was treated with foscarnet and the left with placebo cream six times daily for five days. (courtesy of Dr A-C Ericson.)

responding reaction with acyclovir monophosphate, leading to a high concentration of 2′NDG triphosphate in the infected cell. These properties might explain why 2′NDG is more active in HSV-2 infected mice than acyclovir, although acyclovir is equally active in cell culture (Ashton et al. 1982).

Trifluorothymidine (TFT, trifluridin – Burroughs-Wellcome). The mode of action could be a preferential incorporation into herpesvirus DNA, resulting in short DNA pieces and defective viral proteins (Heidelberger, 1975, Müller, 1979). In contrast to the other thymidine analogues, TFT does not require a viral thymidine kinase for phosphorylation to the monophosphate. TFT inhibits the phosphorylation of dTh and TFT monophosphate prevents the formation of dTMP from dUMP (Müller, 1979). These effects will decrease DNA synthesis due to a decreased dTTP concentration. TFT has a high cellular toxicity and is less selective than the other inhibitors in Fig. 11.9, restricting its use in the clinic to herpes keratitis (where nevertheless it is very useful).

Vidarabine (Adenosine arabinoside, araA - Warner-Lambert). Vidarabine is phosphorylated in both herpesvirus-infected and uninfected cells to a triphosphate. The triphosphate shows a selective inhibition of HSV-1 DNA polymerase in cell-free assays (Müller et al., 1978) and a selective inhibition of viral DNA synthesis has also been noticed in infected cells (Shipman et al., 1976). Other mechanisms could also be involved (See Shannon and Schabel, 1980) but the main antiviral effect seems to be on the viral DNA polymerase, where both a selective inhibition and a partial incorporation are indicated as shown in Fig. 11.15. Vidarabine is rapidly deaminated to ara-hypoxanthine which has less antiherpes activity. This deamination can be reduced by the use of deaminase inhibitors, such as coformycin or co-vidarabine. Vidarabine monophosphate can be used instead of vidarabine with the advantage of a higher water solubility, and the mechanism of antiviral action is probably the same as for vidarabine.

Combinations. The use of combinations of antiherpes drugs has mainly been limited to cell culture experiments. Since there are inhibitors with different modes of action, two advantages with combinations could be expected, namely a synergistic effect and a reduced risk of development of drug resistance (as with the well established principle of treating tuberculosis with two or three drugs). In cell cultures

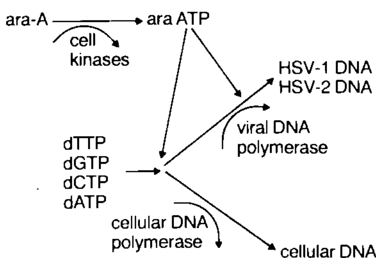


Fig. 11.15. Mechanism of action of vidarabine (araA).

TABLE 11.7.
Effects of foscarnet, separately and in combination with BIOLF-62, acyclovir, and BVDU, on plaque reduction of HSV-1 Patton in HFF cells (after Smith et al., 1982b)

Drug[a]	Virus titre ($PFU/ml \times 10^6$)	Fold titre reduction
BIOLF-62	1.2	260
Foscarnet	52	6.0
BIOLF-62+foscarnet	<0.005	>63 000
BVDU	3.2	98
Foscarnet	52	6.0
BVDU+foscarnet	1.5	210
Acyclovir	98	3.2
Foscarnet	52	6.0
Acyclovir+foscarnet	110	2.9
No-drug control	315	1

[a] The following drug concentrations were used: BIOLF-62, 0.7 μg/ml (10 ED_{50}); foscarnet, 90 μg/ml (5 ED_{50}); BVDU, 0.08 μg/ml (10 ED_{50}); acyclovir, 0.84 μg/ml (10 ED_{50}).

synergistic effects have been reported for Biolf-62 and foscarnet and for BVDU and foscarnet as shown in Table 11.7 (Smith et al., 1982b). Burkhardt and Wigand (1983) have used several combinations of antiherpes drugs and determined the therapeutic effects on cutaneous HSV-1 infections in guinea pigs. Their results show a synergistic effect of topically applied acyclovir and foscarnet. Human studies have not yet been reported, but combinations of antiviral drugs will certainly be tested clinically in the future.

11.9. Summary

The ability of herpesviruses to form latent infections, more or less effectively controlled by immune mechanisms, makes prevention and treatment of disease more complicated than for most other viruses. These latent infections have not been eliminated by antiviral compounds active against herpesvirus replication and moreover, no rational way of affecting latency has been found. It can even be argued that we *need* a latent, occasionally reactivated virus to boost our immunity and that the elimination of latent virus could result in new severe infections of a primary type.

The attempts at prevention of herpesvirus infections by vaccination have not been very successful to date and the intrinsic ability of herpesviruses to avoid the immunological defence might, even in the future, restrict the development of vaccines.

Chemotherapy against herpesvirus infections has received considerable attention and a large number of antiherpes drugs have been developed. This has been facili-

tated by the presence of several viral enzymes amenable as targets for antiviral drugs, the enzymes most commonly involved being viral thymidine kinase and DNA polymerase. It is becoming increasingly evident that the cell culture efficacy of antiherpes drugs often has little relevance to the in vivo efficacy and an understanding of the reasons for this will probably result in the development of more effective drugs. Antiviral drugs with different modes of action are known, making combinations possible both to reduce the risk for resistance development and to increase the efficacy. A continuous rapid development of new antiherpes drugs can be anticipated.

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CHAPTER 12

Herpes simplex virus infections

12.1. Labial and cutaneous herpes simplex virus infections

12.1.1. Clinical aspects

12.1.1.1. Primary infection

Most children are infected with HSV-1 during their first years of life. In this primary infection virus is transmitted from other children or from parents with herpes vesicles and this mostly results in a symptomless infection. In about 10% of the cases the infection, after an incubation period of about 1 week (Hale et al., 1963), develops into a gingivostomatitis, with ulcerations in the mouth or localized vesicles in the perioral area. The gingivostomatitis is normally accompanied by fever and heals spontaneously in 1–3 weeks although virus can be excreted for as long as 3 weeks (Buddingh et al. 1953).

A cutaneous herpes infection can also take place later in life and involve any part of the body. Dentists are sometimes infected in their fingers (herpetic whitlow) when working with patients having cold sores and wrestlers can be infected on the arms and legs (herpes gladiatorum) by an antagonist with herpes vesicles. A primary infection in eczematous children can lead to a widespread involvement of eczematous skin (Kaposis's varicelliform eruption, eczema herpeticum). In most instances the labial and cutaneous herpes infections are caused by HSV-1, but infections with HSV-2 occur and can give the same symptoms.

During a primary infection with HSV the virus passes in the nerves from the infected area to a ganglion where it establishes a latent infection as illustrated in Fig.

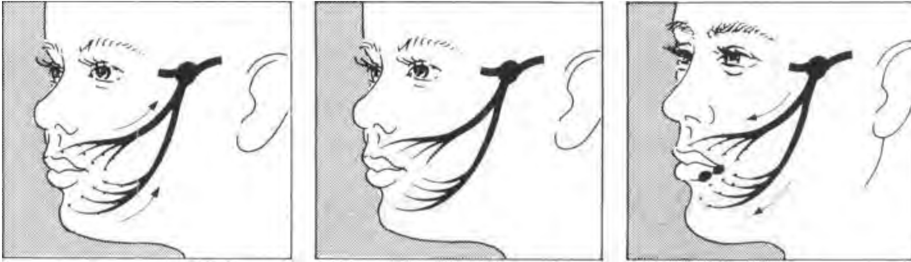


Fig. 12.1. Labial herpes infection. Establishment of latency and a recurrent episode.

12.1. It is presently unclear in what state the latent virus exists, but there is possibly a slow virus replication and a slow release of virus through the nerves to the skin, normally not leading to any symptoms. Thus virus can be isolated from the saliva in 1–5% of asymptomatic adults (Herrmann, 1967, Lindgren et al., 1968). However, in a stress situation, during fever, menstruation, sunburn, or any sort of immunosuppression, virus reaching the skin will cause a recurrent episode of vesicle formation. In an alternative hypothesis, these triggering factors are thought to induce the formation and release of virus from the ganglion and the virus will start to pass down to the skin and cause a recurrent episode. It is not clear whether virus during a recurrent episode also moves back from the vesicles to the ganglion or if it is only a one way transport. The presence in human ganglia of viral mRNA coded by only a part of the genome indicates that an incomplete transcription takes place during latency (Galloway et al., 1982). Aspects of latent infection have been reviewed recently by Klein (1982) and this paper should be consulted for more details.

12.1.1.2. Recurrent infection

A pattern of recurrent episodes normally establishes itself in patients having had a primary HSV-infection with symptoms. In most cases a small number of vesicles are formed in the perioral area (Fig. 12.2). The disease is usually not associated with any constitutional symptoms and the sores heal without scars.

The time course of the recurrent disease (herpes labialis, cold sores) is much shorter than in the primary infection and has been carefully studied by Spruance et al. (1977), Rytel et al. (1978) and Bader et al. (1978). A typical appearance is shown in Fig. 12.3. Vesicles begin to appear as early as 1–2 days after exposure to a triggering factor, mostly in the perioral area. In about 80% of the cases (Spruance et al., 1977) prodromal symptoms like itching or tingling will precede erythema and papules. The time sequence is illustrated in Fig. 12.4 from the work by Spruance et al. (1977). A very rapid progression of the recurrent labial herpes infection is evident. Also, when considering the multiplication and presence of virus in the skin, a very rapid decrease in virus titre with time has been observed as shown in Fig. 12.5. This is important to keep in mind when considering evaluation of antiviral therapy directed against this virus infection.

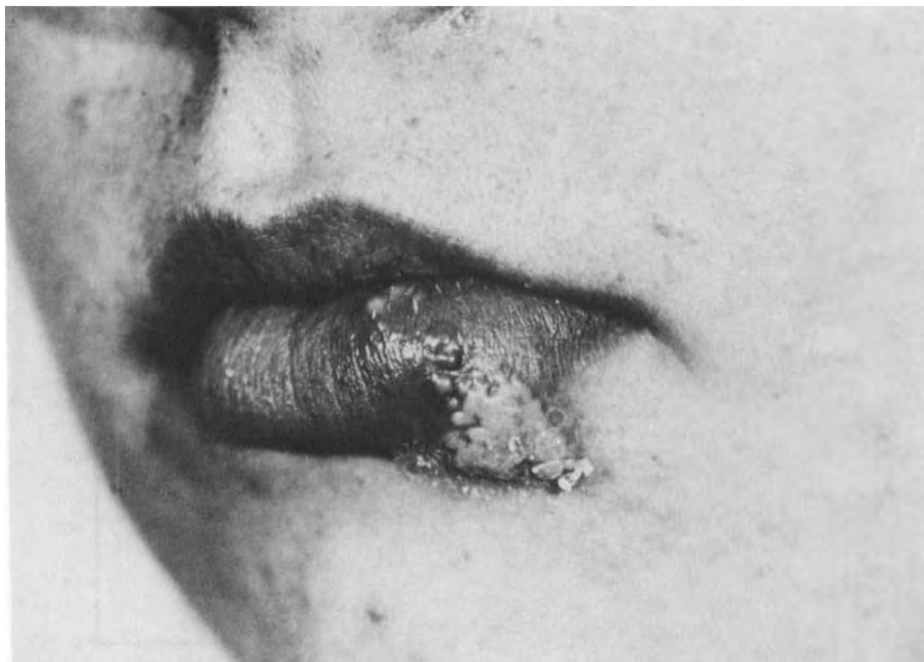


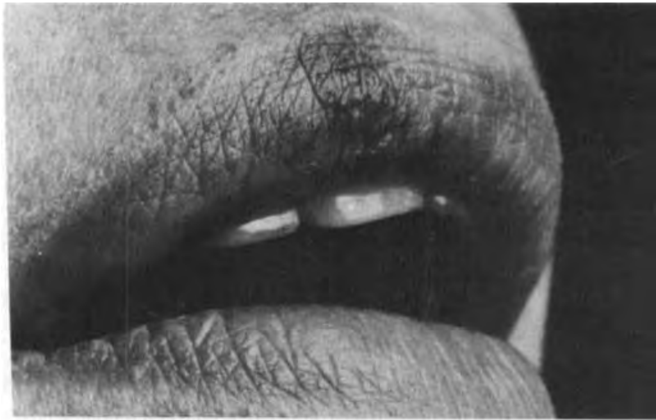
Fig. 12.2. Recurrent labial herpes simplex type 1 infection.

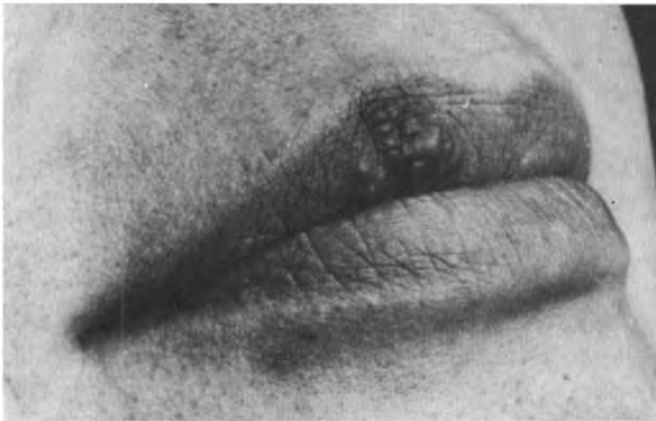
Clinical diagnosis of the disease is easy and does not normally require virus isolation or other laboratory methods. Microscopically, multinucleated cells, ballooning degeneration of epithelial cells and intranuclear inclusion bodies can be observed (Juel-Jensen and McCallum, 1972). Immunofluorescent methods are also available, but the very rapid progress of the disease makes most of the present diagnostic procedures, apart from direct observation, useless in the design of a treatment. This speed is important from the point of view of intervention by specific antiviral proprophylaxis – patients may have to self-initiate treatment or prophylaxis.

12.1.2. Epidemiology

The epidemiology of herpes infections involves problems such as correct typing of the virus, a high frequency of subclinical infections, both primary and recurrent infections, the presence of concomitant HSV-1 and HSV-2 infections in one patient and the possibility of carrying more than one type or strain of virus in the latent form.

It seems clear that transmission of both HSV-1 and HSV-2 occurs by close contact between the susceptible host and a person excreting virus with, and possibly also without, clinical signs of disease. Certainly a substantial number of persons without clinical signs of labial or cutaneous herpes will excrete virus. Both HSV-1





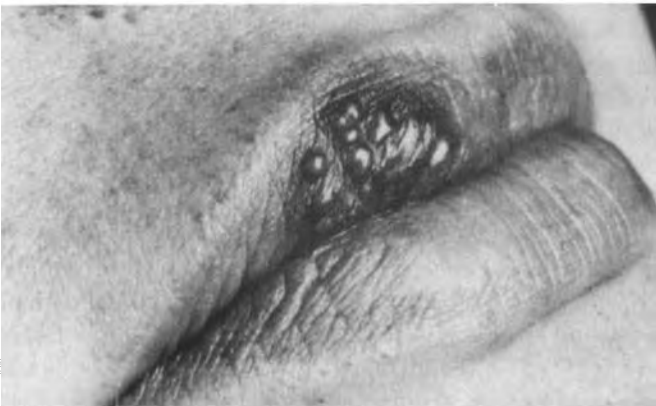


Fig. 12.3. Clinical course of recurrent labial herpes. The pictures were taken on the same day at 8.30 a.m., 10.00 a.m. and 4.00 p.m.

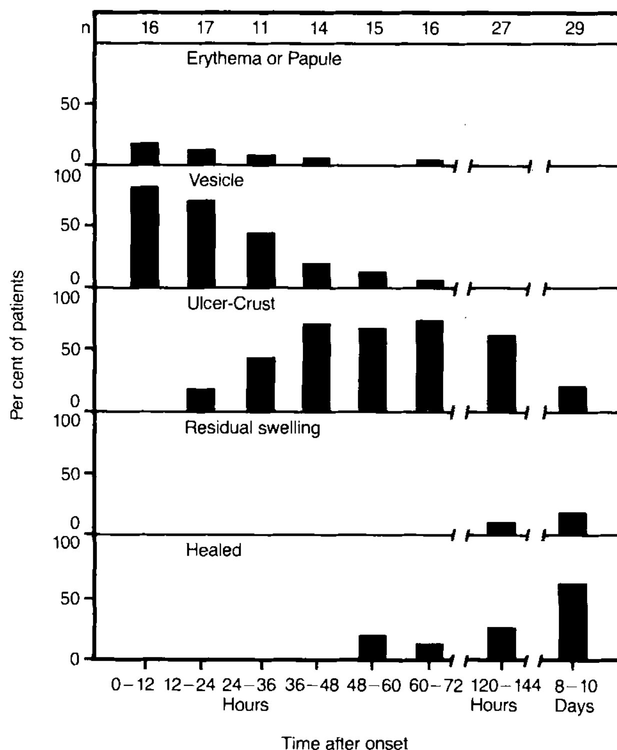


Fig. 12.4. Clinical course of recurrent labial herpes. Frequency of lesion stages at intervals after onset of recurrent labial herpes; n denotes the number of patients. (after Spruance et al., 1977.)

and HSV-2 are labile and will not survive for a long time outside cells, again suggesting close contact between persons as the main method of spread. Epidemic outbreaks in closed communities have been reported but the attack rates have been widely different. Thus, herpes stomatitis has been observed in children's homes at rates of 77% per month (Hale et al., 1963, Juretic, 1966), 56% per 11 months (Anderson and Hamilton, 1949) and 10% per 6 years (Cesario et al., 1969).

The prevalence rate of HSV infections can be determined from symptoms of disease, serology and the presence of latent virus in ganglia. In a study of ganglia from adult cadavers, Baringer and Swoveland (1973) found trigeminal ganglia to contain HSV-1 in 6 of 7 cases. Serological studies indicate that in adults the prevalence rate of antibodies to HSV is 50–100%, the level probably depending both on serological methods used and the study groups selected (Nahmias and Starr, 1977, Rawls and Campione-Piccardo, 1980). Antibodies to HSV-1 are normally acquired during the first years of life (Rawls et al., 1970) and the level of neutralizing antibodies seems not to correlate with the frequency of recurrences (Douglas and Couch, 1970).

The prevalence rate for clinical symptoms has been reported for labial herpes in 1800 students (Ship et al., 1961). In this group 1.9% had lesions once a month, 13%

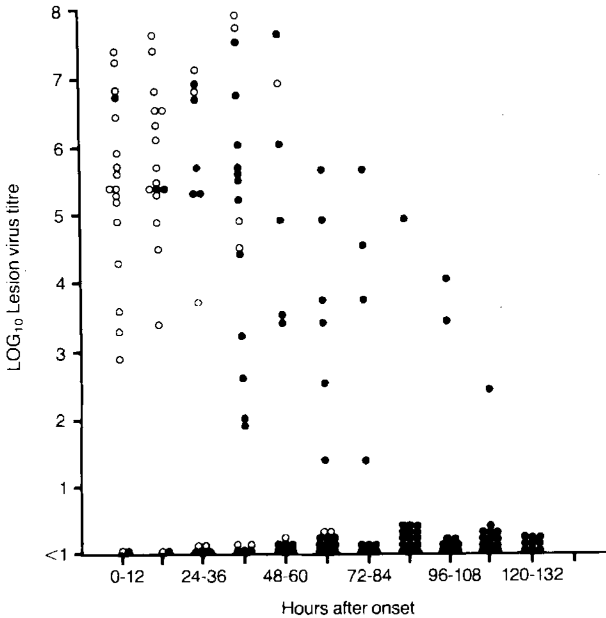


Fig. 12.5. Virus titre from swabs of vesicles and ulcers/crusts in patients with recurrent labial herpes. (after Spruance et al., 1977.)

had lesions at intervals of 2–11 months and 24% had lesions less than once a year. It has been estimated (Overall, 1980) that there are 98 million annual episodes of recurrent labial herpes in the US involving 33% of the population and with an average of 1.6 episodes per year. Surveys in many countries indicate that the disease is common in all geographical areas (Embil et al., 1975, Black, 1975).

The relative importance of HSV-1 and HSV-2 in herpes infections is illustrated in Table 12.1 from Nahmias et al. (1980). In general, HSV-1 predominates in infections above the waist and HSV-2 below, but in local communities this may not be the case and HSV-1 may be isolated frequently from both areas of the body.

12.1.3. Vaccination

Persons with frequent episodes of recurrent labial herpes caused by HSV-1 can have high titres of neutralizing antibodies against the virus (Heineman, 1966). Moreover, an inoculation with virus from labial lesions on one patient into the arm of the same patient resulted in a new recurrent cutaneous infection and not a decrease in the frequency of recurrent labial herpes as hoped (Blank and Haines, 1973). In view of these observations, immunization against labial or cutaneous HSV-1 infections seems likely to be ineffective, but controlled clinical trials will be necessary to investigate the possible efficacy of vaccines.

TABLE 12.1.
Clinical spectrum of infections caused by herpes simplex virus 1 and 2 in newborns and older persons and the type isolated from different sites and clinical conditions (after Nahmias et al., 1980)

	Number of individuals with HSV type		
	Type 1	Type 2	Total
I. Usually mild to moderately severe (persons over 1 month of age)			
A. Urogenital infections			
1. Females (cervic, vulva, vagina, urethra)	34 (7[b])	272 (5[c])	306
2. Males (penis, urethra)	6 (1[b])	187 (1[d])	193
B. Nongenital infections			
1. Gingivostomatitis or asymptomatic (mouth)	131	3 (1[e])	134
2. Herpes labialis (lips)	84	0	84
3. Keratitis and/or conjunctivitis (cornea and/or conjunctiva)	28	1	29
4. Dermatitis			
a. Skin above waist	84 (1[e])	3	87
b. Skin below waist	4 (1[b], 1[e])	72 (6[e], 2[f])	76
c. Hands or arms	13 (1[f])	11 (2[f], 1[e])	24
C. Latent infections (trigeminal or thoracic ganglia)	21 (1[g])	0	21
(sacral ganglia)	0	5	5
II. Usually severe to fatal (persons over 1 month of age)			
A. Meningoencephalitis (brain, spinal cord, CSF)	86	2	88
B. Multiple sclerosis (brain)	0	1	1
C. Eczema Herpeticum (skin, lungs)	11	0	11
D. Generalized disease (visceral organs)	2	1[h]	3
III. Newborns – localized or generalized infection (skin, eyes, brain, CSF, visceral organs)	36	74	110
Total	540	632	1172

[a] Typing is done by microneutralization or direct immunofluorescence tests.
[b] Simultaneous isolation of similar HSV type from mouth.
[c] Simultaneous isolation of type 2 HSV from cervix or vulva and type 1 HSV from lip or mouth.
[d] Simultaneous isolation of type 2 HSV from penile lesions and type 1 HSV from eye.
[e] Simultaneous isolation of same type from genitals.
[f] Laboratory- or hospital-acquired infection
[g] Simultaneous isolation of type 2 HSV from sacral ganglia
[h] Isolated also from brain.

The consideration of an HSV-1 vaccine also poses problems such as should one vaccinate early and try to prevent the formation of a latent natural infection, can vaccination change an already established infection, should one have a DNA-free vaccine, are frequent revaccinations necessary, what will be the effect of an infection

later in life if the vaccine induced immunity has deterioriated, and is there a positive cost-benefit and risk-benefit for such a vaccination?

Herpes simplex type 1 vaccines have been tested and used to some extent but there is, to date, no well-controlled study showing clinical efficacy. Hopefully the present studies using HSV-2 vaccine for the prevention of primary genital disease or for reducing the recurrent disease could indicate same future possibilities for an HSV-1 vaccine. The possibilities for HSV vaccines have been well discussed by Rapp and Adelman (1982) and by Allen and Rapp (1982).

12.1.4. CHEMOTHERAPY

12.1.4.1. Primary infection

This infection offers an easier target for an antiviral drug than the recurrent infection because of the extended course of the disease. The possibility of treating a primary labial herpes infection with an antiviral drug has not been extensively evaluated. It is likely that drugs active against recurrent labial herpes infection will be even more effective actually on a primary infection.

12.1.4.2. Recurrent infection

A large number of remedies have been tested and are used for this recurrent disease, and most of the current usage is based on uncontrolled studies. For the following compounds there are *no* convincing results showing clinical efficacy: topical use of araC, araA, araAMP, ribavirin, 2-deoxy-D-glucose, tromantadine (Viru-Merz®), zinc, urea, tannic acid, oral treatment with lysine, photodynamic inactivation with neutral red, ether, ethanol, high temperature, low temperature and ultrasound. This list is not complete. In some cases the original claims of efficacy have been derived from uncontrolled studies which were subsequently found not to be reproducible. For this reason anecdotal reports concerning other compounds cannot be accepted until they have been supported by double-blind placebo-controlled trials. It is quite obvious that there is a very considerable placebo effect with herpes labialis and this has led to false conclusions in many studies which have not been placebo-controlled or double-blind. Excellent reviews of the different treatments attempted have been presented by Overall (1979, 1980). The first true therapeutic effect of a compound was probably observed by Juel-Jensen and MacCallum (1966), using idoxuridine in dimethyl sulphoxide (DMSO) although the smell of DMSO and the toxicity of idoxuridine makes this treatment unsuitable for widespread use. However, two compounds, foscarnet (Wallin et al., 1979) and acyclovir (Fiddian et al., 1983), have now shown reproducible effects when used topically against recurrent labial herpes infection.

The therapeutic effect by foscarnet is illustrated in Fig. 12.6 where the time to crust formation is compared for a 3% foscarnet cream and a placebo cream. A sig-

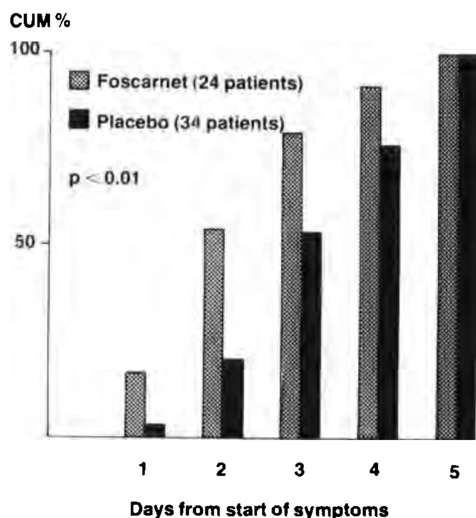


Fig. 12.6. Therapeutic effect of 3% foscarnet cream on recurrent labial herpes. The diagram shows the cumulative percentage of patients who have passed the vesicular stage after six daily treatments with 3% foscarnet or placebo for four days. (J. Wallin, personal communication.)

nificant reduction in time to crust formation is achieved when treatment is initiated early. This was observed also in a cross-over study where two episodes in a single patient, one treated with foscarnet and one with placebo, were compared (Fig. 12.7). It can be concluded from the foscarnet trial that early treatment is important and this correlates well with the rapid course of the disease. Therefore a patient must have the treatment at home in advance to be able to treat early, preferably during the period of prodromal symptoms.

Acyclovir at 5% in a PEG formulation has been tested in large and well-controlled studies (Spruance et al., 1982a) but no therapeutic effect was found when clinical variables were analyzed. However, significant effects *were* observed by Fiddian et al. (1983) using acyclovir in a new vehicle and the results are summarized in Table 12.2.

A note of caution should be added concerning the lack of reproducible clinical effect of the large number of compounds tested in the past. In view of the knowledge from trials with foscarnet (Wallin et al., 1979) and acyclovir (Spruance et al., 1982a, Fiddian et al., 1983) it would seem that earlier clinical trials were designed in a way which would probably not have shown an effect even for an active drug! However, animal studies with nearly all of the above mentioned clinically inactive compounds have also shown a lack of effect or, at most, only a very slight one (Alenius and Öberg, 1978, Alenius, 1980). Foscarnet and acyclovir (Alenius et al., 1982), on the other hand, have been shown quite conclusively to be therapeutically

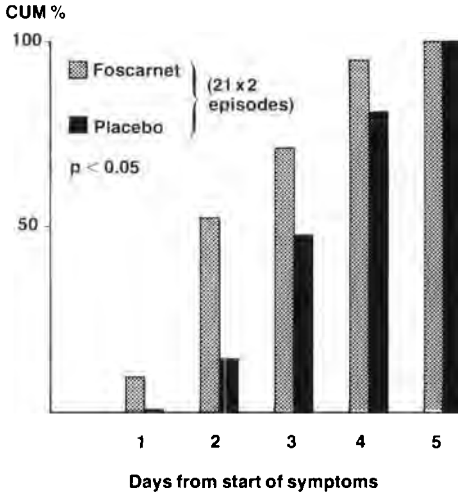


Fig. 12.7. Therapeutic effect of 3% foscarnet cream on labial herpes comparing two episodes in each patient. The diagram shows the cumulative percentage of patients who have passed the vesicular stage. One episode was treated six times per day for four days with foscarnet and one episode with placebo in each patient in this cross-over study. (J. Wallin, personal communication.)

TABLE 12.2.
Effect of 5% acyclovir cream on recurrent labial herpes (after Fiddian et al., 1983)

	First episode			All episodes		
	Placebo (n=24)	Acyclovir (n=24)	P value	Placebo (n=40)	Acyclovir (n=34)	P value
No. of abortive lesions	2	8	0.03	4	9	0.06
No. of new lesions	1	2	0.52	3	2	0.10
No. of days to ulcer or crust	2	1	0.02	2	1	0.04
No. of days to healing	6	1	0.02	6	4	0.01
Duration of itching (days)	1	0.5	0.21	1	1	0.15
Duration of all symptoms (days)	3	1	0.07	2	1	0.11

Treatment was initiated within 24 hours of onset of the attack. After the first treated episode the patients were re-randomized for the next episode.

active in animal studies as indicated in Chapter 11 where the activity of foscarnet and acyclovir is compared to some other antiherpes drugs.

12.1.5. Interferon and immunomodulators

Non-specific immunostimulants such as vaccinia virus vaccine or levamisole have been tested, but there are no results from placebo-controlled double-blind studies

showing any statistically significant effects against HSV infections (Russel et al., 1978, Overall, 1979, Overall, 1980). The use of transfer factor has been reported in an uncontrolled study (Khan et al., 1981).

The only well-controlled study showing any effect of interferon is that from Pazin et al. (1979) where patients undergoing trigeminal root surgery were given a prophylactic treatment with leukocyte interferon. As shown in Table 12.3 this resulted in a reduced frequency of labial herpes lesions. The use of interferon in the clinic for uncomplicated recurrent labial or cutaneous herpes simplex infections seems unlikely in the near future, and indeed, the temperature increase caused by interferon could possibly trigger herpes lesion formation. Overall et al. (1981) and Spruance et al. (1982b) have recently shown that lesions from labial herpes contain high leukocyte interferon titres, thus making it unlikely that exogenous interferon would have any therapeutic effect. Table 12.4 shows the titres of interferon observed by Overall et al. (1981). These important observations should be kept in mind when considering interferon therapy of viral infections, and Spruance et al. (1982b) furthermore observed that the interferon titres correlated with lesion age and that high titres were observed in lesions less than 12 hours old.

12.2. Genital herpes simplex virus infections

12.2.1. Clinical aspects

Genital herpes infections are mostly caused by HSV-2, but HSV-1 causes the same symptoms and has been isolated from genital herpes infections in frequences rang-

TABLE 12.3.
Prevention of reactivated labial herpes simplex infection by prophylactic use of leucocyte interferon in patients operated for tic douloureux (after Pazin et al., 1979)

Group	No. of patients	Manifestations of reactivation		Total reactivated[a]
		Herpetic lesions	Virus in throat wash	
Interferon	19	5 (26%)	8 (42%)	9 (47%)
Placebo	18	10 (56%)	15 (83%)	15 (83%)
Totals	37	15 (41%)	23 (62%)	24 (65%)
P value		>0.05[c]	<0.05[c]	0.049[b]

[a] Lesions or isolated virus or both
[b] Fisher's exact method, 2-tailed
[c] χ^2 with Yate's correction.
7×10^4u/kg/day of HLIF or placebo was given i.m. twice per day for 5 days starting the day before the operation.

TABLE 12.4.
Levels of interferon (IFN) in vesicle fluid from lesions of recurrent herpes labialis (after Overall et al., 1981)

Subject No	Lesion duration (hr)[a]	Vesicle fluid	IFN titre (units)	HSV titre in vesicle fluid (log pfu/ml)
		Quantity (μl)	Vesicle fluid	
1	49	2	63 600	9.0
2	19	3	< 680	<4.8[b]
3a[c]	50	3	17 000	9.0
3b[c]	17	2	8100	6.7
4	25	1	44 200	9.2
5	8	3	25 800	<4.8[b]
6	14	3	1400	<4.8[b]
7	N.A.	1	8000	9.2
8	N.O.	1	18 100	9.1
9	48	7	22 200	8.8
10	4	1	3000	<4.8[b]
11	3	3	28 600	6.8
12	25	1	5000	6.3
13	32	5	6600	6.6
14	29	3	36 700	9.4
15	16	1	10 100	9.4
16	34	2	8100	7.2
17	58	1	54 300	9.1
18	18	1	18 100	9.5
19	9	1	26 100	9.3

[a] Interval between onset of physical sign of lesion and removal of vesicle with calibrated capillary tube.

[b] Lowest quantity of IFN or herpes simplex virus (HSV) detectable with the dilution of specimen used for titration.

[c] Two separate epidsodes in the same patient.

N.A., information not available; first episode of recurrent disease for this patient.

N.O., information not obtained.

ing from 2% (Wallin et al., 1983) to 53% (Chang et al., 1974, Ishiguro et al., 1982) in different patient groups (see Table 12.1). Isolates of both HSV-2 and HSV-1 from the same patient during separate episodes of lesions have been obtained (J. Wallin, personal communication) as well as different HSV-2 strains from different episodes of lesions in the same patient (Buchman et al., 1979). The disease can be subdivided into a *primary* infection in a patient not having antibodies against HSV-1 or HSV-2; an *initial* infection where the patient has antibodies to HSV-1 from a previous labial infection or *recurrent* genital infection caused by activation of latent virus present in the sacral ganglia.

12.2.1.1. Primary and initial infections

The primary infection is most severe and will in women (Fig. 12.8) involve vesicles, rapidly changing to ulcers, on the labia majora and minora, vestibule of the vulva, perineal area, vagina and cervix. In men, lesions occur mostly on the glans penis, prepuce, sulcus and the shaft of the penis. Fever and malaise are common and the primary disease can require hospitalization. The initial disease is thought to be somewhat milder. An initial or primary infection can also be asymptomatic (Rawls et al., 1971), but usually clinical manifestations appear after an incubation period of 3–7 days (Kaufman et al., 1973, Nahmias and Roizman, 1973, Poste et al., 1972, Gardner and Kaufman, 1972). Prodromal symptoms have been observed in women with initial disease, but these might be unusual (Brown et al., 1979). Mild paresthesia and burning may precede the lesions (Overall, 1979). Lesions begin at a localized focus and often spread across the external genitalia and the total number of lesions often exceeds 10. The rectum, bladder and upper genital tract in women can be involved and new lesions may develop while others start healing. A careful study of the sequence of events has been presented by Brown et al. (1979) and the virus titres for 39 women are shown in Fig. 12.9. The virus titres are higher and virus persists longer than in the recurrent infection.

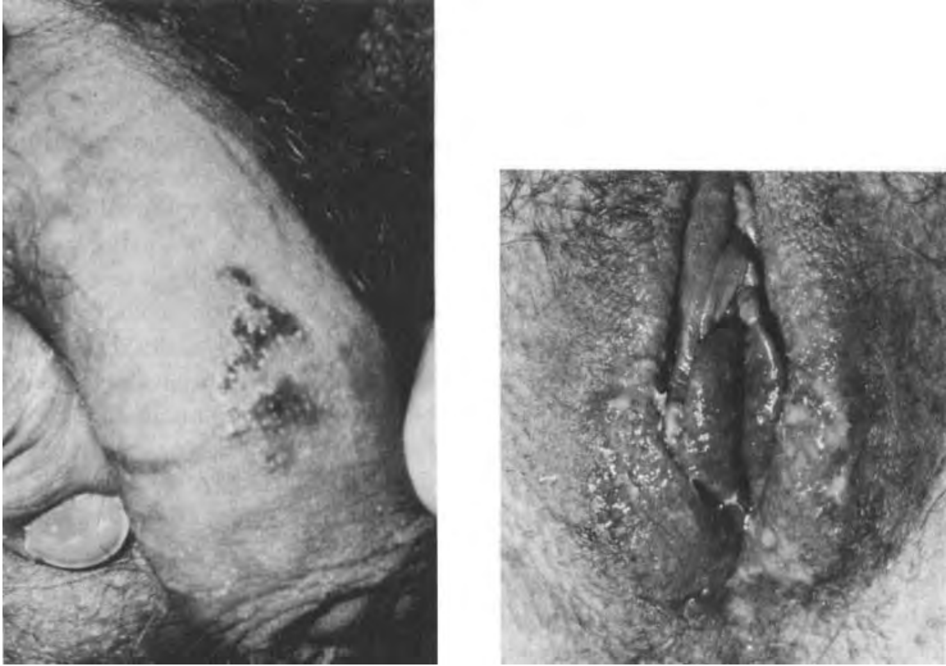
In the normal host, viraemia is probably infrequent, but complications with meningitis can occur (Craig and Nahmias, 1973). The symptoms usually persist for 2 weeks. This increases the possibility of blocking virus replication by antiviral agents in a primary/initial infection as compared to a recurrent episode, assuming that patients start medication equally early.

12.2.1.2. Recurrent infection

The symptomatic first episode of genital herpes is normally followed by recurrent episodes (Fig. 12.8) when the infection is caused by HSV-2 and less frequently when caused by HSV-1 as shown in Fig. 12.10 (Reeves et al., 1981).

The recurrent disease can cause considerable psychological distress but is significantly milder from a clinical point of view than the primary or initial disease. In women the recurrent disease can pass with very minor symptoms and can, for anatomical reasons, be overlooked. Virus can be excreted even in a symptom free state but the highest virus titres are found early during an episode of vesicle formation.

A recurrent episode can be initiated by triggering factors such as menstruation and stress (Guinan et al., 1981). A recurrent episode due to activation of virus latent in the sacral nerves could be confused with a reinfection but this seems not to be a frequent event (Corey et al., 1981). Prodromal symptoms such as itching, burning and neuralgia have been noted in 46% of persons in one investigation (and was similar for women and men) (Brown et al., 1979) and in 85% of the women in another study (Guinan et al., 1981). In an extensive study by Nahmias et al. (1982a) 73% of the males and 84% of the females experienced prodromal symptoms and in 57% of the men and 68% of the women this happened in at least 3 out of 4 episodes.



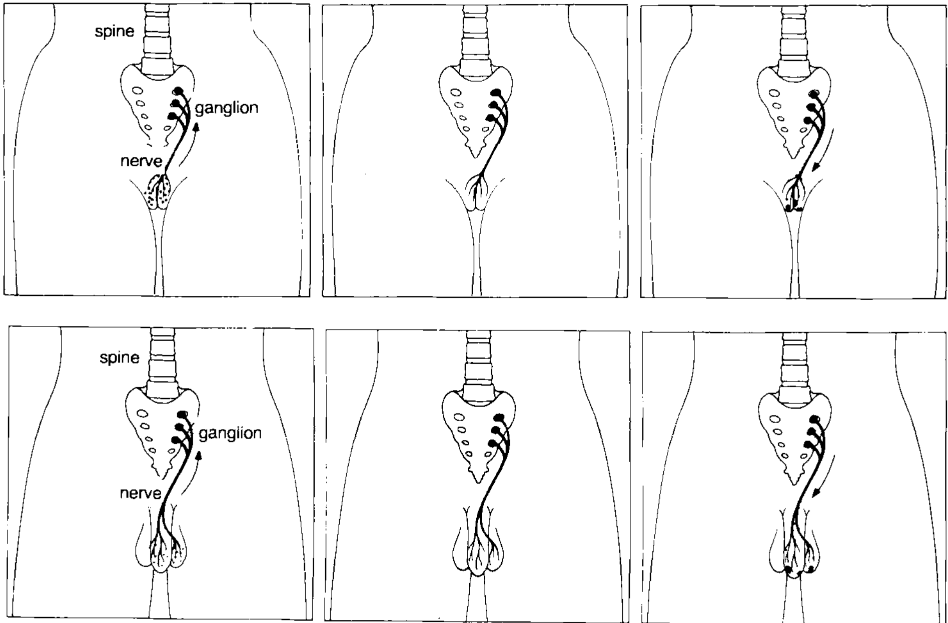


Fig. 12.8. Above, primary genital herpes in a female and a recurrent herpes in a male patient. Below, establishment of latency. (Courtesy of Dr. J. Wallin.)

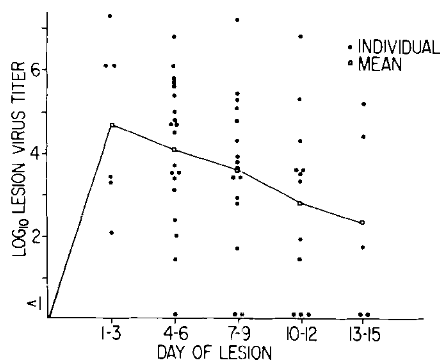


Fig. 12.9. Virus shedding in primary (initial) genital herpes in women. (after Brown et al., 1979.)

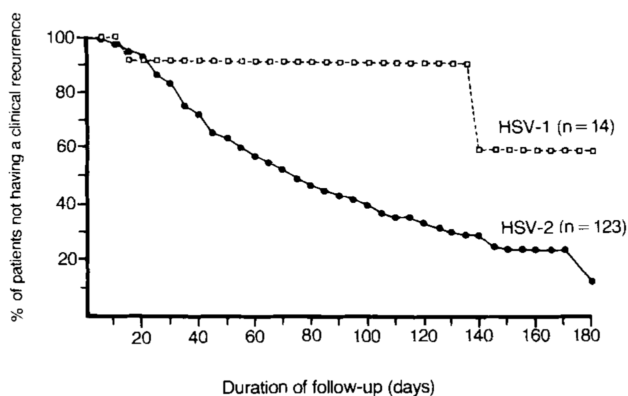


Fig. 12.10. Time to first clinical recurrence in patients presenting with first episodes of genital herpes. The frequency of subsequent recurrences was significantly lower in patients with HSV-1 than those with HSV-2 ($P \leqslant 0.01$, Mantel-Haenszel test). (after Reeves et al., 1981.)

False prodromals, not followed by vesicles, appear to be infrequent (less than 10%). In most cases the prodromal symptoms preceded the development of lesions by more than 24 hours.

Only a few lesions are formed in a recurrent episode and Guinan et al. (1981) found 1–4 lesions in women. On dry skin the lesions develop into ulcers and crusts and then heal. On wet skin areas these different stages are not as distinct and ulcers persist longer and heal without crust formation. The time course of infection in women is shown in Fig. 12.11 from the data of Guinan et al. (1981). High virus titres are detected early during a recurrent episode and decrease rapidly both in men and women as shown in Figs 12.12 and 12.13 from the study by Brown et al. (1979). A recurrent episode has a duration of about one week (Brown et al., 1979, Guinan et al., 1981, Corey et al., 1982a). Cervicitis is uncommon in the recurrent disease and vaginal lesions seem to be uncommon (Guinan et al., 1981). As for recurrent

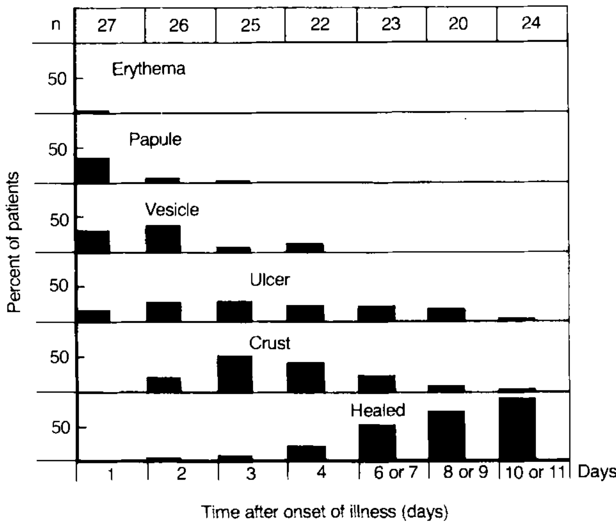


Fig. 12.11. Frequency of appearance of lesion stages during illness in women with recurring genital herpes simplex virus infection. The letter n denotes the number of patients examined on the day or days indicated. (after Guinan et al., 1981.)

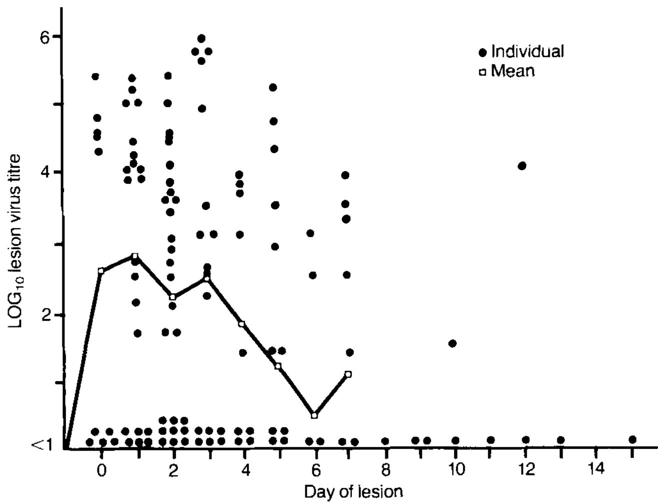


Fig. 12.12. Virus shedding in recurrent genital herpes in women. (after Brown et al., 1979.)

labial herpes, the rapid progress of the disease presents problems for antiviral chemotherapy, from the point of view of the rapidity with which therapy has to be started.

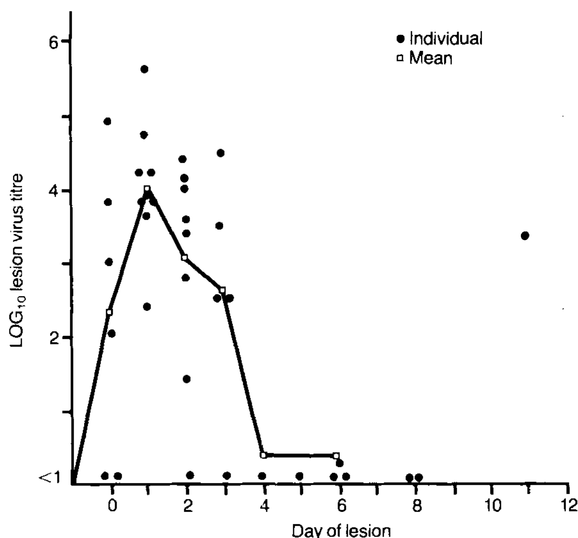


Fig. 12.13. Virus shedding in recurrent genital herpes in men. (after Brown et al., 1979.)

12.2.2. Epidemiology

The epidemiology of genital herpes infections involves the same basic problems as that of labial and cutaneous herpes infections. Infection by one strain of HSV-1 or 2 does not seem to prevent the infection by another strain (Buchman et al., 1979, Wallin, personal comm.). A further potential complication is that minor genetic variations, not easily seen by restriction enzyme analysis of viral DNA (see Chapter 17), have been seen in isolates from the same patient (Maitland et al., 1982). The accuracy of the statistics for genital herpes infections is improving but is still unsatisfactory. The increasing rate of patient consultations with private physicians in the US for genital herpes infections is shown in Fig. 12.14. This increase is probably dependent both on a real increase and on an increased awareness of the disease. There seems to be a greater tendency for genital HSV-2 infections to be recurrent than HSV-1 infections as shown in Fig. 12.10 from Reeves et al. (1981), and for more recurrences of HSV-2 in patients with the highest antibody titres against HSV-2 after the primary infecti)n (Table 12.5).

12.2.2.1. Transmission

The major route of transmission is close personal contact with a person shedding virus. The sexual pattern of transmission is shown not only by the location of the infection but also by the age-specific incidence being highest in young adults (Nahmias et al., 1969, Wolontis and Jeansson, 1977, Ng et al., 1970) especially those with multiple sex partners (Ishiguro and Ozaki, 1978). Transmission of HSV-1 to genital sites by oral-genital contact as well as homosexual transmission of HSV-2 resulting

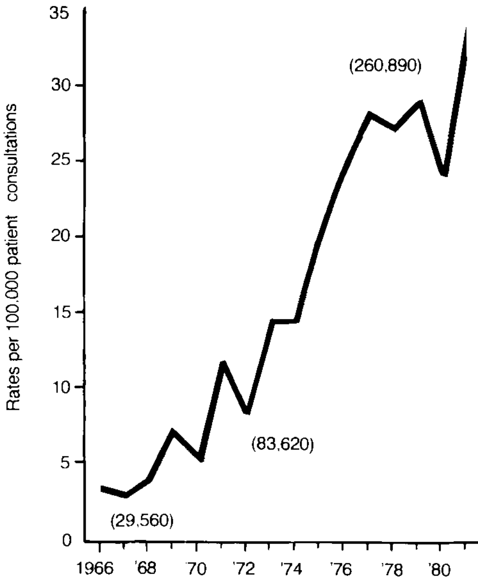


Fig. 12.14. Estimated rates of patient consultations with private physicians for genital herpes infections, United States 1966–1981. Numbers in parentheses are absolute figures. (after Wiesner and Parra, 1982.)

TABLE 12.5.
Relation between development of convalescent-phase neutralizing antibody to HSV-2 after primary genital HSV-2 infection and subsequent recurrence (after Reeves et al., 1981)

Recurrence/No recurrence (No. in group)		Patients seroconverting to HSV-2 No. (%)	Geometric mean titre among antibody seroconverters HSV-2	HSV-1
Recurrence	37	23 (62)	18.2	6.6[a]
No recurrence	23	8 (35)[b]	9.0[c]	7.5

[a] Significantly different from titre for HSV-2 ($P<0.01$, Student's t-test).
[b] Significantly different from values for patients with recurrence ($P<0.05$, chi-square test).
[c] Significantly different from values for patients with recurrence ($P<0.05$, Student's t-test).

in perianal lesions has been reported (Nahmias and Josey, 1976). The transmission can also occur by autoinoculation from one site to another. HSV-2 infections on the hands of medical personnel can occur from contact with infected patients. The virus is labile and therefore spread by infected objects such as towels seems unlikely. The risk of acquiring genital herpes after a single exposure to a partner excreting virus is not clear. A female with a partner having penile herpes probably has a risk of 60–80% of being infected (Nahmias and Starr, 1977, Rawls et al., 1971). The risk for males being infected by females has not been quantitated, nor has the risk

of infection by a symptomless virus-excreting partner been determined. Virus shedding from women, having asymptomatic genital herpes, has been detected in 4–14% of the women studied (Rattray et al., 1978, Adam et al., 1979, Centifanto et al., 1971, and Guinan et al., 1981). In a study on pregnant patients with genital herpes, as many as 43% had asymptomatic virus shedding at some time during pregnancy (Nahmias et al., 1971). Asymptomatic shedding of HSV-2 from men has been reported (Jeansson and Molin, 1970, Centifanto et al., 1972) but was not found in a later study (Deture et al., 1978).

