Cover photograph. Two cytomegalovirus nucleocapsids enclosed in a membrane structure. (Original magnification ×187 500. Photo reproduced by permission of Dr J. Almeida, The Wellcome Research Laboratories, Beckenham, Kent, UK)

Textbook of Medical Virology

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Preface

Virus infections have influenced the life of man throughout history, but knowledge about viruses and the diseases they cause is of relatively recent origin. The expansion of virological knowledge has been markedly determined by the availability of methods for growing viruses under laboratory conditions and for the biochemical characterization of virus particles and molecular events in infected cells. Thus advents such as the introduction of cell culture techniques, the development of techniques for polypeptide analyses and detailed nucleic acid characterization have been of major importance.

It has become increasingly possible to describe pathogenic events in molecular terms although a great deal still remains to be discovered. The comprehensive knowledge available about virus replication now allows a rational approach to the design of antiviral drugs. It can be foreseen that a number of antiviral compounds will be introduced during the forthcoming decade. These drugs will be an important supplement to the currently used immunoprophylactic measures which have already provided some of the major advances in biomedicine. Vaccines also will be used more extensively in the future and new products will be developed.

This book encompasses the whole field of medical virology. The first part of the book describes the structure and chemical composition of virus particles and the biological and immunological activity of individual virus components. The next section analyses the interaction between viruses and cells. The capacity of viruses to enter cells, the modes of replication of DNA and RNA viruses and the mechanism of release of viruses are discussed separately. Before the subsequent section of the book which presents the pathogenesis of virus infections, the effect of viruses on cellular functions and the genetics of viruses are reviewed. The pathogenesis of virus infections include chapters on acute, congenital, persistent, 'slow' and tumorigenic infections and on defence mechanisms against virus infections. Hereafter, separate chapters discuss laboratory diagnosis of virus infections, their epidemiology, and prevention by use of immune prophylaxis and antiviral drugs. The last section of the book concentrates on special virology, focusing in separate chapters on the major virus groups and summarizing the information on viral syndromes in a concluding chapter.

It is our hope that this book will enable the reader to comprehend the disease process in virus infections in man and to understand the methods currently available for diagnosis and prevention.

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1

Viruses – a unique kind of infectious agent

Erik Lycke and Erling Norrby

Our first awareness of the infectious agents which later became known as virus (L. poison) dates back to the turn of the century. It had been observed that there were infectious diseases which were caused by agents lacking the capacity to replicate on artificial substrates and that these agents were smaller than all previously known infectious agents. Unicellular organisms, arranged according to decreasing size and complexity, include protozoas, fungi, bacteria, mycoplasmas, rickettsia and chlamydia. These groups of cellular microorganisms differ distinctly from viruses with regard to many basic properties.

Viruses can only replicate in living cells. As a consequence two separate phases can be distinguished in the life-cycle of a virus. During one of these phases the virus occurs outside cells in the form of a virus particle. This particle is a passive transport vehicle which provides opportunities for a spread of infection both from cell-to-cell within the multicellular organism and between individuals. During the other phase the virus resides inside the infected cell where replication may occur. This replication includes a synthesis of new virus-genetic material and new virus-specific proteins. Other building materials for virus particles as well as the necessary energy and main machinery for the assembly of a virus are provided by the infected cell. Certain unicellular organisms, e.g. rickettsia and chlamydia, replicate in cells but in contrast to the replication of viruses this occurs through a growth and division of the organisms. Furthermore, both rickettsia and chlamydia like other cellular organisms contain both DNA and RNA whereas the genetic material of a virus is represented by either one of these nucleic acids. As a consequence a virus displays a relatively more advanced form of cellular parasitism. Concerning certain intracellular functions of a virus there are similarities with functions carried by some extrachromosomal genetic elements in cells, i.e. episomes and plasmids. However, as distinct from these kinds of cellular genetic material, a virus has an independent extracellular form of transmission.

Viruses occur not only in mammals but also in insects, plants and prokaryotes, e.g. bacteria. From a practical point of view one therefore refers to animal, insect, plant and bacterial viruses. The latter viruses are usually called bacteriophages (Gk. *phagein* = to eat). Certain viruses have a capacity to replicate in completely different hosts, for example both in mammalian cells and in insect cells. Bacteriophages, however, are limited to replication in prokaryotic cells only.

Replication of an animal virus in cells can lead to their destruction. If the replication of a virus in a particular organ is widespread this may lead to such extensive destruction that symptoms of disease appear. Thus, viruses may cause

acute degenerative diseases. Such diseases are common. In industrial societies children in the pre-school age usually have 5–7 virus infections per year. About half of all absenteeism from work and school is considered to be caused by virus diseases. Most often these diseases are of a rather trivial nature such as the uncomplicated common cold.

Every year new variants of known viruses and often completely new viruses are discovered. In the case of some of these newly discovered agents methods for laboratory cultivation are not available and frequently it is not known whether the agent has the capacity to cause disease. In spite of our increasing knowledge of the complex capacity of virus infections to influence cellular and organ functions we still do not have an overall view of the total medical importance of virus infections.

The possibilities of specific treatment of virus diseases are still rather limited but developments concerning preventive treatment, i.e. immune prophylaxis have been a major advance in modern medicine. Smallpox has disappeared from our world. Poliomyelitis has become a very rare disease in many industrial countries. Yellow fever has been brought under control. Vaccines against measles, mumps and rubella provide an opportunity for effective control of these infections and in the foreseeable future there could be effective vaccination against other childhood diseases and against the two major forms of virus-induced hepatitis. In principle it is possible today to produce vaccines against all cultivatable viruses.

During the last few decades it has been found that many viruses can give not only acute infections but also have the capacity to remain in the body and give chronic diseases or dormant infections. A special property of certain viruses is their ability to change the growth characteristics of normal cells into those of tumour cells. The importance of this phenomenon for the emergence of tumours in man and animals is currently subject to intensive studies. It should be emphasized that a majority of all virus infections contracted by man or animals are not apparent, i.e. they do not produce any symptoms. From a biological viewpoint there obviously is no reason for a virus to cause severe disease. Concerning the possible spread of infection it is an advantage if the infected individual does not contract incapacitating disease.

Viruses have been the focus of attention not only because of their importance as disease-causing agents but also because they are interesting subjects for general biological study. Viruses represent the most simplified self-replicating and genetically active elements. The possible evolutionary origin of viruses has been extensively debated. In the absence of hard facts these discussions are usually more speculative than informative. Viruses have been referred to as the 'selfish' gene which during its evolution has acquired capacity to a restricted cell-independent existence and an ability to transfer its genetic information to another cell. It does not seem unlikely that the evolution of viruses has been advantageous also for the evolution of cells and cellular organisms. Genetic material which is important for cells can be transported by use of virus-genetic material as a vehicle within and between genomes of cells. Studies of viruses therefore provide opportunities for an analysis of the basic cellular mechanisms of life.

Our knowledge about viruses has increased dramatically during the last three decades. This is due to the development of practical methods for the cultivation of viruses in cell cultures in the laboratory and to the development of new methods for biochemical characterization of virus products. The total chemical composition of certain viruses is now known and much detailed information concerning the interaction between viruses and cells has been obtained. A continued rapid accumulation of knowledge concerning both the theoretical and the applied aspects

of virology can be predicted. Today we stand on the threshold to an age in which the practical application of antiviral substances in medicine will be seen. Diagnostic knowledge of viral diseases will therefore become of increasing importance and the study of virology will be essential for both practising and trainee medical doctors and other personnel engaged in biomedicine.

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2

The morphology of virus particles. Classification of viruses

Erling Norrby

The survival of organic life is dependent on its capacity to replicate genetic material. The most simplified natural form of a viral infectious agent therefore would comprise a limited amount of nucleic acid with capacity to direct its own replication. This form of infectious agent exists in plants but has not been identified so far in other host organisms. It is called *viroid*. Viroids are composed of a circular form of single-stranded RNA with a molecular weight of about 100 000. It is not known how this nucleic acid can be replicated nor has it been clarified how this kind of agent can cause disease in the plants on which it forms a parasite.