It seems likely that the risk of transmission is highest early during a recurrent episode when the virus excretion is highest (Brown et al., 1979). It is possible that the use of condoms would reduce the risk of infection, but this has not been proven. The use of antiviral drugs such as acyclovir and foscarnet reducing the excretion of virus ought to reduce the risk of transmission, but this has not been proven either.

The immune factors determining the susceptibility of a host to a genital HSV infection are not clear, but reinfection with a new strain is possible even in a host with a recurrent genital herpes (Buchman et al., 1979).

12.2.2.2. Incidence and prevalence

The prevalence of antibodies to HSV-2 varies considerably between different populations and figures of 7–85% have been reported (For review see Rawls and Campione-Piccardo, 1980). In chaste nuns a prevalence of 3% has been reported (Nahmias et al., 1970) while 70% prevalence was found in prostitutes (Duenas et al., 1972, Tantivanich and Tharavanij, 1980). Baringer (1974) found HSV in the sacral nerves of 20% of the cadavers investigated. The prevalence of HSV-2 antibodies in persons of different ages is shown in Fig. 12.15. In one study, the incidence of cytologically detected genital herpes was 3–4% for VD clinic patients, 0.3–1% for patients in other clinics and 0.03–0.09% for private patients. (Nahmias et al., 1976) The incidence of HSV excretion in persons attending a VD clinic was, in one study, 4.4% in men and 5.2% in women and of these 31% males and 44% females did not show any lesions (Jeansson and Molin, 1970). The incidence of genital herpes in pregnant women has been reported to be 0.46% and in non-pregnant women 0.07% (Ng et al., 1970). It has been estimated that there are, in the US, 9 million persons infected with genital HSV and that the number of new cases per year is 300 000 (Overall, 1981). In a large student population genital herpes was more frequent than gonorrhea or syphilis (Sumaya et al., 1980).

12.2.3. Vaccination

The problems facing vaccination strategies against genital herpes are similar to those already discussed for labial herpes. As an example, inoculation with virus from a genital location into the arm of a patient only resulted in a new location

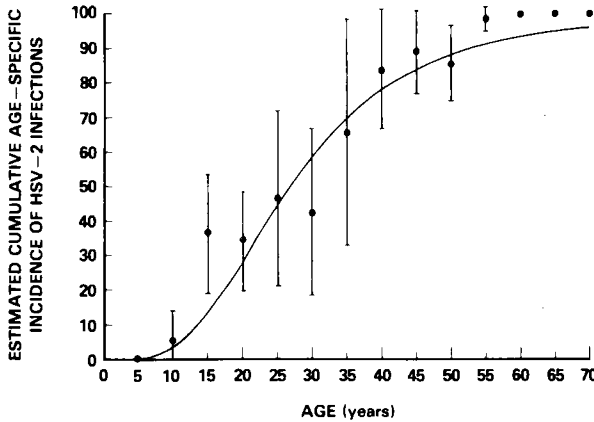


Fig. 12.15. Cumulative age-specific incidence of HSV-2 infections estimated from the data of age-specific prevalence of HSV-2 antibodies in different populations. The continuous line represents the best fit line assuming that the probability density function is a log-normal distribution. The bars represent one standard deviation above or below the value of the mean. (after Rawls and Campione-Piccardo, 1980.)

of a recurrent disease *added* to the old (Goldman, 1961). One difference is that a vaccine against genital herpes could be given later in life than one against labial herpes. A possible target group for vaccination would be partners of persons with known pre-existing latent genital recurrent HSV-2 and HSV-1 infection. Obviously this must be a restricted number of people, but a highly effective vaccine would undoubtedly make an important contribution to public health. An approach to constructing a vaccine would be firstly to identify common antigenic determinants betweeen HSV-1 and HSV-2 viruses (See Chapter 11). A more long term approach would be genetic engineering of immunogenic proteins and synthesis of short peptides (see Chapter 2) and such studies are in progress.

A recent workshop at the National Institute of Allergy and Infectious Disease (NIAID) carefully reviewed the whole concept of vaccines against genital herpes (Allen and Rapp, 1982). The main conclusion from this meeting was that we do not have sufficient basic information about the incidence of the disease, recurrency rate and the immune status of the population and that proposed clinical trials should be evaluated before consideration should be given to further development of vaccine. Several groups are presently developing vaccines and the main issue, whether immunization has any effect or not, should be answered in a few years. Production of a DNA-free vaccine is a lesser problem and progress in this direction by DNA hybrid technology has been reported (Faras and Enqvist, 1983). A modest start has also been made with an HSV-2 vaccine prepared from detergent lysates of human diploid cells infected with virus (Skinner et al., 1978).

12.2.4. Chemotherapy

12.2.4.1. Primary and initial infection

Due to long duration of virus replication in primary and initial genital herpes infections these syndromes should present an easier target for chemotherapy than the recurrent disease. However, few studies have been performed on the primary/initial disease partly due to the difficulty of recruiting a sufficient number of patients early in their disease. In Table 12.6 we list the double-blind placebo controlled trials performed so far, and their results on primary/initial infections. The use of topical ether was not beneficial (Corey et al., 1978). 2-Deoxy-D-glucose was reported to be effective both on initial and recurrent genital herpes (Blough and Giuntoli, 1978) but severe criticism has been raised concerning this study (Corey and Holmes, 1980, Overall, 1981) and in animal models 2-deoxy-D-glucose has been shown to be completely inactive on genital herpes (Kern et al., 1982). The present conclusion seems to be that this drug is ineffective.

The only significant effects reported so far on primary/initial genital herpes have been obtained with acyclovir (Table 12.6). Topical application of 5% acyclovir in PEG was effective in primary but not initial disease, reducing the time for virus shedding and, to a minor extent, the time to crusting and pain (Corey et al., 1982a). The duration of disease or the appearance of new vesicles were not affected. The results are shown in Table 12.7. Part of this study has been reported separately (Corey et al., 1982b). Oral treatment of primary and initial genital herpes disease probably resulted in a better therapeutic effect than the topical treatment, as shown by the study of Nilsen et al. (1982) in Table 12.8. Intravenous administration of acyclovir in patients with primary and initial genital herpes has been effective, although the number of patients is still small (Mindel et al. 1982). The result of this study is shown in Table 12.9.

A new cream formulation of acyclovir has recently been shown to reduce the duration of pain, time to healing, duration of viral shedding and duration of new lesion formation in patients with primary and initial genital herpes (Kinghorn et al., 1983).

12.2.4.2. Recurrent genital infection

As noted above, recurrent genital herpes infection is a common and important disease requiring therapy. The large number of remedies tested has been well reviewed by Overall (1979, 1981). The difficulty in performing clinical trials on recurrent genital herpes has only recently been appreciated and many older trials were designed in a way making evaluation, even of an active drug, impossible. The predictability of animal studies is not yet clear but as more well-controlled clinical studies appear it will hopefully be possible to predict clinical efficacy from animal studies.

TABLE 12.6.
Chemotherapy of primary/initial genital herpes

Drug	Administration	No. patients evaluated	Effects observed	Comments	References
Ether	Topical	35	No effect on pain, lesion healing, virus shedding or time to recurrence	Randomized treatment/ no treatment	Corey et al., 1978
2-Deoxy-D-glucose	Topical 0.19% cream	26	Shorter duration of lesions and virus excretion. Reduction of recurrence rates	Probably no effects. See text	Blough and Giuntoli, 1979
Acyclovir	Topical 5% ointment in PEG	77	Shorter duration of virus shedding, time to crusting of lesions and less pain	Effects shown only on primary infection	Corey et al., 1982a
Acyclovir	Oral 5 × 200 mg/day	31	Shorter duration of virus shedding, pain and itching, time to healing and less new vesicle formation	Primary and initial disease not separated but 22 seronegative for HSV	Nilsen et al., 1982
Acyclovir	Intravenous 5 mg/kg 8 hourly	30	Shorter duration of virus shedding vesicles, new lesion formation, and shorter time to healing	20 patients with primary and 10 with initial infection	Mindel et al., 1982
Acyclovir	Topical 5% cream	49	Shorter duration of virus shedding, pain, time to healing and new lesion formation	Better effect in females than in males	Kinghorn et al., 1983

TABLE 12.7.
Effect of topical acyclovir on first episodes of genital herpes. Acyclovir was used as a 5% ointment in PEG (after Corey et al., 1982a)

Symptom	Mean duration (days) after onset of therapy			
	Primary first episodes		Nonprimary first episodes	
	Acyclovir (n=28)	Placebo (n=23)	Acyclovir (n=28)	Placebo (n=11)
Itching	3.6 (±0.8)[b]	8.0 (±1.5)	3.4 (±2.5)	3.9 (±1.1)
Pain	5.2 (±0.6)[a]	7.0 (±0.7)	6.0 (±0.9)	3.9 (±1.1)
Dysuria	4.4 (±0.8)	5.0 (±0.9)	0.6 (±0.4)	1.3 (±0.3)
Vaginal discharge	6.1 (±0.5)	7.3 (±0.6)	6.8 (±0.8)	3.3 (±1.3)
Viral shedding from lesions	2.3 (±0.4)[c]	5.6 (±0.8)	1.0 (±0.3)	2.5 (±0.9)
Time to crusting of lesions	8.5 (±0.9)[a]	12.9 (±1.3)	6.0 (±1.1)	8.2 (±2.0)
Duration of lesions	11.2 (±1.3)[a]	15.8 (±1.4)	8.9 (±1.7)	13.9 (±3.5)

( ) = S.E.M.
[a] $P < 0.05$, Mantel Cox statistic
[b] $P < 0.01$, Mantel Cox statistic
[c] $P < 0.001$, Mantel Cox statistic

In Table 12.10 we list some double-blind placebo controlled trials where the design makes an evaluation possible. Some studies using IDU in DMSO indicated efficacy (Juel-Jensen and MacCullum, 1966, Parker, 1977) but this was not observed in later studies (Silvestri et al., 1979). The ready incorporation of IDU into cellular DNA, its teratogenic effect and the appearance of new more active antiherpes drugs makes further studies using IDU in DMSO unlikely. Several trials using araA have shown a lack of effect. The use of dyes and photoinactivation have been without effect in controlled trials and should be discontinued because of a possible induction of carcinoma (Berger and Papa, 1977). The objections raised against results obtained using 2-deoxy-D-glucose have been mentioned previously (Corey and Holmes, 1980, Overall, 1981, Kern et al., 1982). Similarly, ether and nonoxynol have not been effective agents (Corey et al., 1978, Vontver et al., 1979).

Two new drugs, acyclovir and foscarnet, have shown effects against recurrent genital herpes in double-blind placebo-controlled studies and both drugs are free of clinical side effects. The results using topical acyclovir (5% in PEG) are presented in Table 12.11 from the study by Corey et al. (1982a) and in Fig. 12.16 from Corey et al. (1982b). The major effect seen was a reduction in the duration of virus shedding. A slight reduction in the time to healing was seen for men but not for women (Fig. 12.16). A reduction in virus shedding was also observed by Reichman et al. (1983). However, these effects do not seem to warrant a routine use of this formulation in patients with recurrent genital herpes (Holmes et al., 1982). No effect on

TABLE 12.8.
Effects of oral acyclovir on initial genital herpes (after Nilsen et al., 1982)

	All patients			Females			Males		
	Acyclovir (n=17)	Placebo (n=14)	P	Acyclovir (n=10)	Placebo (n=7)	P	Acyclovir (n=7)	Placebo (n=7)	P
Viral shedding (days)	1	13	<0.001	1	10	<0.01	1	15	<0.01
Crusting time (days)	4	6	N.S.	4	6.5	<0.01	7	5	N.S.
Assessed healing time (days)	6	11	<0.01	4.5	6	<0.05	7	11	0.06
Averaged healing time (days)	7	11.5	0.08	6	7	N.S.	7.5	14	<0.05
Itching duration (days)	1.5	5	<0.01	1	5	<0.05	2	7.5	N.A.
Pain duration (days)	4	8	<0.001	3.5	7	<0.05	4	9	<0.05
Combined symptom duration (days)	4	9	<0.05	5	8	N.S.	3	9	<0.05
New lesion formation (%)	0	43	<0.01	0	29	N.S.	0	57	<0.05

N.S., Not significant; N.A., Not analysable (too few patients with itching). Acyclovir was given 5×200 mg per day.

TABLE 12.9.
Efficacy of acyclovir given i.v. to patients with genital herpes (after Mindel et al., 1982)

	Primary patients			Females			All patients		
	Acyclovir (n=12)	Placebo (n=8)	P	Acyclovir (n=12)	Placebo (n=12)	P	Acyclovir (n=15)	Placebo (n=15)	P
Viral shedding (all lesions, days)	2.0	8.8	<0.001	2.0	7.5	<0.001	2.0	8.5	<0.001
Healing time (all lesions, days)	9.0	15.0	<0.05	7.0	12.5	<0.05	7.0	14.0	<0.001
Duration of new lesion formation (days)	0.0	2.0	<0.01	0.0	1.5	<0.05	0.0	2.0	0.001
Duration of vesicles (days)	2.5	5.0	N.S.	2.5	4.5	N.S.	3.0	5.0	<0.05
Duration pain (days)	3.5	5.0	N.S.	4.0	4.0	N.S.	4.0	4.0	N.S.
Duration all symptoms (days)	6.3	8.8	N.S.	6.8	7.3	N.S.	6.5	8.5	<0.05

N.S., Not significant. Acyclovir was given i.v. 5 g/kg 8 hourly. A total of 15 doses were given.

TABLE 12.10.
Chemotherapy of recurrent genital herpes

Drug	Administration	No. patients evaluated	Effects observed	Comments	References
Neutral red photo-inactivation	Topical	56	No effect	Possibly harmful	Myers et al., 1975
Ara-A	Topical, 3%	151	No effect		Adams et al., 1976
IDU	Topical, 5%; 20% in DMSO	53	Reduced virus shedding, shorter duration	Not repeated in a later study (Silversi et al., 1979)	Parker, 1977
Proflavine photo-inactivation	Topical	157	No effect		Kaufman et al., 1978
Ether	Topical	46	No effect	Randomized treatment/ no treatment	Corey et al., 1978
Nonoxynol	Topical, ? % cream	69	No effect		Vontver et al., 1979
2-Deoxy-D-glucose	Topical, 0.19% cream	25	Shorter duration of virus shedding and duration of vesicles	Probably not effective. See text.	Blough and Giuntoli, 1979
Ribavirin	Oral, 200 mg 4 times daily	94	Reduced severity and faster healing	No statistical evaluation	Bierman et al., 1981
Acyclovir	Topical, 5% in PEG, 4–6 times daily	111	Shorter duration of virus shedding and pain, accelerated healing		Corey et al., 1982a,b
Acyclovir	Topical, 5% in PEG, 6 times daily	88	Shorter duration of virus shedding		Reichman et al., 1983
Acyclovir	Topical, 5% cream, 5 times daily		Shorter time to healing, decreased formation of new lesions		Kinghorn et al., 1983
Acyclovir	Oral, 5 × 200 mg/days	83	Shorter duration of virus shedding, shorter time to healing, decreased formation of new lesions		Nilsen et al., 1982
Acyclovir	Oral, 5 × 200 mg/day	107	Shorter duration of virus shedding	Study not completely evaluated	Reichman et al., 1982
Foscarnet	Topical, 0.3% cream, 6 times daily	72	Faster healing, fewer days in severe disease, less redness, shorter time with sores (ulcers)	Virus shedding not evaluated	Wallin et al., 1984

TABLE 12.11.
Effect of topical 5% acyclovir in PEG on recurrent genital herpes. Acyclovir was used as a 5% ointment in PEG (after Corey et al., 1982b)

Symptom	Mean duration (days) after onset of therapy			
	Males		Females	
	Acyclovir (n=31)	Placebo (n=35)	Acyclovir (n=20)	Placebo (n=25)
Pain	2.2 (±0.4)[a]	3.2 (±0.7)	1.6 (±0.4)	1.8 (±0.4)
Itching	2.0 (±0.4)	2.0 (±0.5)	2.3 (±0.8)	1.9 (±0.5)
Viral shedding, genital lesions	1.0 (0.2)[b]	2.2 (±0.4)	0.4 (±0.2)	1.1 (±0.3)
Percent forming new lesions	39%	46%	40%	30%
Duration of new lesions	3.3	4.1	4.0	3.7
Complete crusting of all lesions	3.5 (±0.4)	5.0 (±0.9)	4.6 (±0.6)	4.3 (±0.5)
Complete healing of all external lesions	7.6[a] (±0.6)	9.7 (±0.8)	6.6 (±0.7)	5.6 (±1.1)

[a] $P<0.05$, Mantel Cox statistic
[b] $P<0.01$, Mantel Cox statistic
The ointment was given 4 times per day.

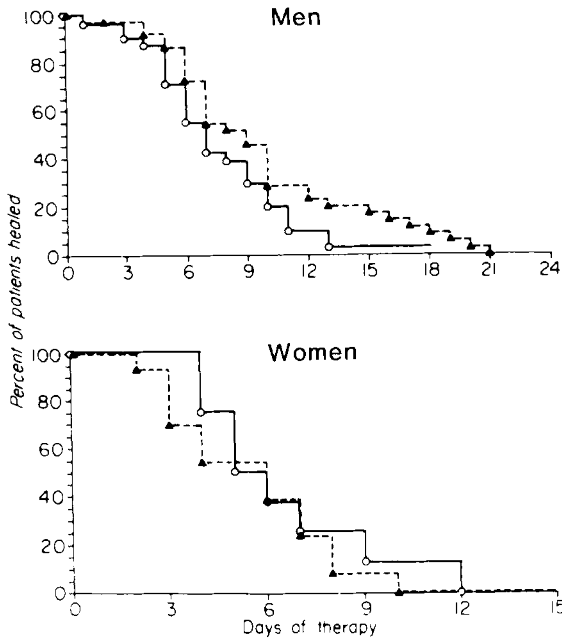


Fig. 12.16. Effect of topical 5% acyclovir in PEG on healing in patients with recurrent genital herpes. The ointment was applied 4–6 times/day. Men: $P=0.03$ for comparison between acyclovir (O—O) and placebo (▲—▲). Women: $P \geq 0.2$ for comparison between acyclovir and placebo. Mantel Cox test. (after Corey et al., 1982b.)

the frequency of recurrences was observed. The new acyclovir cream used for labial herpes (Fiddian et al., 1983) might be better than the PEG formulation and therapeutic effects have recently been reported by Kinghorn et al. (1983) as shown in Table 12.10.

Oral treatment with acyclovir seems to give a better therapeutic effect than topical treatment and the results from Nilsen et al. (1982) are presented in Table 12.12 and Fig. 12.17. The healing-time decreased from 6 to 5 days. A second study on oral therapy with acyclovir (Reichman et al., 1982) has only been partly analyzed and shows a reduced time of virus shedding. Intravenous therapy using acyclovir has not been reported for recurrent genital herpes, but since this type of treatment was about equal to oral treatment in primary/initial herpes it is unlikely that i.v. treatment would be better than oral treatment in the recurrent disease. In any case it would be impractical.

Successful topical treatment of recurrent genital herpes using 0.3% foscarnet cream has been reported by Wallin et al. (1984) and in a preliminary report by Wallin et al. (1982). This study contained one first episode part where patients were compared to each other, and one cross-over part where the effect on two consecutive episodes in one patient were compared. As shown in Fig. 12.18 the time to healing is shorter in patients on foscarnet in the first episode study where patients were compared to each other. This is particularly obvious when the number of patients healed on day 6 are compared. It is obvious that a better effect is seen in men, possibly due to an easier inspection in men than women. The cross-over part of this study is summarized in Table 12.13, where the number of days in a more severe

TABLE 12.12.
Effects of oral acyclovir on recurrent genital herpes (after Nilsen et al., 1982)

	Median time in days					
	All patients			Males		
	Acyclovir (n=41)	Placebo (n=42)	P	Acyclovir (n=32)	Placebo (n=34)	P
Viral shedding	1	2	<0.001	1	2	<0.001
Crusting time	4	4	N.S.	4	4	N.S.
Assessed healing time	5	6	<0.001	5	6	<0.001
Averaged healing time	5	6	<0.01	5	6	<0.05
Itching duration	1	2	N.S.	1	2	N.S.
Pain duration	3	2.5	N.S.	3	2	N.S.
Combined symptom duration	3	3	N.S.	3	2.5	N.S.
New lesion formation (%)	2%	19%	<0.05	3%	18%	0.07

Acyclovir was given 5×200 mg per day
N.S., not significant.

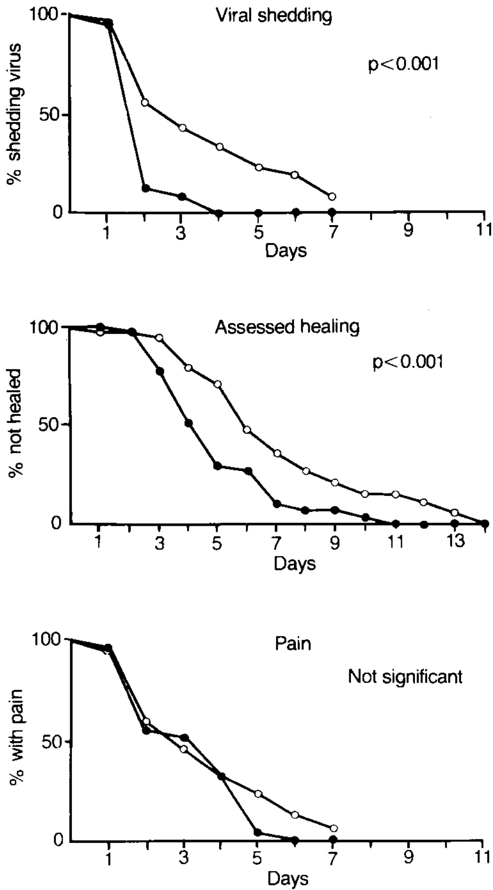


Fig. 12.17. Effect of oral acyclovir, 5 × 200 mg/day, on recurrent genital herpes, in male patients. ●—●, oral acyclovir; ○—○, placebo. (after Nilsen et al., 1982.)

stage of the disease are compared. The most notable effect is, as also shown in Fig. 12.19, the reduction in time with sores (ulcers), which is the most troublesome part of the disease. The effect on virus shedding was not determined in this study.

The therapeutic effects shown by acyclovir and foscarnet have, for the first time, clearly shown that therapy *is* possible and also indicated several important factors in the design of clinical trials with recurrent genital herpes. The relative efficacy of the two drugs is presently unclear.

12.2.5. Interferon and immunomodulators

There is no controlled trial data published showing therapeutic efficacy of interferon on genital herpes. As discussed previously in this chapter, a cutaneous herpes infection rapidly induces high levels of interferon. This is probably also the case

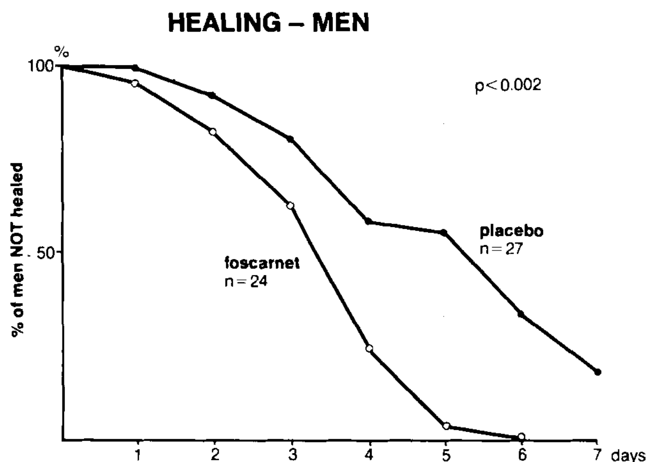


Fig. 12.18. Effect of 0,3% foscarnet cream on the rate of healing of recurrent genital herpes in men. Foscarnet cream or placebo was applied six times per day for five days. (after Wallin et al., 1984.)

TABLE 12.13.
Effect of 0.3% foscarnet cream on recurrent genital herpes (after Wallin et al., 1984)

Variable	Drug at 1st episode	Mean number of days	Drug at 2nd episode	Mean number of days	*P*-value
Redness	Foscarnet	2.0	Placebo	2.4	
	Placebo	2.8	Foscarnet	1.2	<0.01
Swelling	Foscarnet	0.9	Placebo	0.7	
	Placebo	1.3	Foscarnet	0.6	N.S.
Blisters	Foscarnet	0.5	Placebo	1.0	
	Placebo	0.8	Foscarnet	0.7	N.S.
Sores	Foscarnet	1.8	Placebo	3.2	
	Placebo	4.3	Foscarnet	1.1	<0.001
Scabs	Foscarnet	0.3	Placebo	1.2	
	Placebo	0.7	Foscarnet	0.6	N.S.
Healing	Foscarnet	3.9	Placebo	4.7	
	Placebo	5.7	Foscarnet	3.5	<0.01

Mean number of days in a more severe disease stage in 24 cross-over patients on treatment sequences foscarnet-placebo or placebo-foscarnet. Days 1–8. N.S., not significant.

in a genital infection and the further addition of interferon seems to be without rationale. However, a prophylactic use of interferon could possibly be effective. The increasing availability of purified interferon will now make controlled trials possible and hopefully will answer the question whether exogenous interferon is of any use against genital herpes.

Non-specific immunostimulation with smallpox vaccine has been tried in the past

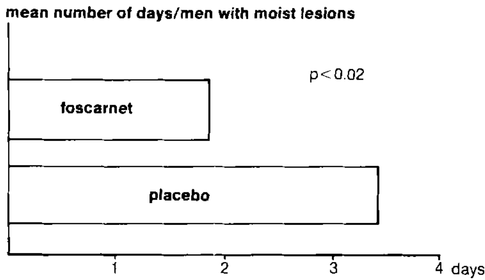


Fig. 12.19. Therapeutic effect of 0.3% foscarnet cream on genital herpes. Reduction of days with ulcers in men with moist lesions. Foscarnet or placebo cream was applied six times per day for five days. (after Wallin et al., 1984.)

but is not effective and should not be used. BCG vaccine is likewise not effective in the treatment of genital herpes (Bierman, 1976, Corey et al., 1976). The use of levamisole, a stimulant of cell-mediated immunity, did not prove to be effective in controlled studies (Bierman, 1978, Chang and Fiumara, 1978). The prophylactic use of levamisole for long periods has been reported to decrease the number of days per month with labial and genital herpes infections (Jose and Minty, 1980) but the number of patients in each group was rather small and side-effects were frequent.

12.3. Neonatal herpes simplex infection

12.3.1. Clinical aspects

Neonatal HSV infections, usually caused by HSV-2, can occur during delivery from a genital tract excreting virus (Nahmias and Visintine, 1976). Transplacental transmission and spread from hospital personnel has also been reported, but is probably less common (Florman et al., 1973, Linnemann et al., 1978). Such neonatal infections have been well reviewed by Whitley and Alford (1979, 1981) and these papers should be consulted by readers requiring specific data. The incidence of neonatal HSV infection has been estimated at 1 in 7500 live births (Nahmias and Visintine, 1974), which indicates that most infants born of mothers excreting virus will *not* develop an overt disease. However, at the present time genital lesions present at the time of delivery, especially in a primary infection, is an indication for caesarian section.

It has been estimated that the risk of a neonatal infection is 4% during a recurrent infection and 50% during a primary infection (Nahmias et al., 1971). The precautions recommended when a newborn is at risk for neonatal herpes simplex have

been summarized by Jarratt (1983). Whitley et al. (1983) has recently pointed out that over 50% of a large series of newborns with neonatal herpes were premature. This indicates that weekly monitoring by vaginal cultures should start as early as 32 weeks in women with a history of genital herpes or having a sexual partner with recurrent genital herpes.

The incubation period of neonatal herpes seems to be 2–20 days and the symptoms can be disseminated or localized as indicated in Table 12.14. The mortality

TABLE 12.14.
Clinical finding in newborns with HSV infections (after Whitley and Alford, 1979)

Clinical findings	Frequency in disseminated disease (1/2 to 2/3 of cases) (%)	Frequency in localized disease (1/3 to 1/2 of cases) (%)
Constitutional		
Fever	21–50	<20
Reticuloendothelial system		
Jaundice	21–50	0
Hepatomegaly	21–50	0
Splenomegaly	20	0
Haematologic system		
Haemolytic or other anaemias	20	0
Bleeding	21–50	0
CNS		
Encephalitis	51–75	51–75
Microcephaly	20	20
Intracranial calcifications	20	20
Meningitis	<20	<20
Eye		
Conjunctivitis	20	20
Keratoconjunctivitis	20	20
Chorioretinitis	20	20
Cataracts	<20	<20
Skin		
Maculopapular exanthem	<20	0
Vesicular exanthem	21–50	51–75
Vesicular enanthem	20	20
Lung		
Pneumonitis	20	0
Heart		
Pericarditis	<20	0
Arrhythmia	<20	0
Lethal outcome	76–100	20
Sequelae		
Psychomotor retardation	51–75	21–50
Visual impairment	20	20

in the disseminated form is more than 75% with brain damage as the main cause of death. High titres of neutralizing antibodies in the infants seemed to result in a more favourable outcome of the infection in one study but not in another (Whitley et al., 1983).

12.3.2. THERAPY

The only therapy with proven efficacy at present is ara-A (vidarabine) given i.v. (Whitley et al., 1980, Whitley et al., 1983). In the first study to be published 56 infected newborns were given 15 mg/kg/day i.v. over 12 hours once per day for 10 days. The infected newborns were grouped according to the type of clinical disease

TABLE 12.15.
Mortality and morbidity in newborns with herpes simplex virus infection according to the type of clinical disease and treatment regimen (at one year follow-up) (after Whitley et al., 1980)

Type of clinical disease	No. of recipients (% total)			
	Vidarabine (ara-A)		Placebo	
	No.	Per cent	No.	Per cent
Disseminated (Dis), total	14		13	
Dead	8	57%	11	85%
Alive	6	43%	2	15%
Normal	2	14%	1	8%
Abnormal[a]	4	29%	1	8%
Localized CNS, total	10		6	
Dead	1	10%	3	50%
Alive	9	90%	3	50%
Normal	5	50%	1	17%
Abnormal[a]	4	40%	2	33%
Combined (Dis+CNS), total	24		19	
Dead	9	38%	14	74%
Alive	15	62%	5	26%
Normal	7	29%	2	11%
Abnormal	8	33%	3	16%
Localized skin, eye, or mouth, total	4		8[b]	
Dead	0		0	
Alive	4	100%	8	100%
Normal	3	75%	5	62%
Abnormal	1	25%	3	38%

[a] One patient in each category had spasticity or hemiparesis only, while the remainder demonstrated combinations of microcephaly, paresis, spasticity, seizures, blindness or deafness.
[b] One patient who died of bacterial septicaemia is excluded.

and the result of the study is shown in Table 12.15. The mortality decreased from 74% to 38% with drug therapy in the group with CNS and disseminated disease, whereas in the group with only disseminated disease the death rate was reduced from 85% to 57%. In the milder localized disease the mortality was reduced from 50% to 10% because of treatment. As is evident from Table 12.15 the sequelae from the disease in the surviving children is a serious problem. It has been emphasized by Whitley et al. (1980, 1983) that the superficial skin infection in 70% of the cases progressed to a more serious disease. In an extended clinical trial Whitley et al. (1983) found that ara-A therapy decreased the mortality in babies with disseminated and CNS disease to 40% (Fig. 12.20). The two dosages used, 15 and 30 mg/kg/day gave the same result in the different types of disease as shown in Fig. 12.21. No serious side effect of the treatment with ara-A was observed. Earlier initiation of treatment, and possibly more effective antiviral drugs or combinations of drugs, might improve the clinical outcome. The well established therapeutic effects of ara-A therapy makes the presence of herpes skin vesicles or recovery of HSV from the skin of newborns an indication for intravenous araA treatment.

12.4. Herpes encephalitis

12.4.1. Clinical aspects

Although rare, herpes simplex encephalitis is considered the most common cause

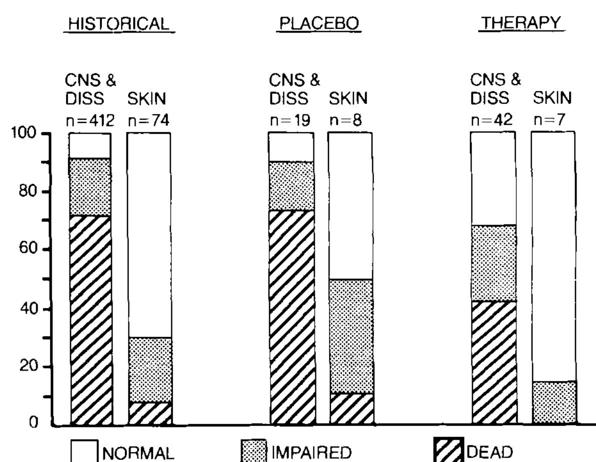


Fig. 12.20. Morbidity in children who had neonatal herpes simplex infection at two years of age or older. Historical data are compared with placebo or vidarabine (ara-A) treated newborns. The types of disease are grouped as CNS and disseminated and as skin involvement. AraA was given either 15 mg/kg/day (16 newborns) or 30 mg/kg/day (23 newborns) intravenously for 10–14 days. (after Whitley et al., 1983.)

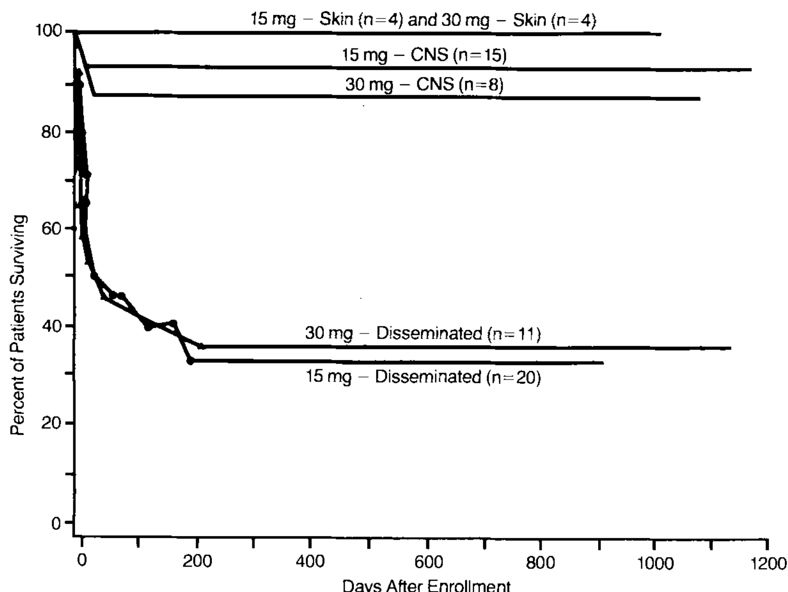


Fig. 12.21. Survival of herpes simplex virus infected newborns according to disease classification and dosage of vidarabine (ara-A). (after Whitley et al., 1983.)

of fatal encephalitis in the US (Meyer et al., 1960). It has been estimated that 1000 cases per year occur in the US (Workshop on the treatment and prevention of herpes simplex virus infections, 1973). The disease starts with headache, fever, behaviour changes and lethargy. This can be followed by more severe neurological signs, often indicating a temporal lobe affection. The disease, if untreated, has a mortality of about 70%. In 88 cases where HSV was isolated and typed, 86 were HSV-1 (Table 12.1) and 2 were HSV-2. Rapid and early diagnosis is an important and difficult task in this disease. A comparison of different diagnostic methods shows direct brain biopsy to be the best method (Nahmias et al., 1982). Direct immunfluorescence of the brain material was positive in 70% of the cases proven positive by virus isolation and gave false positive results in 9% of the negative cases. Other methods were less accurate, and virus identification in brain biopsy material still seems to be the method of choice. However, objections have been raised against this invasive method and with the hopeful advance of more nontoxic treatments it could possibly be avoided in the future (Campbell et al., 1982). Whitley et al. (1982a) have studied the possible identity between virus isolates from orolabial sites and from the brain in eight patients with both labial herpes and herpes encephalitis. The frequency of labial herpes was less than 10% in herpes encephalitis patients. By restriction-enzyme analysis of the viral DNAs it was found that the orolabial and brain isolates were identical in five patients but differed in three (see Chapter 17 for more details of this study). One patient with identical isolates had clear serological evidence of a primary infection.

12.4.2. Therapy

Several treatments have been tried for herpes encephalitis but the only well controlled studies showing efficacy are those of Whitley et al. (1977, 1981) using ara-A intravenously. The critical importance of an early treatment institution is shown in Fig. 12.22, where the dependence of the final condition of the patient on the rapidity of institution of therapy is shown. The prognosis in herpes encephalitis depends significantly both on treatment and the level of consciousness at the time of therapy. It has also been shown that ara-A therapy decreases the virus content in brain tissue (Nahmias et al., 1982b) and that the outcome of the disease was more favourable in patients with a low quantity of virus in the initial biopsy.

The use of ara-A has not resulted in any serious problem of toxicity, certainly compared to the serious nature of HSV infection in the brain, but nevertheless the insolubility of ara-A leads to very large volumes of drug being administered. To overcome this, ara-AMP, which is more soluble, is presently being evaluated for the treatment of herpes encephalitis. Acyclovir is also being tested at present against herpes encephalitis, although it is too early to predict any difference in efficacies. In animal experiments ara-A is probably the most active drug against herpes simplex encephalitis, but acyclovir possibly has the advantage of lower toxicity. This could lead to an earlier treatment without brain biopsy even when the diagnosis is uncertain. Several new inhibitors with good antiviral effects in animal studies are also likely to be candidates for clinical trials in the future.

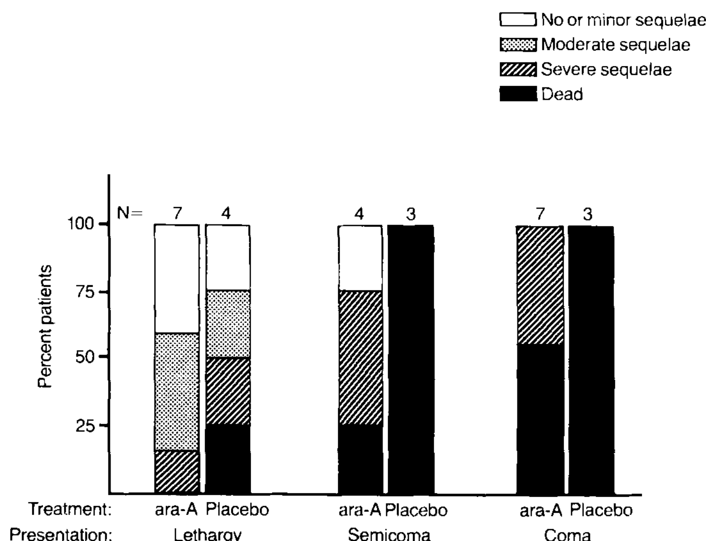


Fig. 12.22. Relation between outcome and state of consciousness at time of ara-A or placebo therapy in biopsy-proven herpes encephalitis. The dosage of ara-A was 15 mg/kg/day or placebo over 12 hours given for 10 days. (after Whitley and Alford, 1981.)

12.5. Herpes simplex virus infections in the immunocompromised host

12.5.1. Clinical aspects

Latent herpesvirus infections have a tendency to be activated during immunosuppression and the result of this can be a very severe infection. An increasing number of patients are nowadays subject to this risk, making the need for antiviral drugs for these diseases urgent. Immunosuppression can result from malignancies in the lymphoreticular system or be dependent on the use of cytotoxic drugs as well as immunosuppressive drugs given to prevent graft-versus-host reactions. Herpes simplex virus infections in immunosuppressed patients are, when symptomatic, most commonly mucocutaneous rather than systemic. In renal transplant patients, 20–66% develop an infection about 1 month after transplantation (Armstrong et al., 1976, Korsager et al., 1975, Luby et al., 1974, Pass et al., 1979). However, the mucocutaneous infections can disseminate or progress into a chronic infection. Even when healing occurs without complications the course of the disease is about twice as long as in the normal host (Muller et al., 1972, Pass et al., 1979, Korsager et al., 1975). HSV infections seem to be more prolonged in bone-marrow transplant than in kidney transplant patients (Meyers et al., 1982, Whitley et al., 1984). Oesophagilitis can occur, but systemic dissemination seems to be rare (Muller et al., 1972, Meyers et al., 1982). Very rarely a dissemination can occur in healthy adults and a few cases have been reported during pregnancy (Hillard et al., 1982). Herpes simplex infections in immunocompromised hosts are generally not fatal.

12.5.2. Therapy

Ara-A has been used for the i.v. treatment of severe mucocutaneous HSV infections in immunosuppressed patients (Chien et al., 1973). In this uncontrolled study 3.3–10 mg/kg/day of ara-A seemed to reduce virus shedding and time to healing for patients with HSV-1 but not HSV-2 infections.

Whitley et al. (1984) have, in a controlled study, evaluated intravenous ara-A (10 mg/kg/day) for treatment of mucocutaneous herpes simplex infections in immunocompromised hosts and found some clinical benefits. The loss of pain was significantly acclerated as well as a decrease of virus shedding, especially in those persons over 40 years of age. However, healing was not significantly accelerated. The results indicate a limited usefulness of ara-A treatment.

In a double-blind placebo-controlled study using topical application of 5% acyclovir in PEG to immunocompromised patients with mucocutaneous herpes simplex infections Whitley et al. (1982b) found a reduction in virus titres but no reduction in time to healing. The prolonged course of this infection ought to make it more responsive to topical treatment than, for example, a normal recurrent labial infection. Intravenous acyclovir has been used in a controlled study as a prophylactic

agent in mucocutaneous herpes simplex infections in immunocompromised patients (Saral et al., 1981). As shown in Table 12.16, when given intravenously 250 mg/m^2 acyclovir prevented the appearance of herpes lesions in bone-marrow transplant patients. However, once the treatment was stopped, oral HSV lesions developed in half of the patients who had received acyclovir. The results at present show that prophylaxis with acyclovir is possible in seropositive patients but that the latent infection is not eliminated. Acyclovir treatment did not prevent the shedding of CMV or adenovirus in this study.

Case reports on the therapeutic use of systemic acyclovir in herpes infections in immunocompromised patients indicate a clinical efficacy (O'Meara et al., 1979, Selby et al., 1979, Teare and Clements, 1980, Heaton et al., 1981 and Straus et al., 1982). A small placebo-controlled double-blind trial of intravenous acyclovir in heart transplant patients showed that acyclovir treated patients were more likely to be culture negative ($P=0.004$) at the end of the trial than placebo treated patients (Chou et al., 1981). The therapeutic efficacy of intravenous acyclovir on mucocutaneous herpes simplex infections in immunocompromised patients has been further studied by Mitchell et al. (1981) and Wade et al. (1982). These plus some further patients have been evaluated by Meyers et al. (1982). In this evaluation, 97 patients without visceral disease were studied and acyclovir was found to shorten virus shedding, lesion pain, lesion scabbing and time to healing as shown in Table 12.17. Patients received either placebo or 250 mg/m^2 acyclovir intravenously over 1 h every eight hours for seven days. The most common adverse reaction, a peripheral vein irritation, was seen in 16% of the patients treated with acyclovir and in 7% of the placebo patients.

Topical treatment using foscarnet has not been reported but ought to be effective since it has been shown to be efficacious in recurrent disease in immunocompetent patients. Intravenous foscarnet treatment of HSV infections in renal transplant patients has resulted in rapid healing of the lesions in an open study (Ahlmén, pers. comm.) and further evaluation is therefore warranted.

Prophylactic treatment with leukocyte interferon and antithymocyte globulin did not prevent herpes simplex infections in renal transplant patients (Cheeseman et al., 1979).

TABLE 12.16.
Herpes simplex virus infections in bone marrow transplant recipients during the treatment period with acyclovir or placebo (after Saral et al., 1981)

Group	No. of patients	No. of infections[a]
Acyclovir	10	0
Placebo	10	7

[a] $P=0.003$.
250 mg/m^2 acyclovir was given prophylactically by the i.v. route every eight hours.

TABLE 12.17.
Influence of underlying disease on efficacy of intravenous acyclovir in immunocompromised patients with mucocutaneous herpes simplex infections (after Meyers et al., 1982)

	Marrow transplant		Other		Effect of (P-value)	
	Acyclovir	Placebo	Acyclovir	Placebo	Illness	Treatment
Virus shedding (days)	2.5	17.0	2.9	12.9	N.S.	0.0002
Pain (days)	9.9	13.6	6.0	10.1	0.02	0.01
Scabbing (days)	13.0	13.8	5.3	13.1	0.02	0.04
Healing (days)	13.9	20.7	13.2	19.4	N.S.	0.08

Acyclovir was given i.v. 250 mg/m^2 every 8 hours for 7 days. The infusion time was 1 hour. N.S., not significant.

12.6. Ocular herpes simplex virus infections

12.6.1. Clinical aspects

The main infectious cause of blindness in industrialized countries is ocular herpes infections, in most cases caused by HSV-1. About 300 000 cases of ocular herpes infections are diagnosed every year in the US and a considerable fraction of these eventually lead to blindness.

A primary infection in a person without antibodies can occur by inoculation of virus from a carrier. It is unclear how often an ocular infection is caused by autoinoculation from a labial herpes infection. After a few days incubation the disease appears as follicular conjuncitivitis and corneal lesions evolve as branching (dendritic) ulcers, which can progress to large geographic ulcers. The disease can also be milder as punctuate keratitis. It is not clear how many primary infections are asymptomatic. Vesicles can also be present on the lids of the eyes, more often in the primary disease than in the recurrent disease. After a primary infection, virus is harboured in a latent stage in trigeminal ganglia and can give rise to recurrent episodes of disease. It has recently been reported that virus possibly could remain latent in ocular tissue, possibly in the retina in mice (Openshaw, 1983).

The risk of a recurrence was found to be 26% within the two first years of the initial disease and 43% if one recurrence has occurred (Carroll et al., 1967, Kaufman, 1981). After two episodes, the probability of a further recurrence within one year was 25% (Shuster et al., 1981). The triggering factors for a recurrent episode are not well defined. It is possible that infection of the cornea can take place through virus in the tear fluid (Kaufman, 1981).

The following different types of recurrent herpes simplex infections have been discussed by Pavan-Langston (1979) in relation to treatment and only essential points are mentioned here:

1. Epithelial infectious ulcers are caused by virus replication and develop into dendritic or geographic ulcers. This is usually accompanied by irritation and photophobia.

2. Epithelial herpes ulcers sometimes appear after the infectious ulcers have passed and are caused by piling of epithelial cells which are unable to move across the base of an ulcer. The treatment of this metaherpetic ulcer is different from that of infectious keratitis.

3. Stromal disease can develop as a viral interstitial keratitis or a disciform oedema. In the first form, virus multiplication and immune complexes probably both contribute to the destruction of the stroma. The disciform oedema, often caused by improper use of steroids, seems to be similar to a delayed hypersensitivity reaction. The stromal disease can occur in combination with epithelial ulcers. Dendritic and geographic epithelial herpetic diseases are illustrated in Fig. 12.23.

Wilhelmus et al. (1981) have reported that after treatment of corneal herpes ulcers, 40% of 152 patients were subject to a recurrent corneal ulcer, 25% experienced stromal disease, 5% had ocular hypertension, 6% had a decrease in visual acuity caused by corneal scarring and 22% had corneal opacity.

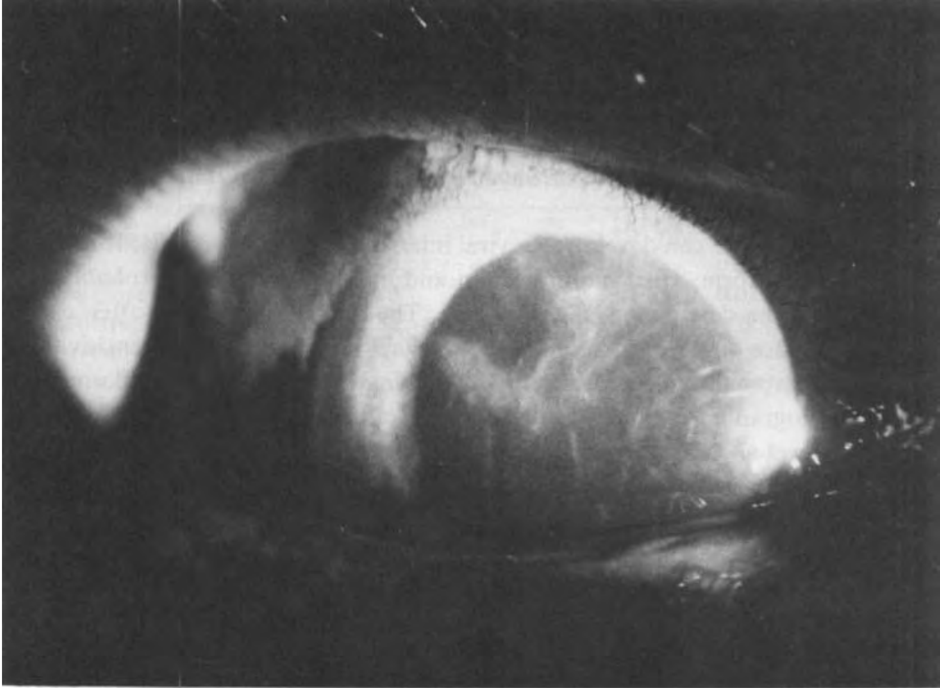
12.6.2. Vaccination

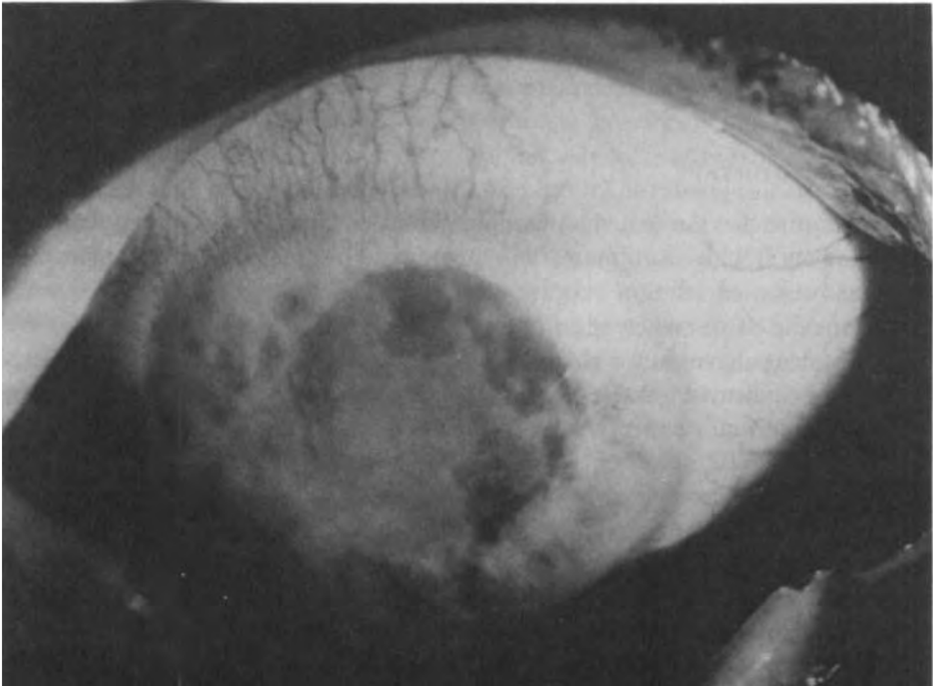
There are no controlled studies on the efficacy of herpes vaccine against herpes keratitis but vaccination has been tried in patients (Shubladze et al., 1978). In rabbits, herpes simplex virus vaccine has a protective effect (Pollikoff, 1970, Metcalf, 1980) but the clinical relevance of this is unclear.

12.6.3. Chemotherapy

Herpes keratitis was the first viral disease to be successfully treated with an antiviral agent, namely IDU by Kaufman (1962). Contrary to some other herpes infections, there has been a correlation between cell culture efficacy of antiherpes drugs and the therapeutic effect on herpes keratitis in animal models as well as in man. Several antiherpes drugs have shown clinical efficacy, mainly when used topically. The use of steroids is indicated only in the case of epithelial trophic ulcers and stromal disease, as they normally encourage virus spread (Pavan-Langston, 1979).

Idoxuridine (IDU, Stoxil®) is available as eye drops and ointment and double-blind studies have shown that IDU has a significant therapeutic effect on dendritic ulcers (Burns, 1963, Paterson et al., 1963, Laibson and Leopold, 1964, Hart et al., 1965). However, other studies (Luntz and McCallum, 1963, Jepson, 1964, Markham et al., 1977) could not find a difference compared to placebo. The discrepancy between the different studies could be due to variables such as patient population, size of the study, time for treatment initiation or registration of effects. Also in studies without significant effect there was still a tendency in favour of a positive clinical





effect for IDU (Markham et al., 1977). It seems likely that IDU has a therapeutic effect on herpes keratitis, although not as impressive as in animal models, and a direct consequence of this is that it is not ethical to perform future studies on any new keratitis treatment as placebo-controlled trials. New drugs must be compared to IDU. Another drug, ara-A was also found to have a therapeutic activity and a local toxicity very similar to that of IDU (Dresner and Seamans, 1975) as shown in Table 12.18. A comparison of ara-A, IDU and placebo indicated that ara-A and IDU were superior to placebo although the difference did not reach statistical significance as shown in Fig. 12.24 (Markham et al., 1977).

A more effective drug than IDU was found in TFT (Wellings et al., 1972), which is more easily water soluble and is very active given as eye drops. Fig. 12.25 shows

TABLE 12.18
Efficacy of ara-A and IDU on herpes simplex epithelial keratitis. Time to re-epithelialize for dendritic and/or geographic lesions (after Dresner and Seamans, 1975)

Time to re-epithelialize by type of lesion	Ara-A patients (n=87)	IDU-patients (n=82)
	Mean no. of days	Mean no. of days
Dendritic	6.67	6.97
Geographic	6.80	6.00
Dendrito-geographic	8.09	11.00
Total re-epithelialized	8.88	7.24

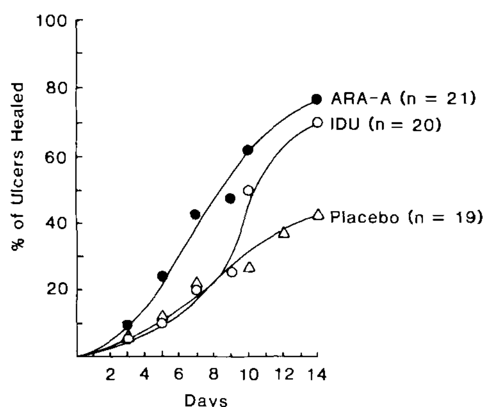


Fig. 12.24. Cumulative frequency curves showing healing rates in three groups of patients with herpes keratitis. The groups (out-patients) were treated with placebo ointment, 3% ara-A ointment or 0.5% IDU ointment four times a day. (after Markham et al., 1977.)

← Fig. 12.23. Appearance of ocular herpes simplex infections, dendritic and geographic ulcers. (courtesy of Dr U. Laurent.)

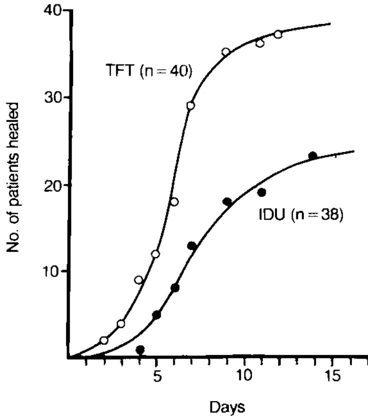


Fig. 12.25. Healing of herpes ulcers treated with TFT and IDU. Cumulative frequency of days required to heal. IDU was given as 0.1% eye drops and TFT as 1% eye drops, 1 drop five times daily. (after Wellings et al., 1972.)

a comparison of TFT and IDU with respect to their therapeutic activity of herpes keratitis. Similar results have also been reported by Pavan-Langston and Foster (1977) who found a healing rate of 96% using 1% TFT drops and 75% using 0.1% IDU drops. Coster et al. (1976) have compared the effects of TFT and ara-A and have not found any significant differences on dendritic ulcers as shown in Fig. 12.26. Amoeboid ulcers might respond better to TFT.

Acyclovir has recently been compared to placebo, IDU, ara-A and TFT for effi-

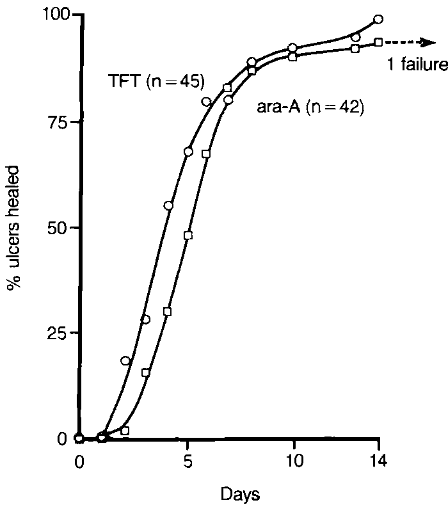


Fig. 12.26. Cumulative frequency graph of rates of healing of dendritic herpes ulcers under treatment with 3% ara-A ointment or 1% TFT eye drops five times daily. (after Coster et al., 1976.)

cacy on herpes simplex corneal ulcers. In two studies comparing 3% acyclovir ointment to placebo ointment a therapeutic effect was observed (Jones et al., 1979, Morgan et al., 1980). Furthermore, comparisons between acyclovir and IDU have shown 3% acyclovir ointment to be better than 0.5% IDU ointment (Collum et al., 1980, Klauber and Ottovay, 1982) but no difference was found between 3% acyclovir ointment and 1% IDU ointment (Coster et al., 1980) as shown in Fig. 12.27.

In three large studies comparing the effect of topical 3% acyclovir ointment and 3% ara-A ointment two of the studies did not reveal any significant difference in the rate of healing (McGill et al., 1981, Laibson et al., 1982). In the study showing a difference in favour of acyclovir ($P \leqslant 0.01$) the acyclovir group contained more primary infections than the ara-A group ($P \leqslant 0.05$) (Young et al., 1982) whereas in the study by McGill et al. (1981) and by Laibson et al. (1982) the primary infections were evenly distributed between the two treatment groups. A comparison between 3% acyclovir ointment and 2% TFT ointment on 59 patients with dendritic ulcers is shown in Fig. 12.28 and did not reveal any significant difference in efficacy, and the same frequency of punctuate keratopathy was seen in both groups (Lau et al., 1982).

Bromovinyldeoxyuridine has been tested clinically as 0.1% eye drops in an open trial and a therapeutic effect is likely on corneal ulcers (Maudgal et al., 1981).

The deep stromal herpes infections have not responded well to topical treatment, probably due to lack of penetration of drugs. Topical application in a model system has shown TFT to penetrate through the cornea faster than IDU or ara-A (O'Brien and Edelhauser, 1977) and this could possibly correlate with the effect reported for TFT on herpetic iritis and uveitis (Sugar et al., 1973, Wellings et al., 1972). A combination of 3% acyclovir and 0.01% betamethasone was better than only acyclovir

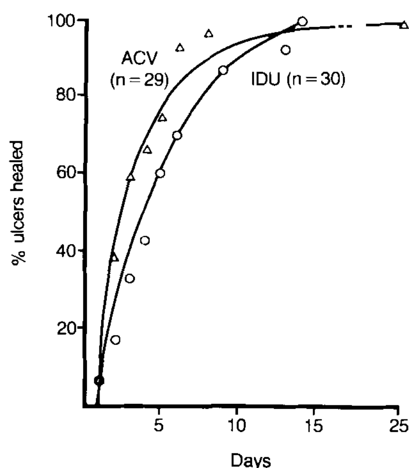


Fig. 12.27. Cumulative frequency graph of the time taken to heal 59 herpetic corneal ulcers (54 dendritic, 5 geographic) treated with 3% acyclovir ointment or 1% IDU ointment. (after Coster et al., 1980.)

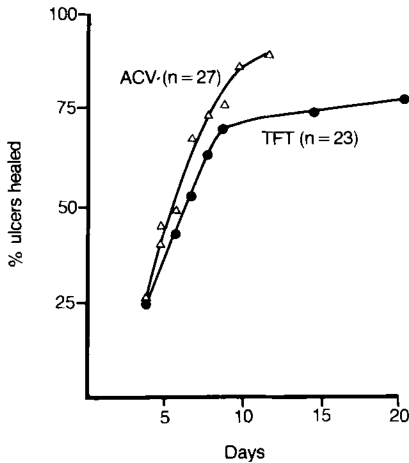


Fig. 12.28. Cumulative frequency distribution of the time taken to heal dendritic herpes keratitis in 27 patients given 3% acyclovir (ACV) ointment and 23 patients given 2% TFT ointment 5 times daily. (after Lau et al., 1982.)

on disciform keratitis (Collum et al., 1983). In an open study BVDU has been reported to have a therapeutic effect on stromal keratitis (Maudgal et al., 1981) and this was also found in open studies using acyclovir (van Ganswijk et al., 1983, Jensen et al., 1982). A systemically useful drug has been sought for the stromal infections. IDU and TFT are too toxic but ara-A has been given i.v. by Abel et al. (1975). The toxicity of ara-A and its insolubility makes the development of alternative drugs highly desirable. Oral use of acyclovir has recently been shown to increase the healing rate of corneal ulcers (Hung et al., 1984).

Some of the properties of the keratitis drugs could usefully be compared. TFT has the advantage of having a good activity when given as eye drops, whereas the low solubility of acyclovir makes the use of eye drops impossible. None of the topically used compounds are active against deep stromal disease. In the studies using IDU, ara-A, TFT and acyclovir no significant differences in local toxicity were noted. Although details about systemic toxicity have not been published, acyclovir would seem to be the least toxic drug at present, although the importance of that for a topical eye treatment is not clear. It is unlikely that development of resistance to acyclovir would lead to resistance to ara-A, and TFT, and moreover, ara-A resistant infections have responded to TFT treatment (Pavan-Langston and Foster, 1977). This makes the presence of several active antiviral compounds with different modes of action a therapeutic safeguard. Several new nucleoside analogues utilizing herpes virus thymidine kinase are possible candidates for use against herpes keratitis, although there is an obvious risk of cross-resistance for compounds utilizing the viral thymidine kinase.

12.6.4. Interferon, combinations of interferon and antiviral drugs, and immunomodulators

Immunomodulators such as levamisole have not been shown to be active even in experimental animal models (Kaufman and Varnell, 1977) and should not be used in man.

Topical use of human leucocyte interferon was not effective by itself in herpes keratitis and did not improve the beneficial effect of debridement (Sundmacher et al., 1978a, Sundmacher et al., 1978b). Debridement seems to be an advantage when combined with chemotherapy and possibly removes viral antigens which could give rise to immunoprecipitates. Results at present indicate that interferon used topically is less active than TFT, but a combination of TFT and interferon is effective (Sundmacher et al., 1978c). Increasing the interferon titre improves the effect of TFT, as shown in Table 12.19. We can conclude that further work is needed to evaluate the true potential of interferon. Interferon inducers, although active in experimental animals, have not been successful in human use to date.

TABLE 12.19.
Reduction of healing time of dendritic keratitis by combination of HuIFN-*α* and 1% TFT eye drops given 5 times/day (after Sundmacher et al., 1981)

Addition to TFT	Reduction in healing time compared to only TFT drops
1×10^{6u} HuIFN-*α*	18%
10×10^{6u} HuIFN-*α*	30%
30×10^{6u} HuIFN-*α*	45%

12.7. Summary

Vaccination against herpes simplex virus infections has not been shown to be efficacious and also involves unresolved questions such as target groups and oncogenic potential of DNA containing vaccine.

Recurrent labial herpes has been treated with a large number of medications but in controlled studies only foscarnet, acyclovir, and possibly IDU (in DMSO), all used topically, have shown any therapeutic effect. Interferon given i.m. has shown a prophylactic effect against labial herpes in a special group of patients, but is unlikely to be useful for the normal sufferer of cold sores.

Primary and initial genital herpes has responded to treatment with acyclovir. Therapeutic effects against recurrent genital infection has only been obtained using foscarnet and acyclovir. The relative merits and drawbacks of these compounds and of topical and oral treatment remains to be determined. A topical treatment will, however, result in a total dose much less than that used orally and is also directed

specifically to the location of virus multiplication.

Neonatal herpes infections and herpes encephalitis can be treated by ara-A given by infusion, although other compounds such as acyclovir are being investigated.

In controlled trials ara-A and acyclovir have shown therapeutic effects against herpes simplex infections in immunosuppressed patients and in addition, compounds such as foscarnet are being investigated.

Ocular herpes simplex infections such as corneal ulcers respond to the topical use of several compounds, such as IDU, TFT, acyclovir, ara-A and a combination of TFT and interferon, but topical treatment of deep stromal infections has been less successful.

Several compounds are presently being evaluated for efficacy against different herpes simplex virus infections and the vital importance of an early institution of treatment in order to achieve therapeutic effects has been recognized.

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CHAPTER 13

Varicella-zoster virus infections

13.1. Clinical aspects

Varicella-zoster virus (VZV) causes, as the name implies, both varicella (chickenpox) and zoster (shingles). An infectious agent common to these two syndromes was first postulated by von Bokay (1909), who found that children could catch varicella from zoster patients (Fig. 13.1). The identity of the viruses causing varicella and zoster was further indicated by transmission experiments (Kundratitz, 1925), growth in cell-culture giving identical cytopathogenic effects (Weller, 1953), immunological cross-reactions (Weller and Coons, 1954), and finally identical restriction enzyme maps of their DNAs (Richards et al., 1979).

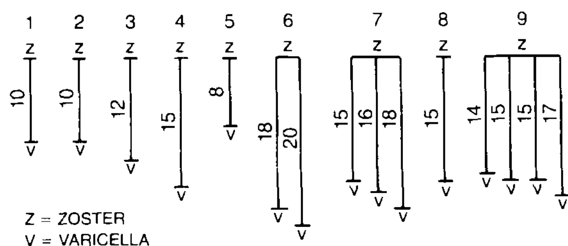


Fig. 13.1. Chickenpox infections caused by contact with zoster patients. The diagram illustrates the incubation period (days) for transmission of infection from 9 zoster patients resulting in 14 episodes of varicella. (after von Bokay, 1909.)

13.1.1. VARICELLA

This is a benign childhood disease which, in the normal child, manifests itself as a vesicular rash emerging as successive crops over 3–5 days. Malaise and fever caused by viraemia often precedes the rash, which first appears on the trunk and then on the face. All stages of papules, vesicles and crusts can be present at the same time. The vesicles formed during the first 1–2 days are clear and have been compared to dewdrops. Crust formation takes 4–5 days and the scabs remain for 1–3 weeks. As many as several hundred vesicles can appear in the young child and this figure tends to be higher in older children. Complications are uncommon, but neurological manifestations such as encephalitis, often with cerebellar signs, can occur (Grose, 1982). The sequence of events following a primary infection is shown in fig. 13.2. In adults the infection can be complicated by pneumonitis (Triebwasser et al., 1967).

On the other hand, in immunocompromised patients (Fig. 13.3) the course of the disease can be severe and prolonged, and, if untreated, associated with a considerable mortality. Feldman et al., (1975), studied seventy-seven cases of varicella in children with cancer. Seventeen children on remission and off chemotherapy did not develop progressive chickenpox, but nineteen of the remaining children had a progressive varicella with visceral involvement, and fifteen of these children developed pneumonitis. Four of the pneumonitis cases died within 3 days.

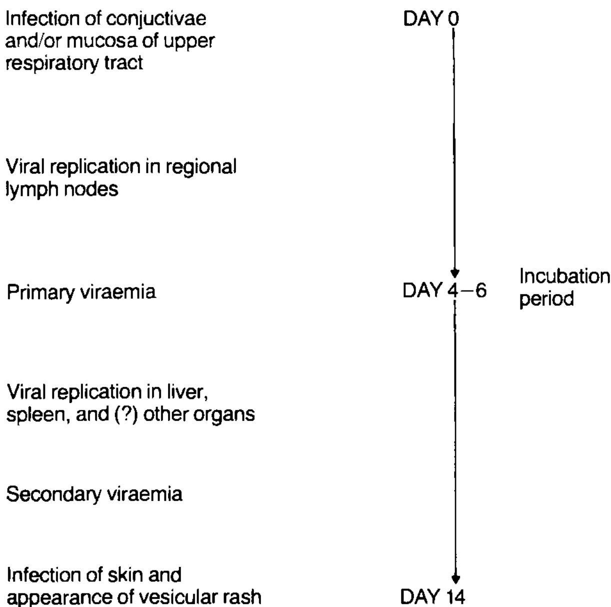


Fig. 13.2. Diagrammatic representation of the probable sequence of events following a primary VZV infection. (after Grose, 1982.)

13.1.2. Zoster

Reactivation of latent VZV harboured in the dorsal root ganglia from a previous episode of chickenpox is thought to initiate zoster. Fever and malaise precedes pain, which is often localized to the area where vesicles will appear a few days after the first symptoms. The distribution of vesicles is usually unilateral, as shown in Fig. 13.4. The areas mostly involved are the same as those affected by chickenpox e.g. head and trunk. The vesicles are normally limited to a dermatome supplied by a dorsal root or extramedullary cranial nerve ganglion. The vesicles are often larger and heal more slowly than in varicella. Virus can be isolated up to a week after vesicle eruption. An episode of zoster can take place with only pain and no vesicle formation, but with a rise in antibody titre (Juel-Jensen and MacCallum, 1972). A severe long-lasting neuralgia is a distressing complication in zoster patients, as well as scar formation in the dermatome. Replication of VZV during zoster is not restricted to a dermatome and a viraemia resulting in secondary appearing chickenpox-like eruptions has been noted in as many as a third of normal zoster patients as shown in Table 13.1 (Öberg and Svedmyr, 1969). The frequency of virus generalization is even higher in patients with malignancies.

Severe manifestations of zoster can occur when activation of VZV in the ophthalmic division of the fifth sensory cranial nerve is involved. The clinical disease (Fig. 13.5) can in these cases be conjuctivitis, keratitis, iridocyclitis and/or glaucoma (Pavan-Langston, 1979). The severity of these infections and the risk for permanently damaged vision calls for rapid therapy.

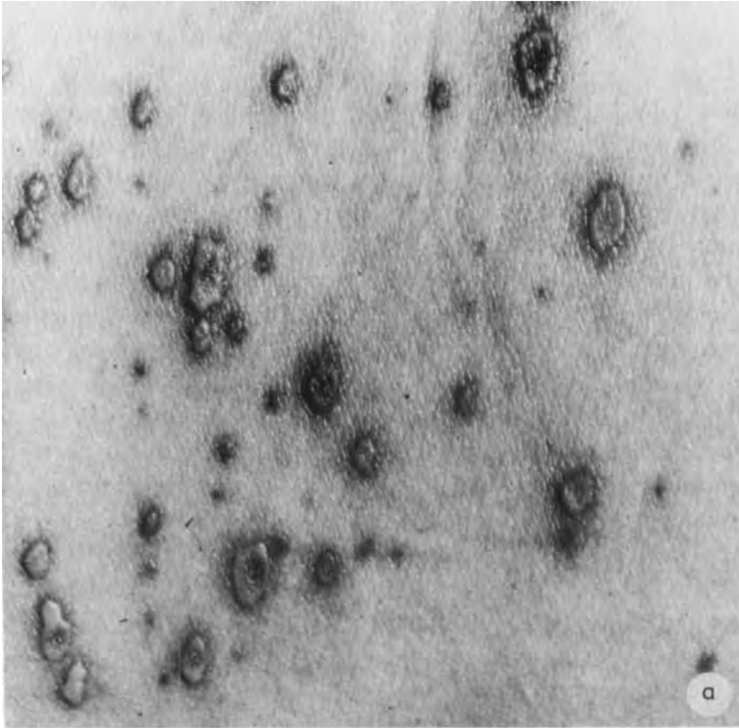
The frequency of complications of herpes zoster is summarized in Table 13.2 from a study by Ragozzino et al. (1982). A total of about 10% of the patients had zoster ophthalmicus but only 20% of these resulted in complications. Postherpetic neuralgia is by far the most common complication.

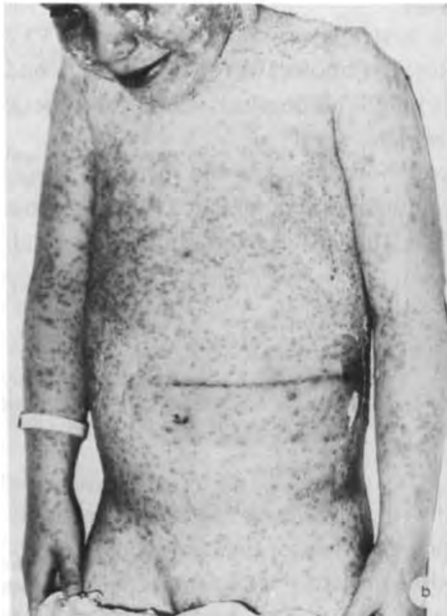
In the immunocompromised patient, zoster is both frequent and severe. The significance of zoster in persons with Hodgkin's disease and bone marrow transplants is shown in Fig. 13.6 from Zaia (1981) and in Table 13.3 from Ragozzino et al. (1982).

13.2. Epidemiology

13.2.1. Transmission and latency

Varicella zoster virus is spread from a patient with varicella or zoster and probably enters the oropharynx mucosa, the upper respiratory tract or the conjunctiva. Infection through the skin is less likely. However, it is presently unclear exactly how the virus is spread from varicella and zoster patients. Virus has not been isolated from pharyngeal or nasal secretions but this might be due to the difficulty in cultivating





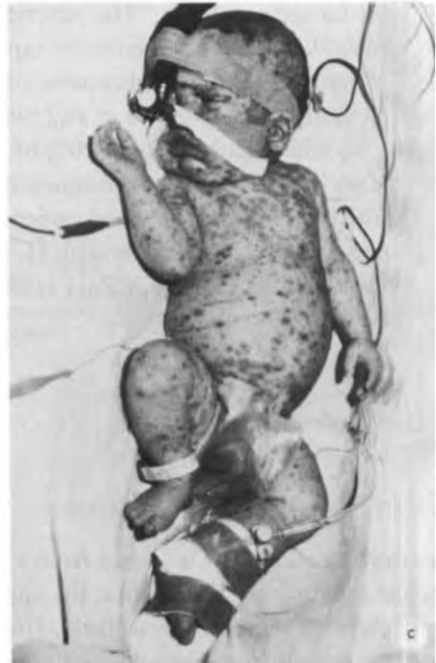


Fig. 13.3. Appearance of a varicella infection. a, Detail of varicella in a normal child. (courtesy of Dr. O. Cars.) b, Varicella in an immunosuppressed child. (courtesy of the late Dr. W.C. Marshall.) c, Neonatal varicella (courtesy of the late Dr. W.C. Marshall.)

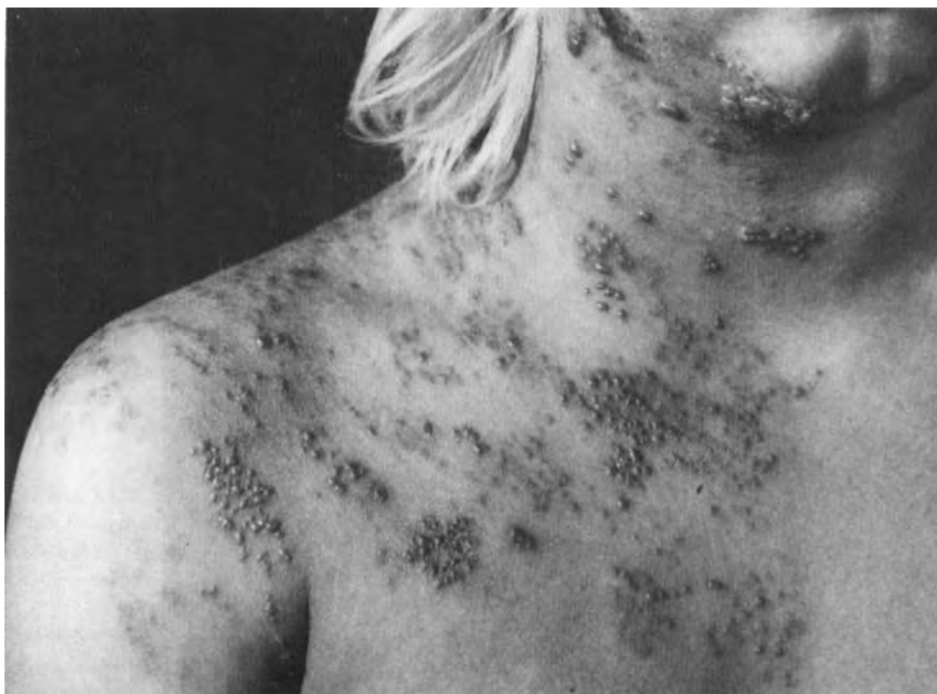


Fig. 13.4. Herpes zoster. (courtesy of Dr. J. Wallin.)

TABLE 13.1.
Distribution of chicken-pox-like generalization in 100 consecutive patients hospitalized for herpes zoster (after Öberg and Svedmyr, 1969)

Patient groups	No. of cases		
	With generalization	Without generalization	Total regardless of generalization
Total, regardless of age or malignancy	35	65	100
50 years	3	12	15
⩾50 years	32	53	85
With malignancy	6	6	12
Without malignancy	29	59	88
50 years	2	11	13
⩾50 years	27	48	75
Age (median), years	74	68	71

VZV from these sources in contrast to the isolation of VZV from vesicles (Brunell, 1981). After transmission, virus replication takes place in a biphasic manner as indicated in Fig. 13.2.

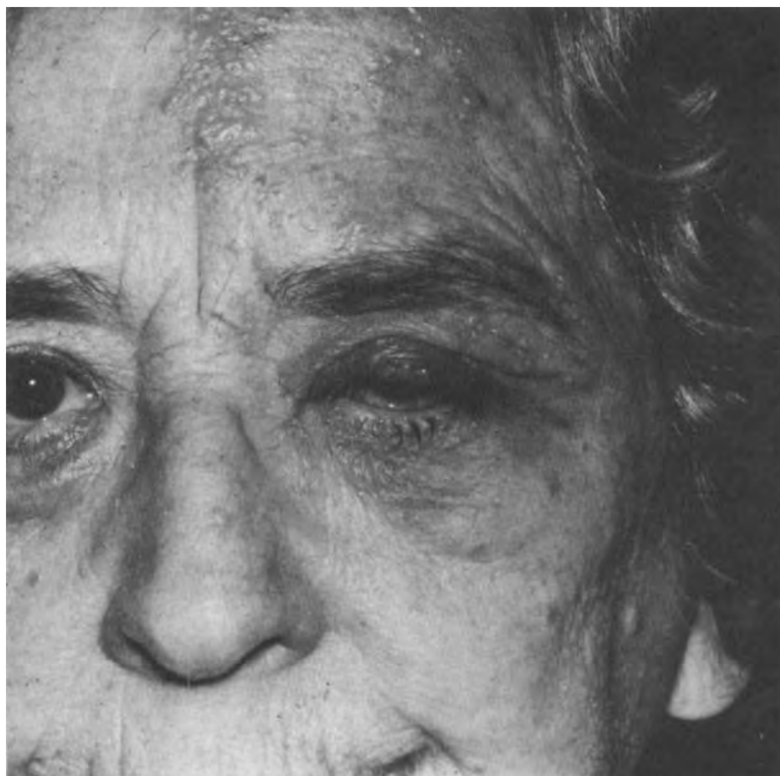


Fig. 13.5. Zoster ophthalmicus in an adult patient. (courtesy of Dr. O. Cars.)

TABLE 13.2.
Complications of herpes zoster observed among 590 residents of Rochester, Minnesota, 1945–1959 (after Ragozzino et al., 1982)

	No. of patients	%
Post herpetic neuralgia	55	9.3
Motor deficit	6	1.0
Herpes gangrenosa	3	0.5
Pneumonia	1	0.2
Meningoencephalitis	1	0.2
Unilateral deafness	1	0.2
Ocular complications	11	1.9
Uveitis	4	0.7
Keratitis	3	0.5
2° glaucoma	2	0.3
Iridocyclitis	1	0.2
Panophthalmitis	1	0.2

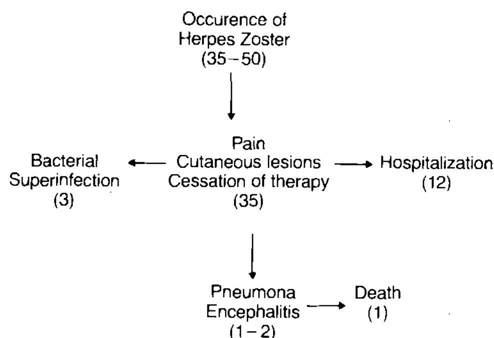


Fig. 13.6. Significance of herpes zoster infections in immunosuppressed hosts. ( ) = estimated number of patients with herpes zoster complications during first year of treatment of 100 high-risk patients with Hodgkin's disease and bone marrow transplantation. (after Zaia, 1981.)

TABLE 13.3.
Risk factors among 590 residents of Rochester, Minnesota, diagnosed with herpes zoster between 1945–1959 (after Ragozzino et al., 1982)

	% of zoster patients
Cancer before diagnosis	6.1
Trauma	1.9
Radiotherapy	1.5
Chemotherapy	0.3
Systemic steroids	0.3
Surgery	0.2

The original suggestion that zoster was caused by activation of latent varicella virus was made by Garland (1943) and activation of latent VZV in dorsal root ganglia was also suggested by Hope-Simpson (1965) based on the dermatomal distribution of a large number of zoster episodes. Viral DNA and RNA has recently been detected in ganglia during an acute infection (Gilden et al., 1983), in ganglia from cadavers without current or recent herpes zoster (Hyman et al., 1983) and in patients dying shortly after a zoster episode (Bastian et al., 1974).

The reports that zoster eruptions preferentially take place in areas subjected to trauma (Juel-Jensen and MacCullum, 1972) and irradiation (Rifkind, 1966), may indicate latency in many different ganglia.

13.2.2. Incidence and prevalence

Varicella and zoster are not reportable diseases in most countries. This fact, and the likely under-reporting where reporting is regulated, lead to an underestimation of the true prevalence levels.

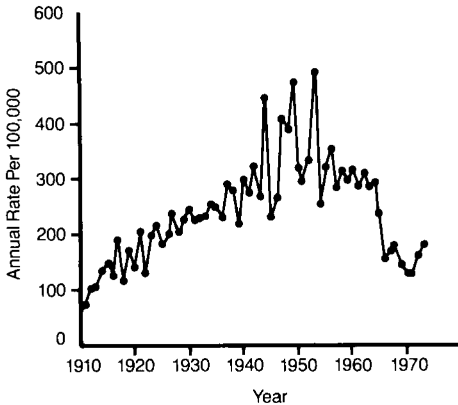


Fig. 13.7. Reported cases of chickenpox per 100 000 population per year in Massachusetts from 1910 through 1973. (after Weller, 1976.)

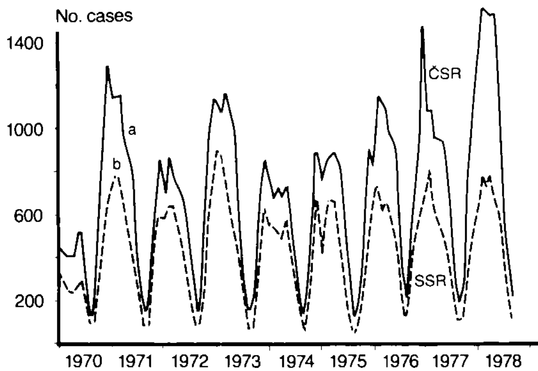


Fig. 13.8. Notified varicella morbidity per 100 000 population per year during 1970 to 1978. a = Czech socialist republic, b = Slovak socialist republic. (after Trlifajova et al., 1980.)

Figs. 13.7 and 13.8 show reported cases of varicella in Massachusetts and in Czechoslovakia, respectively. The average incidence per year in the Czechoslovakian study seems to be about twice that observed in Massachusetts, but differences in reporting could probably explain the difference. The degree of under-reporting is indicated in Fig. 13.9 where the cumulative seropositivity and morbidity are compared. Weller (1976) has estimated that the reported annual incidence rate of 87.3 per 100 000 persons in the US is an underestimation by a factor of 10 to 20. The good correlation between clinical symptoms and seroconversion in varicella epidemics indicates that the discrepancy between serological data and reported diseases is due to inadequate reporting and undiagnosed cases. Most persons will at the age of 15 have antibodies to VZV.

The incidence of zoster in two populations in Czechoslovakia is shown in Fig.

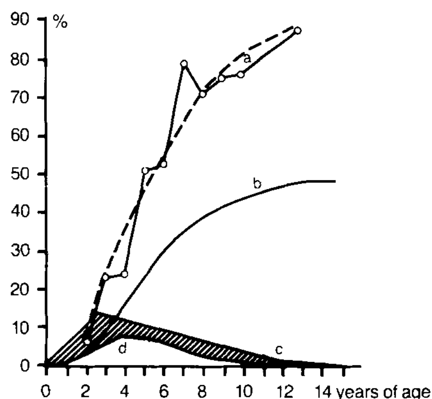


Fig. 13.9. Varicella morbidity in North-Moravia region 1971–1976. a = cumulative seropositivity, b = cumulative notified morbidity, c = % seropositive cases, d = notified morbidity. (after Trlifajova et al., 1980.)

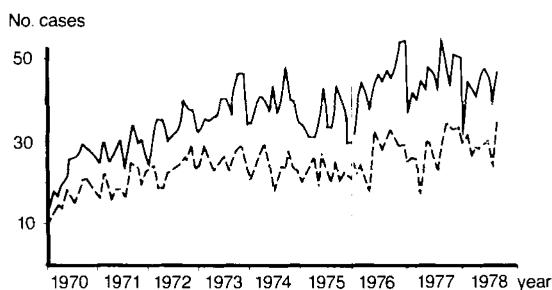


Fig. 13.10. Notified herpes zoster morbidity per 100 000 population per year. Changed notification practice since 1976. (after Trlifajova et al., 1980.)

13.10. These incidences seem to be lower than the overall incidence of 125 per 100 000 reported by Ragozzino et al. (1982) and shown in Fig. 13.11, and of 339 per 100 000 as reported by Hope-Simpson (1965). Differences in population age strongly affect the incidence as shown in Fig. 13.11 since zoster is more prevalent at older age (Ragozzino et al., 1982, Hope-Simpson, 1965).

Varicella shows a pattern with great fluctuations in incidence during the year and the highest numbers are reported for the winter months (Weller, 1976), as also shown in Fig. 13.8. Zoster, on the other hand, does not show any temporal preference.

A strongly increased incidence of zoster is observed in immunocompromised patients as shown in Table 13.4.

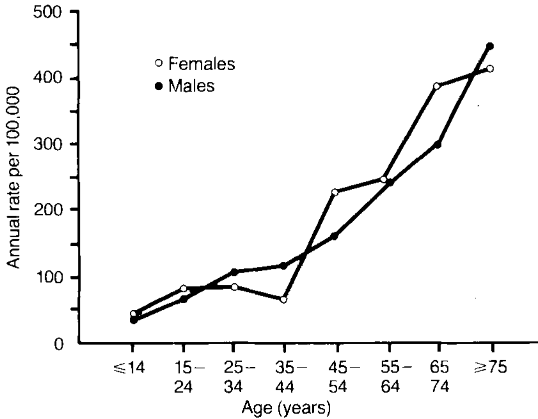


Fig. 13.11. Annual rate of herpes zoster among residents of Rochester, Minnesota, 1945–1959, by age and sex. (after Ragozzino et al., 1982.)

TABLE 13.4.
Incidence of herpes zoster in selected diseases (1955–1974) at NIH (after Mazur and Dolin, 1978)

	Total no. admitted with indicated disease	% with zoster
Hodgkin's disease	549	4.7
Chronic lymphocytic leukaemia	209	2.9
Acute lymphoblastic leukaemia	654	1.5
Systemic lupus erythematosus	334	1.5
Lymphoma	546	1.3
Acute myelogenous leukaemia	396	1.3
Rheumatoid arthritis	740	0.4
Chronic myelogenous leukemia	299	0.3
Diabetes mellitus	1715	0.1
Others	69 262	0.06

13.3. Vaccination

The use of live attenuated VZV vaccine has been investigated mainly by Takahashi and co-workers (Asano et al., 1982) and Plotkin and co-workers (Arbeter et al., 1983). The concept of a live attenuated VZV poses a number of questions concerning target groups and safety. A mass-vaccination programme is clearly not advisable since normal VZV infection in children is a mild disease. Evaluation of vaccine given early in life to prevent subsequent zoster is an almost impossible undertaking since it should involve a very large number of vaccinees and a time period of half a century. The only possible use of a vaccine seems to be in special risk groups,

mainly seronegative children with malignancies or under immunosuppression for transplants. Progressive disease will develop in approximately 30% of children with acute lymphocytic leukaemia who contract chickenpox and the mortality rate in these children with untreated varicella is about 7% (Feldman et al., 1975).

Experience with the Japanese vaccine has been promising, and has clearly demonstrated that an effective degree of sero-conversion and protection against subsequent re-infection is achieved in 90% of vaccinees following parenteral immunization with the Oka strain of VZV vaccine (Takahashi et al., 1981). Takahashi et al. (1981) have reported that of 34 vaccinated children, none developed varicella during a 4 year period involving a total of 21 contact exposures to varicella. In a group of 23 vaccinated leukaemic children and a group of 35 non-vaccinated children with a history of previous varicella the incidence of zoster seemed to be equal (13% versus 11.4%) and the symptoms were mild. In a recent report Asano et al. (1982) have described the influence of vaccine dose and timing when used to protect household contacts against varicella and this data is shown in Fig. 13.12. Several studies have indicated that VZV specific cell mediated immune responses are more important than antibody response in the mechanism of protection against VZV infection. It is possible that immunization with the vaccine may also induce specific mucosal cellular immunity in addition to the development of cutaneous reactivity. However,

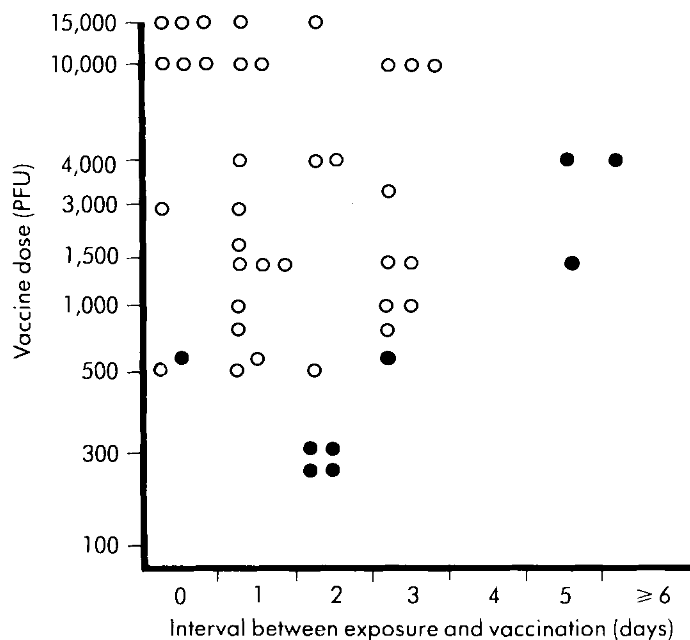


Fig. 13.12. Protective effect of varicella vaccination. ○, no varicella symptoms; ●, varicella symptoms. (after Asano et al., 1982.)

a definite hazard exists when some live vaccines such as polio and measles are given to immunodeficient children and children with leukaemia (see Chapter 2), and hence studies in these children with live VZV vaccine have proceeded with extreme caution. It is not anticipated that such a vaccine would be used on a widespread scale. Also VZV is much more severe in adults than in children and any widespread vaccination, even given a successful vaccine, would leave some adults susceptible to infection. Brunell et al. (1982) administered live varicella vaccine to 23 children with lymphoreticular malignancies. The children were given 500 pfu of the Oka strain of VZV subcutaneously. The vaccine had been passaged in guinea pig embryo cells and human diploid cells. Antibody responses were induced in all vaccinees. A biphasic rash developed in 2 children who failed to develop lymphocytic stimulation to VZV antigen after immunization, although they did develop serum antibody. Thus, a cell mediated immune response may be required to prevent evolution of the late vesicular lesions. The authors concluded that children with leukaemia should be protected against chickenpox as long as they are at risk of increased morbidity from VZV infection. It would seem prudent to immunize children whose chemotherapy has been completed, as well as those still receiving treatment. Routine immunization with vaccine of children at least 1 year from diagnosis and remission might be considered.

A biochemical study compared the restriction enzyme maps of DNAs of VZ virus and VZ vaccine virus, and DNA from the vaccine virus showed a heterogeneous buoyant profile and altered restriction enzyme cleavage patterns, presumably as a result of accumulation of defective viral DNA during cell passage (Ecker and Hyman, 1981). The attenuation procedure for the vaccine strain had been 11 passages in human embryo lung cells at 34°C, 12 passages in guinea pig embryo cells at 37°C and 1–21 passages in human diploid (WI-38) cells at 37°C. Finally, Arbeter et al. (1983) have developed another attenuated strain of VZV (KMcC) which after the 50th passage was tested in healthy children and found both sufficiently immunogenic and safe to initiate clinical studies in leukaemia patients.

13.4. Immunoglobulin

Passive immunization against VZV has been practiced using immune serum globulin (ISG), zoster hyperimmune globulin (ZIG) and zoster immune plasma (ZIP). Ross (1962) studied the effect of ISG on the severity of varicella in children. A decrease in the number of vesicles was observed in the group of children receiving ISG, and in the ISG treated group 42% of the children developed fewer than 20 vesicles while only 1% of the untreated children had fewer than 20 vesicles. The severity of the disease was reduced, but attack rate, incubation period and ability to transmit the disease were not affected.

In order to increase the anti-VZV antibody titre and possibly to have a better

therapeutic effect than with ISG, Brunell et al. (1969) prepared ZIG from plasma from donors with recent episodes of zoster. ZIG was used to determine any prophylactic effect in situations where two susceptable siblings were infected with chickenpox by one child in that family. Brunell et al. (1969) found a protective effect of ZIG, although this trial involved only a limited number of normal children. Attempts to use ZIG after household exposure of immunocompromised children to chickenpox did not prevent infection but seemed to decrease the severity of the disease (Gershon et al., 1974). In another trial involving 43 children at special risk (impaired immunity etc.) Evans et al. (1980) found that ZIG did not prevent the chickenpox infection but it appeared to modify the disease. Another conclusion from this study was that infants born from mothers who had chickenpox or zoster at least five days before delivery had VZV antibodies at birth and did not require ZIG.

Stevens and Merigan (1980) in a double-blind trial compared the efficacy of ISG and ZIG in changing the course of zoster in 97 immunocompromised patients. The globulins were given within 6 days of the onset of zoster. Despite a 100 fold difference in anti-VZV titre no differences between ISG and ZIG were noted in their abilities to prevent dissemination or to reduce post herpetic neuralgia. A recent study by Zaia et al. (1983) indicated that immunoglobulin from donors with high anti VZV titres (VZIG) is slightly better than ZIG and more easily accessible.

The limited availability of ZIG has restricted its use and, moreover, the criteria required for obtaining ZIG from the American Red Cross are presented in Table 13.5.

TABLE 13.5.
Five criteria for the release of varicella-zoster immune globulin (ZIG) from the American Red Cross (after Grose, 1982)

1. One of the following underlying illnesses or conditions:
   - Leukaemia or lymphoma
   - Congenital or acquired immunodeficiency
   - Under immunosuppressive medication
   - Newly born of a mother with varicella
2. One of the following types of exposure to varicella or zoster patient:
   - Household contact
   - Playmate contact ($\geqslant 1$ hr play indoors)
   - Hospital contact (in same two- to four-room or adjacent beds in a large ward)
   - Newborn contact (newborn whose mother contracted varicella within 4 days before delivery or within 48 h after delivery)
3. Negative or unknown prior disease history
4. Age of less than 15 years
5. The request for treatment must be initiated within 72 h of exposure

TABLE 13.6.
Chemotherapy of varicella in immunocompromised hosts

Drug	Administration	No. of patients	Effects	Comments	References
Ara-C	Infusion, 100 mg/m^2 per day	39	Virus dissemination prolonged by ara-C	Should not be used	Stevens et al. (1973) Davis et al. (1973)
Ara-A	10 mg/kg/day, 12 h infusion	32	Reduction in number of days of new vesicle formation (P=0.015)	Placebo-controlled trial	Whitley et al. (1982)
Acyclovir	500 mg/m^2, 8 times/day	20	No effect on time to healing. Possible effect on frequency of pneumonitis (P=0.054)	Placebo-controlled trial. Small groups and not equal in previous ZIG treatment	Prober et al. (1982)

13.5. Chemotherapy

13.5.1. Varicella

The benign course of varicella in otherwise healthy children does not motivate the use of antiviral chemotherapy. However, in immunosuppressed patients, as noted above, varicella can be a progressive and severe disease requiring therapy. Some chemotherapeutic attempts in this direction are listed in Table 13.6. The use of ara-C was shown in controlled studies to make the disease worse (Davis et al., 1973, Stevens et al., 1973), possibly due to immunosuppressive side effects of the drug, and it should not be used any further. A significant reduction in the number of days of new vesicle formation was observed in varicella patients given ara-A (Whitley et al., 1982) and five children with varicella pneumonia experienced a shorter course of disease than expected for untreated patients. One placebo-controlled study (Prober et al., 1982), and some case reports on the use of acyclovir have also been published, but no therapeutic effects were seen at the 5% confidence level and the small size of the study prevents any conclusive statement about efficacy, although the combined results indicate some effect. Further clinical studies with new compounds will have to be performed against ara-A for ethical reasons and not against placebo. A comparision of ara-A and acyclovir will be of interest as well as a trial of BVDU, which in cell-culture is extremely active against VZV replication. None of the known antiviral agents is likely to eliminate latent VZV.

13.5.2. Zoster in the normal host

Herpes zoster is, especially in the elderly, a painful disease which can lead to post herpetic neuralgia and hence the use of therapy is clearly indicated. The therapies tried have aimed both at reducing virus replication and post herpetic neuralgia. Some results of treatment of the acute phase of the disease are presented in Table 13.7.

Idoxuridine has (Table 13.7) been found in several studies to decrease pain and healing time when given topically as a solution in dimethylsulphoxide (DMSO). This is an excellent vehicle for transporting nucleoside analogues through skin but is also is keratolytic and is not permitted for human use in several countries. The observation by Wildenhoff et al. (1981), that trigeminal (facial) but not thoracic zoster responded to IDU remains to be explained. The results from this study are presented in Table 13.8. The influence of IDU concentration is controversial, possibly due to analysis of too small a number of patients when different concentrations have been compared (Dawber, 1974, Juel-Jensen and MacCallum, 1972, Simpson, 1975 and Verbov, 1979).