Infectious nucleic acid

All known animal, insect and bacterial viruses have an extracellular transport form which includes nucleic acid and a protein shell in which this nucleic acid is enclosed. In some cases the particles also include additional structures. Isolated virus nucleic acid, DNA or RNA, may cause infection and initiate a synthesis of complete virus particles. The nucleic acid is infectious, however, only in cases when the complete virus particle does not contain any enzyme(s) needed to initiate replication (*see* Chapter 3). Free isolated *infectious nucleic acid* is an ineffective contagious entity. One single break in the nucleic acid molecule induced by physical or chemical factors will lead to the loss of its infectious capacity. It is therefore of importance to their survival that viral infectious agents have their nucleic acid packed into a protective protein shell during the transport between cells.

Principal aspects of virus particle structure

The composition of a conventional virus can schematically be described as follows. Centrally the particle contains nucleic acid of varying quantity. This nucleic acid is either RNA or DNA, but never both kinds simultaneously. The nucleic acid is surrounded by a protein shell, called *capsid* (from L. *capsa* = box). In the case of many viruses the nucleoprotein complex represents the whole virus particle. The virus particle is referred to as the *virion*. In more complex viruses further (one or more) enclosing structure(s) occur. This component is structurally similar to cellular membranes and is referred to as the *envelope*. An envelope is composed of proteins specific to the virus and lipids and carbohydrates which are taken

preformed from the infected cell. Even the more complex virus particles do not contain organelle structures equivalent to, for example, mitochondria and lysosomes of cells. If strict definitions were applied, a virus should not be called a microorganism. However, for practical reasons, viruses are included in the group of microorganisms.

Virions thus have a relatively simple compostion and, as a consequence, they are small. The largest virions have dimensions of 320 × 270 × 120 nm, a size corresponding to that of certain forms of the smallest bacteria (mycoplasmas), whereas the diameter of the smallest virions is about 20 nm. The difference in volume is 5000-fold. In spite of this variation in dimensions, viruses have common features which motivates their classification as one common category of infectious agent. The limited size of virions allowed a distinction to be made between bacteria and viruses as cellular infectious agents in early studies. Virions were found to be capable of passing through filters which retained bacteria and they were therefore classed as being ultrafiltrable. Furthermore, bacteria were characterized by light microscopy whereas virus particles, because of their limited size, could not be detected. Information about the morphology and dimensions of virions could be clarified firstly through electron-microscopic analysis. Originally it was possible to get only a rough impression of the size and form of virus particles. In 1956 the negative contrast technique for electron microscopy was introduced. Instead of being stained with electron-dense substances, the particles were suspended in a contrast solution. With this technique new possibilities for detailed characterization of virus morphology became available.

Live or dead materia?

During the 1930s it was shown that purified virions of a plant virus could be crystallized. The fact that virus particles were giant molecules with a capacity to crystallize caused extensive discussion about whether a virus should be considered as live or dead materia. The extracellular virus particle which lacks energy-providing systems and has no capacity, or only a limited capacity, for independent metabolism obviously must be considered as a lifeless unit. Since it also lacks capacity for active movement, the transport of virions in time and space from cell to cell is a chance event. If a virion comes into contact with a susceptible cell, however, a sequence of events is initiated which fulfils all definitions of life, i.e. the reproduction of genetic material which is incorporated into new transport particles. The question about live or dead material becomes more complicated when we are dealing with defective viruses which have a capacity to replicate only in cells which concomitantly are infected with another virus (*see* Chapter 12).

Only certain non-enveloped virions can be crystallized. The availability of viral crystals has facilitated three-dimensional analyses by aid of x-ray diffraction. Through these studies it has been possible to shed light upon the interaction between virus nucleic acid and capsid protein.

Structural proteins – symmetry arrangements

A single-stranded nucleic acid can direct the synthesis of a protein which has a size corresponding to about 1/7 of its molecular weight. This fact caused Watson and

Crick, well known for their description of the double helix nature of DNA, to postulate two important principles for the structuring of virus particles. The first principle was that the virus capsid must be built up of repetitive units; the second, that the structure of the capsid should be symmetrical. By use of the two above-mentioned methods of analyses – electron microscopy and x-ray crystallography – and chemical analyses, the correctness of these postulates has been verified. The number and character of the chemical units, *structural proteins*, which are the building stones in virions, have been described for the majority of animal viruses.

Nature generally utilizes symmetrical building principles in the construction of more comprehensive three-dimensional structures. Hereby, information can be spared since the design of the individual building stones can decide their mutual

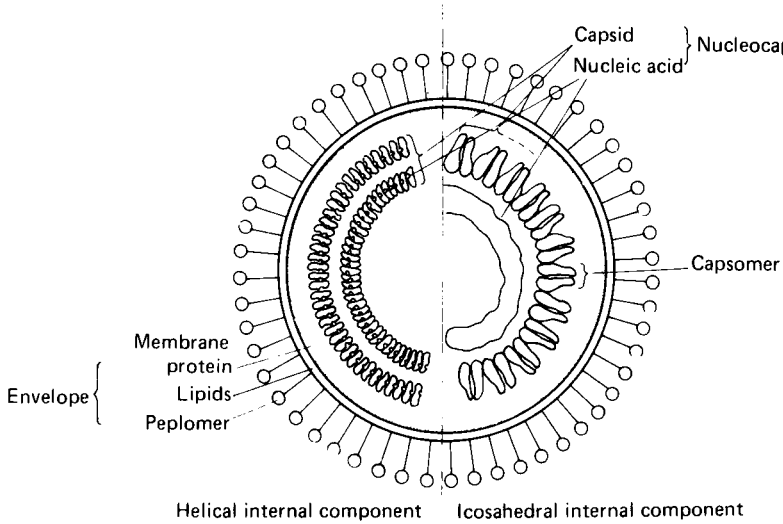


Figure 2.1. Schematic description of the structure of a virus with a helical (left part of the picture) or icosahedral (right part of the picture) internal component (nucleocapsid). The particle in the figure is surrounded by an envelope but many viruses lack this structure. A capsid represents the outermost protective structure in such non-enveloped viruses

relationships and therefore allow a spontaneous assembly via crystallization-like processes. It is characteristic of nature that it alternates unique design and symmetrical arrangements on different levels of the organized biological hierarchy in both plants and animals.

The principle of symmetrical constructions is well illustrated by the design of virus particles. Two different forms of symmetry, *helical* and *icosahedral* have been used for the construction of virus capsids (*Figure 2.1*).

Helical symmetry

Helical (screw-formed) capsid symmetry is used in the construction of rod-shaped plant virions and bacteriophages and the internal structure of some enveloped animal viruses. Among the rod-shaped viruses tobacco mosaic virus (TMV) has

been studied in most detail since it can be obtained in large quantities and crystallized from the juice of leaves from diseased plants. TMV RNA has a molecular weight of 2 million. The nucleic acid winds in a helical form inside a protein helix structure and is thereby protected from external physical and chemical influences.

The protein helix is formed by 2130 units of one single protein with a molecular weight of 18 000. The complex of nucleic acid and protein can be dissociated by addition of alkali. After readjustment to a neutral pH virions are again formed via a spontaneous crystallization process. The interaction between RNA and protein does not seem to have any high degree of specificity since virus RNA can be exchanged for a piece of cellular RNA, for example, in connection with dissociation and reassociation.

Helical structures, which in their appearance are similar to those of rod-shaped plant virions, also occur in animal viruses, e.g. mumps virus. However, in these viruses the helical structure is more flexible and forms a coiled internal component which is enclosed in a membrane in the complete virion (cf. *Figure 2.6a*). An internal component composed of nucleic acid and capsid is called *nucleocapsid*.