In a large and well-controlled study Peterslund et al. (1981) have shown acyclovir given i.v. to have a beneficial effect on herpes zoster. This was also found by Bean

TABLE 13.7.
Chemotherapy of zoster infections in normal hosts

Drug	Administration	No. of patients	Effects	Comments	References
IDU	40% IDU in DMSO, topically	43	Shorter pain and reduced healing time		Juel-Jensen et al. (1970)
IDU	5% and 25% IDU in DMSO, topically	118	Shorter pain and reduced healing time	No difference between 5% and 25%	Dawber (1974)
IDU	40% IDU in DMSO, topically	122	Shorter pain ($P \leq 0.001$), less fever ($P \leq 0.05$), fewer and shorter scab formation ($P \leq 0.05$)	Only effective in patients (42) with trigeminal zoster and not in patients (80) with thoracic zoster	Wildenhoff et al. (1981)
IDU	5% IDU in DMSO, topically	46	Fewer new vesicles ($P \leq 0.01$) and shorter time to decreased pain ($P \leq 0.05$)	Treatment groups somewhat different at start of treatment	Burton et al. (1981)
Rifamycin SV	500 mg/day i.m.	144	Shorter duration of vesicles, scabs and pain as compared to standard treatment	Not double-blind placebo-controlled. Effect of standard treatment not specified nor inclusion criteria in this preliminary report	Bruni et al. (1981)
Acyclovir	5 mg/kg i.v. every 8 hours for 5 days	56	Decreased pain in the acute phase, improved healing rate of lesions. Best response in patients above 67 years of age	No difference in pain between the acyclovir and placebo group at 1 and 3 months after admission	Peterslund et al. (1981), also reported by Esmann et al. (1982)
Acyclovir	5 mg/kg i.v. every 8 hours for 5 days	28	Earlier cessation of vesiculation ($P \leq 0.05$)		Larkin et al. (1983)
Acyclovir	500 mg/m^2 i.v. 3 times/day for 5 days	31	Reduced pain during therapy ($P = 0.02$), improved rate of healing ($P = 0.007$), shorter time of virus shedding ($P = 0.02$)	No effect on post herpetic neuralgia, increased serum creatinine level during acyclovir treatment	Bean et al. (1982)
Acyclovir	3% acyclovir ointment topically, 5 times/day	21	No recurrences when steroids were omitted	Open study on ocular zoster	McGill (1981)

TABLE 13.8.
Treatment of trigeminal and thoracic herpes zoster with idoxuridine. Patients were treated topically with 40% IDU in DMSO or DMSO alone (after Wildenhoff et al., 1981)

	Thoracic herpes zoster				Trigeminal herpes zoster			
	IDU (n=40)	Placebo (n=40)	Total (n=80)	P	IDU (n=20)	Placebo (n=20)	Total (n=42)	P
Neurological evaluation								
Duration of pain (days, median)	21	10			13	⩾30		
Pain ⩽30 days (no. of patients)	19	26	45	N.S.	14	3	17	⩽0.001
Pain ⩾30 days (no. of patients)	20	13	33	N.S.	6	18	24	⩽0.001
Pain ⩾6 months (no. of patients)	3	2	5	N.S.	3	6	9	N.S.
Paraesthesia ⩾30 days (no. of patients)	18	15	33	N.S.	12	16	28	N.S.
Clinical evaluation of skin lesions								
All vesicles dry (days, mean±SD)	7.7	8.5	8.1±3.7	N.S.	5.7	6.7	6.2±2.4	N.S.
New vesicles (no. of patients)	17	17	34	N.S.	7	8	15	N.S.
Temperature ⩾38°C (no. of patients)	13	11	24	N.S.	7	15	22	⩽0.05
Photographic evaluation of skin lesions								
Total scab formation (days, mean±SD)	5.2	4.9	5.0±3.6	N.S.	3.3	5.0	4.1±2.4	⩽0.05
No scabs formed (no. of patients)	7	13	20	N.S.	6	1	7	⩽0.05

N.S., not significant

et al. (1982). The variables affected by acyclovir were pain, rate of healing and virus shedding. As shown in Fig. 13.13 the best response was seen with patients of 67 years or older. Unfortunately, neither study showed any decrease in post herpetic neuralgia. The higher dose of acyclovir used by Bean et al. (1982) caused toxic problems, mainly as an increased creatine level, but this increase was reversible.

The effect of rifamycin is unclear and, moreover, there is no experimental data from preclinical investigations suggesting that it should be active.

Several treatments have been used to decrease postherpetic neuralgia but controlled studies are conspicuous by their absence. The risks and benefits of steroid use have not been sufficiently well evaluated in controlled studies to allow a final

TABLE 13.9.
Chemotherapy of zoster infections in immunocompromised hosts

Drug	Administration	No. of patients	Effects	Comments	References
Ara-A	10 mg/kg/day, i.v.	121	Increased healing time and decreased dissemination (P=0.014) fewer visceral complications (P=0.015), less severe postherpetic neuralgia (P=0.047)	No serious drug toxicity	Whitley et al. (1982)
Acyclovir	15–25 mg/kg/day, 3×3 min infusion for 5 days	14	Not possible to evaluate	Open study	Van Der Meer and Versteeg (1982)
Acyclovir	7.2–43.2 mg/kg/day, continuous infusion, 5 days	14	Not possible to evaluate	Open study	Spector et al. (1982)
Acyclovir	1500 mg/m²/day	94	Less complications of zoster (P=0.02), less progression of disease (P=0.05), faster clearance of virus (P=0.05)	No effect on pain or time to healing	Balfour et al. (1983)
BVDU	7.5 mg/kg/day, orally. Three or four capsules/day for 5 days	4	Not possible to evaluate	Open study	De Clercq et al. (1980)

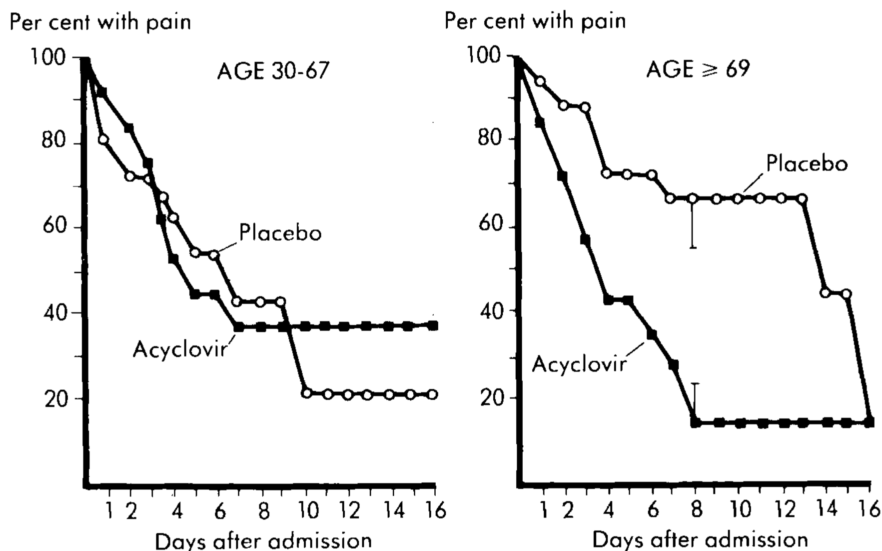


Fig. 13.13. Persistence of pain in herpes zoster patients after acyclovir treatment by days after admission and by age. Patients treated with 5 mg/kg of ACV or placebo, i.v., every eight hours. Age 30–67: 13 ACV treated patients and 11 placebo patients. Age ≥69: 14 ACV treated patients and 18 placebo patients. (after Peterslund et al., 1981.)

conclusion as to usefulness. The use of high-dose prednisone for herpes zoster has been advocated by Nuss (1980).

In the case of ophthalmic zoster a combination of an antiviral agent and steroids seems to be the treatment of choice (Pavan-Langston, 1979). An open study by McGill (1981), indicated that acyclovir could be beneficial when used topically against ophthalmic zoster, but effects from systemic administration have not been reported. It is not possible to discriminate at present between the efficacy of IDU and acyclovir in ophthalmic zoster, but, from a toxicity point of view, acyclovir seems to be preferable. The limitation at present is the need for i.v. treatment which is not practical for zoster in the normal patient. Oral administration and, in the future, the possible use of BVDU might improve the situation.

13.5.3. Zoster in the immunocompromised host

The frequent dissemination and severity of zoster in immunocompromised patients has resulted in several attempts to control this disease by chemotherapy. However, as shown in Table 13.9 the only treatment which, so far, has shown efficacy on healing in a placebo-controlled double-blind trial of sufficient size is ara-A given by infusion (Whitley et al., 1982). Several variables were positively affected by the ara-A treatment in this careful study, and some are shown in Fig. 13.14. It was also found

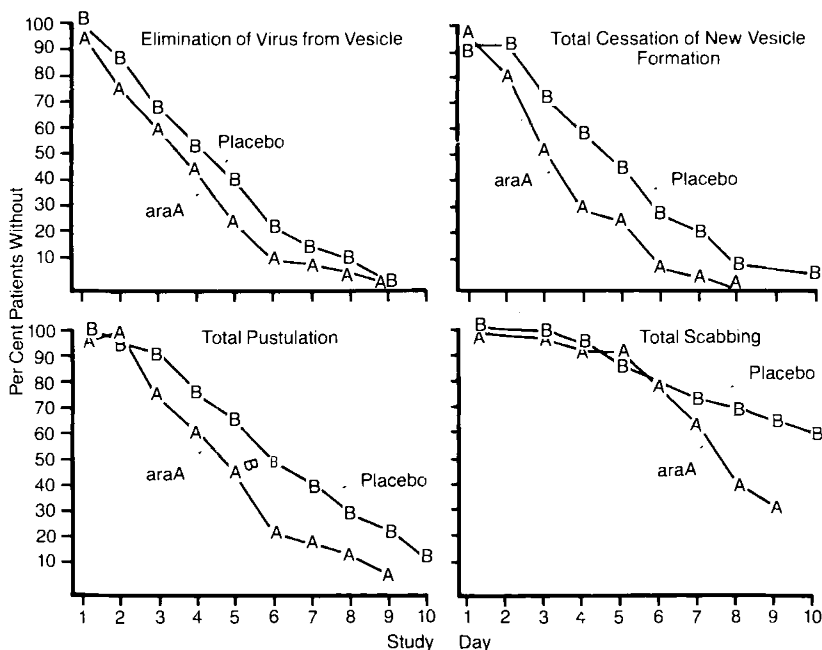


Fig. 13.14. Effect of vidarabine therapy to control complications in herpes zoster in immunosuppressed patients. The patients were given 10 mg/kg/day of ara-A intravenously over 12 hours (63 patients), or placebo (58 patients). For elimination of virus from vesicles $P=0.014$ by generalized Wilcoxon test and $P=0.162$ by log-rank test; for cessation of lesion formation $P=0.002$ by Wilcoxon test and $P=0.011$ by log-rank test: for time to total pustulation $P=0.003$ by Wilcoxon test and $P=0.0068$ by log-rank test; for time to total scabbing $P=0.059$ by Wilcoxon test and $P=0.0068$ by log-rank test. (after Whitley et al., 1982.)

that the percentage of dermatome involvement was significantly lower in the ara-A group, and visceral complications were decreased (Table 13.10). Adverse effects were noted in 18 ara-A patients and 9 placebo patients, but these did not result in drug discontinuance.

Published trials using acyclovir do not permit a proper evaluation of efficacy as compared to ara-A. The serum levels of acyclovir obtained have been adequate for antiviral activity in cell culture but as pointed out elsewhere (Chapter 3) cell culture data are not always relevant for predicting in vivo efficacy. This is also true for the situation with BVDU, which, given orally in an open study reached serum levels 100 times that required for cell-culture efficacy (De Clerq et al., 1980). Foscarnet has been tested in a few VZV patients and has been shown to be active in monkeys with simian varicella (J. Ahlmen, S.S., Soike, personal communications).

The chemotherapy of choice at present for zoster seems to be ara-A, and future trials will have to be performed against this drug, rather than placebo.

TABLE 13.10.
Influence of vidarabine therapy on complications of localized herpes zoster in immunosuppressed patients. Treatment was as described in Fig. 13 (after Whitley et al., 1982)

	Vidarabine (n=63)	Placebo (n=58)
Uveitis (blindness)	1 (0)	3 (2)
Hepatitis	0	3
Encephalitis	2	3
Neuropathy	3 (5%)[a]	11 (19%)[a]

[a] P=0.015 (chi-square test)

13.6. Interferon, transfer factor and immunomodulators

Human leukocyte interferon has been used in the treatment of varicella in children with cancer. However, in a double-blind placebo-controlled study on 18 patients Arvin et al. (1978) did not see any significant effect when giving 4.2×10^4 U/kg or 2.55×10^5 U/kg i.m. every 12 hours. Complications of varicella occurred in 6 of 9 placebo recipients and in 2 of 9 interferon recipients. However, in an extension of this trial involving a total of 44 patients, Arvin et al. (1982) observed significant therapeutic effects. In the second part of the trial the dose of interferon was increased to 3.5×10^5 u/kg/day for 48 hours followed by 1.75×10^5 U/kg/day for 72 hours. When the total patient data was analyzed, interferon was found to reduce the mean number of days of new vesicle formation from 5.3 ± 2.56 to 3.8 ± 1.89 ($P \leqslant 0.05$). In the high dose part of the study 92% of the interferon recipients had no new lesions for 24 hours by day 6 as compared with 45% of the placebo recipients ($P \leqslant 0.025$). There was also a reduction in visceral complications as shown in Table 13.11.

The effect of human leucocyte interferon in cancer patients with zoster has been evaluated by Merigan et al. (1978, 1981). When patients were given a 7 day course of interferon the highest dose used, 5.1×10^5 U/kg every 12 hours, prevented the progression of vesicles in the primary dermatome, and no cutaneous dissemination occurred. The healing time was not changed, but a decrease in post herpetic neuralgia was noted. With lower doses of interferon the therapeutic efficacy was lost and a shortening of the treatment to 48 hours also reduced the efficacy. In monkeys, recombinant type α interferon (HuIFN-αA) has been effective against simian varicella infection (Soike et al., 1983).

Steel et al. (1980) have evaluated the effect of transfer factor in children with leukaemia and who were seronegative to VZV. The transfer factor was prepared from leukocytes from normal persons recovering from chickenpox. Transfer factor was given to 31 children and placebo to 30 children. The two groups were exposed

TABLE 13.11.
Interferon treatment of varicella in children with cancer. Human leucocyte interferon, $4.2 \times 10^4 - 3.5 \times 10^5$ U/kg/day, or placebo, was given intramuscularly (after Arvin et al., 1982)

Finding	Interferon (n=23)	Placebo (n=21)
Mortality		
Acute ($\leqslant$1 week)	0	2
2–3 weeks after onset of varicella	2	1
Visceral dissemination in survivors		
Pneumonia	3	4
Encephalitis symptoms	0	3
Coagulopathy	0	2
Hepatitis, as shown by SGOT	5	4
Episodes of life-threatening dissemination in survivors	0/21	10/18[a]
No. of patients with life-threatening dissemination	0/21	3/18[b]
Incidence of mortality and life-threatening dissemination	2/23	6/21[c]

[a] P=0.18 (Wilcoxon rank-sum test)
[b] P=0.11 (chi-square test with Yates' correction)
[c] P=0.094 (chi-square test with Yates' correction)

to chickenpox to the same extent and 13 in the placebo group were infected, three of whom had progressive chickenpox. Only one patient in the transfer factor group was infected and had a mild disease. The mechanism of action is unclear but it has been pointed out (Kirkpatrick, 1980), that the children before the trial were skin tested with VZV antigen and that the transfer factor might act as an immunological adjuvant.

Finally, the possibility of stimulating the cellular immune system with inosiplex has been investigated by Feldman et al. (1978) in cancer patients with localized zoster, but no therapeutic effect or stimulation of lymphocyte response was detected.

13.7. Summary

Prophylaxis and therapy of varicella infections are rarely needed in the normal person. However, in immunosuppressed children varicella is often severe and experimental vaccines are being evaluated. Passive immunization of groups at risk seems to decrease the severity of infection. Also, ara-A has shown significant effects in children with severe infections. Moreover, IDU used topically in DMSO has shown some activity against zoster and i.v. acyclovir has a beneficial effect. In the immunocompromised host ara-A and acyclovir are beneficial against zoster, and case reports indicate effects by BVDU and foscarnet. High doses of interferon have also given therapeutic effects.

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CHAPTER 14

Epstein-Barr and cytomegalovirus infections

14.1. Epstein-Barr virus infections

14.1.1. Clinical aspects

Most people in the world have been infected by Epstein-Barr virus (EBV). These infections are mostly asymptomatic but will result in latent, persistent infections where the virus can be found in B-lymphocytes. The infection can be symptomatic and vary in severity, but it is rarely fatal. EBV, furthermore, is closely associated with some human tumours, such as nasopharyngeal carcinoma and Burkitt's lymphoma.

14.1.1.1. EBV infections in normal persons

Infectious mononucleosis (glandular fever) is a self-limiting lymphoproliferative disease and the result of an EBV infection. This is characterized by fever, sore throat, generalized lymphoadenopathy and often hepatosplenomegaly. The incubation period (in college students) is 4–7 weeks (Evans, 1960, Hoagland, 1964) and may be as short as 4–10 days in children (Hobson et al., 1958). During the incubation period, malaise and fatigue frequently precede the infectious mononucleosis symptoms. The disease is characterized by lymphocytosis, numerous atypical lymphocytes and the development of a variety of heterophil antibodies.

The frequency of clinical symptoms in one study (Timar et al., 1982) on children hospitalized for infectious mononucleosis is shown in Table 14.1 whilst typical pattern of clinical and laboratory findings for the infection in an adult is shown in Fig. 14.1. The childhood infections are usually mild or inapparent, but in college stu-

TABLE 14.1.
Clinical symptoms in 40 children suffering from infectious mononucleosis. The numbers in brackets indicate the percentage of patients with pronounced clinical symptoms (febrile period longer than one week, confluent tonsillitis, marked cervical lymphadenopathy and generalised swelling of the lymph nodes, hepatomegaly and splenomegaly exceeding 2 cm) (after Timar et al., 1982)

Symptom	Percentage
Fever	95.0% (70.0%)
Tonsillitis	87.5% (50.0%)[a]
Lymphadenopathy	100.0% (50.0%)
Hepatomegaly	95.0% (40.0%)
Splenomegaly	72.5% (37.5%)
Exanthema	30.0%[b]

[a] Four patients had previously undergone tonsillectomy.
[b] Eight patients had previously received ampicillin.

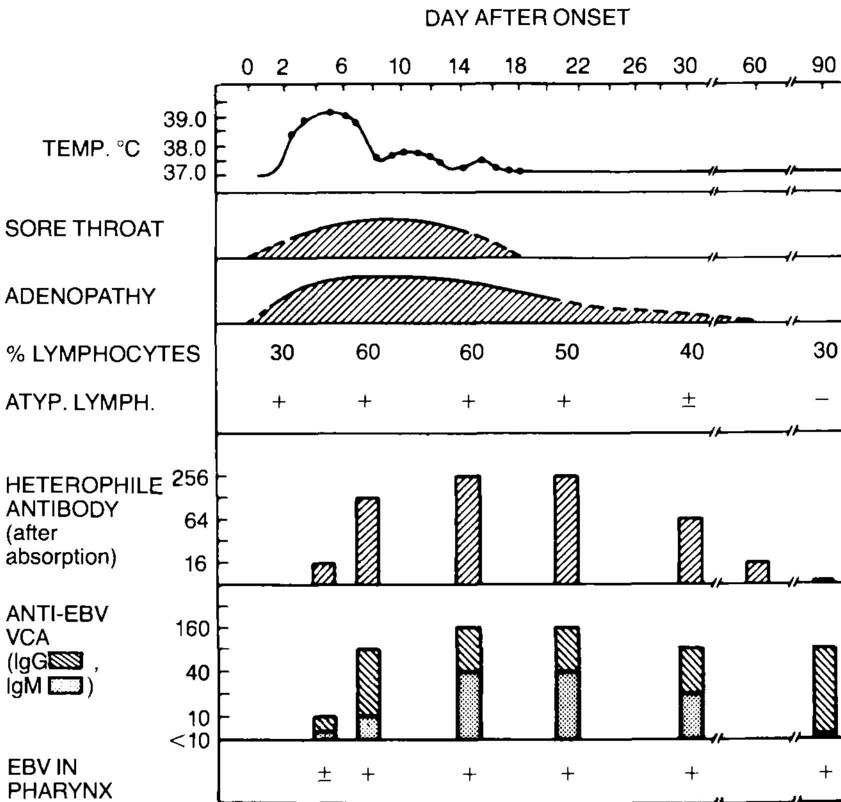

Fig. 14.1. Scheme of symptoms, antibody responses, and EBV oropharyngeal shedding in typical cases of infectious mononucleosis. (after Niederman, 1981.)

dents, about 50% develop clinical symptoms (Sawyer et al., 1971). Reactivation of EBV takes place to a significant extent during pregnancy but seems not to be associated with any adverse effects on the foetus (Fleisher and Bolognese, 1983).

Involvement of the central nervous system is rare but can appear as encephalitis, meningoencephalitis and Guillain-Barré syndrome.

14.1.1.2. EBV infections in immunocompromised persons

The risk of a reactivation of latent EBV resulting in clinical symptoms seems to be less than for the other human herpes viruses. One explanation of this could be that the B-lymphocytes containing EBV are affected or reduced during immunosuppression, whereas the target cells for the other herpes viruses are not. However, both diseases and chemotherapy with immunosuppressive drugs can result in a reactivation of EBV multiplication, and this is often seen as an increased excretion of EBV in oropharyngeal secretions. In renal transplant patients as many as 37% of the patients excrete EBV in the pharyngeal secretions (Cheeseman et al., 1980) and this should be compared to the 17–18% of normal seropositive individuals who excrete virus (Strauch et al., 1974, Chang et al., 1973). It is also evident that increased immunosuppression by, for instance, antithymocyte globulin increases this figure to 83% excreters (Cheeseman et al., 1980). An increased rate of EBV excretion has been observed in rheumatoid arthritis patients on corticosteroids and Aitcheson et al. (1983) found up to 43% excreters as compared to 17% in the control group not on immunosuppressive drugs. The importance of the activation of EBV multiplication for the development of clinical syndromes is not clear, but it is likely that it has a role in the development of lymphoproliferative diseases as exemplified by the high incidence of lymphomas in patients on prolonged immunosuppressive therapy (Kinlen et al., 1979).

14.1.1.3. Burkitt's lymphoma and nasopharyngeal carcinoma

There is a strong aetiological association between EBV and Burkitt's lymphoma and nasopharyngeal carcinoma. These tumours both contain EBV genomes and EBV antigens and the tumour patients have certain unique EBV-related antibodies. It is also clear that EBV in vitro can transform normal human lymphocytes and also induce lymphomas in nonhuman primates. The worldwide prevalence of EBV and yet the restricted geographic concentration of Burkitt's lymphoma to children in equatorial Africa and nasopharyngeal carcinoma to the southern Chinese indicate that the EBV infection is not the sole factor responsible for these tumours. In the case of Burkitt's lymphoma, malaria has been suggested as a possible cofactor. Treatment of Burkitt's lymphoma with cyclophosphamide has been quite successful but we should remember that it is not evident that Burkitt's lymphoma or nasopharyngeal carcinoma could be treated with selective antiherpes agents. (The possibility of using an EBV vaccine in high risk populations will be discussed later.) The association between EBV and tumours has been discussed in numerous papers of

which only a selected few can be referred to here (Burkitt, 1962, Epstein et al., 1964, De-Thé et al., 1969, Henle et al., 1969, 1970, Epstein and Achong, 1979, Kieff et al., 1982, Klein, 1982, Sugden, 1982).

14.1.2. EPIDEMIOLOGY

The prevalence of EBV antibodies in different age groups shows a large variation between different populations and Fig. 14.2 shows the prevalence at age 4–6 years in several countries. There is a clear correlation between economic and hygienic level and prevalence of EBV antibodies. The age at which antibodies are acquired is dependent on socioeconomic conditions as shown in Fig. 14.3. As with other human herpesviruses, EBV is found in all geographic areas of the world.

The prevalence of EBV in the oropharynx is shown in Table 14.2. The high prevalence of EBV in oral secretions explains the efficient spread of the virus. The infection at a low age is mostly asymptomatic. In communities with a later spread of the virus the importance of an oral spread is indicated by Fig. 14.4, where the cases of infectious mononucleosis are presented for different age groups. The name kissing disease seems to be appropriate. In family settings, a 10.5% secondary attack rate among susceptibles has been reported by Joncas and Mitnyan (1970). Wahren et al. (1970) found an antibody increase in 7 out of 21 persons exposed to an index

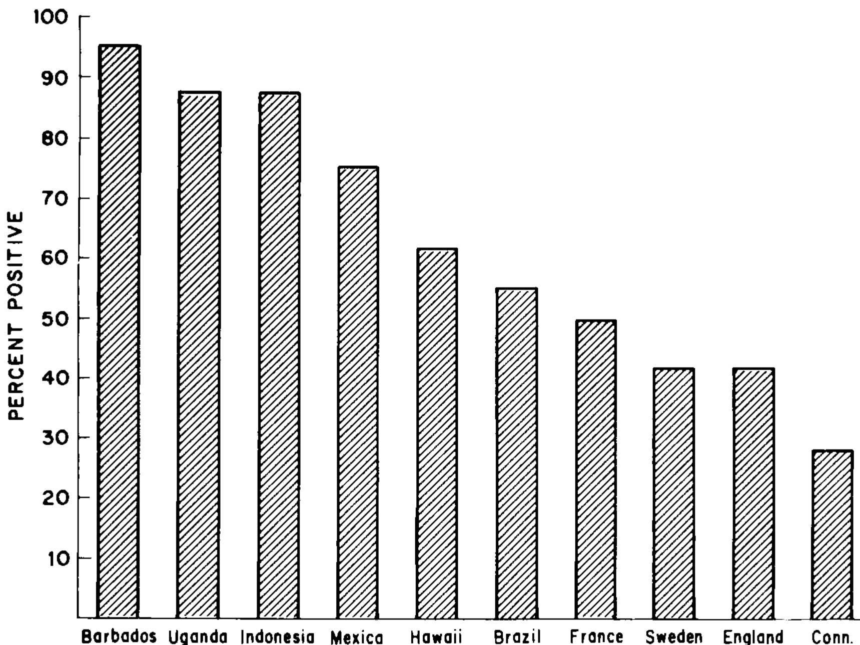

Fig. 14.2. Prevalence of EBV-VCA (viral capsid antigen) antibody by age 4 to 6 years in 10 different population groups. (after Evans, 1981.)

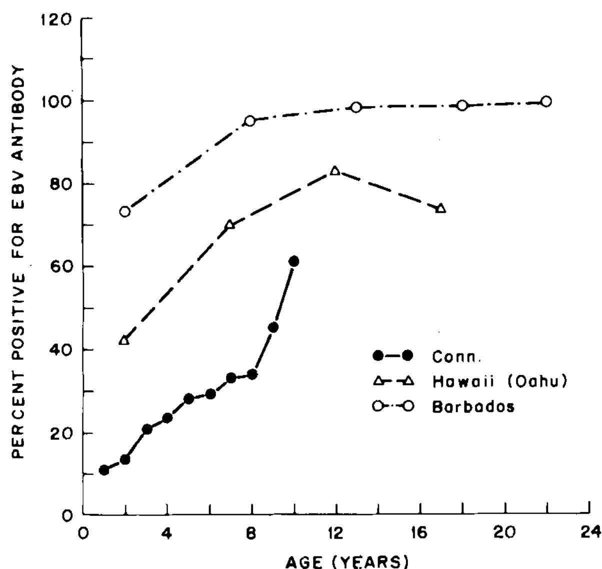

Fig. 14.3. Acquisition of EBV-VCA antibody by age in Connecticut, Hawaii and Barbados. (after Evans, 1981.)

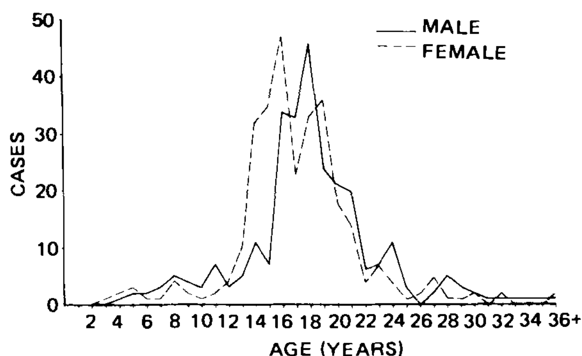

Fig. 14.4. Age and sex distribution of clinical infectious mononucleosis in a community survey in Atlanta, Georgia, USA. (after Evans, 1981.)

case and of 6 persons initially lacking EBV antibodies, 3 seroconverted. A confined environment is not enough to result in a spread of EBV as indicated, for example, by a study in a submarine crew (Storrie et al., 1976).

The incidence of clinical infectious mononucleosis is largely dependent on the age at infection. The incidence rate has been listed in Table 14.3 for some communities and Table 14.4 further shows the high incidence of clinical symptoms in young adults. Although the incidence of clinically important EBV infections is increased in immunodeficient patients the increase is less than for HSV or CMV infections (see Chapters 12 and 13).

TABLE 14.2.
Prevalence of EBV excretion in the oropharynx

Condition	No. tested	Percent positive
1. Infectious mononucleosis[a]		
Days after onset		
0–14	16	81.3
15–28	12	83.3
29–150	11	100.0
>150	3	66.7
	42	85.7
2. Healthy students[a]	41	14.0
3. Patients attending a clinic[b]		
Age		
<1	21	10
1–4.9	43	12
5–9.9	33	15
10–19.9	52	19
20–29.9	56	25
30–49.9	76	17
>50	87	20
	368	18
4. Cancer: All leukaemias and lymphomas[b]	30	50
All solid tumours[b]	30	27

After [a]Chang et al. (1973) and [b]Miller et al. (1973) as modified by Evans (1981).

TABLE 14.3.
Incidence of clinical infectious mononucleosis

Population	Annual rate per 100 000	Reference
General population		
Connecticut, USA	47	Christine (1968)
Atlanta, USA	45	Heath et al. (1972)
Minnesota, USA	200	Henke et al. (1973)
College students		
Average of 19 colleges, USA	840	Centre for Disease Control (1972)

TABLE 14.4.
Infection rates and clinical infectious mononucleosis in young adults during Freshman year

Place	Number in study	Percent suscep- tible	Infection rate in suscep- tibles (%)	Percent clinical infectious mononucleosis	Reference
US Military Academy	1401	36	12.3	27.7	Hallee et al. (1974)
Five English schools	1487	43	12.0	59.1	University Health Physicians (1971)
Yale University	355	49	13.1	74.0	Sawyer et al. (1971)

14.1.3. Vaccination

A vaccine against EBV could be directed against infectious mononucleosis or EBV-associated malignancies. However, the low frequency of symptomatic infectious mononucleosis and its benign course seems to make a vaccine against this disease unnecessary. In some special groups such as college students who do not have EBV antibody it might have a place, but the potential risk of using an oncogenic virus should be kept in mind. A vaccine to prevent Burkitt's lymphoma or nasopharyngeal carcinoma must be confronted with the problem of a population exposed to natural EBV infection at a very early age. In the case of these tumours, malaria control and identification of the cofactor involved in nasopharyngeal cancer seem to be more suitable preventative approaches. No reports have, as yet, been published on the use of EBV vaccine in humans, although experimental studies are in progress, in the UK for example, with cell-associated antigen vaccines.

14.1.4. Chemotherapy

The likely lack of a viral thymidine kinase makes EBV relatively resistant to nucleoside analogues requiring this enzyme for activation. Compounds such as ara-T and foscarnet inhibit EBV in cell culture but have not been tested clinically. We note here that since foscarnet has been used, and possibly is effective against CMV disease it might be worthwhile to test foscarnet against EBV infections.

Acyclovir, which requires a viral thymidine kinase, has been used to treat severe EBV infections in three patients, one with chronic active EBV infection, one with an X-linked lymphoproliferative syndrome and one renal transplant patient (Sullivan et al., 1982, Hanto et al., 1982). The first two patients did not improve as a result of treatment. The third patient showed a decrease in oropharyngeal shedding of EBV and resolution of fever and other symptoms during the first treatment with acyclovir, but treatment of two later recurrences did not prevent a lymphoproliferation of monoclonal B-cells and the patient did not survive.

Chemotherapy of immunocompetent patients with infectious mononucleosis has not been described.

14.1.5. Interferon

Human leucocyte interferon has been given to renal transplant patients and their excretion of EBV has been followed (Cheeseman et al., 1980). As shown in Table 14.5, interferon reduced virus shedding in the group also receiving antithymocyte globulin but not in the group not given antithymocyte globulin. Antithymocyte globulin evidently increased the excretion of EBV (Table 14.5). There are no reports on the effect of interferon on clinical symptoms caused by EBV infections.

14.2. Cytomegalovirus infections

Cytomegalovirus (CVM) is a ubiquitous herpesvirus causing clinical and latent infections in most persons in all geographical areas of the world. Most of the infections are asymptomatic but in some instances they can cause disease of diverse severity. In immunocompromised patients the disease can be life-threatening, and during pregnancy a CMV infection could have severe consequences for the foetus.

14.2.1. Clinical aspects

14.2.1.1. Congenital and neonatal infections
The frequency of congenital CMV infections is 0.5–2.5% of all live births (Whitley

TABLE 14.5.
Oropharyngeal excretion of Epstein-Barr virus in renal transplant patients. The patients were given placebo or 3×10^6 U HuIFN-α i.m. twice weekly. Some patients also received equine derived antithymocyte globulin. The figures show the number of patients excreting virus versus total number of patients in each group (after Cheeseman et al., 1980)

	Antithymocyte globulin	No antithymocyte globulin	Combined
	No.	No.	No.
Interferon group	5/11[a]	3/10	8/21[b]
Placebo group	10/12[a]	3/8	13/20[b]
Combined	15/23[c]	6/18[c]	21/41

P=level of significance by Fisher's exact test.
[a] P=0.07 for interferon-antithymocyte globulin versus placebo-antithymocyte globulin.
[b] P=0.08 for interferon versus placebo.
[c] P=0.04 for antithymocyte globulin versus non-antithymocyte globulin.

and Alford, 1979, Ahlfors, 1982). About 80% of these infections are asymptomatic at birth but in 5–10% they might later on develop into central nervous dysfunctions such as hearing loss and/or mental retardation or minor brain disease (MBD). The risk of intellectual impairment in children with asymptomatic congenital CMV is probably low (Saigal et al., 1982). The clinical findings in infants with symptomatic infections have been summarized by Whitley and Alford (1979) and are shown in Table 14.6. The congenital infections involve both the endoreticular and the central nervous system. CMV infections can, consequently, be an important cause of deafness. It has also been found that in children with severe congenital CMV infections, 40% of the cases had tooth defects while in children with less severe infections only 5% of cases showed tooth defects (Stagno et al., 1982).

Infection with cytomegalovirus during delivery is estimated to occur in 1–5% of live births in the US (Stagno et al., 1975) and these infections are mostly asymptomatic. The long term morbidity has not been determined, but is believed to be lower than in the congenital infection. In addition, infant infections have been reported from intensive care units where blood transfusion seems to be the likely mode of transmission (Ballard et al., 1979).

TABLE 14.6.
Clinical findings in infants with symptomatic congenital CMV infection (after Whitley and Alford, 1979)

Clinical findings	Frequency (%)
Intrauterine growth retardation	21–50
Reticuloendothelial system	
Hepatitis	51–75
Direct hyperbilirubinaemia	51–75
Haemolytic and other anaemias	21–50
Petechiae-ecchymoses	76–100
Hepatosplenomegaly	76–100
CNS	
Encephalitis	51–75
Microcephaly	21–50
Intracranial calcifications	21–50
Eye	
Chorioretinitis	9–20
Congenital malformations	
Inguinal hernias	21–50
First branchial arch derivatives	9–20
Pneumonitis	9–20
Sequelae	
Psychomotor retardation	51–75
Hearing loss	21–50
Visual impairment	0–20

Intrauterine death, hydrocephalus, myocarditis and bone effects are rare.

Viruria occurs both in congenitally and postnatally infected infants (Stagno et al., 1975) and can persist for years. This is not prevented by humoral antibodies and furthermore, immune complex formation resulting in granular deposits in the glomerular basement membranes has been reported. (Stagno et al., 1977). It has been suggested that there is a defect in T-lymphocyte-mediated response to CMV in congenitally infected infants (Schauf et al., 1976, Gehrz et al., 1977, Starr et al., 1977).

14.2.1.2. Infections in children and adults

Most CMV infections in children and adults are asymptomatic but in about 1% of the infections a variety of clinical syndromes appear, such as mononucleosis, hepatitis, respiratory or gastrointestinal symptoms and always fever, but rarely central nervous disease. The mononucleosis syndrome was first described by Klemola and Kääriäinen (1965) as fever, malaise, hepatitis and atypical lymphocytosis in the absence of pharyngitis and adenopathy. The typical time course of the infection has been described by Alford et al. (1981) and is shown in Fig. 14.5. Most cases of heterophil negative mononucleosis are thought to be caused by CMV (Oill et al., 1977).

14.2.1.3. CMV infections in immunocompromised persons

Both primary and recurrent CMV infections can be serious in immunocompromised persons. They are a problem mainly for renal transplant, bone marrow transplant and cardiac transplant patients, for patients with malignancies and for patients with other immunodeficiencies. Betts (1982) has carefully reviewed cytomegalovirus infections in transplant patients, and the clinical variables seen in transplant CMV illness are shown in Table 14.7.

In renal transplant patients, primary infections in seronegative hosts are mostly

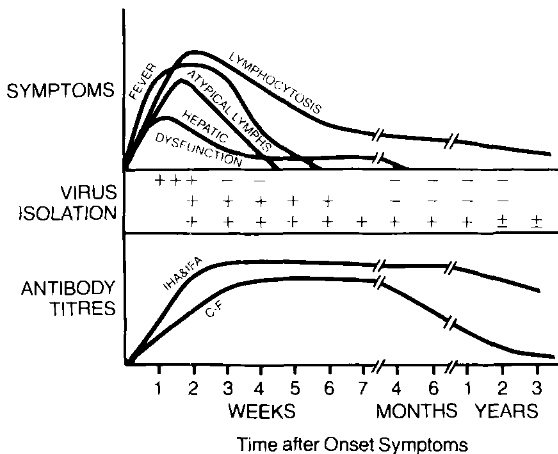

Fig. 14.5. Findings of symptomatic primary CMV infection in normal hosts (mononucleosis syndrome). (after Alford et al., 1981.)

TABLE 14.7.
Clinical parameters occurring in transplant-associated CMV illness (after Betts, 1982)

	Frequency, %
Fever	100[a]
Lymphadenopathy	0
Pharyngitis	0
Hepatomegaly	10–30
Splenomegaly	5–40
Skin rash	5–10
Liver function abnormality	40
Leukopaenia (<2000)	40
Lymphocytosis (>35 000)	5
Pneumonia	10–15

[a] Required to be included.

symptomatic, whilst recurrent infections are mostly asymptomatic. The infections occur 1–3 months after transplantation. The morbidity varies in different centres, possibly depending on the degree of immunosuppression. (Spencer, 1974, Luby et al., 1974, Fiala et al., 1975, Suwansirikul et al., 1977, Peterson et al., 1981, Walker et al., 1982). In the study by Walker et al. (1982) CMV disease was observed in 37% of the patients, and in 46% of the patients seropositive prior to transplantation a reactivation occurred. In 28% of the seronegative patients a primary CMV disease was encountered. CMV is the dominating factor causing fever in renal transplant patients (Peterson et al., 1981) as indicated in Fig. 14.6. Primary infections are more severe and commonly result in pulmonary bacterial and fungal superinfections (Chatterjee et al., 1978). It is not clear if CMV infection is involved in transplant rejection.

In bone marrow transplant patients, interstitial CMV pneumonia is the major cause of mortality in the early period after transplantation and the frequency is highly dependent on the method of immunosuppression, as shown in Fig. 14.7 (Meyers et al., 1982a). The incidence of CMV pneumonia in the study by Meyers et al. (1982a) is shown in Table 14.8 and the mortality was 91% among these patients with CMV pneumonia. In a study by Neiman et al. (1977) among marrow transplant patients the overall mortality due to pneumonia was 42% and in patients not seroconverting to CMV the mortality was 77%. Cardiac transplant patients are also at risk for CMV pneumonia (Rand et al., 1978). The influence of the type of immunosuppression used on the mortality in CMV infections in transplant patients is not clear, but the use of antithymocyte globulin may be a determinant of CMV pathogenicity in marrow (Pass et al., 1980, 1981, Meyers et al., 1982a), and cardiac transplant patients (Preiksaitis et al., 1983).

A donor allograft infected by the recipient virus could alter the antigenic match

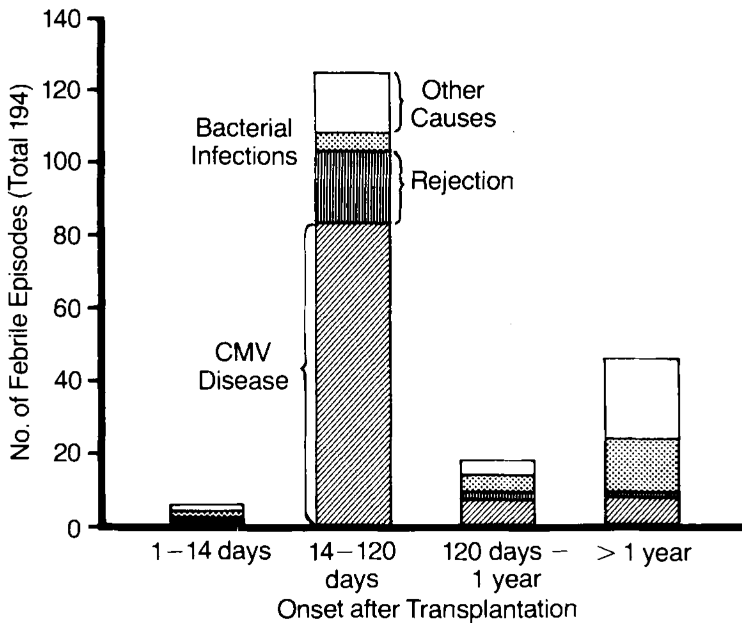

Fig. 14.6. The importance of CMV infections as the cause of fever in renal transplant patients. (after Peterson et al., 1981.)

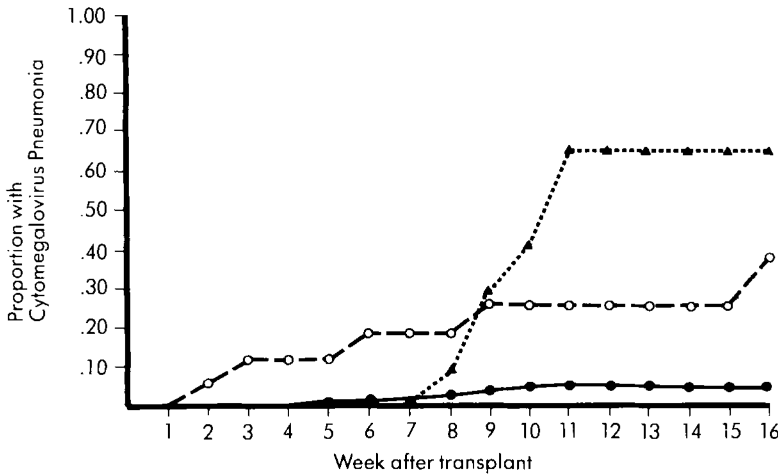

Fig. 14.7. CMV pneumonia in marrow transplant patients after different conditioning regimens. △-----△, 21 patients receiving total body irradiation; ○-·-·-○, 19 patients receiving procarbazine, antithymocyte globulin and cyclophosphamide; ●——●, 129 patients receiving cyclophosphamide. (after Meyers et al., 1982a.)

and possibly lead to an attack by cytotoxic T cells resulting in a graft versus host disease. This has been described in animal models (Zinkernagel and Oldstone, 1976)

TABLE 14.8.
Incidence of non-bacterial pneumonia after marrow transplantation among 525 recipients of allogeneic grafts and 100 recipients of syngeneic grafts (after Meyers et al., 1982a)

Type of pneumonia	Percentage of patients with pneumonia	
	Allogeneic	Syngeneic
Idiopathic	12	11
Cytomegaloviral	16	0
Pneumocystis carinii	6	1
Other viral	3	1

and might conceivably take place in patients (Meyers et al., 1975, Leinikki et al., 1978, Winston et al., 1980).

A severe CMV retinitis can occur in immunosuppressed hosts (Pollard et al., 1980) and this has characteristic fundoscopic findings. Recently this has been reported for AIDS patients (Bachman et al., 1982, Neuwirth et al., 1982). Chorioretinitis is also observed in congenitally infected infants with cytomegalic inclusion disease (Hanshaw and Dudgeon, 1978).

14.2.2. Epidemiology

The prevalence of CMV antibodies shows that this virus is ubiquitous in the world. Fig. 14.8 shows the prevalence of antibodies in persons of different ages in different parts of the world. It is obvious that socioeconomic factors influence the age at which the first infection occurs. Large geographic differences are also seen in the early acquisition of CMV antibodies when serum from cord blood, infants and children aged 4–48 months are analyzed (Krech and Tobin, 1981) as shown in Fig. 14.9.

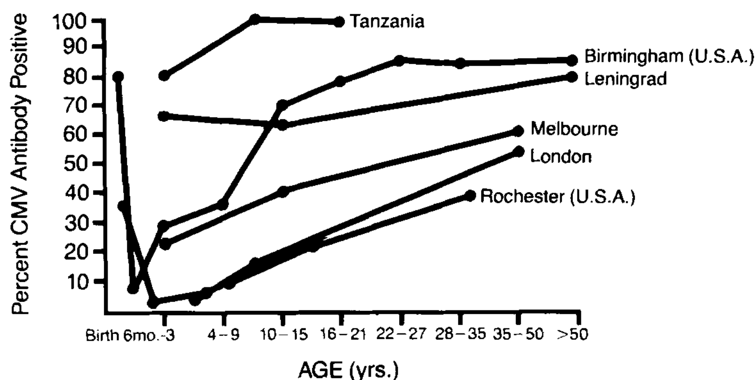

Fig. 14.8. Seroepidemiology of cytomegalovirus infection. (after Alford et al., 1981.)

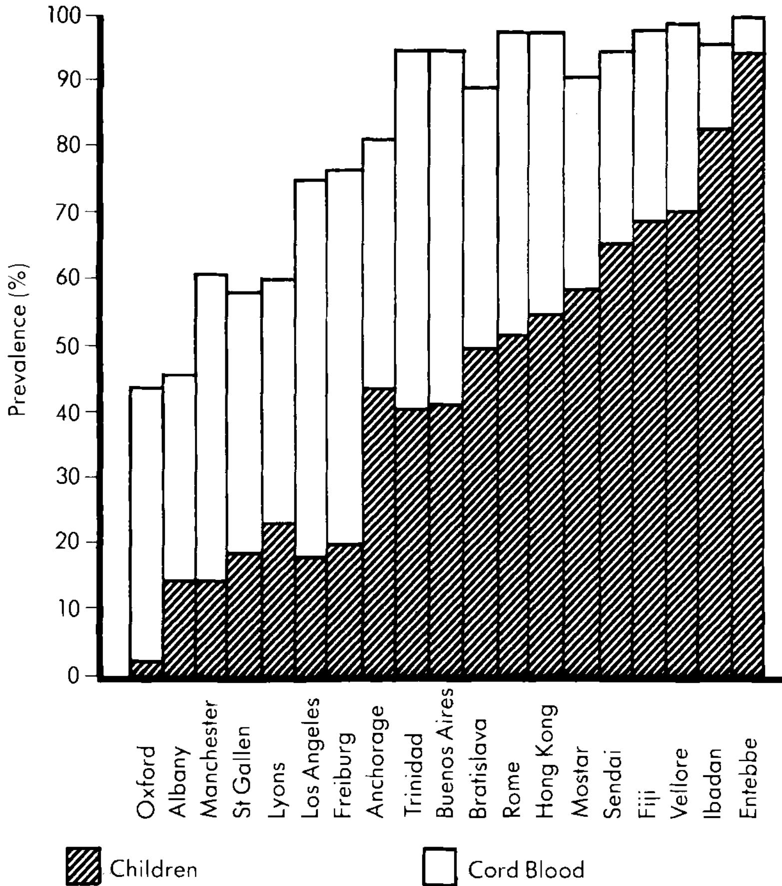

Fig. 14.9. Cytomegalovirus complement-fixing antibody titres in serum from cord blood and from infants and children aged 4–48 months. (after Krech and Tobin, 1981.)

As with other herpesviruses,CMV has been found in all communities analyzed, including remote and isolated tribes (Black, 1975).

The spread of CMV is facilitated by frequent virus excretion by infected persons. The major spreading occurs from mothers to infants and also between children in nursery schools. Virus is frequently excreted in urine, in the cervix, the throat and in breast milk. As an example, Hayes et al. (1972) reported that 27% of an Australian population excreted CMV in breast milk, and high figures were also reported by Alford et al. (1981) as shown in Table 14.9. The frequency of infection is also reflected in virus excretion by infants, as shown in Table 14.10.

In young adults CMV does not seem to spread as effectively as EBV, for example, but in transfused patients, who are seronegative, seroconversion rates of 61–69% have been observed (Purcell et al., 1971, Gold and Nankervis, 1976). Transfusion

TABLE 14.9.
Recurrent excretion of CMV. Rate of shedding into breast milk and other sites in relation to the type of preceding CMV infection (after Alford et al., 1981)

Type of preceding CMV infection	No. excreting CMV/total studied	
	Breast	Other sites[a]
Prior delivery congenitally infected infant	11/ 16	9/ 28
Previous excretion of CMV	8/ 49	13/ 62
Seropositive nonexcretors	19/146	4/ 57
	38/211	26/147

[a] Genital and urinary tract or pharynx.

TABLE 14.10.
Prevalence of CMV shedding in infants

Age	% virus shedding	Location	Study
3 months	24	Helsinki	Granström et al. (1977)
3 months	12	Seattle	Levinsohn et al. (1969)
3 months	5	Manchester	Collaborative study (1970)
3 months	8	Sweden	Ahlfors et al. (1978, 1979)
1 year	60	Japan	Numazaki et al. (1970)
3–12 months	25–50	Birmingham, AL	Alford et al. (1981)

and perfusion increase the incidence of CMV infections and, for example, of 53 patients undergoing open heart surgery 21 had a rise in CMV antibodies and 4 developed a mononucleosis-like syndrome. (Paloheimo et al., 1968). Perhaps not unexpectedly an increased number of units of blood transfused is associated with an increased risk of CMV antibody conversion (Prince et al., 1971).

The presence of CMV in the cervix and in semen indicates that venereal spread could take place. High (7.4%) frequencies of CMV excretion in the urine of homosexual men and CMV antibodies in 94% of this group, as compared to 43% in a heterosexual control group, indicate a sexual transmission in homosexuals (Drew et al., 1981) and indeed the virus is frequently present in the newly described syndrome of acquired immune deficiency (AIDS) in homosexual men, as discussed later in this Chapter.

14.2.3. Vaccination

Vaccines against CMV infections are being developed, mainly with the aim of re-

ducing the number of severe congenital CMV infections. Elek and Stern (1974) used a live attenuated CMV strain (AD 169) in 26 volunteers and detected seroconversion in 25. No virus excretion was detected, the presence of complement fixing antibodies was transient and some delayed reaction at the site of subcutaneous injection was observed. The attenuated AD 169 strain was also used by Neff et al. (1979), who confirmed the immunogenicity and delayed local reaction. Moreover, the vaccine induced antibody titre was maintained for at least one year.

Plotkin et al. (1976) have reported the use of the attenuated CMV strain Towne-125 as a vaccine. This strain also gave rise to an antibody response after s.c. inoculation in volunteers. The local reaction observed with AD 169 was also seen with Towne-125 and a cellular immunity response was observed, although less in renal transplant patients than in normal volunteers (Just et al., 1975, Plotkin et al., 1976, Gehrz et al., 1980, Starr et al., 1981).

The Towne-125 strain has been used to vaccinate seronegative paediatric nurses, who are at some risk of acquiring CMV (Fleicher et al., 1982). As shown in Fig. 14.10 this vaccination resulted in both humoral and cell-mediated immunity lasting at least one year and no virus excretion was detected. It is obvious that to detect any effect of a vaccine on a symptomatic congenital infections a very large number of women of childbearing age must be included in a trial.

Perhaps not unexpectedly, critical concern about the use of CMV vaccine has been raised (See Osborn, 1981). Thus, despite the immunity induced by vaccination, several renal transplant patients subsequently excreted CMV in the urine. The excreted CMV strains differed from the vaccine strain Towne-125 and therefore the vaccination had apparently not prevented an exogenous CMV infection (Glazer et al., 1979). Furthermore, the use of a DNA-containing, potentially oncogenic virus could be of concern as well as the possibility of formation of virus revertants with

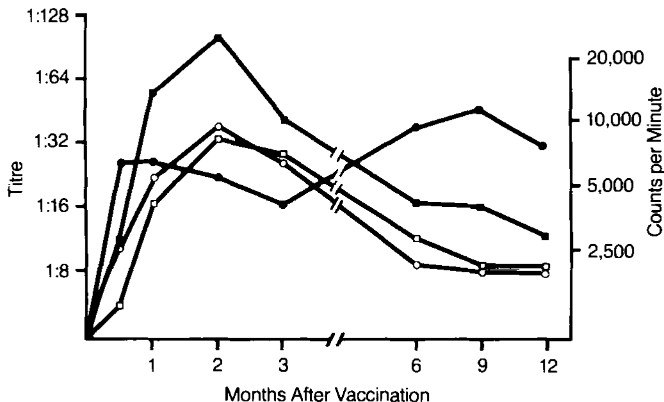

Fig. 14.10. Cellular and humoral immune response to vaccination with CMV strain Towne-125. The results are mean from 10 vaccinees. ○, complement fixation; ■, anticomplement immunofluorescence; □, neutralization; ●, lymphocyte proliferation. (after Fleicher et al., 1982.)

virulence characteristics. Before any general use of CMV vaccine is considered, the possibility of defining high risk groups ought to be further evaluated. The need for a vaccine would also, to some extent, be reduced if effective antiviral chemotherapy was available. Finally, the use of CMV hyperimmune plasma is unclear and will require further study before any conclusions are made about efficacy.

14.2.4. CHEMOTHERAPY

Several nucleoside analogues, such as acyclovir and BVDU, with activity against herpes simplex virus in cell culture require the presence of a viral thymidine kinase for antiviral activity. As CMV lacks a thymidine kinase enzyme, compounds of this type are not likely to be active against this virus. In short, there is at present, no double-blind placebo-controlled study showing therapeutic activity of any compound against CMV infections.

Fluorodeoxyuridine has been used to treat suspected CMV pneumonia in a few children with leukaemia (Cangir et al., 1967). The small size and absence of placebo controls in this study makes it impossible to determine if fluorodeoxyuridine had any effect on the CMV induced infections, although the five treated leukaemic children seemed to respond favourably. Plotkin and Stetler (1970) treated congenital CMV infections with ara-C and reported depression of viruria but no clinical benefit. Moreover, this treatment induced considerable toxicity.

Ara-A has been tried by Chien et al. (1974) to treat severe CMV infections. A transient reduction in viruria was noted in infants and non-immunosuppressed adults, but not in renal transplant patients. Some clinical benefit was noted, but the size of the study did not permit any definitive conclusions about efficacy. Rytel and Kaufman (1976) have also reported ara-A treatment of three renal transplant patients, but without noted clinical benefit. Negative results about the usefulness of ara-A in renal patients was also reported by Marker et al. (1980). Fourteen immunosuppressed patients with CMV retinitis were treated with ara-A by Pollard et al. (1980). The possible clinical benefits observed did not seem to balance the significant toxicity of ara-A.

Acyclovir has been given i.v. to immunocompromised patients with severe CMV infections. Balfour et al. (1982) have reported a small controlled trial which could indicate some beneficial effect for renal transplant patients, but the size of the trial does not permit any clear conclusions. In a trial on 8 bone marrow transplant patients with CMV pneumonia no therapeutic efficacy and a mild toxicity were noted when doses of 400–1200 mg/m^2 were given i.v. (Wade et al., 1982). Four patients with congenital CMV infection were given acyclovir without any clinical improvement (Plotkin et al., 1982). However, as mentioned above, acyclovir must be phosphorylated by a viral thymidine kinase and since CMV lacks this enzyme, it is perhaps not surprising that the clinical trials have been negative.

Trifluorothymidine does not require a viral thymidine kinase for its activity and

is presently undergoing clinical trial. Phosphonoacetate, at least in animal studies, has been shown to be active against murine CMV infections (Overall et al., 1976) but its dermal toxicity even when given i.v. (Felsenfeld et al., 1978, Eriksson and Öberg, 1984) seems to preclude clinical use.

Foscarnet, which inhibits human CMV replication in vitro (Wahren and Öberg, 1979, 1980, Eriksson et al., 1982), has been given intravenously to renal and marrow transplant patients with severe CMV disease. The preliminary results from 60 patients indicate that when foscarnet is given late to bone marrow transplant patients with severe pneumonia the treatment has only a limited effect on mortality, which is partly due to fungal and bacterial infections. However, in immunocompromised CMV patients without severe pneumonia i.v. treatment with foscarnet seems to be clinically beneficial (G. Klintmalm and J. Ahlmén, personal communication), but obviously more extended and controlled trials are necessary to evaluate the true potential of foscarnet for severe CMV infections. Sufficiently high serum levels of foscarnet to inhibit CMV have been obtained without any noted toxicity. One example of a CMV infection in a bone marrow patient treated with foscarnet is shown in Fig. 14.11.

14.2.5. Interferon and immunomodulators

Attempts to use human and bovine transfer factors have given variable results (Paganelli et al., 1981, Jones et al., 1981) and no controlled trials are available for evaluation.

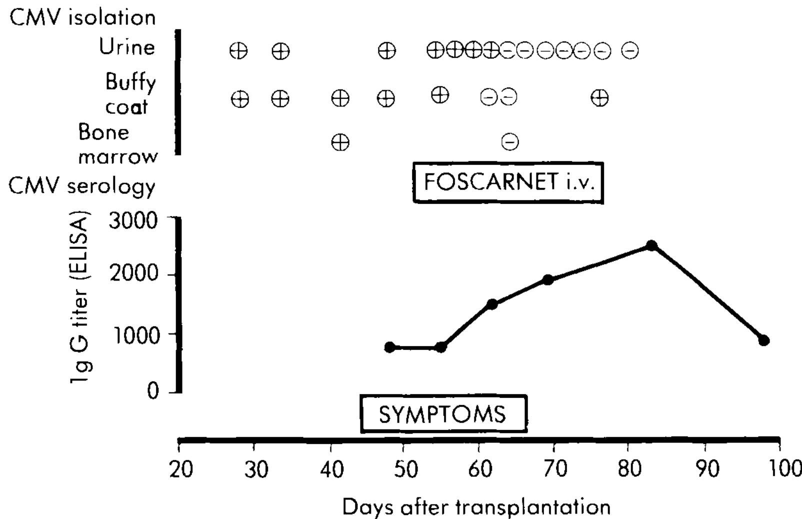

Fig. 14.11. Effect of foscarnet on a CMV infection in a bone marrow transplant patient. (G. Klintmalm, personal communication.)

The prophylactic use of human leukocyte interferon in renal transplant patients has resulted in some reduction in virus shedding and a decreased incidence of viraemia (Cheeseman et al., 1979) as shown in Table 14.11. Interferon did not have any effect on the incidence of CMV syndromes and, moreover, addition of antithymocyte globulin seemed to *increase* the incidence as shown in Tables 14.12 and 14.13.

The use of interferon in bone marrow transplant patients has not been successful (Meyers et al., 1980), and a trial using a combination of leucocyte interferon and ara-A showed that the combination of these agents was neither safe nor effective (Meyers et al., 1982b). In the latter trial virus titres decreased, but there was no clinical evidence of efficacy, and some patients showed declining neutrophil counts and severe neurotoxicity.

14.3. Acquired immune deficiency syndrome (AIDS)

The initial observations that male homosexuals living in certain areas had a high

TABLE 14.11.
Incidence of viraemia in cytomegalovirus infected renal transplant patients treated with human leucocyte interferon (after Cheeseman et al., 1979)

Treatment	Antithymocyte globulin	Non-antithymocyte globulin	Combined[c]
Interferon	5/7	0/4	5/11[a]
Placebo	6/6	3/4	9/10[a]
Combined	11/13[b]	3/8[b]	14/21

[a] $P=0.04$, interferon vs. placebo, by Fisher's exact test.
[b] $P=0.04$, antithymocyte globulin vs. non-antithymocyte globulin.
[c] 95% confidence intervals for combined groups were interferon 0.20–0.75; placebo, 0.65–0.99; antithymocyte globulin, 0.57–0.97; and non-antithymocyte globulin, 0.11–0.71.

TABLE 14.12.
Incidence of cytomegalovirus syndromes in renal transplantation patients treated with human leucocyte interferon (after Cheeseman et al., 1979)

Treatment	Antithymocyte globulin	Non-antithymocyte globulin	Combined[b]
Interferon	4/7	0/7	4/14
Placebo	3/7	1/5	4/12
Combined	7/14[a]	1/12[a]	8/26

[a] $P=0.03$, antithymocyte globulin vs non-antithymocyte globulin, by Fisher's exact test.
[b] 95% confidence intervals for combined groups were interferon 0.13–0.58; placebo, 0.12–0.45; antithymocyte globulin, 0.26–0.74; and non-antithymocyte globulin, 0.01–0.29.

TABLE 14.13.
Relation between the use of antithymocyte globulin (ATG) and the occurrence of CMV pneumonia (after Meyers et al., 1982a)

Type of ATG used (total No. of patients)		% of patients with CMV pneumonia
Prophylactic	(58)	17
Therapeutic	(91)	24[a]
Any	(147)	22
None	(378)	14[a]
Total	(525)	16

[a] $\chi^2=4.90; P=0.05$.

frequency of Kaposi's sarcoma and/or opportunistic infections such as *Pneumocystis carinii* (Pneumocystis pneumonia – Los Angeles. Morbid. Mortal. Weekly Rep., 1981, Gottlieb et al., 1981, Masur et al., 1981, Siegal et al., 1981) have in two years developed into an area of intensive research concerning the acquired immune deficiency syndrome (AIDS).

14.3.1. Clinical aspects

A prodromal illness with long lasting fever, diarrhoea, weight loss and lymphadenopathy has been common in AIDS cases. The disease is characterised by autoimmune disturbances, opportunistic infections especially by herpesviruses, Kaposi's sarcoma, chronic lymphadenomegaly, non-Hodgkin's lymphoma or squamous cell carcinoma (Masur et al., 1981, Centers for Disease Control, 1982, Friedman-Kien et al., 1982, Levine, 1982, Masur et al., 1982, Mildvan et al., 1982, Small et al., 1983, Vieira et al., 1983, Sonnabend et al., 1983). A decreased helper T cell (T_4) and an increased level of suppressor T cell (T_8) as well as increased levels of serum immunoglobulins have also been observed, possibly related to EBV reactivation. A disturbed humoral immunity is indicated by absence of response to deliberate immunization.

14.3.2. Epidemiology

There has been an exponential rise in the incidence of AIDS in the USA, a doubling of cases every six months and an estimate of 20000 victims by 1985 (Nature, News and Views, 1983). By June 1984 about 4000 cases had been reported and about 2000 died. The major risk group is male homosexuals with an excessively large number of partners. Other groups at risk are intravenous drug addicts, haemophiliacs, female sexual partners of AIDS' victims and Haitian emigrants to the USA. Case reports of AIDS in other groups in the population have also appeared. It seems likely

that an infectious agent is spread through intimate contact or blood products. The number of pre-AIDS patients seems to be ten times the number of AIDS cases and AIDS has now been reported from all parts of the world.

14.3.3. Causative agents

The spread of AIDS is compatible with the appearance of a new infectious agent but can also be explained by the presently known viruses and possibly by a combination of known viruses (Sonnabend et al., 1983).

Several viruses have been discussed as causative agents of AIDS. CMV is spread via saliva, semen and urine and 57% of homosexual men have IgM against CMV, as compared to 4% of heterosexual men (Drew et al., 1982). The CMV genome has also been detected in Kaposi's sarcoma (Drew et al., 1982). EBV antibodies were found in all of 95 AIDS patients (Sonnabend et al., 1983) and the EBV genome has been found in B-cell lymphomas (Ziegler et al., 1982). HTLV-III antibodies (Essex et al., 1983) and the HTLV-III genome (Gelmann et al., 1983) as well as HTLV-III particles (Gallo et al., 1983) and LAV particles (Barré-Sinoussi et al., 1983) have been found in AIDS patients (see Retrovirus, Chapter 9). It seems possible that these viruses could upset the immune system and result in the clinical syndromes characteristic for AIDS. It is also likely that HTLV-III and LAV are identical or closely related. A combination of these viruses and other factors might be required for the development of AIDS. The very active research in this area will hopefully, in the near future, explain this new disease.

14.3.4. Treatment and prevention

Once the causative agent(s) and immunological mechanisms resulting in AIDS have been described rational approaches to therapy and prevention can be developed. A number of drugs are presently being tested and the possibility of affecting AIDS by inhibiting the multiplication of HTLV-III and LAV should be considered. Preliminary evidence indicates that foscarnet (Öberg, 1983) inhibits HTLV-III (R. Gallo and P. Sarin, pers. comm.) and LAV (L. Montagnier and J.-C. Chermann, pers. comm.).

14.4. Summary

No therapy or prophylaxis against EBV infections is presently available. Since EBV lacks thymidine kinase the only presently known compound likely to be effective against EBV infections is foscarnet and a trial with that compound against severe EBV infection is now required.

Vaccines against CMV are being developed but although vaccination results in

humoral and cellular immunity this has not prevented subsequent infection by CMV. In general the delineation of target groups for a vaccine is not clear and much will depend therefore on the development of antiviral drugs against CMV.

There is no controlled study clearly showing therapeutic effect of any drug against CMV infection although case reports indicate foscarent to be beneficial in severe CMV infections in immunosuppressed patients.

Therapy and prevention of AIDS can not be rationally devised until the aetiology of the disease is known. The possible involvement of HTLV-III and LAV should be considered in selecting treatment regimens today and foscarnet seems to be worth testing.

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CHAPTER 15

Poxvirus infections

In a vehicle carrying two children with smallpox, Ali Maow Maalin, a cook at the Merca Hospital in Somalia, contracted the last endemic smallpox infection on the 12th of October 1977 while directing the vehicle from the hospital to the leader of the local smallpox surveillance team (Deria et al., 1980).

Smallpox has for thousands of years been a most feared disease. It has left its marks on the 3000 year old mummy of Ramses V and it was the deadly cargo of the conquistadore ships landing in Mexico. The successful global vaccination campaign resulted, in 1977, in the last natural case of smallpox. Three years later WHO officially declared that smallpox had been completely eradicated from the earth. The smallpox virus is still present in a few freezers in special designated laboratories. The eradication of smallpox will be described at some length as the first, but hopefully not the last, example of an eradication of a severe human disease. The virology of the poxviruses has been reviewed by Joklik (1966), Baxby (1977), Moss (1978), Fenner (1979), and smallpox was recently reviewed by Behbahani (1983).

15.1. The viruses

Poxviruses are the largest and most complicated viruses of humans and they more resemble 'reduced' bacteria than viruses. Fig. 15.1 shows the vaccinia virus, which is structurally very similar to smallpox virus (variola). The virions are brickshaped or ovoid, 300–450 nm × 170–260 nm and have both an envelope and a coat enclosing lateral bodies and a core structure. The core structure contains a dsDNA with a M.W. of $85-240 \times 10^6$. The G + C content is very low, about 36% for vac-

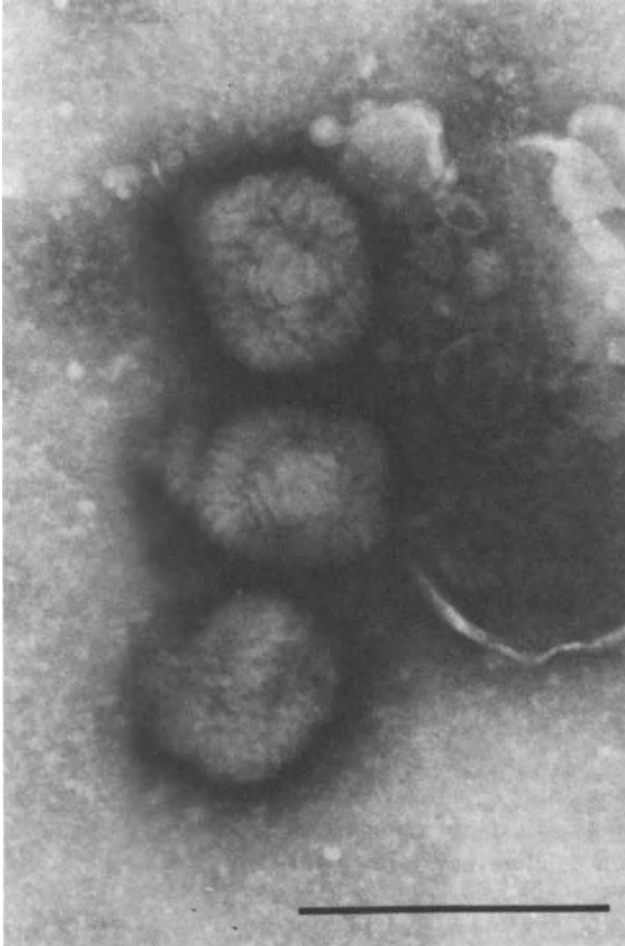


Fig. 15.1. Pox virus. The bar represents 500 nm in this EM picture. (courtesy of Dr. L. Svensson.)

TABLE 15.1.
Poxviruses affecting humans

Genus	Virus
Orthopoxvirus	Smallpox (variola)
	Vaccinia
	Monkeypox
	Cowpox
Parapoxvirus	Orf
	Milkers nodes
	Molluscum contagiosum

cinia virus DNA. The genetic material is enough to code for a few hundred proteins, and more than one hundred have been detected. As shown in Table 15.1 there are poxviruses from three genera infecting humans. Cowpox and monkeypox viruses are orthopoxviruses which also can infect humans. The virions contain 10 major antigens of which one cross-reacts with most poxviruses. There is also extensive serological cross-reactivity within each genera of poxvirus. Although vaccinia and variola virions differ in only one antigen, the restriction enzyme cleavage maps of their DNAs differ.

The relatedness and also important differentiations between variola, monkeypox and vaccinia have been analyzed at DNA level (Esposito et al., 1978). The virus-induced polypeptides can also be analyzed in order to identify poxviruses (Harper et al., 1979). The laboratory differentiation between variola, monkeypox, vaccinia and cowpox is especially important since they can cause similar symptoms.

15.2. Replication and molecular biology

The poxviruses generally have a narrow host range. After penetration into a cell the virus particles are degraded in several steps and the viral DNA is released in the cytoplasm. This process requires the synthesis of viral proteins. The poxvirions contain a DNA dependent RNA polymerase and this enzyme transcribes about 14% of the genome, resulting in the synthesis of viral proteins necessary for the uncoating of the viral DNA. The viral DNA replicates and is transcribed in the cytoplasm. The two DNA strands are linked covalently at both ends.

Many enzymes participate in the poxvirus replication and several are probably virus coded, such as a DNA dependent RNA polymerase (transcriptase), a DNA polymerase, a poly A polymerase, a thymidine kinase, a DNase, a DNA ligase and enzymes required for the capping of viral mRNA. Enzymes induced by vaccinia virus are listed in Table 15.2. The number of viral proteins made is not known, but more than 100 have been found in virions (Essani and Dales, 1979). Extensive modification of virus proteins occurs such as glycosylation, phosphorylation and proteolytic processing. Mature virus particles are released from microvilli or by cellular disruption. Genetic recombination can occur within genera.

15.3. Clinical aspects

Smallpox (Variola)

This has probably been the most devastating disease in history and can be traced back as long as 5000 years in Asia. It is now, fortunately, only of historic interest. The virus is spread by airborne droplets from an infected person to the upper airways of another person. The incubation time is 12–13 days and the virus spreads

TABLE 15.2.
Vaccinia virus enzymes

Enzyme	Reference
(Guanine-7)-methyltransferase	Boone et al. (1977)
Ribose-2′-O-methyltransferase	Boone et al. (1977)
Protein kinase	Paoletti and Moss (1972)
Thymidine kinase	Kit et al. (1977)
DNA dependent RNA polymerase	Kates and McAuslan (1967)
DNA dependent DNA polymerase	Citarella et al. (1972)
Poly A polymerase	Brakel et al. (1974)
Guanylyltransferase	Boone et al. (1977)
Endoribonuclease	Paoletti and Lipinskas (1978)
DNase	Rosemond-Hornbeak et al. (1974)
Alkaline protease	Arzoglou et al. (1979)
Nucleoside triphosphatase	Paoletti et al. (1974)
Polynucleotide 5′-triphosphatase	Tutas and Paoletti (1977)
Polynucleotide ligase	Sambrook and Shatkin (1969)
DNA nicking-closing enzyme	Bauer et al. (1977)

from the mucosa to lymph nodes, resulting in a viraemia. The acute disease is characterized by fever, headache and fatigue and followed by the formation of vesicles. In contrast to varicella (Chapter 13), the vesicles in a smallpox patient develop all at the same time. In surviving patients the vesicles crust and the crusts are lost after about three weeks of disease. Characteristic scars remain after the disease. The mortality varied from 1 to 40% in different parts of the world depending on different subtypes of smallpox, Variola major giving the more severe disease and Variola minor (alastrim) the less severe. Several epidemics were devastating, such as that in Iceland in 1707 when 36% of the total population died in a single year. The fatality rates for some countries are shown in Table 15.3.

Orf virus infection
This is caused by a virus normally infecting sheep. Persons in contact with sheep can be infected and often the orf infection appears as a single lesion on the hands or on the face. The lesion heals in about one month. No virus specific prophylaxis or treatment is available, but there is a vaccine for sheep.

Milker's nodes
This bovine poxvirus differs from cowpox but can also be transmitted to persons handling cows. The symptoms are similar to those of an orf virus infection. The lesions heal without scar in about one month. No specific vaccine or antiviral drug has been reported.

TABLE 15.3.
Case-fatality rates for smallpox in different geographic areas

Country	Years	No. of cases	No. of deaths	Fatality rate (%)
India	1974–1975	2826	575	20.3
Vaccinated				6.2
Unvaccinated				26.5
West Africa	1967–1969	5628	540	9.6
Ethiopia	1972–1974	21 250	243	1.1
Botswana	1972	1 059	2	0.2

Data are from the World Health Organization.

Molluscum contagiosum

The infectious agent for this disease is an unclassified poxvirus with a world-wide distribution. It is spread by contact and can infect skin and mucous membranes. The transmission is often venereal in adults. After an incubation period of a few weeks, small papules form and grow to about 10 mm in 2–3 months and then regress in about half a year without scar formation. No vaccine or antiviral drug has been reported.

Monkeypox

This monkey virus can infect humans and occasional cases are reported in Africa. The symptoms resemble smallpox but the secondary spread among humans is fortunately less efficient, with attack rates of about 4% in contrast to smallpox with attack rates of about 40%. Immunization with vaccinia gives good protection.

Cowpox

This virus can spread from cattle to the hands or arms of persons handling cattle. The symptoms resemble a primary vaccinia vaccination. The virus differs both from vaccinia virus and milkers nodes virus, but it is antigenically related to vaccinia.

15.4. Global eradication of smallpox

Several biological features of the smallpox virus itself favoured the final successful attempt to eradicate smallpox, including the absence of an animal reservoir, and absence of any recurrent infections in man, together with an effective and stable vaccine. The WHO intensified the smallpox eradication programme following a suggestion in 1958 by the USSR at the Eleventh World Health Assembly. WHO co-ordinated world-wide efforts from 1966 onwards and provided the expertise, finance (5% of WHO's budget in the early years) and continuing stimulus which were

all required to bring about the final demise of this notorious infection. In that year, for example, 46 countries recorded 131 697 smallpox cases although this probably represented as little as 1% of the real number of cases. Four endemic areas were present namely, Africa, Asia, Indonesia and Brazil. Large financial contributions were made initially by the USSR and USA but eventually 26 countries became contributors. By 1973, 80% of the smallpox vaccine was being produced in the endemic countries and another advance came in 1968 when the bifurcated needle was introduced, soon becoming the standard method for vaccination. Fenner (1982) has recently summarized the problems of vaccination in two of the most difficult countries, namely Ethiopia and India and he has emphasized how the lessons learnt could be applied when other diseases such as measles or polio become, in their turn, the next in line for eradication. These lessons encompass knowledge of the biology of the virus, foibles of human nature, technology and mass communication and exertion. An interesting observation made is that links between field workers and researchers were, contrary to general opinion, essential for the final eradication campaign. Thus, the laboratories were able to develop more rapid and accurate diagnostic methods, and continuing research also solved some essential unsolved problems such as whether monkey pox could mutate to variola. Restriction endonuclease analysis of genomes of strains of orthopoxvirus, monkey poxvirus and whitepox mutants showed no evidence that these viruses could mutate or revert to variola.

Biologically, eight features of smallpox favoured its eradication (Table 15.4). Importantly, the general severity of the disease justified a major effort to eradicate the virus. Throughout the world and over the centuries only one serotype of smallpox has ever been identified and, moreover, orthopoxviruses all cross react serologically. A heat stable freeze dried vaccine was developed in the early 1950s by L. Collier at the Lister Institute, thus obviating the need for refrigeration and 'cold chains'. Potency was retained for periods of greater than 1–2 months at 37°C and the vaccine was very cheap. All vaccine was donated or produced locally. The smallpox virus was furthermore restricted to humans, with no animal reservoir (unlike influenza or arboviruses, for example, Chapters 7 and 5). It was also an important

TABLE 15.4.
Biological features of smallpox that favoured its eradication

1. Severe disease
2. No subclinical cases
3. Infectivity accompanies rash
4. Recurrent infectivity unknown
5. Only one serotype
6. Availability of an effective stable vaccine
7. Seasonality
8. No animal reservoir

factor that subclinical smallpox did not occur in unvaccinated individuals and that patients did not spread the disease during the incubation period (unlike measles, polio and influenza). Smallpox is not as contagious as measles, chickenpox or influenza and transmission required a closer contact with patients. The average smallpox case did not infect more than 2 or 3 other people. If patients who had smallpox were surrounded by vaccinees or immune persons the chain of transmission was broken. Finally, unlike herpes virus or hepatitis B virus for example, carrier states or latency were unknown with smallpox.

Many invaluable lessons can be learnt from the smallpox eradication campaign and Fenner (1982) highlights the most important of these. Success of a vaccination programme should not be estimated solely in terms of *number* of vaccinees because many vital groups may be left out e.g. newborn babies. Also it was not considered possible to vaccinate all 650 million people in India. Mass vaccination had to be combined with surveillance and containment. Therefore available resources were concentrated on areas with smallpox cases. Moreover, the reporting system had to be accurate. Identification of smallpox and containment of foci rather than 100% vaccination was the key strategy. To give an idea of the scope of the operation in India, in 1974, 100 epidemiologists were in the field at any one time, and at this period there were 8403 individual outbreaks with 11 000 cases of smallpox. 28 mobile teams were established in addition to 2800 field workers. A searcher enquiring about smallpox became a familiar figure in every village and rewards were offered for notification of cases. Smallpox patients were removed by flying squad to infectious diseases hospitals and neighbours vaccinated. Fortunately, identifying the disease needed no great diagnostic skill. 'Search weeks' were initiated and teams visited every household to break the chain of transmission. By May 1975 the disease had been conquered in India. A major task had been overcoming ignorance and prejudice. For example, so rooted was smallpox in the everyday life of India that the disease had its own Goddess, Shitala Mata (Fig. 15.2). Temples dedicated to the smallpox goddess are dotted around the country and it was believed that she spilled grain from a basket on her head every time she shook it and each grain turned into a smallpox pustule. Victims survived if she used water from the pitcher in one hand to clean the spilt grain, but did not survive if she used the broom which was in the other. Some people worshipped smallpox cases as being blessed by the goddess and so spread the infection.

Ethiopia illustrated other problems. Most of the 28 million inhabitants lived in rural areas and communication was poor, with most trained health people in the urban areas. Half the population lived more than a day's walk from any accessible road. Civil war and famine compounded the problems. Moreover, variola minor, the endemic type of smallpox, was not considered to be a major national public health problem in Ethiopia itself. Initially emphasis was placed in surveillance, notification and containment and so it is not surprising that reported cases *rose* from 722 in 1970 to 26 329 in 1971. Introduction of more vehicle transport (Fig. 15.3)

Fig. 15.2. The Indian Goddess of smallpox, Shitala Mata. (from World Health, 1980.)

and helicopters meant that by 1975 containment was feasible throughout the whole country and by 1976 the disease had been conquered. For two further years teams worked to ensure no cases had been missed. In many countries a reward was offered and tens of thousand of cases of chickenpox, measles and other rash diseases were

Fig. 15.3. Eradication of smallpox, the practical side. (from World Health, 1980.)

reported by villagers in hopes of collecting a reward. Thousands of specimens were sent to the WHO diagnostic centres is Moscow (USSR) and Atlanta (USA) but none proved to be smallpox.

The eradication programme also illustrates dramatically how important were political and social conditions and D.A. Henderson, director of the campaign between 1967 and 1976, has emphasized that the success of many national programmes often 'hung by a thread'. During the first 4 years of the West African programme there were 23 changes of government in the 18 countries. "Smallpox eradication was achieved but was just barely achieved." The final success can be illustrated by Fig. 15.4 which shows the cover of the World Health of May 1980 announcing that smallpox is dead and by Fig. 15.5 one of the pages from that issue summarizing the achievement, and emphasizing how little this campaign cost.

More recently a new direction has been taken by WHO and other International Agencies since the International Conference on Primary Health Care in Alma Ata, USSR in 1978 (see also Chapter 1). The Alma Ata conference in 1978 introduced

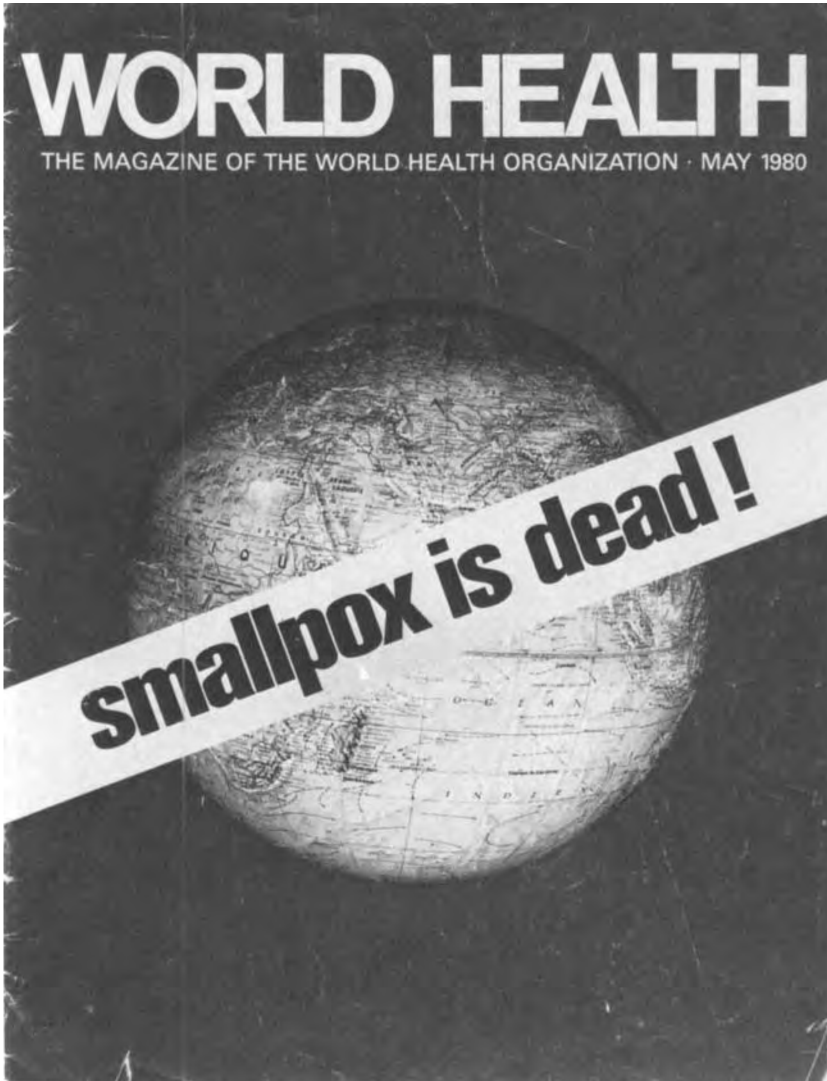


Fig. 15.4. The cover page of World Health announcing that smallpox had been eradicated.

the slogan aim of 'Health for all in the year 2000' (see Chapter 1) and outlined seven basic components of primary health care, including immunization, control of diarrhoeal and respiratory disease, maternal and child health with family planning, nutrition and potable water. Infectious viral disease plays a major and central role in this grand strategy and although immunization is highlighted as a major prophylactic approach nevertheless the use of antiviral drugs could, in the future, play a major role with certain viral diseases, particularly respiratory viruses. However, it is possible that a programme to eliminate a particular disease such as measles or

polio would not enjoy the international support required. Additionally, people in remote or poorly developed areas co-operated willingly in the smallpox programme but they may not be so enthusiastic about eliminating diseases such as polio or measles. It may well be the case that no other candidate viral disease for global eradication exists. We may have to be content with 'national' or 'area' eradication. Commenting on the 'Health for all by the year 2000', Dr. H. Mahler, Director General of WHO said "The present realities of the Third World are simply unacceptable. There is little joy in life nor any kind of justice for a child condemned to disease or early death because of the accident of birth in a developing country. Nor is there any rationale that can defend a system that continues to withhold the gift of health and care from 9/10ths of a nation's population. Smallpox eradication is a sign, a token of what can be achieved in breaking out of the cycle of ill health, disease and poverty. It comes as a glimpse into the future, an intimation of a viable new order of things, in which world health, meaning health for the *world*, will have central significance in an upward spiral of economic and social progress." (World Health, 1980).

15.5. Production of smallpox vaccine

Although smallpox has been eradicated from the world, batches of vaccinia vaccine (for prevention of smallpox) are still prepared and stored in some countries. Details of production, for example, are worth reiterating as an example of a highly successful virus vaccine, yet which, because of the relatively uncontrolled production methods and standardization would probably not be licensed today if proposed for a new vaccine. Currently used vaccine strains of vaccinia virus are neither cowpox nor mutants of variola virus, although they may be hybrids of these. The strains induce only mild dermal and systemic reactions as estimated by clinical observation in man. Jenner's original virus was maintained by arm to arm passage but this technique was discontinued after 1881, when large scale preparation of vaccine in calves was instituted. Up to the 1970s calves or sheep were used for vaccine production, the latter often preferred because of freedom from tuberculosis, small size, and docility (Turner, 1970). Under anaesthesia one flank is clipped and shaved and, after washing, lightly scarified with 8–10 ml of seed virus applied with a sterile scapula. Four days later the animals are exsanguinated, the vaccinated area is washed and dried with sterile towels. 50–100 g of pulp is removed by scraping with a large spoon and stored in liquid nitrogen. Each g of tissue contains up to 10^{10} virions and would thus represent approximately 2000 doses of vaccine. Treatment of the pulp with phenol at room temperature reduces bacterial counts without greatly diminishing virus titre. Virus may be easily extracted by fluorocarbon. Following examination for bacterial contamination and potency the material is used for glycerolated liquid vaccine (40% glycerol) and freeze dried. Virus is quantitated by titration on the

HOW MUCH DID IT COST

to send a man to the Moon?

Between 1961, when President John Kennedy gave a directive to set a man on the Moon and bring him back "before this decade is over", and the successful landing in August 1969 of two men in the Sea of Tranquillity and their safe return to Earth, the US space agency NASA is estimated to have spent

US $24,000 million

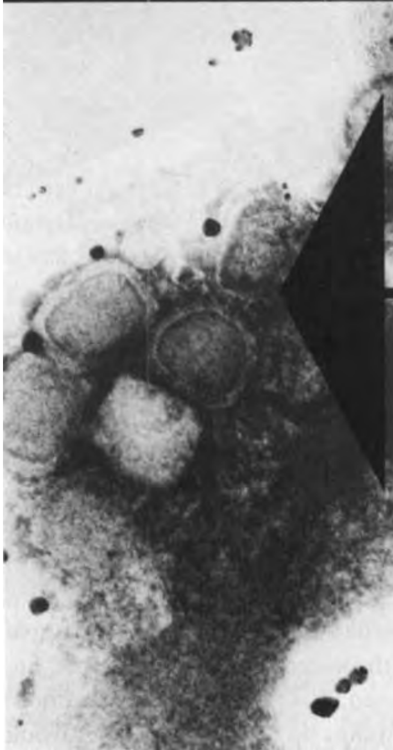
to wipe out smallpox from the Earth?

Between 1967, when WHO ordered its intensified Smallpox Eradication Programme into action, and 1980, when the Thirty-third World Health Assembly endorsed the final disappearance of this disease from the Earth, the total cost of eradication was

US $300 million

HOW MUCH WILL IT SAVE?

The estimated saving *every year* to all countries when smallpox vaccination is abolished worldwide is *US $1,000 million*



HOW MANY VICTIMS WERE THERE?

In the one year 1967, official health statistic returns showed there were 131,697 cases of smallpox. But the figures showed only a tiny fraction of the real suffering. It is estimated that in that year there were

over 10 million cases of smallpox in the world

HOW MANY PEOPLE DIED?

It is estimated that, in 1967, the death toll was

about two million people

HOW MANY PEOPLE WORKED TO SAVE THE WORLD FROM SMALLPOX?

The total number of national staff, in over 40 countries, who worked in the Smallpox Eradication Programme was *200,000 men and women*

The total number of international staff, from more than 70 countries, was *about 700 men and women*

HOW MANY SHOTS OF VACCINE?

Estimated total number of doses of smallpox vaccine used in the global programme: *2,400 million*

Total produced by endemic countries: *2,000 million*

Total distributed by WHO: *400 million*

HOW MANY BIFURCATED NEEDLES?

Between 1967 and 1976 WHO supplied over 40 million needles to the programme. The needle was inspired by the basic sewing machine needle, the loop being ground down to produce the pronged fork. The advent of the bifurcated needle brought major savings in the quantity of vaccine required.

Fig. 15.5. A summary of financial and practical aspects of the smallpox eradication campaign. (from World Health, 1980.)

chick chorio allantoic membrane and 1 ml of vaccine should contain more than 10^8 ID_{50} of virus and less than 10^3 microbial contaminants (none of them pathogens). It is unlikely, therefore, that this traditional smallpox vaccine would pass licensing requirements which could be required today! A new interesting proposal for the use of vaccinia as a vector for other virus antigens such as hepatitis B, polio or influenza is discussed in Chapter 2.

15.6. Chemotherapy

The need for chemotherapy against poxvirus infections in man has almost disappeared since the eradication of smallpox and the decreased use of vaccinia virus for vaccination. The rare orf and milkers nodes infections are not primary targets for antiviral chemotherapy. However, molluscum contagiosum is frequent enough to call for therapy but since it cannot be grown in cell culture or in animals the possibilities of developing drugs against this disease are very distant, and no work has been reported in this direction. Poxviruses induce a large number of enzymes (Table 15.2) including DNA polymerase and thymidine kinase of potential interest as targets for antiviral drugs. These enzymes have not been used in any published systematic search for inhibitors.

Possibly the first antiviral compound described in the literature was p-aminobenzaldehyde thiosemicarbazone which Brownlee and Hamre (1951) found to inhibit the multiplication of vaccinia virus. Among similar structures investigated, Bauer and Sadler (1960) found 1-methyl-1H-indole-2,3-dione-3-thiosemicarbazone (methisazone, Marboran®, Fig. 15.6) which is active against several different viruses in cell culture (Bauer et al., 1970). The mechanism of action of methisazone against poxvirus is not known in detail. It is not virucidal and does not affect virus adsorption. It is possible that the metal chelating properties of methisazone are of importance. The structure-activity relations for this type of compound are illustrated in Table 15.5 where the N-substituents are varied.

Vaccinia infections

The complications resulting from vaccination against smallpox in persons with eczema or immunological deficiencies have been treated with methisazone and gammaglobulin. Both these modalities seem to have therapeutic effects (see Bauer,

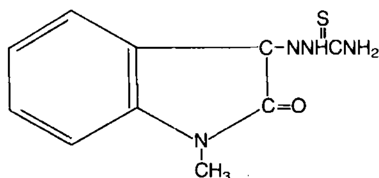


Fig. 15.6. Structural formula of methisazone.

TABLE 15.5.
Structure-activity relations for the anti-vaccinia activity of N-substituted isatin β-semithiocarbazones in cell culture (after Bauer and Sadler, 1960)

N-substituent	Relative antiviral activity
None	100
Methyl	202
Ethyl	286
Isopropyl	44
Propyl	28.5
Pentyl	3.4
Hydroxymethyl	42
1-Methyl-4-trifluoromethyl	48.4
2-Hydroxyethyl	204
Acetyl	87
Ethoxycarbonylmethyl	0

1977). The availability of vaccinia immune globulin (VIG), prepared from revaccinated military personnel, will decrease as vaccination decreases. No controlled study on the efficacy of methisazone on vaccinia infections has been reported.

Smallpox

Methisazone has been used as a prophylactic agent in contacts exposed to patients suffering from smallpox. Bauer et al. (1969) conducted a prophylactic trial in Madras where contacts of variola major cases were given methisazone according to three different schedules. The results from this trial is summarized in Table 15.6 and clearly show that methisazone gave a significant ($P<0.001$) protection. However, in prophylactic trials carried out subsequently by Heiner et al., (1971) no significant effects were observed although there was a trend in favour of methisazone (Table 15.7). The difference in outcome could possibly be explained by different dosing of the drug. The last study was carried out using 2×3 mg methisazone with a short interval which might have induced vomiting and concomitant loss of drug. Two

TABLE 15.6.
Effect of methisazone treatment on the incidence of contact cases of smallpox. Methisazone was given prophylactically as 2×3 g/day, 2×1.5 g/day or 3 g/day for 4 days (placebo was not generally given) (after Bauer et al., 1969)

Group	Treatment	Contacts	Cases	Deaths	Case incidence (%)
1	Treated, all dose levels	2292	6	2	0.26
2	Not completed	318	12	2	3.77
1+2	Total treated	2610	18	4	0.69
3	Not taken	150	11	3	7.33
4	Not offered	2560	105	18	3.99
3+4	Total untreated	2710	116	21	4.17

TABLE 15.7.
Effect of methisazone on attack rates in contact cases of smallpox. Adults were given two daily prophylactic doses of 3 g at intervals of four to six hours (doses for children were reduced) (after Heiner et al., 1971)

Treatment	Contacts	Cases	Deaths	Attack rate (%)
Methisazone				
Full dose	229	6	1	2.6
Incomplete dose	13	0	0	0.0
Vomited	20	1	0	5.0
Total methisazone	262	7	1	2.7
Placebo	260	13	2	5.0
Other untreated				
Refused	26	1	0	3.8
Absent	22	0	0	0.0
Total untreated	308	14	2	4.5

daily, well separated, doses of 3 g methisazone for 4 days might be expected to be optimal.

No therapeutic effect of methisazone or any other compound against smallpox has been reported. A double-blind study by Rao et al. (1969) showed no therapeutic effect of methisazone. It is possible that treatment institution in many patients may have been too late to be expected to change the course of the infection.

15.7. Summary

The global eradication of smallpox by vaccination is a milestone in preventative medicine. The smallpox vaccine also protects against monkeypox. Passive immunization can prevent complications from vaccination against smallpox. The antiviral compound in methisazone has a prophylactic, but not therapeutic, effect against smallpox.

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World Health, May 1980.

CHAPTER 16

Hepatitis virus infections

Jaundice has been known as a symptom of serious disease since ancient times. Several virus infections can result in jaundice or hepatitis but only a few viruses are characterized as hepatitis viruses. At least three distinct hepatitis viruses have been found and some of their properties are listed in Table 16.1. Although hepatitis A virus is a picornavirus (Chapter 4) it is discussed in this chapter together with hepatitis B which is a DNA virus, the δ-agent and the non A-, non B hepatitis viruses. This should not be taken as an indication of similarities in the molecular biology of these viruses but is rather an adherence to the normal clinical grouping of these viruses. The differential clinical diagnosis of the hepatitis viruses has presented considerable problems. The hepatitis viruses have been the subject of several excellent reviews and these should be consulted by readers requiring more detailed information (Zuckerman, 1975, Cossart, 1977, Vyas et al., 1978, Zuckerman and Howard,

TABLE 16.1.
Hepatitis viruses

Properties	Virus				
	Hepatitis A	Hepatitis B	δ-Agent	Non-A, non-B hepatitis	
				Transfusion	Epidemic
Viral nucleic acid	RNA	DNA	RNA	?	RNA?
Chronic infections	–	+	+	+	–
Helper virus	–	–	Hepatitis B	–	–

1979, Gerety, 1981, Bianchi et al., 1980, Sherlock, 1980, Szmuness et al., 1982, Deinhardt and Gust, 1982).

16.1. Hepatitis A virus infections

16.1.1. The virus

Hepatitis A virus is a small RNA virus classified as human enterovirus 72 (Gust et al., 1983). It is 27 nm in diameter (Fig. 16.1), has no envelope or lipids and contains a single stranded infectious RNA with a molecular weight of 2.5×10^6 as indicated in Table 16.2. The virus is fairly stable to heat, ether and acids. One serotype has been described and the nomenclature of viral antigens and antibodies is listed in Table 16.3. The virus can be grown, although with some difficulty, in cell cultures, and can, apart from man, infect chimpanzees and some monkeys. The molecular biology is not well investigated at present but the virus replication is resistant to actinomycin D indicating the presence of a virus specified RNA dependent RNA polymerase. The replication is expected to be similar to that of other picorna viruses. (Chapter 4 summarizes replication strategy.)

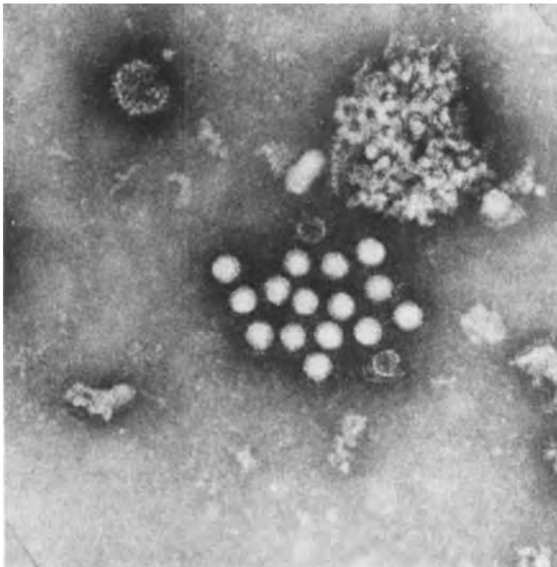

Fig. 16.1. Hepatitis A virus. EM picture. Diameter of virus particles, 27–29 nm. (courtesy of Dr. B. Tufvesson.)

TABLE 16.2.
Hepatitis A virus

Component	Molecular weight	Other properties
RNA	$2.25–2.8 \times 10^6$	Infectious, single stranded Contains poly A
Polypeptides VP1	$30–33 \times 10^3$	
VP2	$24–26 \times 10^3$	
VP3	$21–23 \times 10^3$	
VP4	$7–14 \times 10^3$	

TABLE 16.3.
Nomenclature of Hepatitis A virus antigens and antibodies

Hepatitis A virus	HAV
Hepatitis A virus antigen	HAAg
Antibody to HAV	Anti-HAV
IgG antibody to HAV	Anti-HAVIgG

16.1.2. Clinical aspects

The clinical onset of a HAV infection is more abrupt than an HBV infection but the symptoms are similar. The incubation period (average 4 weeks) is shorter and the fever is higher in the case of HAV infections and the duration of elevated transaminase is shorter, about 1–3 weeks. The HAV induced acute inflammation of the liver, manifest as jaundice, nausea and vomiting, is similar to the symptoms caused by HBV infection. However, HAV infections are often mild and do not lead to chronic infections and only very rarely to the hepatocellular necrosis sometimes seen in HBV infections. The presence of virus and appearance of antibodies during a HAV infection are shown in Fig. 16.2.

16.1.3. Epidemiology

Hepatitis A virus is mainly spread by faecal contamination of drinking water or food and the infections are often epidemic. The virus can be accumulated from contaminated water by oysters and clams and its spread is facilitated by a good biological stability of the virus in water. Infections caused by HAV are common and it has been estimated that about 50% of the adult population in industrialized countries have antibodies to HAV. In epidemics, attack rates of up to 50% have been observed among adults eating infected oysters (Gard and Alin, 1957). In contrast to HBV, transmission of HAV by contaminated needles or syringes or by blood is rare. There seems to be only one serologic type of HAV and it causes an immunity

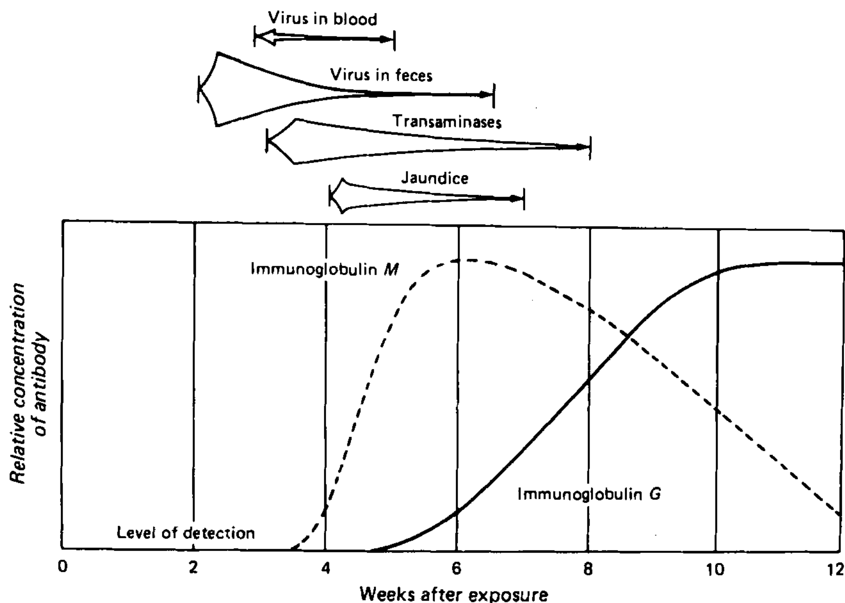

Fig. 16.2. Presence of virus, antibodies and symptoms during hepatitis A virus infection (after Hollinger and Dienstag, 1980.)

lasting for decades. A drop in prevalence rate has been noted in industrialized countries and HAV is on the point of disappearing in Scandinavia and Switzerland for example, with 1–5 infections/1000 persons per year. In the Mediterranean region, 75 infections/1000 per year for example are reported from Greece.