Icosahedral symmetry

The icosahedron is one of the classical five Platonic bodies. It is composed of 20 triangular facets combined so that the structure has 12 corners (vertices) and 30 edges (*Figure 2.2*). The building stones in an icosahedral shell are put together in accordance with strict mathematical rules. They can be placed in edges and corners

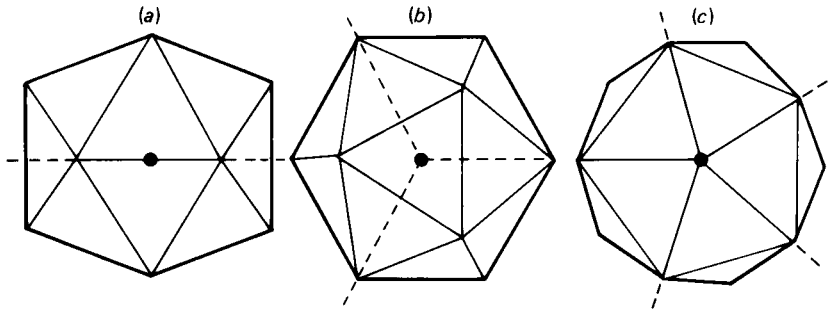


Figure 2.2. The appearance of an icosahedron viewed from 2-fold (*a*), 3-fold (*b*) and 5-fold (*c*) axes of symmetry. Five of the total number of 20 uniformly sized triangular facets meet at each of the 12 vertices

and on the triangular facets or only within the latter. Disregarding the location there is a rule saying that the total number of structural units must be a multiple of 60. The structural proteins of the capsid have a molecular weight which varies between 15 000 and 130 000. Individual molecules cannot be morphologically identified when they form a part of a capsid. However, groups of structural units can be identified and such morphological units are called *capsomers* since they represent a part of the capsid. In occasional cases capsomers are formed by 2 or 3 structural units and the whole capsid may contain for example 60 capsomers (*Figure 2.4*). The most common situation is that the icosahedron is formed by a combination of 12 capsomers each containing 5 structural units localized at the vertices of

the capsid and in addition a varying but fixed number of capsomers containing 6 structural units. Different theoretically possible numbers of capsomers are summarized in *Figure 2.3*. It is of interest to note that nature has used a large number of the different theoretically possible lower capsomer numbers. *Figure 2.4* gives examples of virions with 72 and 252 capsomers. The number of different structural components increases with increasing size of virions. Larger capsids enclose nucleic acid combined with one or more proteins in a structure occasionally referred to as *core structure*.

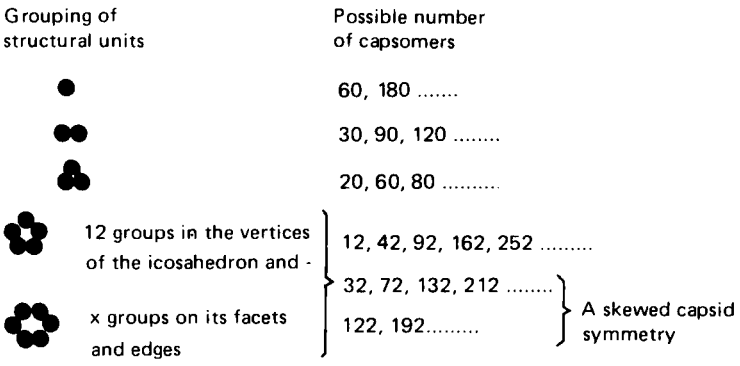


Figure 2.3. In connection with formation of an icosahedron, the building stones, *structural proteins*, can be used either in an isolated form or in different groupings. Such groupings of structural proteins can be identified as morphological units in the electron microscope. This morphological unit is called a *capsomer*. The number of building stones in an icosahedral structure is determined by the triangulation number and this number in turn is determined by the formula $T = H^2 + HK + K^2$, in which H and K are integers. The number of structural proteins is always $60 \times T$. In cases where the capsomers represent pentamers and hexamers of structural units, a total number of capsomers in a capsid is $10T + 2$

The virus envelope

In principle a virus capsid can increase in size in an unlimited fashion with the addition of an increasing number of non-vertex capsomers. However, increase in size reduces the stability of the structure and further accentuates the risk of incorrect assembly. For this reason, perhaps, animal viruses with a diameter exceeding 80 nm usually have another enclosing structure. This structure has a membrane-like character and is referred to as the *envelope*. An envelope can enclose a nucleocapsid with helical or icosahedral symmetry (*Figure 2.5*). The envelope has a similar composition to membrane structures of cells. On the inside of the membrane there is a stabilizing skeleton protein, also called matrix protein, and on the outside there are projections of varying size and form depending upon the kind of virus. The morphologically identifiable projections are called *peplomers* (Gk. *peplos* = drape). Each peplomer is composed of a few structural units. The envelope appears not to be the loose sack-like structure which was originally believed. It seems that the peplomers are located in the envelope and have a certain

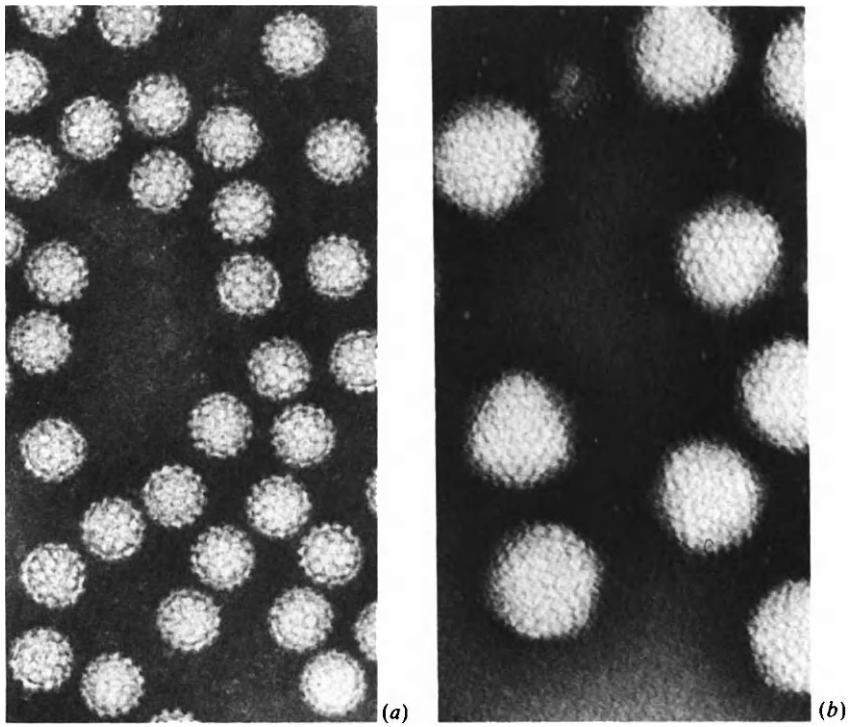


Figure 2.4. Electron microscopic picture of two different non-enveloped icosahedral viruses. Papilloma virus (*a*) has 72 capsomers and adenovirus (*b*) has 252 capsomers in the capsid. (The photograph of papilloma virus was reproduced by permission of Dr J. Almeida, The Wellcome Research Laboratories, Beckenham, Kent, UK. Magnification ×201 000)

symmetrical relationship to each other. Furthermore, the peplomers communicate through the lipid layer with the matrix protein and this protein in turn is in direct contact with the nucleocapsid which, when it has a helical structure, is wound up in a strictly organized fashion.

Most animal viruses have a rounded form. This holds true both for non-enveloped and enveloped viruses. Two of the largest kinds of viruses, however, have a test-tube (bullet)-like and brick-like form with rounded edges, respectively. The latter kinds of particles have both an envelope and an internal membrane.

Classification of viruses

The subdivision of a group of biological entities should reflect their mutual evolutionary relationships. However, the mechanism for evolution of a virus is not known. Since the virus is a cellular parasite it is obvious that the first primitive cells must have arrived on the scene before viruses made their entrance. Two major mechanisms of the origin of viruses have been discussed. One possible mechanism is that the virus represents a cellular structure which has acquired independence. This structure thus would have developed a capacity to occur in a stable particulate form, to be transmitted from cell to cell, and also to initiate its own replication. The

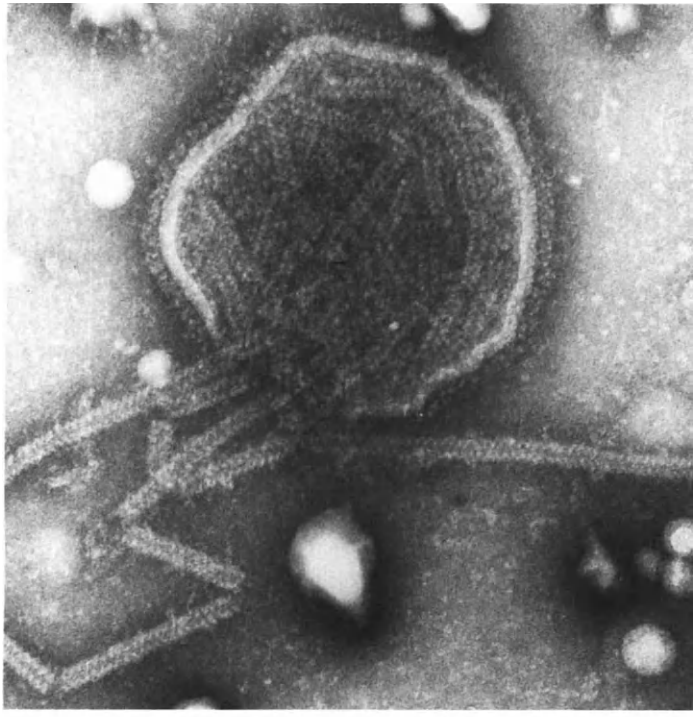
other possible mechanism is that the virus has derived from more complex organisms through a retrograde (backwards) evolution. Primitive bacteria which discovered that there was a certain comfort in replicating in nucleated cells might as a consequence of increasing laziness have made themselves extremely dependent on the metabolism of cells. Certain data indicate that different viruses may have different evolutionary origin. In spite of this it is worthwhile to jointly classify all viruses. The different possible variations in genome strategy and particle structure for such a relatively simplified infectious agent as a virus must of necessity be rather restricted and, independently of evolutionary origin, principal similarities will dominate over the dissimilarities.

Originally viruses were divided into groups on the basis of their ways of spreading and taking into account the organ in which they preferentially initiated an infection. Thus, for example, a grouping into enteric viruses, respiratory viruses and viruses transmitted by arthropods (segmented invertebrates, e.g. blood-sucking insects), *ar*thropod-*bo*rne arboviruses, was made. This method of classifying viruses still has a certain relevance regarding the syndromes and epidemiology of virus diseases. However, since there is a large number of biologically different viruses which can cause, for example, respiratory infections, other properties must also be considered in the classification.

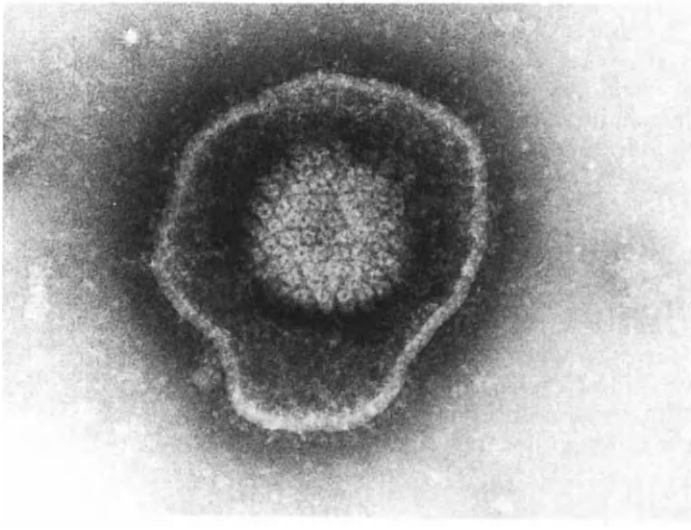
The best means of achieving a practical classification of viruses has been to concentrate on the morphological and gross chemical features of virions. Primarily the following parameters are used for identifying different groups.

1. Kind of nucleic acid; either DNA or RNA.
2. Kind of capsid symmetry; either cubical or helical.
3. Virus envelope; present or absent.
4. Additional characteristics of the capsid; in the case of a helical capsid, the diameter of this structure, and in the case of an icosahedral nucleocapsid, the number of capsomers.

By considering these different properties it is possible to identify all the major groups of animal viruses (*Figure 2.6*). A useful classification of viruses of a different host origin also can be achieved. In many cases the grouping obtained by use of these properties is verified by the existence of unique biochemical features shared between members within individual groups. Many virus groups contain a large number of members which in turn can be divided into subgroups. It is therefore necessary to use several hierarchial levels in the classification. From a practical point of view the following levels are utilized: *family, genus, type (species)*. Regrettably the term 'group' is used in daily language to cover both families and genera. The definition of the term 'type' (species) has been and still is a matter for debate. A practical definition is to identify two virus strains as belonging to the same type if an infection with one of the strains provides immunological protection against a subsequent infection by the other strain. Thus it is by use of immunological techniques that a virus type is defined. Different strains of a certain virus type occasionally display a different capacity to cause disease both from a quantitative and qualitative point of view. It is therefore of interest to characterize a virus isolate by more refined serological techniques or by other methods. An example of the latter is the characterization of the nucleic acid of viruses, e.g. by fragmentation of virus DNA by restriction enzymes and determination of the number and sizes of the fragments obtained. By such procedures different *subtypes* of a virus may be identified.



(*a*)



(*b*)

Figure 2.5. Electron microscopic pictures of two different enveloped viruses. Since the envelope in both particles is damaged the nucleocapsid can be identified. The nucleocapsid is helical in parainfluenza virus (*a*) and icosahedral (162 capsomers) in herpes simplex virus (*b*). (Photos reproduced by permission of Dr J. Almeida, The Wellcome Research Laboratories, Beckenham, Kent, UK. Magnification (*a*) ~×160 300 and (*b*) ~×210 800.)

Type of nucleic acid: RNA

Capsid symmetry: Icosahedral — Helical

Envelope: Absent — Present

Virus family	Picorna	Reo	Toga	Retro	Orthomyxo	Paramyxo	Bunya	
Morphology								
Size nm	25	70–80	40–60	100–120	80–90	120–150	90–120	
Examples of members or group (genus)	Entero (polio, cox-sackie, echo) Hepatitis A Rhino	Rota	Yellow fever, RSSE, Rubella	Leukaemia Sarcoma	Influenza A,B,C	Mumps Measles Parainfluenza RS		

Figure 2.6. Summary of structural properties of different RNA and DNA viruses

Introduction to different families of animal viruses

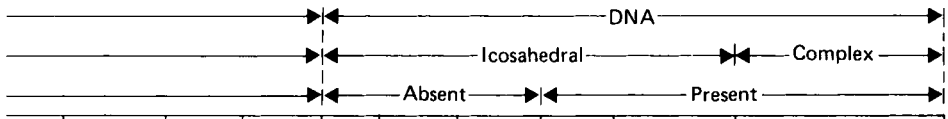
In the following a summarized description of the different families of animal viruses (cf. *Figure 2.6*) is given. A more detailed description will be found in Chapters 25–33, which discuss each family separately. Strictly, virus families should have the suffix *viridae*, but in the following the suffix 'virus' will be employed since it is of daily usage.

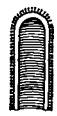
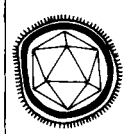
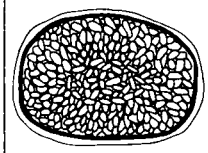
Picornavirus

This group comprises a large number of viruses which have the common feature of being small (It. *pico*) – 25 nm – and containing RNA. Among different genera in this family of viruses can be mentioned enteroviruses (intestinal viruses) and rhinoviruses (nasal viruses). Best known among enteroviruses are the three types of poliomyelitis viruses. Rhinoviruses are responsible for the major part of all common colds. The virus causing infectious hepatitis, hepatitis A, also belongs in this family.

Reovirus

Despite the fact that the name of this family of viruses derives from '*re*spiratory', they have not been found to give respiratory infections. The family includes several genera of which rotaviruses (morphology like a spoked wheel (L, *rota* = wheel); *see Figure 20.1*) have been found to have a considerable importance in intestinal



	Arena	Corona	Rhabdo	Parvo	Papova	Adeno	Unclassified	Herpes	Pox
									
	90–120	80–120	50 × 180	20	45–55	70–80	40–45	150–200	120 × 270 × 320
			Rabies		Papilloma Polyoma SV40		Hepatitis B	Herpes simplex Varicella-zoster Cytomegalo Epstein-Barr	Variola Vaccinia Molluscum contagiosum

infections in man. Like picornaviruses, reoviruses do not have an envelope. They contain double-stranded RNA divided into 10 fragments, have a diameter of 70–80 nm and a capsid composed of 92 capsomers.