16.1.4. Vaccination and immunoglobulin

An inactivated HAV vaccine has been prepared from infected marmoset liver and this vaccine had a prophylactic activity in marmosets (Provost and Hilleman, 1978). Since HAV can now be grown in human diploid cells (see Szmuness et al., 1982) and has also been cloned in and found to express viral antigens in *E. coli* (von der Helm et al., 1981) there is a definite possibility of preparing a useful vaccine in the near future. The presence of, so far, only one serological strain and a long lasting immunity makes the prospects of development of a vaccine very promising. The short immunogenic peptide approach (Chapter 2) may be fruitful.

Passive immunization by use of immune serum globulin (ISG) from pooled human plasma gives protection in 80–90% of cases when given within 1–2 weeks of exposure to HAV. Prophylactic use does not prevent the infection, but reduces it to a mild or subclinical course (Weiland et al., 1979). Doses of 0.02 – 0.1 ml/kg have been given every 4–6 months as a prophylaxis, but the immune status of the person

should be checked to avoid unnecessary and prolonged administration of immunoglobulins.

16.1.5. Chemotherapy

The likely presence of a viral RNA polymerase should make it possible to develop antiviral agents against HAV, but no selective drug has been described to date. Picornavirus inhibitors such as guanidine and HBB fail to inhibit HAV (Siegl and Eggers, 1982). Ribavirin has been used clinically against acute viral hepatitis (Ayrosa-Galvao and Castro, 1977) but the possible efficacy remains to be established and in cell culture ribavirin is not effective against HAV (Widell et al., unpublished data). A double-blind placebo-controlled study evaluating isoprinosine in children with HAV hepatitis revealed no therapeutic effects (Welch et al., 1982).

16.2. Hepatitis B virus infections

The spread of hepatitis by contamination of infected blood was noted as early as 1885 during vaccination against smallpox, and during the second world war it became a problem when batches of yellow fever vaccine containing icterogenic serum produced many infections (Chapter 5). It was not until 1965 that Blumberg discovered the Australia antigen (hepatitis B virus surface antigen) in the serum of an aborigine and this led to the identification of hepatitis B (Blumberg, 1977).

16.2.1. The virus

Plasma of patients with hepatitis B virus (HBV) infection contains spherical and filamentous particles of different sizes. These are constituted of HBV antigens. The infectious human HBV particles have a diameter of 42 nm (Dane particles) and a structure schematically shown in Fig. 16.3. The nomenclature of the antigens of HBV and their corresponding antibodies are listed in Table 16.4. The surface antigen, HBsAg, formerly the Australia antigen, has several antigenic determinants, resulting in several subtypes of HBV of which the four main ones are adw, adr, ayw and ayr. The core structure contains two antigens, c and e, and a partly single stranded circular DNA with a length of 3250 bases. The core also contains a DNA polymerase activity. The physical structure and the genome organization of HBV DNA is shown in Fig. 16.4. It has been suggested that the particles with partially double-stranded DNA are interfering noninfectious virus and that only particles containing supercoiled DNA are infectious (Ruiz-Opazo et al., 1982).

Recently hepatitis viruses have been reported in the woodchuck (Summers et al., 1978) the Chinese duck (Mason et al., 1980) and the ground squirrel (Marion et al., 1980). These hepatitis viruses, as indicated in Table 16.5, are similar to human

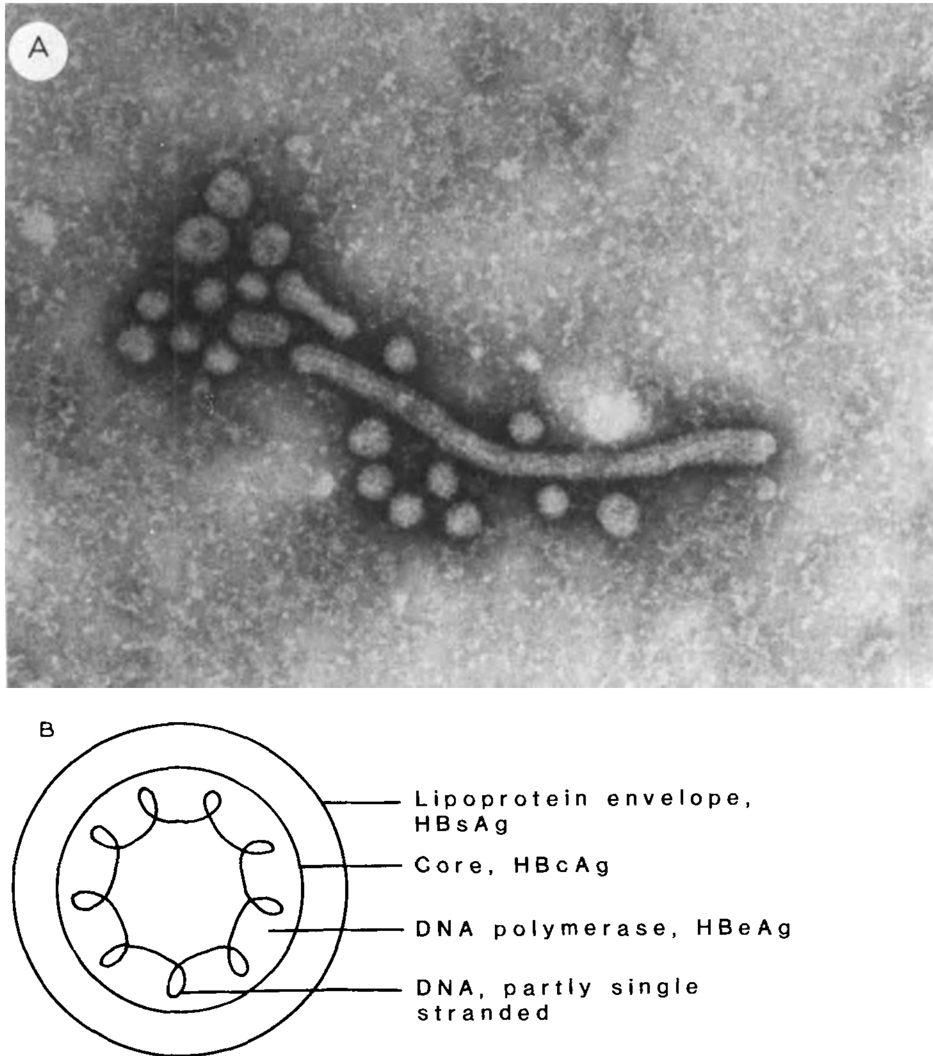

Fig. 16.3. Hepatitis B virus particles. A, EM picture, magnification 195000×. Both spherical 42 nm virus particles (Dane particles) and structures (22 nm spherical and elongated) consisting of HBsAg are shown. (courtesy of Dr. D. Hockley.) B, Schematic structure of hepatitis B virus.

hepatitis B virus and probably constitute a new group of viruses, the hepadenaviruses.

16.2.2. Replication and molecular biology

It has not been possible to cultivate hepatitis B virus in cell cultures, and this has

TABLE 16.4.
Nomenclature of hepatitis B virus antigens and antibodies

Hepatitis B virus	HBV
Hepatitis B virus surface antigen	HBsAg
Hepatitis B virus core antigen	HBcAg
Hepatitis B e antigen	HBeAg
Antibody to HBsAg	Anti-HBs
Antibody to HBcAg	Anti-HBc
Antibody to HBeAg	Anti-HBe
Hepatitis B immunoglobulin	HBIG

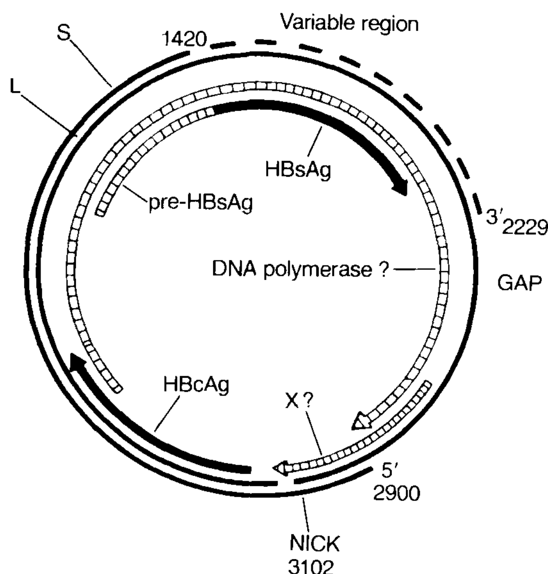

Fig. 16.4. Genetic and physical map of the HBV genome. The two DNA strands (L and S), the variable region and the gap in the double-stranded structure are indicated as well as the coding regions for some viral proteins. (after Delius et al., 1983.)

made it difficult to study its replication (for a review see Tiollais et al., 1981). However, recent results indicate that cultured human lymphoblastoid cells from a case of acute hepatitis produce a low number of virus particles with properties of HBV (Romet-Lemonne et al., 1983). As shown in Fig. 16.4 the genome is composed of a long strand of 3182 nucleotides and a shorter strand of variable length and with a covalently linked protein to its 5′ end. The 5′ position of the S-strand seems to be fixed and the 3′ end variable. An analysis of stop codons and reading frames suggests that the four open regions of the L-strand code for proteins and that the S-strand does not. The reading frame for P would then overlap other regions. The

TABLE 16.5.
Hepadenaviruses (after Deinhardt and Gust, 1982)

Properties	Virus HBV	WHV	GSHV	DHV
Present in blood	+	+	+	+
Double-shelled virion	+	+	+	+
Virion diameter (nm)	40–45	40–45	47	40
Core diameter (nm)	27	27	30	27
Circular DNA, partly single stranded	+	+	+	+
Nucleotides in DNA (number)	3182	3330	3200	3000
DNA homology with HBV (%)	100	3–5		
DNA polymerase in virion	+	+	+	+
Cross reacting core antigen	+	+		+

HBV, human hepatitis B virus; WHV, woodchuck hepatitis virus; GSHV, ground squirrel hepatitis virus; DHV, duck hepatitis virus.

positions of the genes for the surface protein, the core protein and the polymerase are indicated in Fig. 16.4. The DNA polymerase in the capsid can elongate the short strand. It is possible that the DNA polymerase is coded by the P region of the genome. A protein kinase activity has also been found in the core structure (Albin and Robinson, 1980) and it can be speculated that this kinase could affect the polymerase activity. A reverse transcriptase activity has been detected in duck hepatitis virus (Summers and Mason, 1982), possibly implying an RNA intermediate during replication.

The HBV genome seems to be integrated in hepatoma cells and this viral DNA is methylated in contrast to HBV DNA from virions or infected tissue (Miller and Robinson, 1983). It is less clear if it is also integrated in cases of acute hepatitis and chronic active hepatitis with the implication that the viral DNA polymerase activity might not be required for virus production. This would then prevent the successful use of antiviral agents directed against the HBV DNA polymerase activity. However, in chronically HBV-infected chimpanzees, viral DNA seems to be nonintegrated (Monjardino et al., 1982) and in asymptomatic patients hepatitis B, nonintegrated viral DNA is frequently found (Kam et al., 1982). The mode of the replication of HBV will require further studies to delineate the possible ways of interfering with viral replication.

16.2.3. Clinical aspects

A hypothetical scheme of the pathogenisis of HBV infection, and the clinical and serological reactions to an HBV infection are shown in Fig. 16.5. The liver is the

target organ for the HBV infection and the clinical syndromes are similar to those caused by hepatitis A virus. It is possible that the damage to the liver cells is not caused primarily by direct cytopathic effects but by the formation of immunocomplexes and indeed the appearance of symptoms coincides with the rise in antibody formation (Fig. 16.6).

Prodromal symptoms are common during the 1–2 weeks before the icteric (jaundice) phase. These can consist of malaise, nausea, vomiting and a distaste for smoking, and the symptoms are often flu-like. The icteric phase is characterized by dark urine and jaundice. This reaches a peak in 1–2 weeks and is followed by a recovery phase of 2–4 weeks. The liver is usually enlarged. A fulminant and often lethal hep-

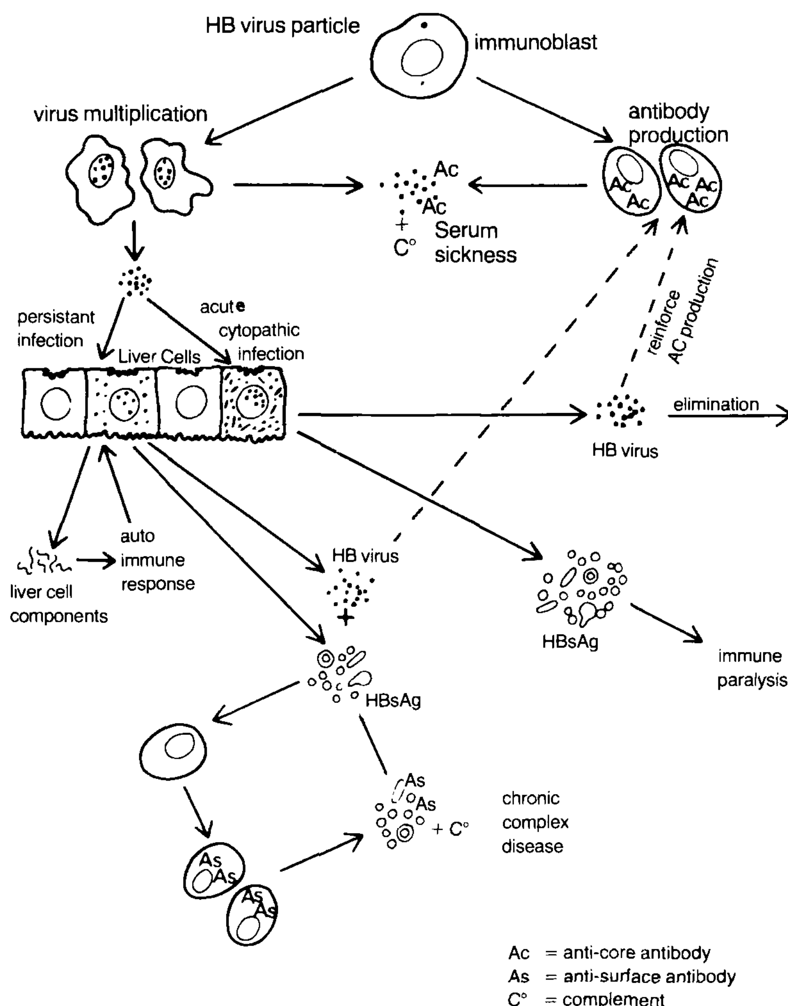

Fig. 16.5. A hypothetical scheme to explain the pathogenesis of hepatitis B. (after Cossart, 1977.)

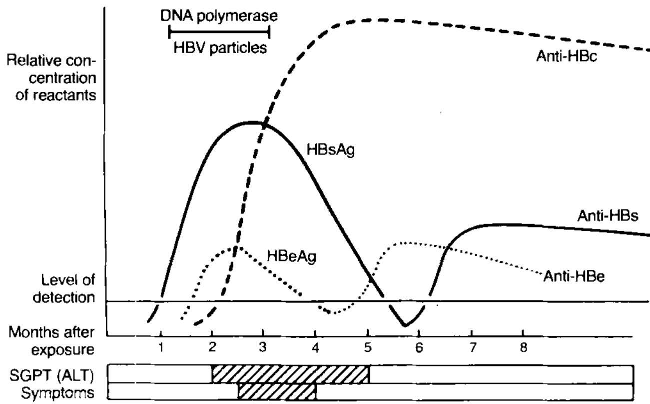

Fig. 16.6. Clinical and serological events occurring in a patient with acute hepatitis B. (after Hollinger and Dienstag, 1980.)

atitis with a rapid necrosis and decrease in the size of the liver can occur, but is rare (1–2% of the cases). The infection is often subclinical and unrecognized in childhood.

In 5–10% of the cases a chronic disease develops. When this is persistent the prognosis is good and complete recovery normally occurs in a few years. In the case of chronic active hepatitis the disease is more severe and prolonged and can develop into cirrhosis. A geographic correlation between a high prevalence of chronic HBV and a high prevalence of liver carcinoma is a further important aspect of HBV infections.

An interpretation of the presence of serological markers of HBV is presented in Table 16.6.

16.2.4. Epidemiology

The main route of transmission of HBV is by parenteral spread, usually by transfusion of contaminated blood, contact with infected blood in hospitals or the use of shared needles by drug abusers. Peroral and sexual transmission as well as congenital and neonatal infections are also ways of infection. In the countries with the highest prevalence of hepatitis, transmission from mother to babies appears to be the single most important factor for the high prevalence of HBV infections. The incubation period is 6–25 weeks.

The frequency of chronic HBV carriers is 0.1–0.5% in Western Europe and USA, 1–2% in South America and Southern Europe, 3–5% in North Africa and the Soviet Union and 6–20% in South Africa and South East Asia. The geographic distribution of carriers is shown in Fig. 16.7. It has been estimated that there are a total of 200 million persons with chronic HBV infection in the world.

TABLE 16.6.
Interpretation of the presence of combinations of serological markers of the hepatitis B virus in infected patients (after Deinhardt and Gust, 1982)

HBsAg[a]	HBeAg	Anti-HBe	Anti-HBc	Anti-HBs[b]	Interpretation	Infectivity of blood
+	+	−	−	−	Incubation period or early acute period during hepatitis B	high
+	+	−	+	−	Acute hepatitis B or *chronic carrier*[c]	high
+	−	+	+	−	Late during hepatitis B or chronic state	low
−	−	+	+	+	Convalescent from acute hepatitis B infection	none
−	−	−	+	+	Recovered from past hepatitis B infection	none
−	−	−	−	+	Immunized without infection, repeatedly exposed to HbsAg without infection, or recovered from past hepatitis B infection	none
−	−	−	+	−	Recovered from past hepatitis B infection with undetectable anti-HBs[a], early convalescent or chronic infection	questionable

[a] All positive for HBsAg are acutely or chronically infected with HBV.
[b] All positive for anti-HBs are immune to hepatitis B.
[c] The titre of anti-HBc and/or the immunological class of the anti-HBc may differentiate between the convalescent phase, persistent carrier, or chronic infection.

16.2.5. Vaccination and immunoglobulin

The discovery of the 'Australia antigen' and its identification as the surface antigen (HBsAg) of HBV (Blumberg, 1964, Blumberg et al., 1967) was the starting point for the development of vaccines against HBV. Since it has not been possible to grow HBV in cell culture or in easily available animals, serum from HBV-patients has to be used as the starting material for viral antigen and vaccine production. An im-

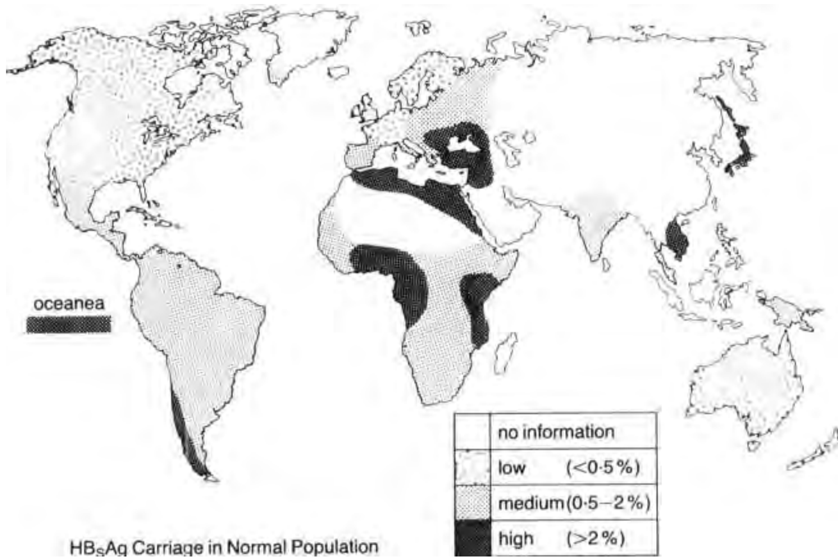

Fig. 16.7. Hepatitis B carrier rates in different countries. (after Cossart, 1977.)

portant step towards an HBV vaccine was taken by Krugman et al. (1971) who used heat inactivated HBsAg positive serum to immunize anti-HBs negative children at high risk of being infected by HBV. Protection to an artificial challenge infection was observed. Subsequently, purified HBsAg vaccines have been developed by several groups (Hilleman et al., 1975; Purcell and Gerin, 1975; Maupas et al., 1976) and the use of chimpanzees was introduced to ascertain the efficacy and safety of these vaccines (Purcell and Gerin, 1975). The vaccine developed by Maupas et al. (1976) has been given to more than 2500 persons in high-risk settings. After three monthly injections 91% of staff and 62% of haemodialysis patients showed anti-HBs responses (Maupas et al., 1978; Maupas et al., 1981) as shown in Table 16.7. The kinetic patterns of this response are shown in Fig. 16.8, the type 2 pattern being the most common. The presence of adjuvant significantly increased the immune response. The frequency of hepatitis B infections was clearly lower in vaccinated subjects than in nonvaccinated subjects as shown in Table 16.8.

In a study on the prevention of an early HBsAg carrier state in children in Senegal, Maupas et al. (1981) found an anti-HBs reponse in 94.5% of the children seronegative before immunization and, importantly, maternal anti-HBs did not interfere with this immunization. The incidence of HBsAg carrier state was reduced by 85% in susceptible children in a twelve month follow-up after vaccination as shown in Table 16.9. In another study in Senegal, Barin et al. (1983) found that vaccination of HBsAg positive children aged between 3 and 24 months was safe but inefficient. Therefore, in a society with a very high prevalence of chronic carriers, the first dose of HBV vaccine might have to be given during the first hours of life.

TABLE 16.7.
Anti-HBs response to HBV vaccine in dialysis staff members and patients (after Maupas et al., 1978)

	Ward staff		Patients	
	previous contact with HBV	free of any contact	previous contact with HBV	free of any contact
Total subjects	21	106	9	37
Primary response	2	97	1	23
Anamnestic response	19	0	8	0
No response	0	9	0	14
Anti-HBs response (% positive)	100	91	100	62

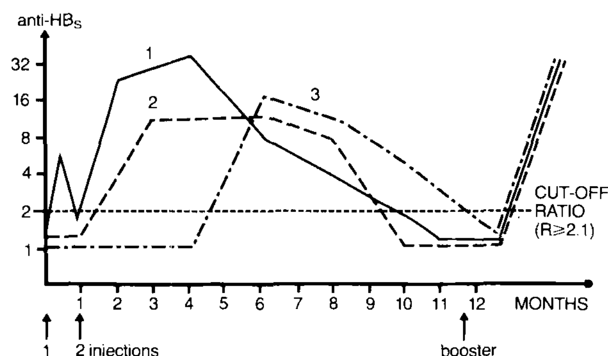

Fig. 16.8. Kinetics of primary humoral anti-HBs response to HBV vaccine. Three different patterns were found. (after Maupas et al., 1978.)

The highly purified formalin-inactivated vaccine developed by Hilleman et al. (1975) at Merck Sharp and Dohme has been used in a placebo-controlled double-blind randomized trial in 1083 homosexual men from New York and has been found to be safe, immunogenic and efficacious (Szmuness et al., 1980; Szmuness et al., 1981a). This vaccine gave a more than 95% antibody response after three doses as shown in Fig. 16.9. This immunological response significantly reduced the hepatitis attack rates when compared to the placebo group, and the protective efficacy increased with increasing number of doses as shown in Tables 16.10 and 16.11. The vaccine dose was 40 μg but it may be possible to reduce this to 20 μg. There was an indication that vaccination was also partly effective when given post-exposure. It seems possible that a combination of vaccine and hepatitis immunoglobulin could be a useful approach in post-exposure situations (Szmuness et al., 1981b).

TABLE 16.8.
Hepatitis B infections in vaccinated and nonvaccinated subjects (after Maupas et al., 1978)

	Ward staff			Patients		
	Vaccinated		Non-vacci-nated	Vaccinated		Non-vacci-nated
	primary anti-HBs response	non-respon-sive		primary anti-HBs response	non-respon-sive	
Total subjects	97	9	24	23	14	31
Transient HBs antigenaemia without hepatitis	5	0	4	1	0	2
Acute hepatitis B						
with jaundice	0	2	8	0	2[a]	2[a]
without jaundice	0	2	0	0	0	0
Chronic hepatitis B	0	0	0	0	5	21

[a] These patients developed icterus and became HBsAg chronic carriers.

TABLE 16.9.
Incidence of HBsAg carrier state in HBV vaccine and control groups 12 months after immunization, in relation to HBV status before immunization (after Maupas et al., 1981)

Serology before immunization	Control group No.		HBV vaccine group No.			Protective efficacy rate[a] (PER, %)
rate[a] (PER)	Tested	HBsAg+	Tested	HBsAg+	P	
Seronegative	195	14	238	4	≤0.005	76
Anti-HBc alone positive	46	9	47	0	≤0.005	100
Total (HBsAg and anti-HBs negative)	241	23	285	4	≤0.0001	85
Anti-HBs positive	12	0	24	0	N.S.	0
HBsAg positive	14	11	26	14	N.S.	32

[a] Percentage reduction of HBsAg carriers in HBV vaccine group compared with control group. N.S., not significant.

Purcell and Gerin (1975) have prepared a vaccine which seems to be similar to the MSD vaccine (Purcell and Gerin, 1978).

A vaccine developed at the Institut Pasteur in Paris has also been used in controlled, but limited, clinical trials where it seems to be effective, both in patients and staff in haemodialysis units. (Crosnier et al., 1981a, 1981b). The results from the studies are shown in Fig. 16.10 as the disease free rates.

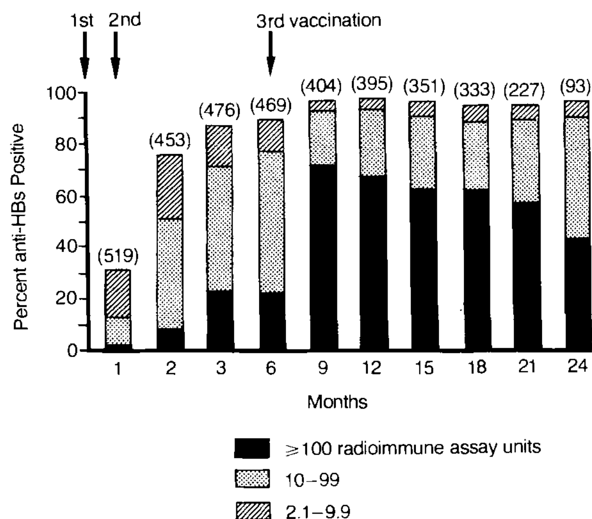

Fig. 16.9. Immunological response (anti-HBs) to hepatitis B vaccine. (No), number tested. (after Szmuness et al., 1981a.)

TABLE 16.10.
Efficacy of hepatitis B vaccine (Heptavax B).
Attack rates at various endpoints (cumulatively for 735 days) and protective efficacy rates (PER) for hepatitis B vaccine or placebo given to 1083 homosexual men from New York (after Szmuness et al., 1981a)

Endpoint[a]	Placebo group		Vaccine group		*P* value	Protective efficacy rate (PER) (%)
	No.	Rate	No.	Rate		
Hepatitis B	63[b]	17.6	7[b]	1.4	≤0.0001	92.1
HBV infection with ALT ≥45 IU/litre	77	21.3	13	2.7	≤0.0001	87.3
HBsAg positive	90	23.5	12	2.6	≤0.0001	88.9
Anti-HBc conversions alone	32	11.9	16	3.4	≤0.01	71.5
All HBV infections excluding anti-HBc	96	25.6	14	3.2	≤0.0001	87.5
All HBV infections, including anti-HBc	127	34.5	31	6.4	≤0.0001	81.4

[a] Not mutually exclusive.
[b] One case which occurred after day 735 not included.

TABLE 16.11.
Protective efficacy rates (PER) according to number of hepatitis B vaccine (Hepatovax B) doses given to homosexual men in New York (after Szmuness et al., 1981a)

Endpoint vaccine dose	Placebo group		Vaccine group		P value	Protective efficacy rate (PER) (%)
	No. of events	LTAR[a]	No. of events	LTAR		
Hepatitis B						
Following 1st dose	57	17.0	6	1.2	⩽0.001	91.8
Following 2nd dose	53	16.3	5	1.0	⩽0.001	93.9
Following 3rd dose	49	15.6	0	0	⩽0.001	100.0

[a] LTAR, life table attack rate.

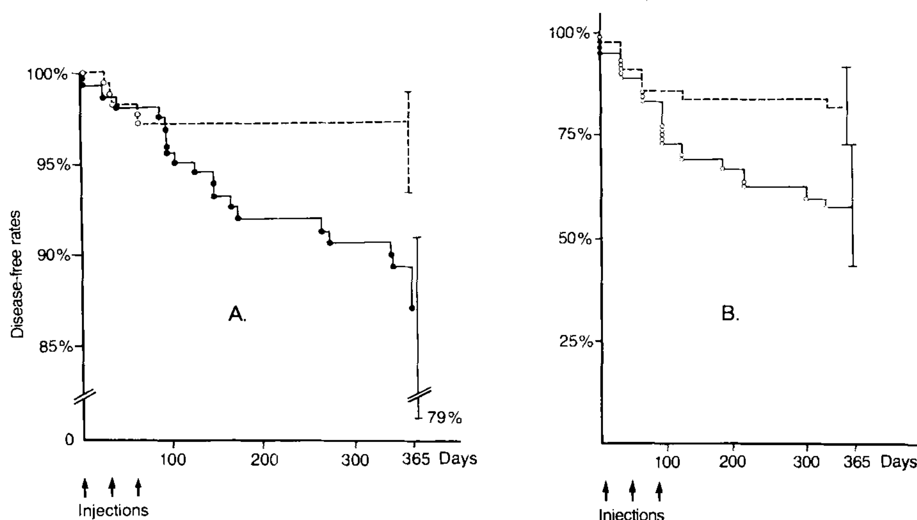

Fig. 16.10. Effect of HBV vaccination in dialysis staff (A) and patients (B). Disease-free curves by the Kaplan-Meier method. ----, Vaccine group; ——, Placebo group. (after Crosnier et al., 1981a and b.)

The possibility of making a hybrid virus of HBV and vaccinia and using this for HBV vaccination is a new and interesting possibility reported by Smith et al. (1983) and discussed further in Chapter 2.

Finally, development of synthetic polypeptide vaccines against hepatitis B is another way to solve the problem of obtaining sufficient amounts of viral antigen (Zuckerman, 1977). The sequence of the 25 000 molecular weight surface protein of HBV has been determined and parts of this sequence have been synthesized in an attempt to obtain immunogenic sequences. Oligopeptides from the first 149 aminoacids in HBsAg have been found to be immunogenic in animals and could,

in the future, be of value for human use (Lerner et al., 1981, Hopp, 1981, Dreesman et al., 1982, Prince et al., 1982).

Preparations of immunoglobulins (IG) with high titres of anti-HBs have not been useful in cases of established chronic hepatitis B, but have prophylactic use in cases of exposure to HBV. Using high-titre hepatitis B immune globulin (HBIG) Prince et al. (1978) found, in a double-blind randomized trial, that HBIG reduced the incidence of infection with HBV, although the course of the disease that did occur in persons receiving HBIG was not changed. A use of HBIG is indicated in the case of exposure to blood which is known or suspected to contain HBsAg and the USPHS recommendations for this use are shown in Table 16.12.

16.2.6. Chemotherapy

The possibility of using hepatitis B virus enzymes as targets for antiviral drugs has been explored to only a limited extent. A few compounds that have been found to inhibit HBV DNA polymerase in cell free assays are listed in Table 16.13. Of these compounds only chloroquine has been used in clinical trials, and as pointed out above, an inhibitor of HBV DNA polymerase is not likely to affect the copying of the viral genome if it has been integrated into the cellular genome. This prediction might have been correct in the case of a chronic wood chuck hepatitis infection which could not be successfully treated with foscarnet despite serum levels of foscarnet sufficiently high to inhibit the wood chuck hepatitis B virus DNA polymerase in vitro (Nordenfelt and Werner, 1980, Nordenfelt et al., 1982). However, it is still possible that an inhibitor of HBV DNA polymerase might prevent the spread

TABLE 16.12.
Guide for the use of hepatitis B immunoglobulin from USPHS

Summary of postexposure prophylaxis of acute exposures to HBV

Exposure	HBsAg testing	Recommended prophylaxis
HBsAg positive	—	HBIG (0.06 ml/kg) immediately and 1 month later
HBsAg status unknown Source known:		
High risk	Yes, if results can be known within 7 days of exposure	IG (0.06 ml/kg) immediately, and if – TEST POSITIVE – HBIG (0.06 ml/kg) immediately and 1 month later or if – TEST NEGATIVE – Nothing
Low Risk	No	Nothing or IG (0.06 ml/kg)
HBsAg status unknown Source unknown:	No	Nothing or IG (0.06 ml/kg)

From Infectious Diseases, 22 Nov., 1981.

TABLE 16.13.
Inhibitors of hepatitis B virus DNA polymerase

Compound	Fifty percent inhibition (μg/ml)	Reference
Chloroquinine	480	Hirschman and Garfinkel (1978)
Chlorpromazine	250	Hirschman and Garfinkel (1978)
Ethidiumbromide	3.2	Hirschman and Garfinkel (1978)
Foscarnet	6	Nordenfelt et al. (1980)

of the virus to uninfected cells and, with a long treatment, anticipating immunological destruction of HBV antigen-producing cells, lead to the elimination of a chronic active hepatitis. The chemotherapy of hepatitis B infections has been usefully reviewed by Smith and Merigan (1982).

16.2.6.1. Ara-A and ara-AMP

Several clinical trials utilizing ara-A and its more soluble derivative ara-AMP have been reported and some are listed in Table 16.14. The number of patients in these trials have been limited which makes a statistical evaluation of each trial difficult, but the accumulated evidence clearly indicates some therapeutic effects. One example of the reduction in HBV DNA polymerase activity in serum is shown in Fig. 16.11 and one example of a reduction in HBsAg is shown in Fig. 16.12. The necessary dose of ara-A required is not clearly established but a rather high toxicity and low solubility seems to prohibit more than 15 mg/kg/day and 5 mg/kg/day seems to be less effective than 10 mg/kg/day, as indicated in Fig. 16.13.

The low solubility of ara-A has made long i.v. infusions necessary, but the introduction of ara-AMP with a highly increased solubility made i.m. or intermittent i.v. administration possible (Weller et al., 1982a) and indicated that a long course of 5–10 mg/kg/day of ara-AMP in three patients permanently reduced the level of virus particles, measured as HBV DNA in the blood (Fig. 16.14) and thus reduced the infectivity. The reduced dosing seemed to eliminate the problem with thrombocytopenia observed with higher doses of ara-AMP or ara-A. A seroconversion to anti-HBe occurred in all these three patients, which should be compared to the annual seroconversion of less than 5% in the same geographic area (Weller et al., 1982a). The results from Hoofnagle et al. (1982) indicate that a 10 day course using ara-AMP is not enough to induce a permanent amelioriation of infection and disease activity in the majority of patients with chronic hepatitis B. The long term effects of ara-AMP on liver function and inflammation, and neurological effects need to be further evaluated, but a prolonged course of ara-AMP treatment seems to hold some promise for the treatment of chronic hepatitis B infections.

TABLE 16.14.
Effect of ara-A and ara-AMP on chronic hepatitis B virus infections

Study design	Effects	Reference
2 patients, 15 mg/kg/day, ara-A, 9–14 days	Reduction of HBV DNA polymerase in serum, temporary in one patient (Fig. 16.11). Disappearance of HBsAg in one patient	Pollard et al. (1978)
4 patients, 10 mg/kg/day, ara-A, 2×5 days	Reduction of HBV DNA polymerase in serum, permanently only in one patient and then associated with fall in HBsAg	Chadwick et al. (1978)
7 patients, HBeAg and DNA polymerase positive, and 6 anti-HBe positive, DNA polymerase negative, treated with 10–15 mg/kg/day, ara-A, 2×5 days	4 treated patients had transient reduction in HBV DNA polymerase activity. 3 treated patients permanently lost HBV DNA polymerase activity and also HBeAg. HBsAg was reduced. No effect on anti-HBe positive patients. No change in controls. See Fig. 16.12	Bassendine et al. (1981)
6 patients, 5–15 mg/kg/day, ara-A, 2×7–21 days	3 patients had reduction in HBV DNA polymerase activity during treatment. 1 patient showed permanent loss of HBV DNA polymerase and HBeAg and reduced titre of HBsAg but not formation of anti-HBs	Scullard et al. (1981)
5 patients, 5–10 mg/kg/day, ara-A, 5 days, 1–5 courses	5 patients had reduced HBV DNA polymerase and HBeAg during treatment. Temporary effect in 2 patients and effects lasting 2–3 months in 3 patients	Watanabe et al. (1982)
8 patients, 5–15 mg/kg/day, ara-AMP, 5–34 days, 1–2 courses/patient	5 patients given a short course of therapy had transient reduction of HBV DNA polymerase. 3 patients on longer treatment lost HBV DNA polymerase, serum viral DNA and HBeAg. HBsAg decreased and anti-HBe developed in these 3 patients	Weller et al. (1982a)
6 patients, 10–15 mg/kg/day, ara-AMP, 10 days	Transient decrease in HBV DNA polymerase and HBV DNA. No permanent effect on the disease	Hoofnagle et al. (1982)

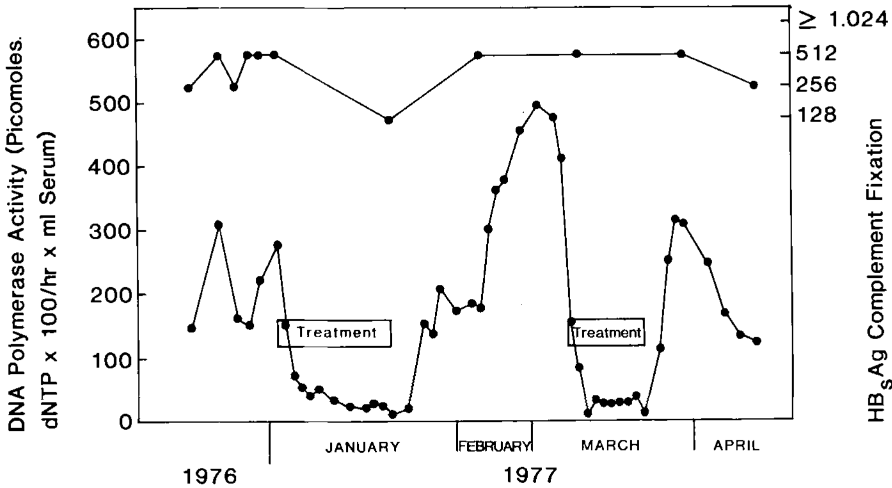

Fig. 16.11. Treatment of chronic hepatitis B with ara-A (vidarabine). Effect of ara-A (15 mg/kg/day) on hepatitis B virus DNA polymerase activity and HBsAg in serum. (after Pollard et al., 1978.)

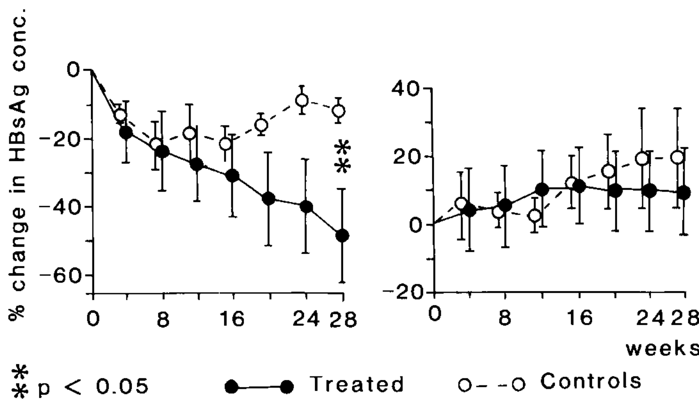

Fig. 16.12. Treatment of chronic hepatitis B with ara-A. (See Table 16.14). Left, percent change in HBsAg after ara-A treatment compared to controls, in HBeAg-positive patients; Right, percent change in HBsAg after ara-A treatment compared to controls, in anti-HBe-positive patients. (after Bassendine et al., 1981.)

The mechanism of action of ara-A and ara-AMP has not been elucidated (see Chapter 11) but it has been assumed that ara-A and ara-AMP are phosphorylated by cellular enzymes to ara-ATP which possibly could have an effect on the synthesis of HBV DNA. Neurological side effects for weeks after therapy and also bone marrow depression makes the search for less toxic compounds necessary.

16.2.6.2. Other compounds

Ribavirin (Virazole) has been used in a controlled trial on chronic HBV patients

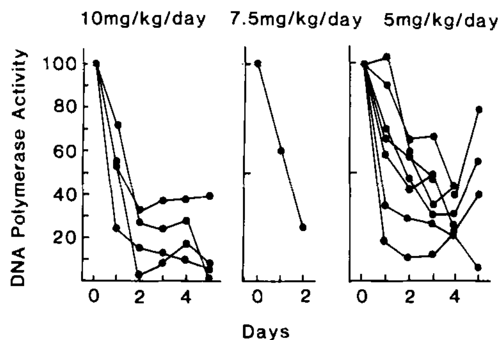

Fig. 16.13. Per cent reduction of DNA polymerase activity after the administration of varying doses of ara-A to hepatitis B patients. Each curve represents one patient. (after Watanabe et al., 1982.)

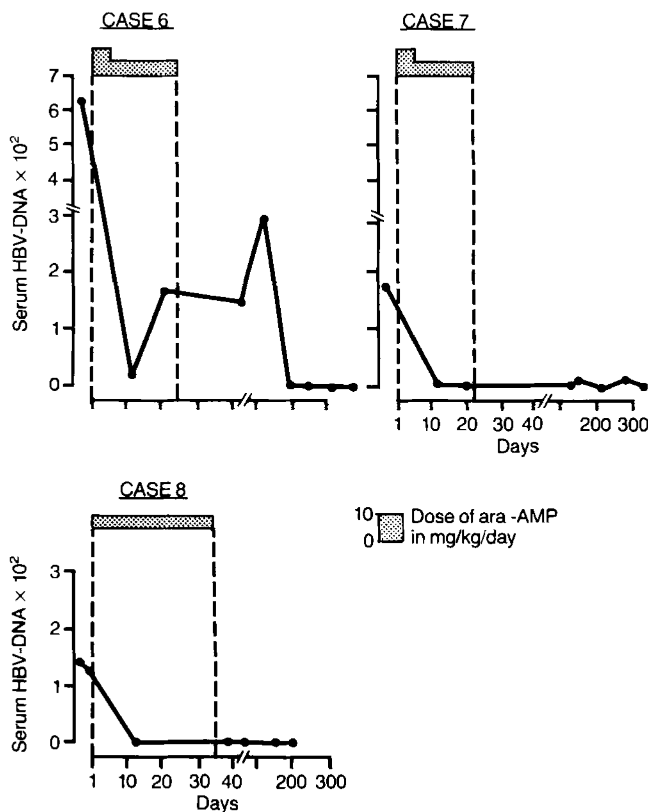

Fig. 16.14. Serum HBV DNA in three patients with chronic hepatitis B infections treated intramuscularly with ara-AMP 12 hourly. (after Weller et al., 1982a.) The amount of DNA was measured by hybridization to ^{32}P cloned HBV DNA.

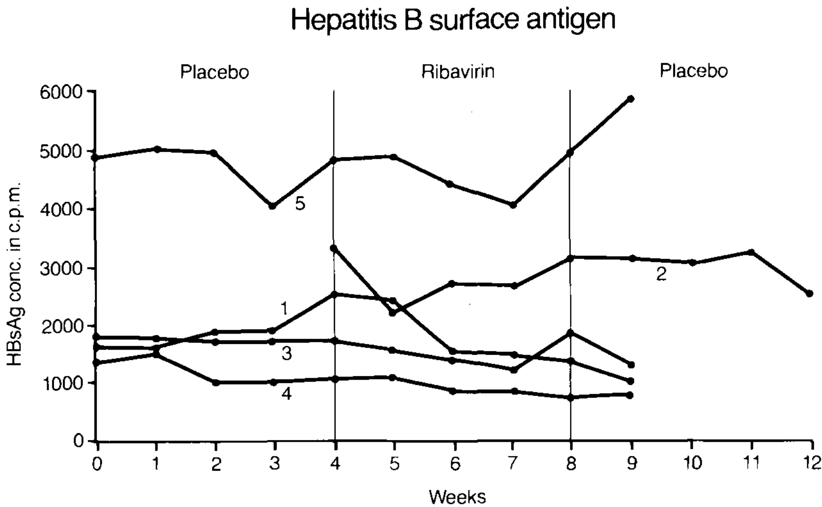

Fig. 16.15. Serial HBsAg titres measured by radioimmune assay during sequential control period and ribavirin treatment (800 mg/day). Patients are numbered 1 to 5. (after Jain et al., 1978.)

but no significant effects were observed (Jain et al., 1978) when 800 mg of the drug was given daily for 4 weeks as shown in Fig. 16.15. This dose also seems to cause some toxic problems. In a double-blind trial on 100 patients with acute virus hepatitis, (+) cyanidanol-3 was found to lower serum bilirubin and accelerate the disappearance of HBsAg (Blum et al., 1977) but this effect has not been confirmed. No reports showing effect of (+)cyanidanol-3 on chronic HBV have appeared and it is not known whether the observed effect was due to specific antiviral activity.

Acyclovir has been given to six patients with chronic HBV infections (Weller et al., 1982b, Weller et al., 1983). A transient decrease in HBV DNA polymerase and HBV DNA in two patients treated with 10 or 15 mg/kg 8-hourly suggested an inhibition which was not observed in two patients treated with 5 or 7.5 mg/kg. In contrast to herpes simplex virus, HBV does not induce a viral thymidine kinase and will thus not phosphorylate acyclovir. A transient increase in urea and creatinine was seen in the two patients given 10 mg/kg 8-hourly as an i.v. bolus.

The recently observed low toxicity for foscarnet (see Chapter 11) given i.v. to CMV and HSV patients and the inhibition of HBV DNA polymerase by the compound (Nordenfelt et al., 1980) indicate that a clinical trial using foscarnet against active hepatitis B might be worthwhile.

Steroids do not seem to be necessary in the treatment of chronic persistent hepatitis but have been used in the case of chronic active hepatitis. Since steroids appear to enhance virus replication their long term use in chronic active hepatitis seems, in most cases, doubtful or even harmful (Damjanovic and Brumfitt, 1980; Smith and Merigan, 1982).

16.2.7. INTERFERON AND IMMUNOMODULATORS

It has been suggested that patients with both acute and chronic hepatitis infections might have a deficient interferon production (Hill et al., 1971, Tolentino et al., 1975, Kato et al., 1980, Levin and Hahn, 1982). This deficiency could be a rational reason to use interferon in the treatment of hepatitis B infections. It is also possible that interferon might prevent infection of new cells.

Table 16.15 summarizes clinical trials using human leukocyte interferon (HuIFN-α) and human fibroblast interferon (HuIFN-β). No reports on the use of immune interferon are as yet available. The use of HuIFN-α seems to result in a clinical benefit, especially with long treatment periods. Varying results have been reported for

TABLE 16.15.
Treatment of chronic HBV infections with interferon

Study design	Effects	Reference
7 patients, 4.2×10^5–1.2×10^7 U/day, HuIFN-α, s.c. or i.m., 100 days	3 patients lost Dane particles and HBeAg. 3 patients lost Dane particles only during treatment	Greenberg et al. (1976) Merigan and Robinson (1978)
1 patient, 10^7 U alt. days, HuIFN-β, i.m., 14 days	Decreased HBV antigens in liver biopsy	Desmyter et al. (1976)
5 patients, 2–8 × 10^6 U/day or twice weekly, HuIFN-β, i.m., 21 days	No effects observed	Weimar et al. (1977)
2 patients, 10^7 U/day, HuIFN-β, s.c. and i.m., 14 days	Dane particles persisted	Kingham et al. (1978)
3 patients, 3×10^6 U/day, first HuIFN-β, then HuIFN-α, i.m., 14–20 days	No effect by HuIFN-β, when on HuIFN-α a permanent reduction of HBV DNA polymerase in one patient and transient in two. No change in HBsAg	Weimar et al. (1979)
1 patient, 10^6 U/day, HuIFN-β, i.m., 82 days	HBV DNA polymerase, HBeAg and HBcAg disappeared. Reduced HBsAg	Dolen et al. (1979)
8 patients, 1–3 × 10^6 U/day HuIFN-α, i.m., daily 5 weeks–5 months	Decrease in HBV DNA polymerase and HBeAg in 1 patient. Transient decrease in HBV DNA polymerase in 4 patients	Scullard et al. (1979)
16 patients, 2–10 × 10^6 U/day HuIFN-α, s.c., 5 months or 5–20 × 10^6 U/day in 10–14 day courses repeated up to 4 times	Decrease in HBV DNA polymerase during treatment in all patients. 4 patients remained DNA polymerase negative and lost HBeAg	Scullard et al. (1981)
16 patients; 8 controls, 8 received 12×10^6 U/day HuIFN-α, i.m., first week, then half dose for 6 weeks	Transient drop in HBV DNA polymerase. Leucopenia in 6 of 8 treated	Weimar et al. (1980)

HuIFN-β but the impression is that it is less effective than HuIFN-α (Weimar et al., 1979, Smith and Merigan, 1982).

The production of different types of interferon by recombinant DNA techniques will make it possible to carry out larger controlled trials using high concentrations of interferon. This will hopefully define the optimal dose level, length of treatment and type of interferon. Severe side effects of interferon have been reported (Sachs et al., 1982) and might limit the dosing of interferon. Some of the side effects observed in hepatitis B patients are shown in Table 16.16. Interferon inducers have not been used and those presently available seem to be too toxic.

Transfer factor has been used clinically in attempts to correct defective cell-mediated immune response to HBsAg. The results so far have not shown any long term therapeutic effect on chronic hepatitis B (Smith and Merigan, 1982).

The immunomodulating compound levamisole has been used in uncontrolled studies in patients with chronic hepatitis and lowered HBsAg titres, and disappearance of HBV DNA polymerase were reported (De Cree et al., 1974, Fattovich et al., 1982). However, Chadwick et al. (1980) did not observe any therapeutic improvement due to levamisole treatment. Virus persisted and the patients remained HBsAg positive but there was an enhanced cell-mediated response to HBsAg and an increased lysis of infected cells. A use of levamisole in acute hepatitis might lead to a fulminant course and is not recommended at present (Par et al., 1977).