Togavirus (L. *toga* = mantle).

This family has been formed by combination of two genera deriving from a larger group of hundreds of insect-borne viruses and rubellavirus and related viruses from animals. The two first genera are called *alphavirus* – previously arbovirus group A – and *flavivirus* (L. *flavus* = yellow), since an important member is yellow fever virus – previously arbovirus group B. Insect-borne togaviruses may give different forms of severe meningitis. Togaviruses contain linear RNA and represent the smallest (40–60 nm) enveloped forms of viruses.

Retrovirus

The name of this family derives from the fact that the virus particles contain the enzyme reverse (L. *retro*) transcriptase. These medium-sized (100–120 nm) viruses are composed of linear RNA enclosed in an icosahedral shell which is surrounded by an envelope. The family includes several members which are divided into a number of genera among which can be mentioned *oncoviruses* (L. *oncus* = tumour) which can give leukaemias and sarcomas in certain animal species and *lentivirus* (L. *lentus* = slow) which can cause a slow virus infection in sheep.

Orthomyxovirus

The name of the family alludes to the affinity which its members have for certain mucopolysaccharides which form a part of the receptor structure for these viruses on the cellular surface. The virus contains 8 pieces of linear single-stranded RNA combined with a helical nucleocapsid (diameter 8–9 nm) enclosed in an envelope. The total diameter is 80–90 nm. The group includes the genera influenza A, B and C of which A is responsible for recurrent epidemics with a global extension.

Paramyxovirus

These viruses show similarities to ortomyxoviruses but they contain RNA in a larger quantity and in one single piece. Furthermore, their nucleocapsid has a diameter of 17–18 nm and the virion has a total diameter of 120–150 nm (*Figure 2.5a*). Some members of this family are important in human medicine, e.g. mumps virus, measles virus and certain respiratory viruses. Among the respiratory viruses may be mentioned respiratory syncytial (RS) virus which can give infections in young children. It is possible that this virus in the future may be allocated to a separate family partly because it has a nucleocapsid with a diameter of 12–13 nm.

Bunyavirus (from *Bunyamwera* = an African community).

These viruses were previously classified as arboviruses. Since they are medium-sized (90–120 nm) and contain three pieces of linear RNA associated with a helical nucleocapsid enclosed in an envelope (*see Figure 33.1* concerning morphology) they now form a family of their own. Members of this family cause a spectrum of diseases in both animals and man.

Arenavirus (L. *arena* = sand)

The name of this family derives from the fact that the virions include a number of cellular ribosomes which in the electron microscope appear like grains of sand. They are medium-sized (90–120 nm), enveloped viruses containing RNA divided into three pieces. The detail structure of virions has not as yet been elucidated. Their natural host is rodents and under special conditions the infections can be transmitted to man and cause severe disease, e.g. Lassa fever.

Coronavirus (L. *corona* = crown)

The name of this family has been given to designate the pattern of the clublike peplomers which radiate from the envelope. They are medium-sized viruses (80–120 nm) which contain RNA and have a structure which, to a major extent, has not been clarified (*see Figure 33.2* concerning morphology). Many members in this group can cause common cold in man.

Rhabdovirus (Gk. *rhabdos* = rod, striation)

The internal structure, which in the electron microscope appears striated, has given the name to this family. Rhabdovirus is one of the large RNA viruses (150–180 nm).

The nucleic acid is in one piece and is combined with a helical nucleocapsid. It is surrounded by an envelope and the particle has a test-tube-like form (*see Figure 33.3* concerning morphology). The rhabdovirus family includes two genera. The member of one of these genera is rabies virus which can give a fatal disease in man. The infection is transmitted from animals.

Parvovirus

This group of small (20 nm)) DNA viruses has received its name from L. *parvus* = small. Hitherto no virus in this group has been proven to give disease in man. Certain intestinal viruses may however turn out to belong to this family. Preparations of adenoviruses (*see below*) occasionally contain a parvovirus that only can replicate in adenovirus-infected cells. This parvovirus is called *adenoassociated virus* (AAV). Parvovirus is the only family with virions containing single-stranded DNA.

Papovavirus

The family name derives from the initial letters of the names of the three original members of the family: *pa*pilloma (wart virus), *po*lyoma (a virus that gives several kinds of tumours in mice) and '*va*cuolating agent' (a monkey virus which produces vacuolating changes in infected cells). The latter virus is generally referred to as SV40 (simian virus 40). Papillomavirus and the other members of the family represent two separate genera in which the virions have different diameters, 55 and 45 nm, respectively. All viruses in the family have the same principal composition, however; circular DNA combined with cellular histones and surrounded by a capsid with 72 capsomers (*Figure 2.4a*). In man papovaviruses may cause warts and in addition certain other unusual diseases. The importance of these viruses concerning the appearance of tumours in man and animals is currently being studied.

Adenovirus

The original isolates were made from lymphoid tissue in the hind part of the nasal cavity, the adenoid, hence the name adenovirus (Gk. *aden* = gland). These medium-sized viruses (70–80 nm) contain linear DNA enclosed in a capsid with 252 capsomers (*Figure 2.4b*). The vertex capsomers are specialized and carry a projection. Adenoviruses occur in all species and in man 38 different types have been identified. Some adenoviruses from patients with intestinal infections do not grow in cell cultures. The viruses can give a number of different infections, e.g. in the respiratory tract and in the eyes. In animal systems it has been found that certain human adenoviruses can induce tumours.

Hepatitis B virus

This virus which can cause serum hepatitis has not been classified. However it probably should belong to a separate family since it contains circular DNA combined with a core structure and surrounded by a lipid-containing structure. The

outer coat does not seem to have a structure corresponding to that of the envelope of other viruses. The diameter is 40–45 nm (*see Figure 30.1* concerning morphology).

Herpesvirus (Gk. *herpein* = to creep).

These large viruses (150–200 nm) contain a linear DNA packed into an icosahedral capsid with 162 capsomers surrounded by an envelope (*Figure 2.5b*). A number of the members of this family cause important diseases in man. They may give vesicular skin diseases such as varicella (herpes zoster) and herpes simplex infections. Cytomegalovirus may cause fetal damage when it occurs as a prenatal infection and Epstein–Barr (EB) virus is the cause of infectious mononucleosis (glandular fever). A possible relationship between cervical cancer and infections with herpes simplex virus type 2 has been discussed.

Poxvirus

The name of this family refers to the kind of skin changes caused by the virus, i.e. the pocks which are vesicular changes containing a cell-rich fluid. Poxviruses are the largest (320 × 270 × 120 nm) and the most complex kinds of viruses. They contain a comparatively large amount of linear DNA enclosed in a complex capsid which in turn is surrounded by both an inner membrane and an envelope (*see Figure 10.6*, page 91, concerning morphology). Poxviruses occur in all species. Smallpox virus has now been eradicated.

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3

The biochemistry of virus particles

Ulf Pettersson

Non-enveloped virions are composed of nucleic acid, which is either DNA or RNA, surrounded by a capsid composed of one or many proteins. Enveloped viruses in addition are enclosed by a membrane composed of virus-specific proteins and carbohydrates and lipids, derived from the host cell.

Our knowledge about the biochemistry of viruses, particularly concerning the detailed structure of their nucleic acids, has increased dramatically in the last decades. In the last few years the field of molecular biology has been transformed by the discovery of restriction enzymes in prokaryotic cells, which can be used as a valuable experimental tool, the introduction of recombinant DNA technology, and the development of methods for analysing base sequences of nucleic acids. Selected genes can now be isolated and multiplied and large quantities of their corresponding messenger-RNA (mRNA) as well as protein product can be accumulated. This new methodology is readily applicable to the biochemical characterization of viruses and it has in many cases been developed for the study of virus-genetic material.

Virus-DNA

General properties

The DNA of viruses has the same basic properties as DNA in eukaryotic cells and bacteria. During the last decade, a relatively detailed knowledge about the organization of viral genomes has been obtained. The complete nucleotide sequence of DNA from the bacteriophage ØX174 and from certain papovaviruses has been determined and from the nucleotide sequence it has been possible to determine the exact position of individual genes.

The base composition of virus-DNA varies extensively both between different virus families and also between members within families. The relative guanine –cytosine (GC) content of DNA has a marked influence on the buoyant density of the nucleic acid and centrifugation techniques can therefore be used in some systems to separate cellular and viral DNA or DNA from different viruses.

Viral genomes vary markedly in size (*Table 3.1*). The members of the parvovirus family have the smallest amount of genetic material and contain single-stranded DNA with a molecular weight of 1.5×10^6, whereas the largest genomes are found in the poxvirus family with a molecular weight of about 1.5×10^8. Measured in terms of nucleotides parvovirus-DNA contains 4500 whereas poxvirus-DNA

contains more than 200 000 base pairs. One gene for a medium-sized polypeptide chain corresponds to about 1000 base pairs which means that the smallest viruses contain genetic information for 4–5 genes, whereas the most complex virus genomes theoretically could encode 200 genes.

However, this reasoning has been found to be too simplified since one DNA sequence can be utilized for the formation of more than one protein product. One mechanism is a shift in the reading frame; since the genetic language does not include any separation marks between triplets a given sequence of DNA theoretically can be translated into three different proteins depending upon where translation of the nucleotide sequence is initiated. Another mechanism is the

TABLE 3.1. Size and type of nucleic acid in different viruses

Virus	*Type of genome*	*Molecular weight* $\times 10^{-6}$
Parvovirus	Single-stranded linear DNA	1.2–1.8
Papovavirus	Double-stranded circular DNA	3.0–3.5
Adenovirus	Double-stranded linear DNA	23
Herpesvirus	Double-stranded linear DNA	100–150
Poxvirus	Double-stranded linear DNA	150–200
Picornavirus	Single-stranded RNA	2.5
Togavirus	Single-stranded RNA	4.0
Retrovirus	Single-stranded RNA	2.5–3.0 (2 similar units)*
Rhabdovirus	Single-stranded RNA	3.5–4.5
Paramyxovirus	Single-stranded RNA	5.5–7.5
Orthomyxovirus	Single-stranded segmented RNA	3.9–4.9 (8 segments)
Reovirus	Double-stranded segmented RNA	12–15 (10 segments)

* A retrovirus genome is composed of a 70S RNA, which after denaturation is separated into two identical units each with a size of $2.5–3.0 \times 10^6$. In addition small cellular RNA molecules occur in the virus particle

post-transcription phenomenon of *splicing*. This phenomenon means that parts (*introns*) of the original transcript are eliminated, whereas other parts (*exons*) are combined and retained in the mature form of mRNA. Certain mRNA precursors can be spliced in many different fashions leading to the appearance of several different protein products. It has been found that the small virus genomes have a very compact organization. The genes are closely packed together and occasionally they overlap so that a segment of DNA is utilized for coding of two separate polypeptides. However, this compact arrangement does not seem to be a characteristic of larger viruses and of cells. An arrangement of this kind most likely reflects the way a virus has evolved. The capsid of small virus particles has not allowed any expansion in the size of the genome and therefore these viruses have been forced to maximize the utilization of their genome.

Viruses occasionally contain single-stranded DNA

Many virus genomes have an anatomy which in certain respects is different from that of genetic material in ordinary cells (*see Figures 3.1* and *3.2*). Already in the 1950s it was shown that DNA from the bacteriophage ØX174 has a single-stranded

nature. Since then a number of viruses have been discovered to contain single-stranded genomes and among animal viruses this type of DNA is found in members of the parvovirus family. However, certain parvoviruses can pack both DNA strands but only in separate virions. As a consequence DNA extracted from the virus particle resumes a double-stranded nature relying on the fact that the strands

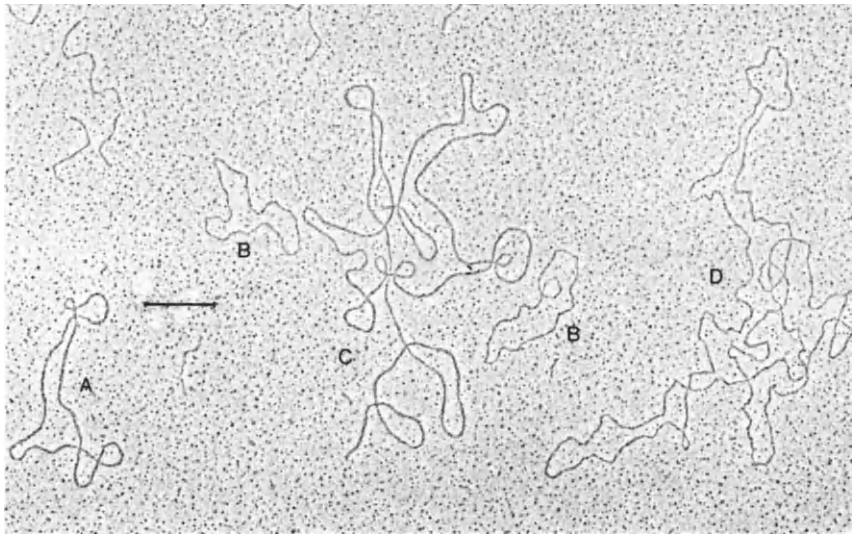

Figure 3.1. Electron microscopic picture showing different types of DNA molecules. A. Double-stranded circular DNA from the bacteriophage PM2. B. Single-stranded circular DNA from the bacteriophage ØX174. C. Linear double-stranded molecule from adenovirus. D. Single-stranded circle of adenovirus-DNA. The ends of the last named molecules are connected by inverted repeated endsequences (cf. *Figure 3.3c*). (Magnification ~×15 300)

renature and form double-stranded structures during the extraction process. When single-stranded DNA molecules are going to replicate, a double-stranded so-called *replicative intermediate* of DNA is first formed. This is necessary since the basic principle for replication of DNA is that a chain of DNA can be formed only by use of a complementary chain of DNA as a template.

Superhelical DNA

The DNA of some viruses are circular. The reason for this is probably that the circular form offers certain advantages in connection with replication and that the circular DNA lacks free ends. The latter property means that the DNA is protected against attack from certain DNA-degrading enzymes, known as exonucleases. Covalently closed double-stranded DNA circles are superhelical as is illustrated in *Figure 3.2*. Because of the special physical properties of these structures they can be isolated by centrifugation techniques.

Linear virus-DNA molecules frequently have special sequences at their ends

It has been long known that linear genomes in bacteriophages frequently have unusual base sequence arrangements in their ends. These structures appear in

many cases to play a role in connection with the replication of DNA. Thus, for example, bacteriophage λ has cohesive (sticky) ends, which means that single-stranded complementary tails extend from each end of the λ-DNA. These complementary ends make it possible for λ-DNA to form a circular structure which seems to be important in connection with DNA replication. It is frequently found that the ends of virus genomes contain repeated sequences which may have the same orientation or may be inverted as for example in the DNA of adenoviruses. An inverted repeated sequence will allow, consequently, the denatured DNA to form single-stranded circles (*Figure 3.2*).

One additional unusual structure is found in the ends of vaccinia virus-DNA. It has been long known that this DNA can not be denatured in the same way as other linear DNA molecules. Recently, it has been found that the two polynucleotide chains are covalently linked to each other in both ends of the DNA molecules. Thus the whole vaccinia virus-genome represents a large circle and since the two chains cannot be separated from each other DNA will renature very rapidly after denaturation.