16.2.8. Combination therapy

An attempt to combine the therapeutic effects observed using interferon and ara-A has been described by Scullard et al. (1981). Three types of responses to treatment

TABLE 16.16.
Interferon side effects (non-haematological) in hepatitis B patients. HuIFN-α, $1-2\times10^7$ U/day, was given s.c. for 1–21 days (after Sacks et al., 1982)

Symptom	No. of affected/total[a]
Initial fever (38°C or greater)[b]	45/113
Fatigue and malaise	33/113
Diffuse myalgia	9/38
Hair loss	8/38
Nausea and vomiting	3/38
Depression	3/38
Weight loss	
Greater than 5% of pretreatment weight	4/38
Greater than 10% of pretreatment weight	0/38

[a] The total number treated was 38 patients or 113 treatment courses.
[b] Of 45 patients, 11 had fever to 39°C.

were found. (I) Permanent loss of HBV DNA polymerase activity, HBeAg and HBsAg from serum, and disappearance of HBcAg and HBsAg in liver tissue. (II) Permanent loss of HBV DNA polymerase activity, persisting but lower HBsAg, and HBeAg slowly decreasing to undetectable levels in serum, reduction or disappearance of HBcAg but not HBsAg in liver tissue. (III) Transient decrease in HBV DNA polymerase activity, persisting HBeAg and unchanged HBsAg in serum, no change in HBcAg or HBsAg in liver biopsy specimens. Table 16.17 shows the outcome of treatment with ara-A, HuIFN-α and a combination of ara-A and HuIFN-α. The amount of interferon and ara-A used is indicated in Tables 16.14 and 16.15 (Scullard et al., 1981). A better effect was obtained by combination therapy than with the drugs used separately, and a better response in females than in males was also observed.

A sequential combination of 10 mg/kg/day ara-A and chloroquine 200 mg/day has been used to treat chronic HBV patients. A transient reduction in HBV DNA polymerase during ara-A administration was observed but the treatment with chloroquine did not have any effect (Thomas et al., 1980).

Ara-AMP and HuIFN-α have also been used in a sequential fashion in chronic HBV patients (Smith et al., 1981, Smith and Merigan, 1982). This study showed that long term treatment with alternating HuIFN-α and ara-AMP given i.m. resulted in a transient fall in HBV DNA polymerase in four patients and a permanent fall in two other patients. Four patients at 7.5 mg/kg ara-AMP had severe side effects and treatment was stopped, but 5 mg/kg was tolerated in the other six patients.

Treatment of persistent HBV infection in chimpanzees using combinations with HuIFN-α, ribavirin and ara-A resulted in temporary effects (Zuckerman et al., 1978).

TABLE 16.17.

Influence of treatment regimen and sex on outcome of 43 courses of antiviral therapy with human leukocyte interferon and/or adenine arabinoside in 32 patients with chronic hepatitis B virus infection (after Scullard et al., 1981)

Regimen	Response no. patients		
	Type I	Type II	Type III
Single-agent			
Female patients	2	2	3[a]
Male patients	0	1	14[a]
Combination			
Multiple cycles	1	6	9[a]
Interferon and one course of adenine arabinoside	0	0	5

[a] $P \leqslant 0.01$.

16.3. δ-agent infections

The structure of the δ-agent is shown in Fig. 16.16. The δ-agent is a defective RNA virus, and requires the presence of HBV for its multiplication. The HBsAg is used as a coat for the δ-agent and the viral RNA has no or little homology to HBV DNA. The δ-agent is always associated with HBV and has been found in both acute and chronic hepatitis. In general, the δ-agent seems to be involved in the most severe hepatitis cases. Epidemiological studies have shown the δ-agent to be present world-wide but to be especially abundant in the Mediterranean area. Only one serotype is presently known. Chronic δ-agent patients seem to have a high prevalence of anti-HBe and to lack markers of HBV synthesis.

The mode of transmission of δ-agent is probably the same as for HBV, since a δ-agent infection resulting in disease requires a concomitant transmission of HBV or transmission to a host with concurrent HBV infection. Apart from man, chimpanzees can be infected by δ-agent. No vaccine or chemotherapy against δ-agent has been reported and it seems possible that it can be prevented by controlling HBV. The properties of δ-agent have been reviewed by Deinhardt and Gust (1982) and Rizzetto et al. (1982).

16.4. Non-A, non-B hepatitis infections

As indicated in Table 16.1 there are at least two types of hepatitis non-A, non-B (HNANB) which are caused by viruses not antigenically related to HAV or HBV. The direct evidence for at least two types was found by Tsiquaye and Zuckerman (1979) in transmission studies in chimpanzees. It has also been shown that persistent carriers (chimpanzees) of HBV can be infected by infusion with HNANB (Tsiquaye et al., 1983). It is possible that this superinfection exerts a suppressive effect on the persisting virus. The diagnosis of HNANB is made by exclusion of other types of hepatitis, and HNANB infections may account for about 20% of clinical hepatitis cases. When HBV is controlled, HNANB can make up as much as 90% of the transfusion hepatitis.

Transfusion HNANB infections are less severe than those caused by HBV but

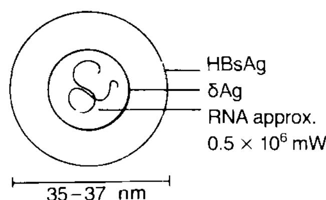

Fig. 16.16. Viral components of the delta agent.

can also develop into chronic infections. Chimpanzees and marmosets can be infected by HNANB but the molecular properties of the agent(s) are not known.

Epidemic HNANB infections have similarities to HAV infections but the agents are not serologically related. The virus(es) is thought to be spread by contaminated water, and a faecal-oral spread is likely. The absence of chronic infections resembles the situation with HAV infections. No serological tests are presently available.

No therapy or prophylaxis is presently available for use against HNANB infections. The essential features of HNANB viruses have been reviewed by Deinhardt and Gust (1982), Gerety (1981) and Gerety (1982).

16.5. Summary

Passive immunization against hepatitis A reduces the infection to a mild or subclinical course. Vaccines are likely to emerge in the next few years. No antiviral drug has shown significant clinical effects against hepatitis A infection.

Immunization of persons at high risk of contracting hepatitis B infections has been successful but the amount of vaccine available will be limited until it is prepared by hybrid DNA technology or peptide synthesis. It remains to be established if vaccination is effective when given post-exposure. High titre immunoglobulin has been useful for prophylactic but not for therapeutic use. Ara-A and interferon might have some therapeutic effect against chronic hepatitis B, but a better treatment is needed.

Development of a vaccine or the use of chemotherapy against hepatitis non-A, non-B infections will first require a better virological characterization of these agents. Control of the δ-agent can probably be achieved by hepatitis B vaccine or chemotherapy.

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CHAPTER 17

Molecular epidemiology and development of drug resistant viruses

17.1. Introduction

Random genetic mutation can occur at a very high frequency with a number of RNA viruses (Holland et al., 1982, Brand and Palese, 1980) and with influenza virus, for example, this may occur at a frequency as high as 1 in 100 000. This means that mutants could possibly emerge during a single infection in an individual and, given a selective advantage, such mutants could predominate in the offspring virus. Two important selective pressures which are already known are specific antibody and antiviral inhibitors. Recent studies using monoclonal antibodies have highlighted the extent of antigenic variation among a number of viruses other than influenza, including rabies (Wiktor and Koprowski, 1980), measles (Birrer et al., 1981) and picornaviruses (Minor et al., 1980). Similarly, mutants with acquired resistance to antiviral agents have been described with almost every virus-drug combination so far investigated (reviewed by Herrmann and Herrmann, 1977, Field, 1983, Barrera-Oro and Melnick, 1961, Burns et al., 1982, Collins et al., 1982, Heider et al., 1981, Katz et al., 1974, Klein and Friedman-Klein, 1975, Oxford et al., 1970, Renis and Buthala, 1965). Will acquired drug resistance to viruses constitute a clinical problem of major significance, similar to the situation experienced in the antibacterial world? Before we examine specific examples it will be worthwhile to make some general observations. Firstly, one should not conclude that if mutants emerge easily in culture in the laboratory that they will also emerge in clinical practice. Fusidic acid is such an example, where bacterial mutants are selected in vitro, but are not frequently isolated in the clinic (Williams and Kruk, 1981). Secondly, it is possible that drug resistant virus mutants may be less virulent than the parent virus or in-

duce a changed disease pattern, and this has been established with certain TK^- mutants of herpes simplex virus (Field and Wildy, 1978, Sibrack et al., 1981). Another potential source of confusion is the assumption that treatment failures may be due to drug resistant virus. This may not be the case and particular examples should always be confirmed by laboratory experiments. In the near future, sensitive tests will have to be developed for laboratory screening of large numbers of viruses for drug sensitivity in much the same way as bacteria are now tested using semi-automated equipment. In this regard, it will have to be borne in mind that when viral specimens are investigated they may also contain small concentrations of drug which could apply selective pressure in vitro. Also, very small changes in drug sensitivity of the virus may be clinically significant, but may be missed in a crude laboratory screening experiment. Particular cell types may be more suitable than others in any of these tests. A useful 'side effect' of investigations of drug resistant mutants is that the mutants may aid in an understanding of the drug action itself. With herpes viruses, TK^- mutants provide a useful test for novel antiviral agents since resistance implies that activation of the drug requires TK, and this reasoning was applied in preliminary studies of the mode of action of BVDU. Finally it should be remembered that clinical specimens are very likely to contain a mixture of drug sensitive and resistant viruses.

How much diversity already exists among virus populations to antiviral agents? A considerable degree of heterogeneity to inhibition by individual drugs may preexist the use of such agents in man. Thus, drug resistant influenza viruses (resistant to rimantadine) have been isolated in Berlin, although the compound has not been used in that country, as an antiviral. This resistance might be a result of random mutations occurring in gene 7 (coding for M protein) which is known to be involved in the mode of action of the compound (see Chapter 7). As regards herpes viruses, it is well known that viruses with a range of sensitivities to acyclovir can be isolated from the community (Table 17.1).

In a sense, therefore, the two aspects to be considered in this chapter of genetic heterogeneity and drug resistance should be considered in tandem. We shall firstly investigate the degree of genetic heterogeneity in commonly occurring viruses and secondly examine specific instances of drug resistance to viruses, both in the laboratory and in the clinic.

One must admit that the question of *prevention* of drug resistance has hardly been considered and one could immediately think of two opposing view points. Should one use a minimum effective dose of drug or, alternatively, a high dose? Could drug combinations with compounds with different modes of action (e.g. acyclovir and foscarnet against herpes virus) be used, as in the case of treatment of tuberculosis with streptomycin and isoniazid? Should certain antivirals be restricted to more serious illnesses and not be used for more trivial infections on a wide scale? The latter question is relevant to the use of the newer anti-viral compounds against herpes labialis versus life threatening herpes infections in the immunocompromised per-

TABLE 17.1.
Fractions of various HSV strains which are resistant to 10-μmol concentrations of acyclovir (ACV) (after Smith et al., 1980)

HSV type	HSV strain[a]	Total virus titre (PFU × 10^6/ml)[b]	Drug-resistant virus titre[c] (PFU × 10^6/ml)	% of drug-resistant virus
1	227	2.4	0.67	20.0
1	Patton-Original	26.0	1.6	6.2
1	MacIntyre	27.0	1.3	4.8
2	EL-3	4.0	0.15	3.8
2	Palmer	0.40	0.015	3.8
2	EL-4	0.36	0.005	1.4
2	EL-7	0.11	<0.001	<0.91
2	2-G	0.70	<0.001	<0.14
2	EL-6	0.79	<0.001	<0.13
1	EL-12	3.3	0.003	0.09
1	EL-19	28.0	0.02	0.07
2	EL-1	1.4	<0.001	<0.07
1	225	15.0	0.008	0.05
1	EL-15	7.5	<0.001	<0.01
1	KOS	90.0	<0.001	<0.001

[a] All strains except Patton, MacIntyre, Palmer and KOS were fresh isolates; none had ever been exposed to ACG.
[b] PFU, Plaque-forming units
[c] Drug-resistant virus is defined as that which formed visible plaques when incubated for 48 h in the presence of 10 μmol of ACG.

son. Most hospitals now have a strict antibiotic policy for bacterial infections, with certain antibiotics held in reserve. The 'front line' antibiotic can then be switched when a degree of resistance becomes apparent. It would be a pity not to heed the lessons of antibacterial therapy and have to 're-discover' every problem again for antivirals.

Finally, with herpes virus a further problem of latency looms. Some people are persistently infected in the ganglion cells and the virus is activated perhaps monthly, or erratically. If a drug is used to treat such a re-activation and a drug resistant virus sub-population is selected, could this colonize a new ganglion with a drug resistant virus or, alternatively, are ganglions already infected for life? It is apparent that many more basic problems are going to be exposed and anticipated in this chapter than are going to be solved.

Firstly, we shall attempt to consider how biologically and genetically different various viruses are as they circulate in the community. How much genetic variation exists in influenza, picorna or herpes viruses and is this variation also present in the 'working' protein products of the genetic information i.e. phenotypic variation? Clinical observation and common sense tells us already that some viruses are appar-

ently more variable than others, e.g. influenza can alter its antigenic phenotype with relative ease and thus escape neutralizing antibodies. Many serotypes of common cold viruses exist, whereas, as far as is known, many fewer serotypes of herpes virus exist. But is the field epidemiological situation much more complicated than this? Even within a number of influenza viruses of the same antigenic subtype can genetic variants exist and flourish with other attributes such as faster replication rate, easier spreadability, low immunogenicity and so on? Antigenic variants of measles virus exist but why are they not detected easily in the community? Is there a range of herpes type I viruses or influenza viruses or picornaviruses with different virulence characteristics?

It is into this diverse genetic pool of viruses that we are planning to introduce new antiviral agents, which may exert strong selective pressures. Will a Pandora's box of new viruses be stirred up and released or could we perhaps even end up with more attenuated viruses which would almost be akin to vaccine strains in their reduced virulence? So, in a brief way, we would like to survey a range of viruses to see how important genetic variation may be. Such studies have only been in progress for 5 years at the most and so are still in their infancy. The chapter must be speculative to this degree, but already interesting data are emerging which tell us that infectious agents have many tricks up their sleeve.

17.2. Genetic and phenotypic variation among viruses: 'molecular epidemiology'

As alluded to above, for the first time new technologies now enable virologists to study small genetic and antigenic differences between field isolates of animal viruses at a higher level of discrimination than has been possible using conventional serological techniques and post-infection antisera. The most important of these laboratory techniques are monoclonal antibodies, peptide mapping, fingerprinting of whole RNA virus genomes, restriction enzyme analysis of virus DNA genomes, cloning of genes and rapid sequencing of DNAs and RNAs. In particular, these techniques can be applied to studies of genetic variation among animal viruses (reviewed by Palese and Roizman, 1980). Genetic and phenotypic heterogeneity may be of considerable practical importance in attempts to control certain virus diseases by chemo or immuno prophylaxis. Thus, certain naturally circulating viruses may be resistant to antiviral drugs or may differ in virulence, antigenic or biological properties which may be accentuated in the presence of an antiviral compound, allowing the emergence of virions causing new disease patterns. These aspects will be considered in some detail, firstly for influenza virus, since more is known about genetic and phenotypic variation in influenza A than probably any other virus, and then for representative viruses of other groups such as polio, rotaviruses, alphaviruses and herpes virus. In general, we can note that a considerable degree of heterogeneity is detected in all these viruses.

17.3. Influenza

An important and unique biological property of influenza noted in the earliest genetic studies with the virus and described earlier in this book (Chapter 7) is that of genetic reassortment. In essence, when two influenza viruses co-infect a cell, exchange of each of the eight RNA segments can occur, leading to 2^8+2 'offspring' virions. This genetic reassortment (commonly and incorrectly called 'recombination' and the offspring called 'recombinants' – a term to which however we shall adhere) occurs in the laboratory, in animal models, and in nature, and allows the gene pool of human and animal influenza A viruses, for example, to be extended. In terms of the evolution of influenza A viruses in humans, the genes coding for the HA and NA antigens are of particular significance, because antibody to these two proteins confers a degree of immunity to virus infection and spread.

17.3.1. Field isolates from the community

Studies of influenza A and B viruses circulating in the community have shown that quite extensive genetic and phenotypic variation occurs. Biological variation including changes in virulence can be detected. Thus, a number of recently isolated influenza A viruses of both H3N2 and H1N1 antigenic subtypes have a temperature sensitive (*ts*) phenotype (Oxford et al., 1980, Chi-Ming Chu et al., 1982). Seventeen of twenty-six influenza A virus isolates of the H1N1 antigenic subtype and two of eleven virus isolates from the 1977–78 season exhibited a *ts* phenotype (Oxford et al., 1980). In our studies, viruses isolated from the same city and from a single person have varied considerably in the phenotypic *ts* character. Laboratory studies of artificially induced influenza *ts* mutants have demonstrated clearly that such mutants are attenuated for man and that the shut-off temperature is related to attenuation, and this applied also to the naturally occurring viruses. The occurrence of non-*ts* and *ts* viruses in nature presumably means that influenza A viruses of varying virulence even within a single antigenic subtype are circulating in the community. This virion heterogeneity may result in a variable response to any attempts to control the virus by vaccine or chemoprophylaxis.

In a series of detailed studies (Nakajima et al., 1980) the degree of genetic variation in influenza A virus was established (by oligonucleotide mapping) between three H1N1 viruses isolated in a single year within a period of several months. When a mixture of oligonucleotides of USSR/90/77 and A/Hong Kong/123/77 viruses were co-electrophoresed, 5 additional USSR/90 spots appeared in the mixture. The minimum number of base changes estimated between A/USSR/90/77 and A/HK/123/77 is 8 and between A/USSR/92/77 and A/USSR/90/77 (isolated in the same country) is 6. Approximately 44 oligonucleotides were analyzed representing a total of 1077 nucleotides, corresponding to 7.0% of the total genome (M.W. 4.9×10^6). Thus, a minimum base frequency difference between large oligonucleo-

tides of the viruses is 8/1088 or 0.7%. The mapping technique can be used to trace the origin of viruses and establish how particular variants circulate.

In the study of H1N1 viruses circulating in Japan in 1978–79 Nakajima et al. (1980) concluded that at least 4 types of H1N1 viruses could be distinguished by the oligonucleotide pattern. The first type was prevalent throughout Japan from January to March 1978, whereas the second and third types caused outbreaks in the next winter of 1978–79. The fourth type was a recombinant and caused influenza outbreaks in the winter of 1979. Thus, even within a single antigenic subtype (H1N1) a genetically heterogeneous group of viruses was circulating and causing influenza outbreaks. At present it is not known if the genetically different viruses differed in their virulence and 'spreadability' and these are important questions to be answered by further studies. In detail, in the group I viruses the RNAs of two viruses isolated in a single area, A/Kumamoto/1/78 and A/Saga/4/78 had identical maps, but A/Akita/1/78 from a different area of the country had a slightly different pattern of oligonucleotide spots. Finally, A/Yamanashi/28/78, which appeared towards the end of the epidemic, showed further spot differences. Only two viruses isolated in the next winter were examined and these were similar to each other and distinct from strains in Group I described above and more related, in fact, to A/USSR/92/77 virus. Thus, it was improbable that they were derived directly from the group I 1978 viruses, but had probably been re-introduced into Japan. This has important implications as regards drug resistant viruses for example and how they could spread rapidly from country to country. Nine influenza virus strains isolated in 1979 over a period of 3 months and isolated from different parts of Japan were nearly identical in oligonucleotide pattern. The spot pattern was very similar to that of A/California/45/78 virus, and this probably indicated that the Japanese strains were derived from this virus, which was isolated first in the USA. The latter virus was shown to be a recombinant that had HA, NA, M and NS genes from an H1N1 parent and P1, P2 and P3 and NP genes from the H3N2 parent. Certainly, the potential of these different viruses to spread varied considerably, and the early 1978 viruses and the California/78 viruses spread rapidly to all Japan. The late A/Kumamoto strains failed to cause extensive outbreaks.

17.3.2. Influenza isolates from circumscribed outbreaks

As an example of this approach, in a recent study (Oxford et al., 1983) we have used monoclonal antibodies to influenza B virus HA to examine in detail the antigenic composition of a series of virus isolates from a single school outbreak of influenza. Between January and March 1983 there was an outbreak of influenza B virus infection at Christs' Hospital School, Surrey (J. Smith, PHLS, Guildford, personal communication). The school is reasonably isolated from the local town and represents a semi-closed community with the boys boarding at the school in dormitories of 25–30 boys and mixing during the daytime at lessons. Studies of virus evolution

during a single virus outbreak can therefore be carried out with less likelihood of new introductions of viruses from the local community as the epidemic proceeds. The general course of the influenza B outbreak is illustrated in Fig. 17.1, where it is apparent that the major wave occurred approximately a week after the first cases were noted. Approximately 200 out of 800 boys fell ill and from these over 100 influenza B virus isolates were obtained by the Public Health Service Laboratory at Guildford.

The viruses were established as an antigenically heterogeneous group and 13 distinguishable antigenic patterns could be detected. Analysis of the first viruses isolated in the school showed several antigenic groupings to be present (Table 17.2) and these were detected thereafter at different times throughout the outbreak. In control experiments, to ensure that the viruses were not single laboratory artefacts, antigenic analysis of multiple clones (approximately 70) of a simple virus isolate failed to show a heterogeneous population of virions. Whilst, in addition, we concluded from an analysis of multiple reisolates of the same virus from a throat swab that the antigenic differences detected above were significant and related to the actual outbreak.

In parallel studies, we examined the polypeptide profile of a number of viruses isolated from Christs' Hospital and other schools. Examination of Fig. 17.2 shows

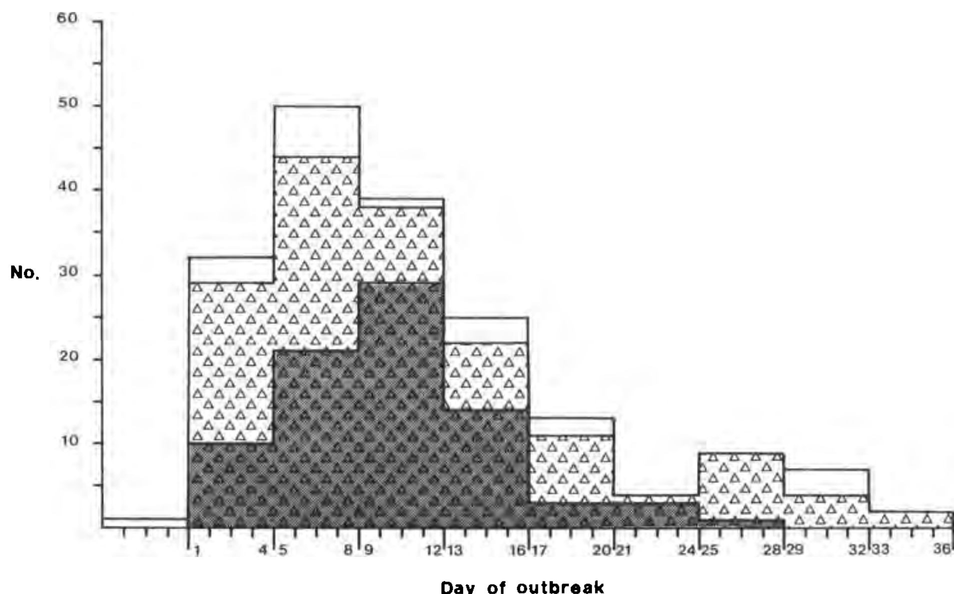


Fig. 17.1. Epidemiological characteristics of an influenza B outbreak at Christs Hospital. Clinical cases of influenza occurred over a 40–day period. The numbers of cases of clinical influenza not confirmed in the laboratory □, clinical cases confirmed in the laboratory (▲) and the number isolations of influenza B virus (△) are shown for 4–day periods. Day 1 (D1) of the occurrence of the outbreak is taken as the first day on which confirmed cases of influenza occurred.

TABLE 17.2.
Serological analysis of HA of influenza B viruses isolated in school and community epidemics, using monoclonal antibodies (after Oxford et al., 1983)

Arbitrary antigenic grouping	Prototype isolate	No of viruses in each antigenic group		Serological reactivity with monoclonal antibody no.											
		Christ's Hosp. (53)	St Edmund's (6)	113/2	124/4	128/2	134/1	146/1	152/2	160/1	162/1	195/3	206/2	238/4	280/2
I	B/Eng/317/82	3	–	<	<	<	<	<	<	<	<	3200	<	<	<
II	319/82	5	–	<	<	<	<	<	<	<	<	6400	<	6400	<
III	288/82	10	–	<	1600	<	<	<	<	<	<	>6400	<	6400	<
IV	281/82	16	–	<	<	<	<	1600	<	<	<	>6400	<	>6400	<
V	138/82	1	–	<	<	<	<	3200	<	<	<	6400	<	>6400	4800
VI	339/82	2	–	<	1600	<	<	1600	<	<	<	>6400	<	>6400	<
VII	340/82	2	–	<	1200	<	<	<	<	<	800	>6400	<	>6400	<
VIII	165/82	5	–	1200	4800	<	<	<	<	400	1600	>6400	<	800	<
IX	137/82	1	–	3200	6400	<	<	<	<	1600	1200	>6400	<	2800	800
X	167/82	5	–	1600	1600	<	<	1600	<	800	800	>6400	<	>6400	<
XI	325/82	1	–	1600	3200	<	<	3200	1600	1600	<	>6400	<	>6400	<
XII	139/82	1	–	800	6400	<	<	4800	800	800	600	6400	<	>6400	<
XIII	252/82	1	–	3200	>6400	<	<	>6400	2800	3200	3200	>6400	<	>6400	600
XIV	B/Eng/143/82	–	1	800	3200	<	<	<	<	<	800	>6400	>6400	6400	3200
XV	147/82	–	3	4800	>6400	4800	>6400	6400	6400	2400	600	>6400	>6400	>6400	>6400
XVI	145/82	–	1	1600	>6400	<	6400	6400	3200	1600	<	>6400	1600	>6400	1600
XVII	146/82	–	1	800	>6400	<	1600	<	800	800	<	>6400	>6400	>6400	>6400

Note that approximately 50% of isolates from Christs Hospital school formed two antigenic groups (III and IV)

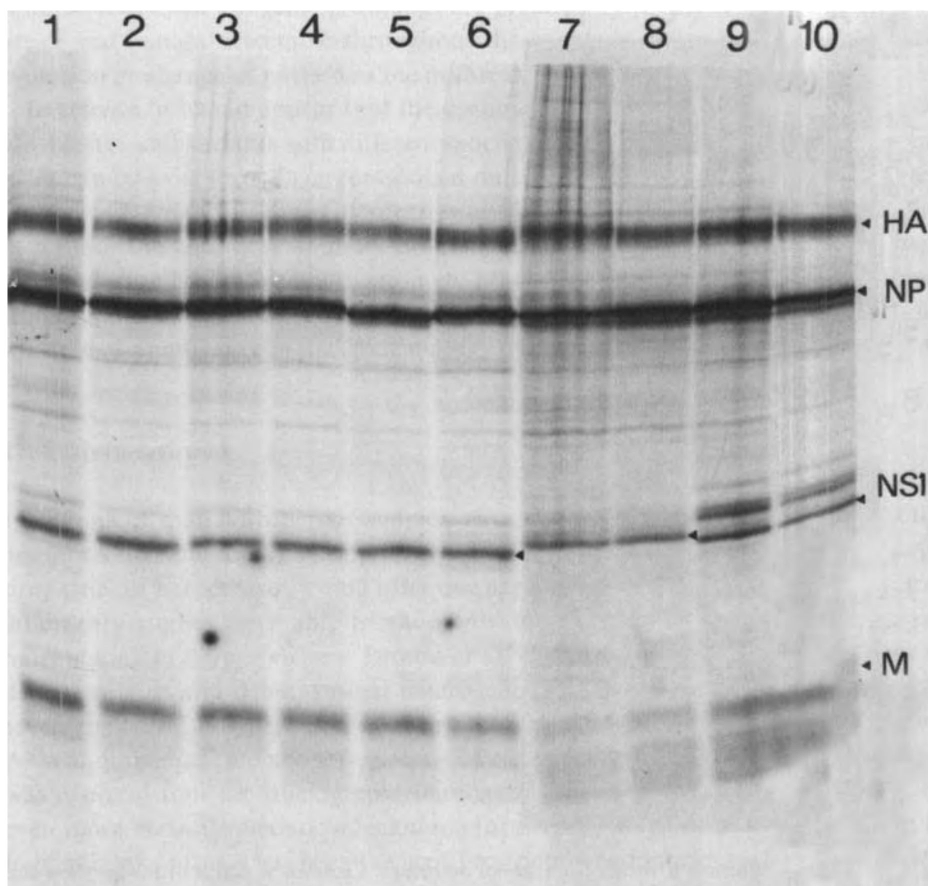


Fig. 17.2. Polypeptides of influenza B viruses from school outbreaks. Lanes: 1, B/E/320/82 (school A); 2, B/E/317/82 (school A); 3, B/E/316/82 (school A); 4, B/E/322/82 (school A); 5, B/E/319/82 (school A); 6, B/E/318/82 (school A); 7, B/E/143/82 (school B); 8, B/E/144/82 (school B); 9, B/E/83/82 (school C); 10, B/E/163/82 (school C). School A, Christs' Hospital; school B, St. Edmund's; school C, Rugby.

that influenza B virus isolates from simultaneous outbreaks in 3 different schools could be distinguished on the basis of electrophoretic migration rate differences in NS1 polypeptides or RNA patterns (Fig. 17.3). Within a single school only very minor differences were detected in electrophoretic characteristics of NP, HA, M and NS1 polypeptides. At present we attribute electrophoretic differences to mutations in the respective genes, so resulting in substitution of hydrophilic for hydrophobic amino acids in the virus polypeptides. Such amino acid substitutions would result in increased or decreased binding of SDS to the polypeptides and hence differences in electrophoretic mobility in the high resolution SDS gels.

Thus, even in an influenza B virus outbreak in a single school, antigenic variants

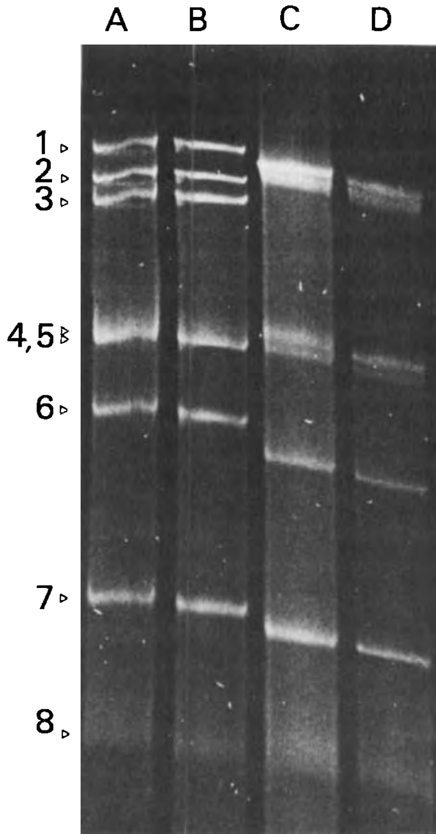


Fig. 17.3. RNA of influenza B viruses from school outbreaks. Channels A and B of the gel show the migration of RNA of viruses isolated from Christs Hospital School, whilst channels C and D show RNA of viruses isolated from Rugby School in an outbreak occurring at the same time.

can co-exist and spread. It is not known at present if the outbreak was caused by several distinguishable viruses or whether mutation occurred in the gene coding for HA during the outbreak itself. The most likely explanation is that the initial infection in one boy resulted in the excretion of a genetically heterogeneous collection of virions which were then cloned in natura among the remaining boys in the school. In certain boys with a high level of antibody and perhaps cell mediated immunity, no infection would result, whilst in others partial immunity with antibody to cross reactive antigenic determinants would give strong selective pressures, so resulting in emergence of virions with antigenically different HAs, in the same way as detected in vitro when cloned influenza virions are suppressed with monoclonal antibodies and antigenic mutants emerge at a rate of approximately 1 in 10^5 parental virions (Yewdell et al., 1979, Natali et al., 1981). The above hypothesis is further substantiated by antigenic analysis of viruses according to the day of isolation: it

is noted that most antigenic groupings are present at the first few days of the outbreak and remain detectable throughout the epidemic, there being no detectable evolution or change of pattern as the outbreak proceeds.

In essence, it would appear that the genome of influenza viruses seems remarkably 'plastic' and variants with different biochemical, antigenic and biological differences can co-exist (even in circumscribed outbreaks) and spread in the community. A true estimate of this diversity must await the analysis of many more strains, but in spite of the considerable practical and theoretical problems of mutations happening during laboratory cultivation etc., the overall reproducibility of the above studies indicate that we are dealing with a tremendous degree of genetic variation, which is of significance in the epidemiology of the virus.

17.4. Herpes viruses

Infections of man with herpes simplex virus are known to vary in respect to the anatomic site, and to virulence. The existence of intratypic differences in the genetic properties of herpes virus would offer one explanation for this variation and earlier laboratory studies were able to show intratypic biological differences in plaque morphology of herpes viruses. Pereira et al. (1976) analyzed 53 HSV-1 isolates of diverse origins and demonstrated heterogeneity in the electrophoretic mobility of seven structural polypeptides, allowing the isolates to be divided into 19 groups. As with influenza (see above), analysis of electrophoretic mobility of polypeptides was a useful tool for tracing epidemiological patterns of infection. However, an even more versatile analytical technique for investigations of molecular epidemiology of herpes viruses has been the use of restriction endonucleases for DNA fingerprinting. With such genome complexity even a minimal degree of mutation would be enough to create a complicated genetic heterogeneity in these herpes viruses. More than 150 HSV-1 and HSV-2 isolates from different clinical and geographic origins have been analyzed by Buchman et al. (1980). Electrophoretic separation of fragments of DNA (cleaved by using restriction endonucleases) of related and unrelated isolates revealed that repeated isolates from a single site in a single individual (isolates more than 12 years apart) or from sexual partners were indistinguishable. In addition, epidemiologically unrelated isolates were easily distinguishable from each other. These authors concluded that mutations occurred during the evolution of HSV and that virus variants accumulated and persisted in the population, presumably without displacing each other. Since HSV viruses are latent and only multiply periodically it can be assumed that intimate contact is required for cross-infection. Viral variants would tend to be perpetuated within clusters of individuals defined by the existence of close personal contact. The epidemiology at first sight is thus very different to that described above with influenza, where virus variants appear to exist side by side and presumably spread rapidly together in the

community, some eventually being displaced by others. In a similar study Lonsdale et al. (1980) analyzed 44 ganglion isolates from 21 individuals using restriction endonucleases (Table 17.3). Multiple isolates from a single ganglion (6 cases), isolates from different ganglia from a single individual (7 cases) and isolates from left and right ganglia from a single individual (7 cases) were the same virus strain (Fig. 17.4). It would appear that the latent virus in ganglia obtained from the same individual comes from the same initial infection. A study of the restriction enzyme profiles of lip lesion isolates obtained from four individuals on two occasions showed that

TABLE 17.3.
Herpes simplex virus type 1: Isolates from human ganglia (after Lonsdale et al., 1980)

Isolate	Age	Sex	Cause of death	Number of isolates	Ganglion
USA–1	17	M	Trauma	1	Right Trigeminal
USA–2	65	M	Trauma	1	Left Trigeminal
USA–3	51	F	Multiple Sclerosis	1	Right Trigeminal
USA–4	40	M	Trauma	1	Right Trigeminal
USA–5				1	Left Trigeminal
USA–6	18	M	Trauma	1	Right Trigeminal
USA–7				1	Left Trigeminal
USA–8	78	F	Lymphocytic	2	Left Trigeminal
USA–9			Lymphocytic		
USA–10 to 12	40	M	Trauma	1	Right Trigeminal
				2	Left Trigeminal
USA–13 to 15	46	F	Trauma	1	Right Trigeminal
				1	Left Trigeminal
USA–16	25	M	Trauma	1	Right Trigeminal
USA–17	26	M	Trauma	1	Right Superior Cervical
USA–18 to 24	38	M	Trauma	3	Right Trigeminal
				1	Left Trigeminal
				1	Right Superior Cervical
				2	Left Superior Cervical
USA–25	25	M	Trauma	1	Right Trigeminal
USA-26				1	Right Vagus
USA–27	24	M	Drug overdose	1	Left Trigeminal
USA–28				1	Right Trigeminal
USA–31 to 39	59	M	Trauma	9	Left Trigeminal
JAP–1	53	F	Renal Failure	1	Left Trigeminal
JAP-2	6	F	Leukaemia	1	Left Trigeminal
JAP–3	67	M	Liver Cirrhosis	1	Right Trigeminal
JAP–4	73	M	Cancer: Stomach	1	Right Trigeminal
JAP–5	65	M	Cancer: Lung	1	Right Trigeminal
JAP–6	61	F	Polycystic Kidney	1	Right Trigeminal
JAP–7	74	F	Hepatoma	1	Left Trigeminal

See Fig. 17.4 for analysis of DNA of isolates

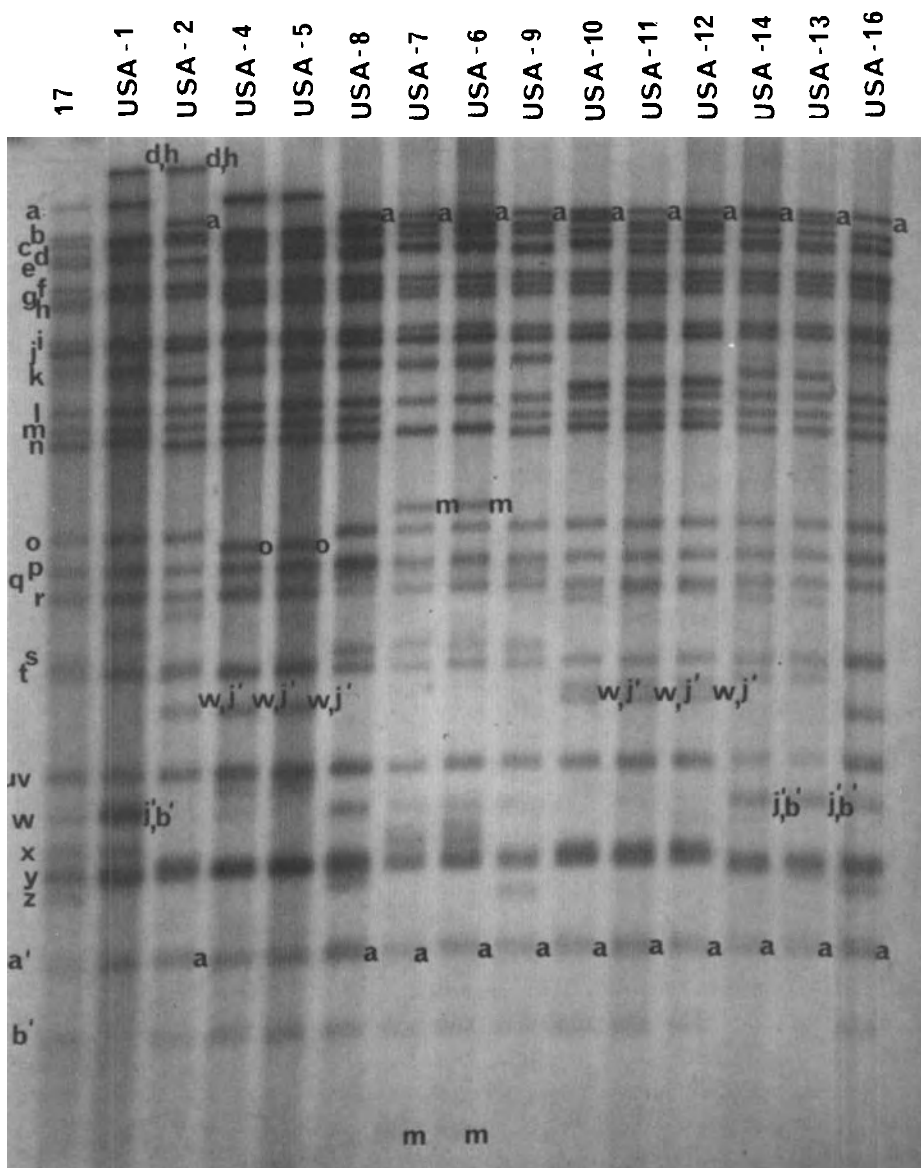


Fig. 17.4. Analysis of DNA of HSV-1 isolates using restriction endonucleases. For key of isolates, see Table 17.3. (after Lonsdale et al., 1980, and figure courtesy of Dr. Chany.)

the virus isolate from each individual could be distinguished. Variations in the restriction endonuclease profiles were not confined to the loss or gain of restriction sites, since variability was also observed in the mobility of certain restriction fragments which could have arisen due to sequence translocations or to local sequence

inversions around an asymmetrically located restriction site. The similarity between strains isolated from labial vesicles and brain biopsies from encephalitis patients is discussed later in this Chapter (page 663) and also in Chapter 12.

17.4.1. Genetic variation among cytomegalovirus (CMV) isolates

Genomic complexity of human CMV is one of the largest among various DNA viruses. With such genome complexity and widespread distribution, even a minimal frequency of mutation will be enough to create a complicated genetic heterogeneity in this virus (Huang and Pagano, 1974).

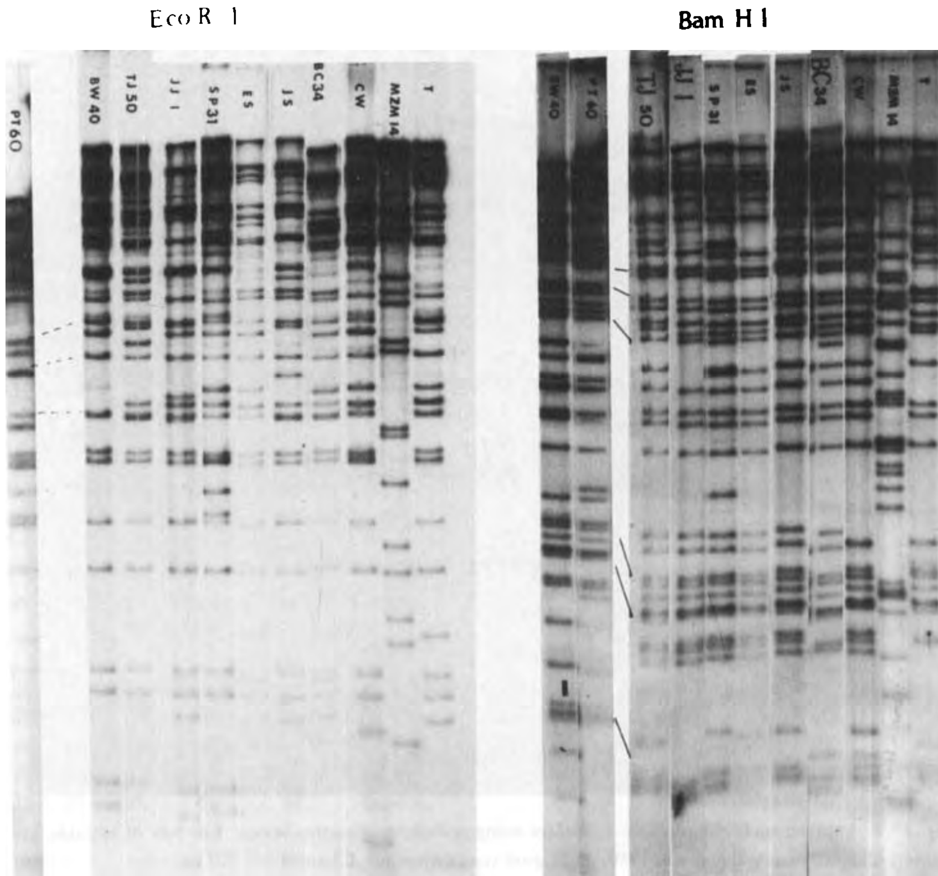


Fig. 17.5. Analysis of DNA of cytomegalovirus isolates using restriction endonucleases. ^{32}P-labelled viral DNA was purified from extracellular virus. Viral DNA (2×10^4 cpm; approximately 1×10^4 cpm per μg) in 20 μl of TBS (Tris-HCl 0.05 M, pH 7.4 and 0.15M NaCl) was digested with 5 μl (5 units) of *Eco*RI or *Bam*HI for 2 to 4 hours at 37°C. The DNA was then subjected to 1% agarose gel electrophoresis. MZM 14 is a chimpanzee isolate; it is shown here for comparison. The rest of the CMV strains are isolated from various epidemiologically unrelated families. (after Huang et al., 1980, and figure courtesy of Dr. E. Huang.)

Restriction enzyme digestion of DNA from various CMV isolates yielded distinct fragment patterns for each isolate. Considerable matching of fragment patterns and comigrating fragments were found among various human isolates; however, no two epidemiologically unrelated isolates have identical fragment patterns (Fig. 17.5). No obvious similarity of restriction fragment patterns is found between human CMV and nonhuman CMV isolates (In Fig. 17.5, MZM 14 is a chimpanzee CMV).

A study of interstrain nucleic acid homology revealed that human CMV strains analyzed shared at least 80% DNA sequence homology with the prototype virus AD-169. The degree of homology existing among human CMV strains appears greater than that between HSV type I and type II (which is about 47 to 50%). CMV strains isolated from the genital tract cannot collectively be distinguished from strains isolated from other sites in sequence homology, as is the case with HSV 1 and 2. (The heterogeneity in restriction enzyme fragment patterns among various strains may merely reflect the rearrangement of the gene(s) order due to recombination as well as certain insertions and deletions of minor gene sequences.) Antigenic heterogeneity among human CMV strains has also been demonstrated by complement fixation, neutralization tests, and by electroimmunodiffusion (Huang et al., 1976, Weller et al., 1960).

Huang et al. (1980) analyzed virus strains isolated from 4 groups of subjects by restriction enzyme fragmentation analysis. Patients included recurrent maternal infections, consecutive congenitally infected infants born to the same family, donor and recipients in blood transfusion and organ transplants and persistently infected individuals.

In order to elucidate whether the recurrent infection is mainly caused by reinfection with a heterologous CMV strain, or by reactivation of the strain causing the primary infection Huang et al. (1980) analyzed the DNA restriction patterns of viruses isolated from eight recurrently infected individuals. Among these eight cases, 6 of them showed almost identical DNA restriction patterns in the repeated isolates when the DNA was analyzed with EcoRI, BamHI and XbaI or HindIII enzymes.

In the other two cases, one showed certain modifications in EcoRI restriction sites. The data implied that the majority of recurrent maternal infections are due to the reactivation of the pre-existing latent virus, although a small portion of recurrent infections might be due to the reintroduction of a new virus strain. Viral DNA sequences appeared to be relatively stable in many cases during several years (2–5) of in vivo residence; however, some minor modifications or variations do gradually occur in some cases.

Studies by the same authors of the DNA restriction patterns of viruses isolated from consecutive congenitally infected infants were performed to investigate the mode of virus transmission and any pattern of recurrency. Huang et al. (1980) found that three pairs of congenitally infected infants born 2 to 4 years apart to the same mothers had identical isolates. Virus strains isolated from the genital tract, throat or urine of 6 women one to four years before or after delivery were compared

with strains isolated from their offspring. In five out of these six cases, viruses recovered from the mother were identical to those recovered from those congenitally or neonatally infected babies. In one case, a minor difference in EcoRI patterns of the mother (JM) and the baby (JS) was found; it is possible that a modification in EcoRI restriction site in the mother's virus recovered 3 years after delivery occurred later in vivo. From the degree of similarity between EcoRI restriction patterns of baby's virus (JS) and mother's virus (JM) and their complete identity in BamHI patterns, Huang et al. (1980) concluded that these two strains were genetically related and of the same origin.

Active cytomegalovirus infection occurs very frequently in organ transplant recipients and in patients receiving blood transfusions during surgery (see Chapter 14). This active infection might be caused either by the reactivation of latent virus of the recipient or the introduction of a new virus strain carried by the organ or blood donor. Huang et al. (1980) analyzed one pair of viruses that were isolated from urine specimens taken from a blood donor and a recipient who became infected with CMV subsequent to transfusion. The CMV infection in the recipient was not associated with clinical illness. By analyzing DNA restriction patterns of viruses isolated from donor (198D) and recipient (198R) with EcoRI and BamHI, it was found that two viruses were not genetically related, which implies that the active infection in this organ recipient was more likely caused by reactivation of the recipient's latent virus via a graft versus host reaction. Huang et al. (1980) also studied a family with three children suffering from haemophilia B who were persistently infected with CMV. Virus strains isolated from child RA on 3/20/73, and 3/27/74 were similar to strain isolates from child WA on 10/17/73. This indicates that before 3/27/74, at least RA and WA were infected by the same virus strain. After 9/25/74, a new virus strain was introduced into this family; all three brothers were infected by this new strain of HCMV. Restriction enzyme patterns of virus isolated after September 1974 from 3 brothers were identical, except for a minor modification in the pattern of the last isolate from RA (9/1/76). Huang et al. (1980) hypothesized that the three members of this family were first infected by a strain of CMV. Thereafter, a new virus was introduced in the middle of 1974. Due to the lack of cross protection by the former immune response, the new virus strain superinfected these three brothers and consequently established a persistent infection. The original virus was not detected after the new strain was introduced, perhaps because the original virus diminished to low titres which could not be detected in the face of the high titre of the new virus strain.

17.4.2. Virulence of herpes viruses

Although considerable progress is being made in our understanding of the 'molecular epidemiology' of both herpes and influenza viruses, the correlation between virus virulence and spread and the precise genetic composition at the genome level (i.e.

genetic determination of virulence) remains to be elucidated. A detailed study to define viral functions responsible for disease patterns caused by herpes simplex virus has used laboratory produced recombinants to locate a portion of the genome which co-segregates with particular ocular disease patterns (Centifanto-Fitzgerald et al., 1982). A laboratory-produced recombinant F(MV)D produced a pattern of dendritic lesions in the rabbit eye characteristic of the donor HSV-1 (MV) virus. Other recombinants produced the typical elongated dendritic lesions characteristic of the recipient HSV-1 (F) virus. The viral functions affecting the ocular disease pattern mapped between 0.70 and 0.83 map units in HSV–DNA within the BGlII F DNA fragment. The data verified clearly that ocular disease patterns produced by wild virus isolates may vary, and are characteristic of each isolate.

As a further application of sensitive fingerprinting techniques one can ask whether a herpes virus causing a CNS disease is identical to virus infecting oro-labial sites in the same patient. Whitley et al. (1982) analyzed paired test isolates from 8 patients in an attempt to answer this question. A total of 92 patients with biopsy-confirmed HSV encephalitis were investigated for excretion of virus from peripheral sites.