Virus-RNA

General properties

At an early stage it could be shown that certain groups of viruses have genomes which contain RNA exclusively. In addition Gierer and Schramm in 1956 could show that RNA from TMV is infectious. This proved beyond doubt that genetic information can be stored in the form of RNA. Also, among RNA viruses there is a considerable variation in the size of genomes even though this variation is not as large as among DNA viruses (*Table 3.1*). The smallest RNA viruses have a genome with a molecular weight of only 4×10^5. However, this virus (satellite tobacco necrosis virus) is defective and can replicate only in the presence of a helper virus, tobacco necrosis virus. Among animal viruses with RNA genomes, the smallest representatives are found in the picornavirus family, the members of which have genomes with a molecular weight of about 2.5×10^6. The largest representatives among RNA viruses, retroviruses and paramyxoviruses, have genomes with a molecular weight of about $5.5–7.5 \times 10^6$.

The number of gene products whose synthesis an RNA virus might direct was originally estimated from the absolute quantity of nucleic acid in the virus. However, it has been found recently that the phenomenon of splicing occurs not only during transcription of DNA viruses, but also in connection with the formation of mRNA from certain RNA viruses. Thus in this situation also one segment of the genome may direct the synthesis of more than one protein.

Small infectious agents containing only RNA also occur in nature and they have been found to cause severe diseases in plants. This group of agents are called *viroids* and they lack a protein shell and contain only a circular RNA molecule with a size of 300–400 nucleotides. The exact mechanism for replication of these agents has not been clarified. They appear to lack capacity to direct the synthesis of any protein. It has been speculated that they may represent free introns.

When an RNA virus is going to replicate, it first has to form a double-stranded replicative form which then can serve as a template for synthesis of progeny-RNA. RNA chains, like DNA chains, have a fixed polarity. The polarity of the viral genome may be identical with the polarity of mRNA, which is used during

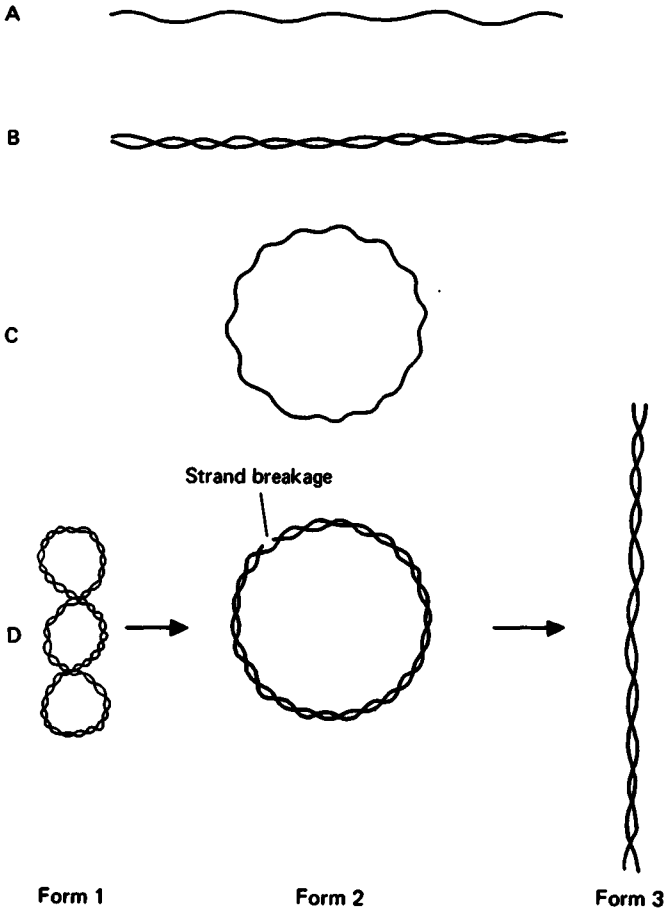

Figure 3.2. Many different kinds of DNA molecules occur in different viruses. This diagram gives some examples. A. Linear single-stranded DNA molecule of the kind found in parvoviruses. B. Linear double-stranded DNA molecule occurring in herpes virions and adenovirions. C. Single-stranded circular DNA which is found in the bacteriophage ØX174. D. Covalently closed double-stranded DNA circles present in papovaviruses. This type of DNA has special physical properties. Sedimentation analysis of DNA of this kind shows that it does not represent a homogenous class but includes three different components, form 1, form 2 and form 3 DNA. Form 1 DNA which sediments the most rapidly of the three forms is covalently closed DNA. This DNA differs from a normal circular structure since it is twisted and such a structure is said to be superhelical. The DNA becomes superhelical because it contains an incorrect number of turns in relationship to the number of base pairs in the DNA molecule. Normally, one turn of a DNA helix includes 10 base pairs. If the conditions are different the molecules try to compensate by twisting and become superhelical. Hereby, a tension is established in the molecule, which is maintained since the molecule is covalently bound.
If a breakage in either of the strands in the molecules is introduced, free rotation is allowed between the strands, which leads to the superhelical structures being lost and the molecule being converted to form 2 DNA (relaxed form). Breakage in both DNA strands at the same point gives a linear form of DNA (form 3) which sediments most slowly.

In cells there are enzymes which can open and close superhelical DNA, so-called 'nicking and closing' enzymes. These enzymes can change the number of turns in superhelical DNA by occasionally opening and closing one of the DNA strands. The DNA in the infected cell is attached to histons. The histons form nucleosomes, which introduce an incorrect number of turns in the helix. When the nucleosomes are removed during extraction of DNA, the incorrect number of turns is retained because of the nature of the molecule

replication. Chains of RNA with such a polarity are usually referred to as 'plus strands'. If a virus genome is complementary to the mRNA which is used in connection with virus replication, it is instead referred to as a 'minus strand'. RNA viruses are grouped according to certain properties of their genome (*see* Chapter 8).

Specific structures in viral mRNA

A virus exploits to a major extent the pre-existing machinery in infected cells for its own macromolecular synthesis. As a consequence viral mRNA should have the same principle structure as mRNA in normal cells (*Figure 3.4*). Most mRNA in higher organisms has a 5′ end which is modified through methylation reactions. This modified part of the mRNA is called *cap* and a corresponding structure is usually present in virus-mRNA. However, members of the picornavirus family represent an exception since it has been shown that, for example, poliovirus RNA has the nucleotide pUp in its 5′ end without any methylated structure. Instead, a protein is covalently bound to the 5′ end of poliovirus-RNA and this protein is removed before the translation of RNA. The importance of this protein for replication of polio-RNA is not yet known. RNA viruses which have negative-strand genomes have to form their mRNA through transcription. This is achieved with the aid of an RNA polymerase which is present within the virus particles. In addition the virus particles also contain guanyl and methyl transferases which are necessary for the formation of cap structure on the mRNA.

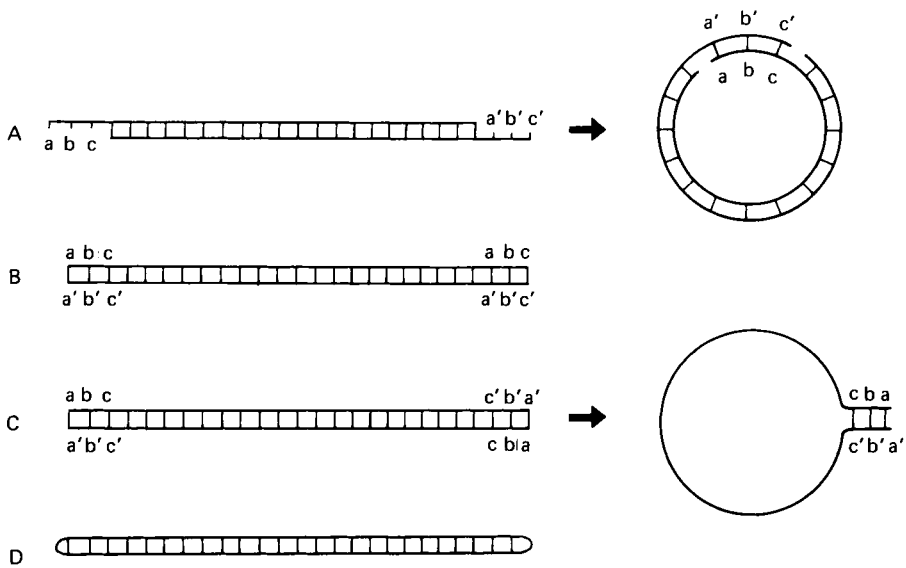

Figure 3.3. Viral DNA molecules frequently have specialized structures in their ends, which are illustrated. A. Virus-DNA may have cohesive ends which allow the DNA to circularize during replication. B. Other kinds of virus-DNA have a terminal repeated sequence, terminal redundancy. C. Certain kinds of virus-DNA have an inverted repeated sequence in their ends. Consequently circular forms of single-stranded DNA can be formed. D. Vaccinia virus-DNA has both of its ends covalently closed