Eight patients excreted herpes simplex viruses from oral or labial sites (Table 17.4). Five patients had identical oral or labial and brain isolates according to re-

TABLE 17.4.
Laboratory and clinical findings in patients with herpes simplex virus encephalitis.[a] (after Whitley et al., 1982)

Case No.	Age Yr.	Sex/Race	History of previous HSV infection	Sites of virus isolation		Reciprocal serum antibody titres[b]	
				Brain lobe	Peripheral	Acute	Convalescent
Identical HSV–1							
1	14	F/B	Neg	Left temporal	Mouth	Neg	256
2	53	M/W	+(R.L.)	Left temporal	Lip	64	1280
3	60	F/B	+(R.L.)	Right temporal	Lip	26	485
4	53	M/W	+(R.L.)	Right temporal	Lip	20	512
HSV–2							
5	0.5	M/W	Neg	Right temporal	Mouth	16	N.A.
Nonidentical HSV–1							
6	71	M/W	+(R.L.)	Right temporal	Lip	66	226
7	45	M/W	+(R.L.)	Left temporal	Lip	205	N.A.
8	35	F/W	Neg	Left temporal	Lip	8	52

[a] HSV–1 and HSV–2 denote herpes simplex virus Types 1 and 2. R.L. denotes recurrent labial herpes, and N.A. not available.
[b] Neutralization titre to homologous virus type.
See Fig. 17.6 for analysis of DNA of isolates.

striction-enzyme analyses; four had herpes simplex virus type 1, and one had herpes simplex virus type 2. Three other patients had nonidentical virus isolates from the lip and brain. Examples of the patterns of digested DNA are shown in Fig. 17.6. Minor differences resulting from variable fragments did not discount identity. As shown in Fig. 17.6, the patterns were identical for Cases 1, 2 and 5, but differed in Case 6 (slots 7 and 8). Of the four patients with identical herpes simplex virus type 1 isolates, one patient (case 1) was considered to have a primary infection, because antibodies were not present in the acute-phase serum and there was no history of recurrent herpetic infection. The other three patients, cases 2 to 4, had a history of recurrent herpes labialis and had herpes simplex virus antibodies in their acute-phase serum samples. A six-month-old child (case 5) was found to have identical oral and brain herpes simplex virus type 2 isolates. It was not possible to differen-

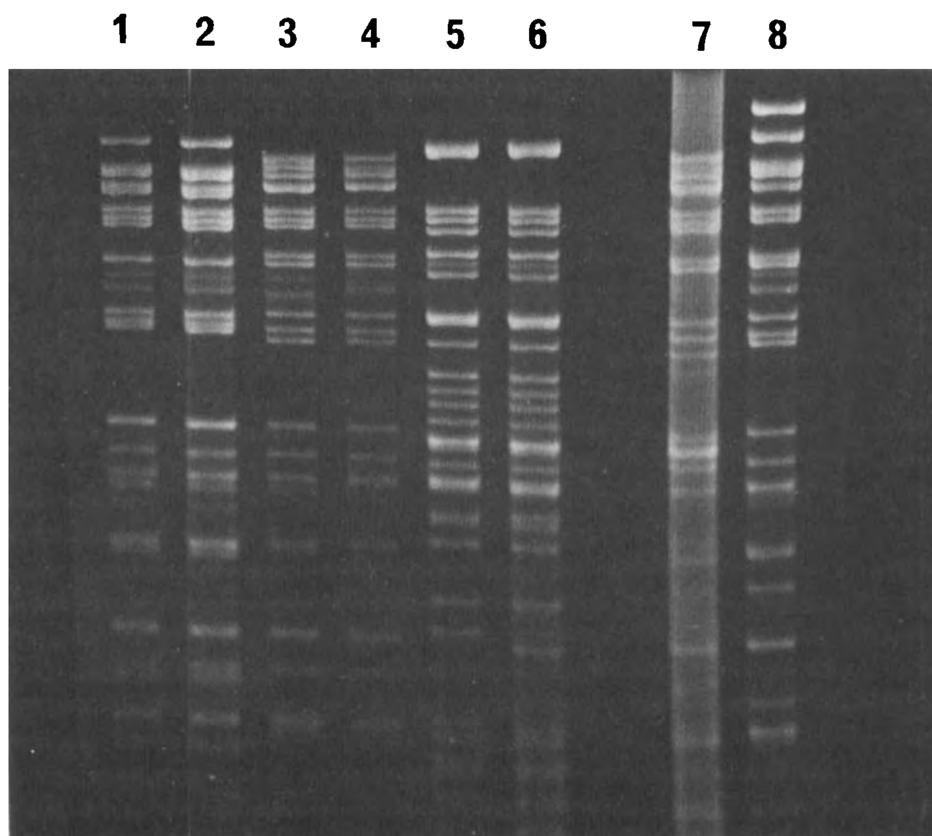


Fig. 17.6. Analysis of DNA of HSV from encephalitis. Slots 1 and 2 refer to mouth and brain isolates from Case 1, Slots 3 and 4 to lip and brain isolates from Case 2, Slots 5 and 6 to mouth and brain isolates from Case 5, and Slots 7 and 8 to brain and lip isolates from Case 6. The patterns are identical in Cases 1, 2 and 5 but different in Case 6. (after Whitley et al., 1982, and figure courtesy of Dr. R. Whitley.)

tiate between a recent infection or reactivation of a neonatally acquired infection. Of the three patients (cases 6, 7 and 8) in whom the paired virus isolates were both type 1 yet different by fingerprinting, cases 6 and 7 had a history of recurrent herpes labialis and had high levels of antibodies in their acute-phase serum, whereas case 8 reported no history of herpetic infection and had low (but not negative) antibody levels in acute-phase serum. Thus, it was difficult to determine whether this patient had a recurrent or a primary infection. In summary, the authors found that only one patient had an unequivocal primary infection with viral isolates that were identical; that five patients had a history of recurrent labial infection – three with identical paired isolates and two with nonidentical isolates, and that two patients had no history of recurrent herpetic infection yet had antibodies in their acute-phase serum, indicating prior subclinical infection (one pair was identical, and the other nonidentical). Herpes simplex encephalitis is thought to occur at a prevalence of one case per million per year – a frequency considerably lower than that of mucocutaneous herpetic infections. Four nonexclusive hypotheses (Whitley et al., 1982) might explain the disparity between the frequencies of herpes simplex encephalitis and mucocutaneous infection; (1) the herpes simplex viruses that cause encephalitis may be unusually neurovirulent; (2) certain patients may have an undefined inborn or acquired host immunological deficiency that facilitates involvement of the brain; (3) subclinical brain involvement may follow primary infection and be subject to clinical or subclinical reactivation later in life; (4) access of the virus to the brain may be a chance event occurring via a neurotropic route after either primary or recurrent infection.

17.5. Rotaviruses

Serological analysis has demonstrated a minimum of four human rotavirus serotypes, but the complete extent of this antigenic variation in human and animal isolates is not yet known (Chapter 9). More recently, several groups (Kalica et al., 1981, Rodger et al., 1981) have examined the electrophoretic mobility of the dsRNA of these viruses to search for evidence of genetic variation (Fig. 17.7). Initially it was assumed that gel electrophoresis would be useful for distinguishing viruses isolated from different hosts but genetic diversity was soon noted among isolates of each animal species. In a rather extensive study, Rodger et al. (1981) identified 19 different human rotavirus electropherotypes among 188 samples circulating in hospitals between 1973 and 1979. When the occurrence of the 19 different electropherotypes was arranged according to the date of collection of the sample it was apparent that the 17 types from children did not occur at random throughout the period 1973–1979. Rather, they showed a sequential appearance, with a limited number of electropherotypes present at any given time. One electropherotype was often predominant in any given period and there was no apparent difference in electropherotypes

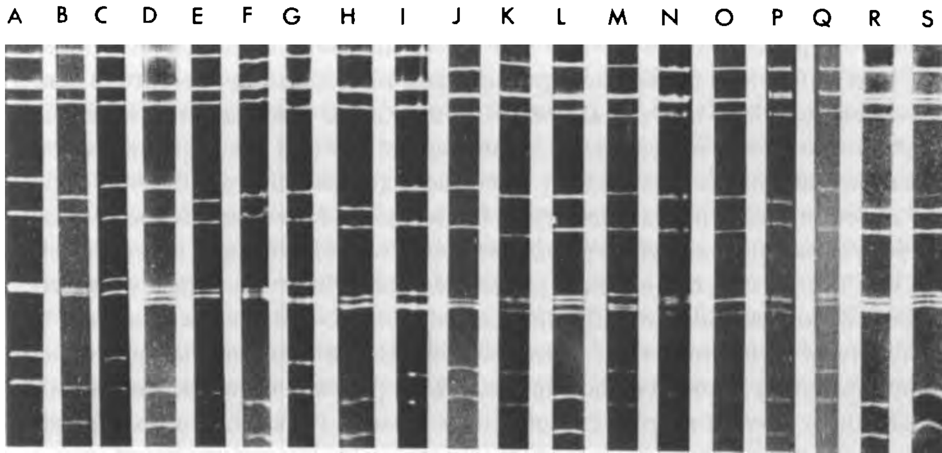


Fig. 17.7. Rotavirus electropherotypes. These 19 different human rotavirus electropherotypes were found among 188 virus samples circulating in hospitals between 1973 and 1979 (Rodger et al., 1981). (figure courtesy of Dr. I. Holmes, University of Melbourne.)

present in children admitted to two hospitals during a period of 1973–1975. Commonly encountered electropherotypes did not appear to change annually although it should be noted that all samples were from children admitted to hospital with acute gastroenteritis and different viruses could also circulate and not be detected causing mild and asymptomatic infections. In general therefore an epidemiological picture is obtained of a constantly changing population of rotaviruses in the community, which, by analogy to influenza described above, may indicate antigenic drift and shift. Also in common with influenza, a number of electropherotypes may be present at any one time in a single city thus potentially allowing dual infections and genetic reassortment. Again, as with influenza, electrophoretic analysis of field isolates of rotaviruses provides more detailed data than serology alone. As an example of this, four of the different electropherotypes examined in the study had all been serotyped as type 2 rotavirus.

17.6. Flaviviruses: Saint Louis encephalitis viruses (SLE)

Flaviviruses are characterized as a group by their structural and biochemical similarities (Chapter 5) but nevertheless within the subgroups differences among strains occur in virulence for laboratory animals and antigenicity. Electrophoretic techniques for the analysis of viral RNAs have begun to play an important role in analysis of genetic variation in flaviviruses. Thus, Trent et al. (1981) have described genetic diversity of SLE strains isolated between 1963 and 1980 from various sources and localities throughout the USA (Table 17.5). In general, the results indicated

TABLE 17.5.
Clustering of Saint Louis encephalitis (SLE) virus isolates by oligonucleotide fingerprint similarity, animal virulence, and geographic distribution (after Trent et al., 1981)

Geographic Area/Epidemiology	Number of strains tested	Percent of strains in cluster	Virulence		
			High	Intermediate	Low
Central and Atlantic States/ Epidemic	17	88 (15/17)[a]	+		
Florida/ Epidemic	5	80 (4/5)[b]	+		
Florida/ Enzootic	2	100 (2/2)			+
Western United States/ Endemic-Epidemic	6	67 (4/6)[c]		+	
Central and South America/ Enzootic	9	89 (8/9)[c]	+	+	+
South America/ Enzootic	3	100 (3/3)[c]			+

[a] Oligonucleotide average similarity coefficient of 0.75 or greater with MSI-7 (virulent strain, Mississippi 1963).
[b] Oligonucleotide average similarity coefficient of 0.75 or greater with TBH-28 (Florida isolate, 1963).
[a] Oligonucleotide average similarity coefficient of 0.75 or greater with BFS-4876 (California isolate, 1963).

that there was a marked degree of similarity among virus isolates of similar virulence characteristics from the same geographic area (Figs. 17.8, 17.9). SLE isolates which are serologically indistinguishable can nevertheless be grouped according to the differences of oligonucleotides. The biochemical classification correlates well with geographical location ('topotype' or geographic genotype), virulence for mice, and epidemiological factors such as vector species. Strains from a geographical area within a 5–10 year time period are more like each other than they are to strains from other areas. During an SLE outbreak, viruses of similar genotype are present throughout the entire geographical area. Also by comparing the 1963 isolates with 1974 isolates from the same area it is apparent that the SLE genome of viruses in a specific area changes over a period of time and distinct new genotypes emerge as the predominant variant. Certainly the viruses change within a 15 year period.

17.7. Poliovirus

Polioviruses are divided into 3 serotypes, but within each serotype exist numerous

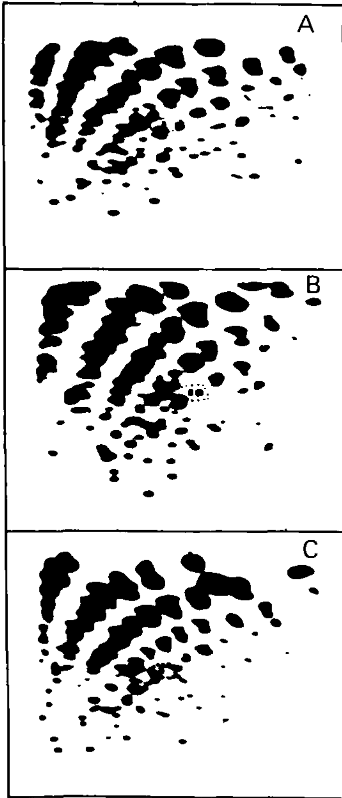


Fig. 17.8. Oligonucleotide fingerprints of RNase T_7 digests of SLE virus (North and Central American isolates). A, MSI-7; B, TBH-28; C, BFS-4876. See Table 17.5 for key. (after Trent et al., 1981, and figure courtesy of Dr. D. Trent.)

subtypes distinguishable by serological techniques (Chapter 4). More recently a number of field isolates of type 1 and type 3 polioviruses have been analyzed by SDS PAGE and oligonucleotide mapping. The latter technique is of particular value because isolates of attenuated virus vaccine origin can be distinguished from 'wild' strains. Moreover, recent work has established that genomes of both vaccine strains and epidemic strains evolve at measurable rates during human intestinal passage (Nottay et al., 1981). This is in marked contrast to in vitro virus passage, which appears to be non-selective for poliovirus. Oligonucleotide mapping has been used by the above group of workers in an interesting study to estimate molecular evolution of type I poliovirus during a single epidemic. In April 1978 paralytic polio occurred in the USA in a non-immunized religious community, apparently transmitted from the Netherlands to Alberta and Ontario and thence to Pennsylvania. The last case was in June 1979, and the total number of reported cases was 133, with 101 paralytic cases and 32 cases of aseptic meningitis (Table 17.6). Oligonuc-

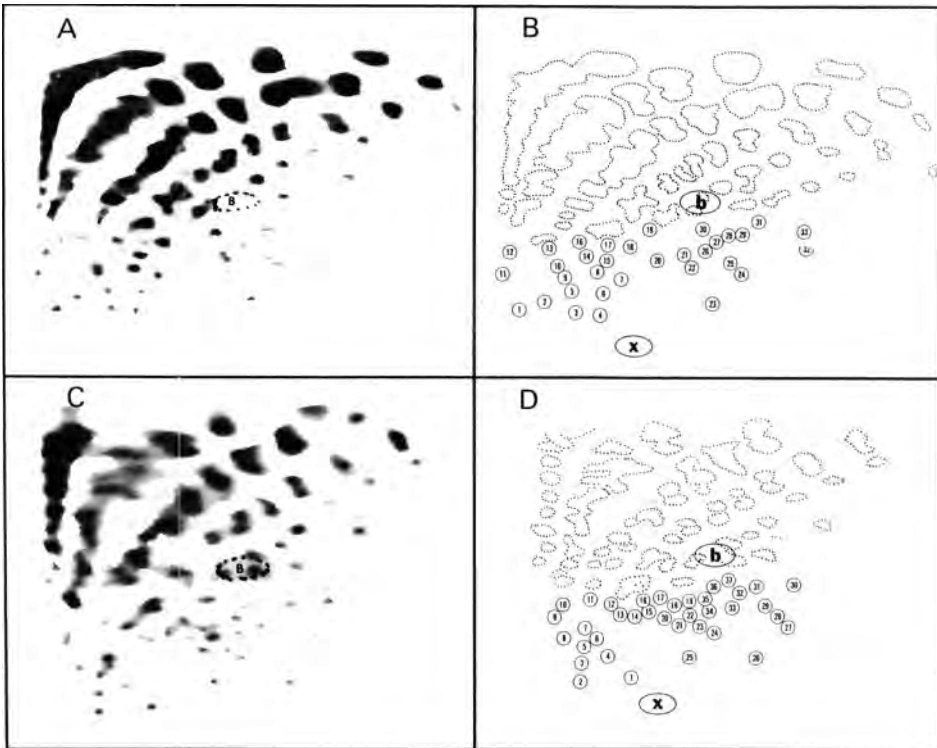


Fig. 17.9. Oligonucleotide fingerprints of RNase T_7 digests of SLE virus (South American isolates). Isolates 78V-6507 (A) and CorAn 9275 (C) are shown with schematic drawings of each of the oligonucleotide fingerprints (B and D). The large oligonucleotides of each virus have been arbitrarily assigned numbers for reference purposes. (after Trent et al., 1981, figure courtesy of Dr. D. Trent.)

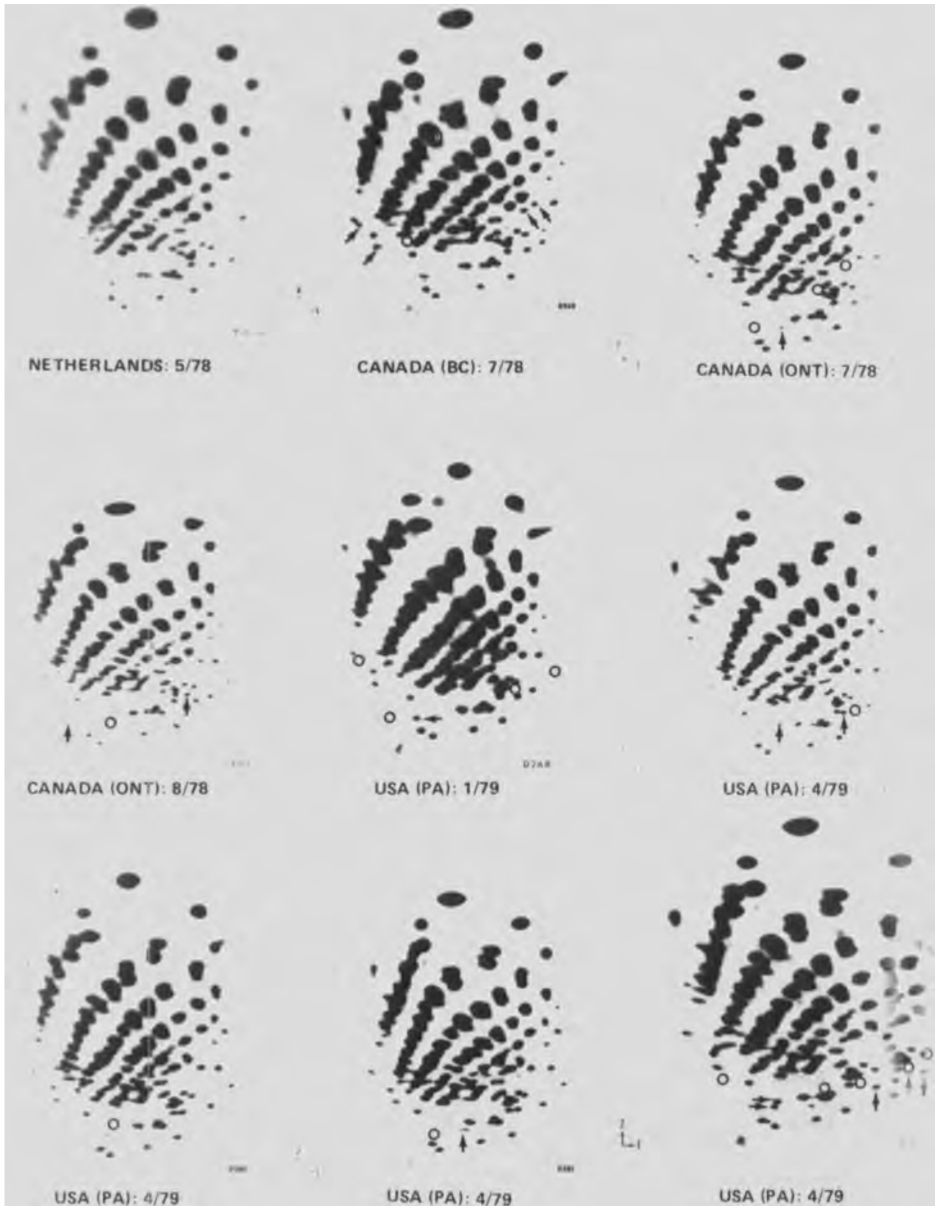
leotide maps of representative strains are shown in the Fig. 17.10 and are arranged in chronological order of isolation beginning with the first Netherlands epidemic isolate (P78-56, May 1978) and ending with a post epidemic isolate (D770, June 1979). The 18 maps are very similar in appearance but small differences were detected in the spot patterns and no two patterns were identical. The most similar maps were isolated from members of the same family, with only a single spot difference. Often several differences are seen from one spot pattern to the next showing the 'plasticity' of the polio genome during person to person passage. There was also the suggestion of highly mutable regions of the genome or, alternatively a dynamic and moveable equilibrium between pre-existing mutants in the virus population since, of course, these techniques measure the 'typical' pattern of a whole population of virions. Comparison of the maps of the earliest and last virus gave an estimate of the minimum rate of genome evolution, which was estimated to be about 1–2% of the genome.

TABLE 17.6.
Polio type I isolates from Netherlands-USA outbreak (after Nottay et al., 1981)

Isolate	Isolation		Patient data	Epidemiologic and clinical background
	Location	Date		
P78–56	The Netherlands	5/78	21m, M	First isolate from 1978 epidemic in The Netherlands; paralytic illness. Strain Elspeet 78–9030
D 510	British Columbia	7/78	26y, M	Paralytic illness. Contacted visitor from the Netherlands
D 511	Ontario	7/78	14y, M	Paralytic illness
D 513	Ontario	8/78	15y, M	Paralytic illness
D 268	Pennsylvania	1/79	22y, F	Index case of 1979 epidemic in the United States, paralytic illness
D 518	Pennsylvania	4/79	24y, F	Third case, 1979, United States, paralytic illness
D 580	Pennsylvania	4/79	–, F	Family contact of third case, not ill
D 581	Pennsylvania	4/79	13y, M	Fourth case, 1979, United States, paralytic illness
D 805	Pennsylvania	4/79	34y, M	Fifth case, 1979, United States; nonparalytic illness (aseptic meningitis)
D 579	Pennsylvania	5/79	36y, F	Seventh case, 1979, United States; nonparalytic illness (aseptic meningitis)
D 598	Wisconsin	5/79	15y, F	Family contact of eighth case; not ill
D 871	Wisconsin	5/79	4y, M	Ninth case, 1979, United States; paralytic illness
D 658	Iowa	5/79	11y, F	Tenth case, 1979, United States, paralytic illness
D 790	Iowa	5/79	16y, M	Eleventh case, 1979, United States; paralytic illness
D 873	Wisconsin	5/79	2y, M	Family contact of twelfth case; not ill
D 930	Pennsylvania	5/79	2y, M	Thirteenth case, 1979, United States; paralytic illness
D 663	Missouri	6/79	20y, F	Fourteenth case, 1979, United States; paralytic illness
D 770	Michigan	6/79	4y, M	Member of Amish community in which no poliomyelitis occurred; not ill
P78–57	South Carolina	10/77	3m, M	Paralytic illness with onset 2 weeks after receipt of oral polio vaccine

Isolate	Isolation		Patient data	Epidemiologic and clinical background
	Location	Date		
1–2181	Idaho	10/77	14m, M	Hospitalized with diagnosis of encephalitis or poliomyelitis. No history of polio vaccination
D 310	New York	1/79	7m, M	Hospitalized with pneumonia and muscle weakness. Received second dose of oral polio vaccine 2 months before. Not considered a case of poliomyelitis
1117	Kuwait	1977	–	Isolated from a case of paralytic illness in Kuwait. Strain 04958
1116	The Netherlands	1971	5y, M	Isolated from a case of meningitis during epidemic in Staphorst. Strain Schraa 1 C
1115	The Netherlands	1971	7y, M	Isolated from a case of meningitis occurring near Staphorst. Patient had received oral polio vaccine about 3 weeks before. Strain ZZ-2
I–2171	New York	10/77	34y, F	Fatal case of paralytic illness. No history of recent polio vaccination
I–2137	New York	10/76	47y, F	Hospitalized with diagnosis of possible Guillain-Barre syndrome. No history of recent polio vaccination
I–2170	Florida	11/77	1y, F	Paralytic illness. Child of migrant workers from Mexico
I–2177	Bahama Islands	10/77	4y, M	Paralytic illness. No history of polio vaccination
I–2162	Dominican Republic	4/77	–	Paralytic illness. No history of polio vaccination
I–2191	Nicaragua	6/77	5y, M	Paralytic illness. No history of polio vaccination
1077	Nicaragua	5/78	–	Paralytic illness. No history of polio vaccination
I–2127	Greece	5/76	4m, M	Paralytic illness
I–2178	Turkey	10/77	3m, –	Paralytic illness
137	Ohio	8/78	11y, M	Fatal case of paralytic illness (paralysis of throat). No history of polio

In contrast with the marked biochemical similarities of the above closely related epidemic strains, oligonucleotide maps of epidemiologically unrelated strains were distinct. A large number of distinguishable type 1 strains exists world wide as shown by fingerprinting. Isolates from different epidemics within the same country (P78-56, 1978 and 1116, 1971) showed no genetic relationship to each other (Fig. 17.11).



Also, contemporary isolates from neighbouring countries with endemic polio, generally appeared different (I-2177, I-2162 and I-2191 or I-2127 and I-2178, Fig. 17.10). During a short period several genetically unrelated type I strains were found

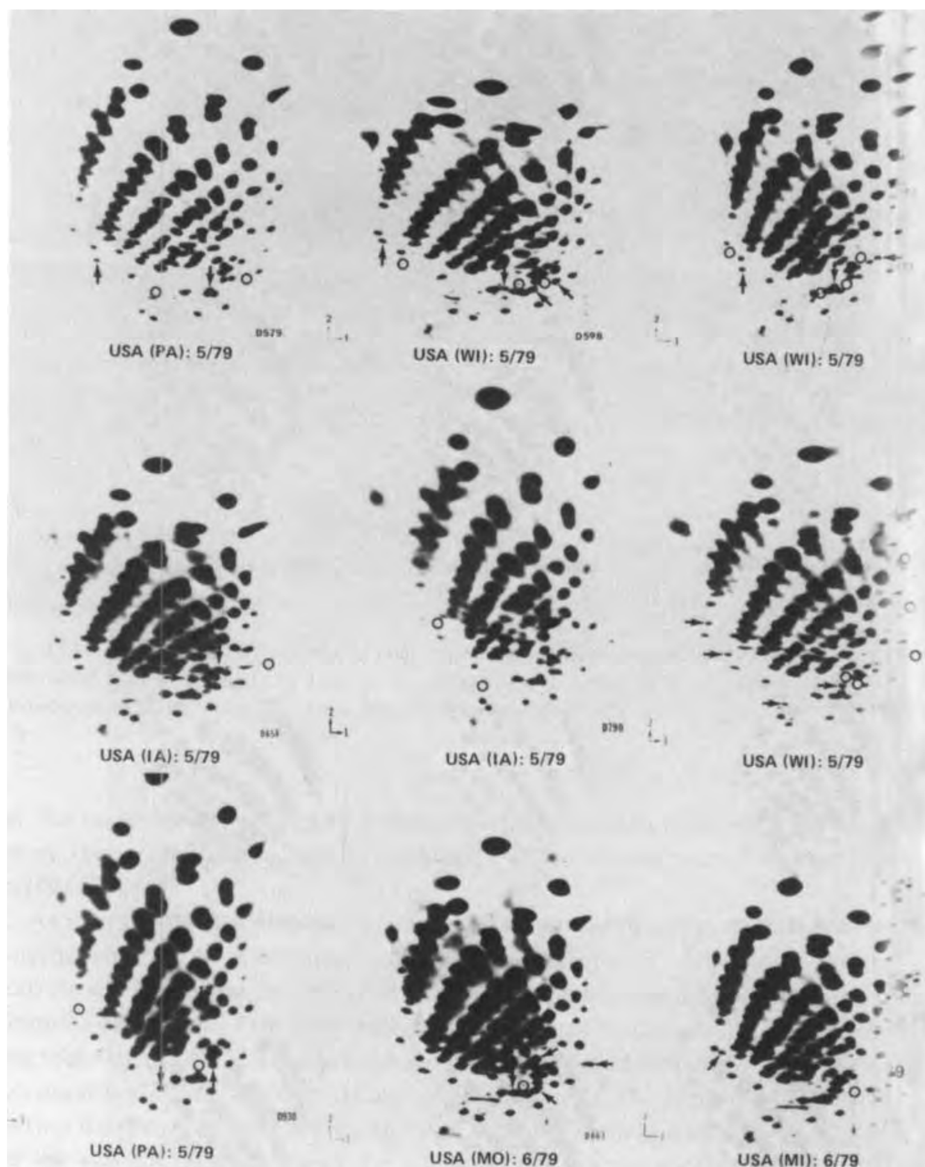


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Fig. 17.10. Oligonucleotide maps of type 1 isolates from 1978–79 polio epidemic (Netherlands–Canada–U.S.A.). Maps are arranged according to the date of strain isolation. Arrows (new spots) and open circles (missing spots) represent differences in each pattern from the next earlier map. (after Nottay et al., 1981) (figure courtesy of Dr. O. Kew, CDC Atlanta.)

Fig. 17.11. Oligonucleotide maps of type I polioviruses independently isolated in different countries. (after Nottay et al., 1981) (figure courtesy of Dr. O. Kew, CDC Atlanta.)

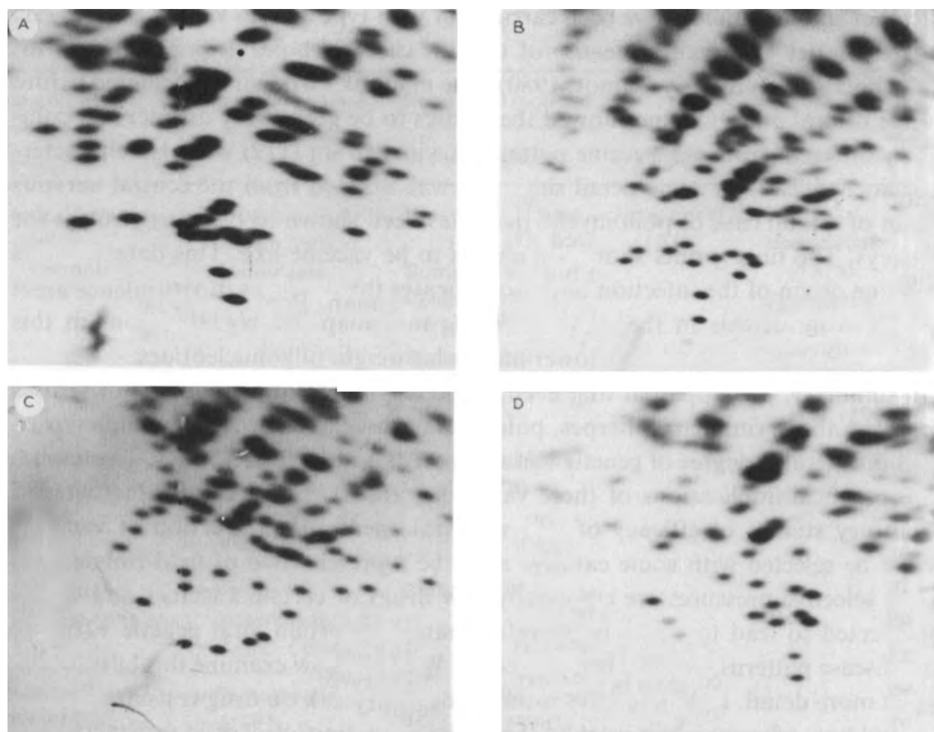

Fig. 17.12. Oligonucleotide mapping of polio type 3 vaccine and vaccine associated viruses. A, Sabin attenuated vaccine strain (Leon Type 3); B, wild polio type 3 virus (UK); C, wild polio type 3 virus (Bombay) and D, vaccine related virus. (figure courtesy of Dr. P. Minor.)

in the same country. Even in geographically small countries such as Nicaragua more than one epidemic strain could be isolated in one year (compare maps of I-2191 and 1077).

As regards the usefulness of the mapping technique to detect differences between vaccine strains and 'wild' strains of polio, the fingerprints of the vaccine strain LSc 2ab closely resembled that of its wild parent Mahoney and a similar number of differences distinguishes the field isolates from the oral vaccine strain. The fingerprinting technique is useful for identifying viruses in cases of suspected vaccine associated paralysis. Thus, the fingerprints of the isolate I-2181 from a 14 month infant with a diagnosis of encephalitis and poliomyelitis showed a close similarity to that of the vaccine virus, although the infant had no known contact with vaccine and serological tests had indicated a non-vaccine like polio virus as the causative agent. The virus isolate was unlikely to have been a recent isolate of unattenuated Mahoney because if wild progeny of Mahoney virus were existent today the virus fingerprints would differ significantly from those of parental virus.

Rather similar studies have been carried out with type 3 polio viruses (Fig. 17.12) since the latter are often the cause of the few cases of polio in populations with a high degree of immunity (Minor, 1980). The maps of 3 wild viruses isolated before the use of oral polio vaccine showed the viruses to be genetically distinct and completely different from the vaccine pattern. A virus strain (119) was also characterized biochemically in some detail since this was isolated from the central nervous system of a fatal case of poliomyelitis and has been shown to be neurovirulent for monkeys. The fingerprints showed the virus to be vaccine-like. This data suggests a vaccine origin of the infection but also indicates that changes in virulence are not necessarily detectable in the oligonucleotide map. Presumably mutations in this case could have occurred in the lower molecular weight oligonucleotides.

In summary, it is apparent that even in the few representative groups of viruses presented above (influenza, herpes, polio, rotaviruses, flaviviruses and alphaviruses) a considerable degree of genetic variation exists among field isolates. Two immediate practical implications of these variations are (i) viruses chosen for detailed laboratory studies of efficacy of new antiviral agents or production of vaccines should be selected with some care, so as to be representative of field isolates; (ii) strong selective pressures are exerted by new drugs or certain vaccines and would be expected to lead to emergence or dominance of certain viral genetic variants. New disease patterns could thus be expected. We shall now examine this latter question in more detail. How extensive is the laboratory work on drug resistance in viruses and how relevant is this work to the clinical situation? Will drug resistance constitute a dominating problem with an extended use of antivirals?

17.8. Early studies of drug resistance to viruses

Probably the first description of virus drug resistance was by Melnick et al. (1961) who described enterovirus mutants resistant to guanidine inhibition (Table 17.7). Drug resistant poliovirus emerged after a single passage in tissue culture and drug resistant virions were also detected in experimentally infected monkeys undergoing drug treatment. It was even possible that the rapid development of drug resistance in the monkeys resulted in a reduced in vivo efficacy of the compound. An interesting aspect of further studies with poliovirus was the documentation of guanidine *dependent* mutants of polio (Loddo et al., 1962) which required the presence of guanidine for growth. In a similar way drug resistant mutants to vaccinia virus and thiosemicarbazones were obtained after two passages in tissue culture (Appleyard and Way, 1966) and mice. The resistant viruses retained their pathogenicity when re-inoculated into animals. Early studies (Oxford et al., 1970) showed that amantadine resistant influenza A viruses could be selected by passage of virus in vivo in mice treated with very high (150 mg/kg/day) doses of the drug. Before passage the influenza A virus was inhibited by 0.3 μg/ml amantadine whereas after a single pas-

TABLE 17.7.
Acquired resistance to antiviral compounds

Compound	Alternative names	Resistance reported		
		In tissue culture	In animals	In man
5-iodo-2'-deoxyuridine	idoxuridine (IUDR), Stoxil, Kerecid, Herpid, etc.	Renis and Buthala (1965) Buthala (1964) Centifanto and Kaufman (1965) Smith (1963)	Jawetz et al. (1970)	Hirano et al. (1979) Jawetz et al. (1970) Isobe et al. (1982)White et al. (1968)
9-β-D-arabinofuranosyladenine	adenine arabinoside, Ara-A, Vira-A, Vidarabin	Coen et al., (1982) Gauri (1979)		
1-β-D-arabinofuranosylcytosine	cytosine arabinoside, Ara-C, Cytarabin	Buthala (1964)		
5-trifluoromethyl-2'deoxyuridine	trifluorothymidine (TFT)	Guari (1979) Field et al. (1981)		
9-(2-hydroxyethoxymethyl) guanine	acyclovir (ACV), acycloguanosine, Zovirax	Coen and Schaffer (1980) Field et al. (1980) Schnipper and Crumpacker (1980)	Field (1982) Smith et al. (1980)	Burns et al. (1982) Crumpacker et al. (1982) Sibrack et al. (1981) Wade et al. (1982)
E-5-bromovinyl-2'-deoxyuridine	(BVDU)	Field and Neden (1982)		
2'-fluoro-5-iodo-1-β-D-arabino furanosylcytosine	(FIAC)	Trousdale et al. (1981)		
Phosphonoacetic acid	(PAA)	Honess and Watson (1977) Klein (1975) Klein and Friedman-Klien (1975)		
Phosphonoformic acid	Foscarnet (PFA)	Eriksson and Öberg (1979)		

TABLE 17.7 *(continued)*

Compound	Alternative names	Resistance reported		
		In tissue culture	In animals	In man
N-methylisatin-β-thiosemicarbazone (and closely related compounds)	Methisazone Marboran		Appleyard and Way (1966) Katz et al. (1974b).	Appleyard and Way (1966)
adamantanamine hydrochloride	amantadine Symmetrel		Cochran et al. (1965) Ilyenko (1975) Oxford et al. (1970b)	Oxford et al. (1970a) Oxford et al. (1970b) Heider et al. (1981)
guanidine			Loddo et al. (1962) Horodniceanu et al. (1963) Melnick et al. (1961)	Barrera-Oro and Melnick (1961)

Note: for these detailed references, see Field, 1983.

sage in the presence of the drug 6 μg/ml of amantadine was required to inhibit replication of some isolates. After six passages in vitro most strains were completely resistant to amantadine and the related drug rimantadine. The drug resistant viruses were still virulent for mice.

More recently a number of laboratories have investigated the genetic basis of amantadine resistance by producing recombinants between amantadine resistant and amantadine susceptible influenza A viruses. (Scholtissek and Faulkner, 1979, Lubeck et al., 1978, Hay et al., 1979, Hamzawi et al., 1981). In this way transfer of drug resistance can be correlated with transfer of a particular gene or group of genes. At present the results from different laboratories are somewhat conflicting, although several groups agree that gene 7 (coding for matrix protein) appears to co-segregate with amantadine resistance. Hay et al. (1979) analyzed the recombinants for gene composition and susceptibility or resistance to amantadine. A limited number of recombinants were analyzed but a correlation was noted with drug resistance and gene 7. The interpretation, however, is complicated by the observation that using different in vitro techniques the same influenza A virus may appear inhibited or resistant to the drug (Scholtissek and Faulkner, 1979). In addition, data on determinants of influenza virus virulence (Mayer et al., 1973, Ogawa and Ueda, 1981) or plaque properties suggest a multi-gene linkage with these properties. More recent data has suggested that inhibition of fusion mediated by HA may explain the antiviral activity of amantadine (see Chapter 7).

TABLE 17.8.
Characteristics of several mutants of herpes simplex virus selected for resistance to acyclovir (after Field, 1983)

Virus strain	TK induction (% wild type)	Probable nature of resistance	ED_{50} (μg/ml) measured in BHK cells						i.c. Inoculation of mice (p.f.u./LD_{50})
			ACV	BVDU	IUDR	TFT	TFT[e]	PAA	
C1(101)[a]	100	—	0.1	0.03	1	0.3	2	4	1
C1(101)TK⁻[c]	<1	TK-defective	7	>10	>10	0.2	>10	5	2×10^2
C1(101)TK⁻ P7	<1	TK-defective+ DNA pol	30					100	2×10^4
C1(101)P_2C_6	2	TK-defective	40	1–>10	>10	0.2	>10	20	$>10^2$
C1(101)P_2C_5	100	DNA pol	25	0.03	1	0.3	0.6	2	3
SC16[b]	100	—	0.02	0.01	0.2			<10	7
SC16R_5C_1	<1	TK-defective	7	>10				<10	5×10^4
SC16R_9C_2	3	TK-defective+ DNA pol	>50	>10				70	$>10^5$
SC16B1[d]	<1	TK-defective	1	5	>10			<10	$>10^3$
SC16S1	30	TK-altered	3	0.2	0.5			<10	60
SC16B3[d]	>100	TK-altered+ DNA pol?	0.03	>10	>10			<10	24
SC16 Tr7	>100	TK-altered	6	0.1	0.3			<10	50

[a] Wild type HSV-1 strain
[b] Wild type HSV-1 strain
[c] Selected for resistance to BUdR, not ACV; also known as 'B2006' or 'MDK'
[d] Selected for resistance to BVDU, not ACV
[e] Test performed in BU-BHK (TK^-) cells
ACV, acyclovir; BVDU, bromovinyldeoxyuridine; IUDR, idoxuridine; TFT, trifluorothymidine; PAA, phosphonoacetic acid; BUDR, 5-bromodeoxyuridine

It is difficult to envisage how matrix protein could influence HA induced fusion unless binding of M to HA occurs so resulting in a configuration change in HA. The data is obviously conflicting at present and further work is required both with recombinants and also to investigate fusion events with these varying influenza viruses. It is quite possible that several gene products are involved and that the product of gene 7 may exert a dominant effect whilst other proteins (e.g. HA) may have 'helper' activity. This would be more consistent with current ideas of cooperation of multiple genes in determining biological characteristics of influenza viruses.

At present very little field work is being carried out to search for rimantadine or amantadine resistant viruses in contacts or in persons being treated for influenza. This is an important aspect of field investigations since it is quite likely that amantadine resistance could spread by recombination. Some influenza H3N2 and H1N1 viruses circulating at present in the community are known to be recombinants (see above) containing genes of both virus sub-types (Ghendon et al., 1981) and thus intra or inter-typic recombination is probably occurring with a relatively high frequency.

A detailed study of rimantadine resistance has been reported by Heider et al. (1981) from Berlin. The group looked at the sensitivity of 21 influenza A isolates, all H3N2. The tests were set up in a biological way, looking at these virus strains versus a series of concentrations of amantadine and rimantadine, using a haemadsorption test. The cells were pretreated with amantadine and rimantadine for half an hour, infected with the viruses, incubated overnight and haemadsorbed the next day. The majority of the viruses were relatively sensitive to rimantadine. A few of them fell into a group that were highly sensitive and two, both isolates from Berlin, were almost totally resistant. The only possible doubt concerning the validity of the data is the rather flat nature of the dose response curve with the sensitive strains examined using this test system. Rimantadine has not been used at all in the East German community to date as an antiviral, so there is a remote possibility that someone with Parkinson's disease and being treated with amantadine was infected with an influenza virus. The other possibility is that the German authorities are using live attenuated influenza vaccine strains with A/PR8/34 (H1N1)-like genes in them, including gene 7. So there is a possibility of gene 7 from PR8 (an amantadine resistant virus), for example, moving around by reassortment in some of those strains, and that could be conferring a degree of amantadine resistance on some of the viruses. Recently occasional amantadine resistant field strains of H3N2 and H1N1 viruses have been detected in the UK (Chapter 7).

17.9. Drug resistant herpes viruses – newer antivirals

As mentioned briefly in the Introduction, we can anticipate herpes virus mutants with lesions in the TK enzyme and the DNA polymerase enzyme resulting from the use of the newer antivirals (acyclovir, foscarnet, BVDU) which target towards one or other or both of these virus induced enzymes.

TABLE 17.9.
Resistance of HSV PAA^R mutants to acyclovir (after Schnipper and Crumpacker, 1980)

Virus	ID_{50} μM	TK activity (%)
HSV-1 KOS (parent)	0.72	100
PAA^R-5 (9)	23.0	105
PAA^R-K	8.2	90
HSV-2 (strain HG52)	1.1	100
PAA^R-B1 (strain HG52)	7.2	109

17.9.1. Thymidine kinase (TK) mutants

An early characterized HSV mutant was C1(101) B2006 with no detectable TK activity and selected using BUDR (Dubbs and Kit, 1964). TK defective mutants are resistant to all TK mediated antiviral drugs including idoxuridine, acyclovir and BVDU but not to foscarnet or TFT, or vidarabine (Table 17.8).

Any of the TK mediated drugs may be used to select for mutants with TK$^-$ phenotype, and TK$^-$ mutants generally emerge after a single passage. Clinical isolates may contain up to 5% of variants resistant to idoxuridine, BVDU or acyclovir.

PAA resistant strains of HSV-1 have been obtained by virus passage in vitro in the presence of 100 μg/ml of PAA (Schnipper and Crumpacker, 1980). After two serial passages one of the PAA mutants selected (PAA^R-K) had an efficiency of plating in PAA (100 μg/ml) of 0.05 compared to 0.004 for the parent virus (Table 17.9). The PAA^R viruses were also resistant to acyclovir. These resistant viruses induce normal levels of TK activity and therefore the PAA^R phenotype presumably is the result of a mutation in the structural gene for viral DNA polymerase.

PAA resistant markers segregate as single determinants among recombinant progeny of conventional crosses in genetic experiments and have been located within regions of less than 1.3 or 2.6 kilo base pairs at approximately 40.2 to 41.0 map units of the HSV-2 and HSV-1 genomes. The coding region for the determinants of PAA resistance can be accommodated within the coding region of the major polypeptide of the virus DNA polymerase (a polypeptide of molecular weight 180 K coded for by less than 4.6 kilobases).

In an interesting study Smith et al. (1980) demonstrated that 6 of 15 herpes viruses initially had 1% or more infectious virions capable of forming plaques in the presence of 10 μmol of acyclovir (Table 17.1). It would appear that the earlier in vitro studies of the antiviral activity of acyclovir were carried out using the most sensitive laboratory virus (KOS) and the most sensitive cell type (VERO). If experiments are carried out using human diploid cells and other strains of HSV-1 then the therapeutic index of acyclovir is lower. This emphasizes the future importance of developing suitable standardization methods for antivirals, an area conspicuous at present for lack of investigation.

Mutants with lesions affecting the DNA polymerase enzyme can also be selected using foscarnet and these are sometimes, but not always, co-resistant to acyclovir (which, interestingly gave early evidence that acyclovir had at least two sites of action viz. TK and DNA polymerase enzymes). Some acyclovir resistant DNA polymerase mutants are fully sensitive to foscarnet (e.g. $C1(101)P_2C_5$) (Table 17.8).

At present most work has been carried out with acyclovir resistant mutants and some interesting and applicable points are highlighted. In general 5 types of mutant with the following properties can be isolated:

a. induce low or non detectable levels of TK
b. induce altered TK
c. induce altered DNA polymerase
d. combination of (a) and (c)
e. combination of (b) and (c)

but mutants in group (a) are most easily isolated and probably occur naturally in isolates with a frequency of 0.1%. They may be 100–1000 times less sensitive to inhibition by acyclovir. It is probably the case that a real degree of heterogeneity exists among these TK deficient viruses with different virion populations exhibiting different (but reduced) degrees of TK activity. In contrast, mutants with *altered* specificity of TK or DNA polymerase are less commonly isolated and are selected in serum starved cells where TK^- variants do not grow, so allowing emergence of other mutants. Mutants with double lesions in both TK and DNA polymerase enzymes are usually highly resistant (e.g. C1(101) TK^- P7 and SC16 R_9C_2). They tend to emerge after lengthy passage in the presence of high concentrations of drug.

One should not immediately conclude however that mutants arise with equivalent ease in vivo because at present evidence exists to suggest that they do not – so certain selective conditions in vivo must be different. Probably one of the more interesting observations is that TK deficient variants of HSV are not able to replicate so well in the skin and neural tissue and may have a 200 fold reduction in virulence. However, mutants in category (b) or (c) above more or less retain their virulence characteristics in laboratory model infections.

Now that acyclovir is being used on a wider scale in the clinic it is not surprising that drug resistant herpes viruses have been isolated, particularly from immunosuppressed patients who may have protracted infection and virus shedding (Burns et al., 1982). The drug resistant viruses characterized to date have been TK defective. Acyclovir resistant viruses were isolated from two bone marrow transplant patients after treatment with acyclovir. The first patient was a 14-year-old boy with acute lymphocytic leukaemia. After cyclophosphamide and irradiation treatment an HSV positive lip lesion developed and he was treated with 250 mg/m^2 acyclovir body surface area intravenously every 8 hours for 7 days. After 4 days on acyclovir the lesions began to crust and heal. On the sixth and seventh days of therapy, no virus was cultured from the lesions, which continued to heal. Virus was, however, isolated from the throat 1 and 2 weeks after therapy, although no new lesions were ob-

served. Seven isolates of HSV obtained before and during acyclovir therapy had normal susceptibility to the drug in vitro with ID_{50} values of 0.07 to 0.31 μg/ml. Virus isolates from the throat 1 and 2 weeks after completion of acyclovir therapy were markedly less sensitive to the drug with ID_{50}s of 2.4 to 5.5 μg/ml.

Patient 2 was a 50-year-old man with acute myelofibrosis and myeloid leukaemia. On the day before transplantation an active herpetic infection developed on his upper lip and left nostril. By 8 days after transplantation, lesions continued to progress and the patient became hoarse. Laryngoscopic examination revealed herpetic-like vesicles on the epiglottic and laryngeal surfaces. The patient was then started on acyclovir at a dose of 250 mg/m^2, intravenously every 8 h for 7 days. Repeat laryngoscopic examination the next day revealed no progression of the laryngeal lesions and by the second day of therapy the patient's voice was normal, with the nostril and lip lesions showing crusting and healing. The lesions were virus negative. 3 days after completion of acyclovir therapy, while lesions continued to heal, virus was again shed in the oropharynx and the ID_{50}s of these isolates (3.3 to 16.1 μg/ml) were significantly higher than those of earlier isolates (0.04 to 0.46 μg/ml) from this patient.

In the long term it would be surprising if herpes viruses did not adapt by mutation to circumvent particular inhibitors but such mutation could well upset the delicate host-parasite relationship which herpes viruses often enjoy. Unexpected and new patterns of disease could then occur. However, to conclude on an optimistic note, the increasing number of drugs with different modes of action might make it possible to reduce a problem of resistance development by using drug combinations.

17.10. Summary

Biochemical studies of virus genomes (both RNA and DNA viruses) have shown that a remarkable degree of genetic diversity occurs. Similarly, antigenic analysis of many human pathogenic viruses using monoclonal antibodies has highlighted antigenic variation among DNA and RNA viruses. Selective pressures exerted by vaccines and antivirals would be expected to allow the selection and emergence of 'new' viruses, perhaps causing modified disease syndromes. Certainly drug resistant viruses can be selected with ease against all the existing antivirals.

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