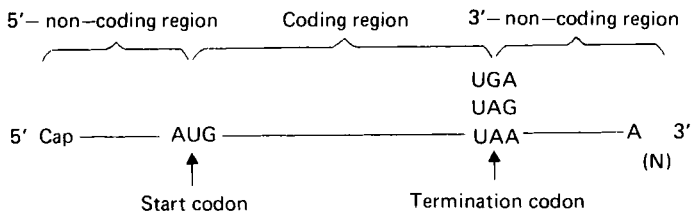

Figure 3.4. The structure of eukaryotic mRNA. In the 5' end of the molecule there is a cap structure. The coding part, which during translation is responsible for the formation of a polypeptide chain, is located between the initiation codon (AUG) and the termination codon (one of the codons UAA, UGA or UAG). A 50–200 nucleotide long segment of poly(A) is added to the mRNA molecule after transcription. In the 5' non-coding region, there are signals which play a role in the initiation of the translation reaction

As with cellular mRNA, animal virus-mRNA usually contain a poly(A)-segment in their 3' end. One exception to this rule is mRNA from reovirus. In most cases poly(A)-segments are added post-transcriptionally by use of a poly(A)-polymerase of host cell origin. However, in RNA viruses with a plus-strand genome, the poly(A)-segment pre-exists in the viral RNA. When the viruses are replicated the plus strand is transcribed into a minus strand of RNA and this as a consequence ensures that the latter strand will contain a segment of poly(U). During the synthesis of new virus-mRNA, this poly(U) segment will be transcribed to a matching poly(A) segment. Thus, in this particular case, poly(A) segments are not added by means of a poly(A)-polymerase.

Virus proteins

General aspects

Certain viruses such as TMV have a very simple composition and contain only one kind of protein which forms the capsid enclosing the nucleic acid (Chapter 2). Animal viruses contain at least three different polypeptides and certain viruses like herpesvirus and poxvirus have a relatively complex structure and contain more than 20 different polypeptide structures. As a rule, the structural proteins of viruses are coded according to the viral genome, although exceptions to this rule have been noted. Many virus components are poorly soluble during non-denaturing conditions, a fact which has complicated their characterization. An exception to this rule are the structural components of adenoviruses which, conseqently, have been the subject of detailed analysis.

The amino acid composition of proteins in virions does not show any remarkable differences from that of other known proteins. By use of modern technology it is frequently easier to determine the amino acid composition of a virus protein indirectly by analysis of the nucleotide sequence of the gene directing its formation rather than by direct amino acid sequence analysis. Frequently one finds markedly basic proteins resembling cellular histones associated with virus-DNA. In fact, papovaviruses contain cellular histones bound to their DNA, whereas adenoviruses synthesize their own basic proteins. The enveloped viruses contain special glycoproteins on the outside of the envelope. The glycosylation is not a virus-specific

event but is brought about by cellular enzymes. The protein occurring on the inside of virus envelopes, frequently referred to as the matrix protein, is highly hydrophobic and as a consequence is very difficult to dissolve in aqueous solution.

Methods to purify and concentrate virus proteins

Many virus components are insoluble under non-denaturing conditions, which complicates the analysis of their structure. The development of the technique of SDS-polyacrylamide gel electrophoresis has markedly improved the situation (*Figure 3.5*). This technique makes it possible to circumvent the problem of poor solubility of proteins since SDS (sodium dodecyl sulphate, a strong detergent) is bound to the proteins whereby they are denatured and simultaneously made soluble.

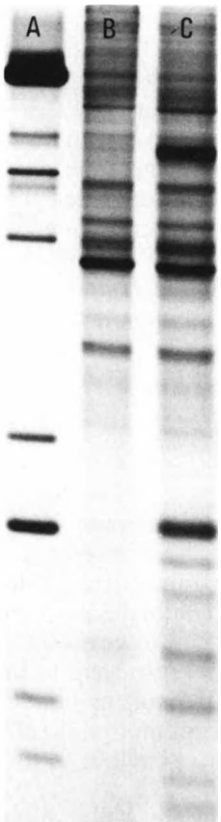

Figure 3.5. Analysis of virus proteins by SDS-polyacrylamide gel electrophoresis. Radioactively labelled proteins have been denatured by SDS and separated by polyacrylamide gel electrophoresis in the presence of SDS. The polypeptides appear as distinct bands which can be identified by autoradiography and the relative electrophoretic mobility of peptides is correlated to their molecular weight. A. Polypeptides from adenovirus type 2 virions. B. Polypeptides in an extract from uninfected HeLa cells. C. Polypeptides in an extract from adenovirus-infected cells. In the extract which is harvested early after infection, many distinct virus-specific polypeptides can be discerned. (Photo: H. Persson)

In addition the method has the advantage that the electrophoretic mobility of polypeptides in an SDS-containing polyacrylamide gel is correlated to their molecular weight. This fact is frequently utilized in order to obtain the approximate molecular weight of polypeptides by comparing the electrophoretic mobility in SDS polyacrylamide gels of other polypeptide chains of known molecular weight. Regrettably the technique does not provide accurate results for molecular weights of proteins which bind exceptional quantities of SDS, which is a particular problem

with glycosylated or phosphorylated proteins. Frequently, viral polypeptides are denoted with reference to their relative rate of migration in SDS-polyacrylamide gels. The largest virus polypeptide is frequently referred to as VP1 (virus protein 1), the second largest VP2 etc. Alternatively, polypeptides are designated from their estimated molecular weight expressed in kilodaltons. Thus for example a polypeptide with an estimated molecular weight of 72 000 is frequently referred to as a '72K polypeptide'. An additional disadvantage with the SDS-polyacrylamide gel technique is that the proteins are heavily denatured by the SDS treatment which complicates functional studies. However, in certain cases, SDS can be removed by slow dialysis and an enzymatic activity can for example be regained. Also denaturing compounds other than SDS have been used for the characterization and size determination of viral structural proteins. Examples are guanidine-HCl and urea.

Concerning the analysis of complex viruses like herpesviruses and poxviruses or the analysis of viral polypeptides in total cell extracts, the ordinary SDS polyacrylamide gel technique does not provide sufficient resolution for the simultaneous identification of all virus components. Recently a two-dimensional technique was developed. This technique has an extremely high resolving power which makes it possible to separate practically all polypeptides available in a living cell. The method combines the SDS-polyacrylamide gel electrophoresis in one dimension with isoelectric focusing in the second dimension. The former technique separates proteins in accordance with their size, whereas isoelectric focusing primarily separates according to charge.

Viral components which are soluble under physiological conditions can be purified and characterized by conventional separation techniques such as ion-exchange chromatography, exclusion chromatography with Sephadex or agaros or other separation techniques described in biochemical textbooks.

Many viruses contain enzymatically-active proteins

Originally the main function of the virus capsid was considered to be the protection of the nucleic acid against physical and chemical influences and the promoting of contact between virus and cell receptors. This description is correct when applied to picornaviruses and togaviruses, for example. However, it was shown in 1967 that poxvirus particles contain an enzymatic activity, an RNA polymerase which can transcribe parts of the viral DNA and mRNA. Since this discovery a number of enzymatic activities have been demonstrated in virus particles of different origin. All RNA-containing viruses with a negative-strand genome of necessity contain an RNA-dependent RNA polymerase since replication can occur only after transcription of the virus-genome into mRNA with a correct polarity. There is no enzyme in uninfected cells which can carry out such a transcription. A viral enzyme which has been the object of considerable interest is the reverse transcriptase found in retroviruses. This enzyme has the capacity to transcribe RNA to DNA and the enzyme plays a central role during the replication of retroviruses when they synthesize DNA copies which after circularization can become integrated into the DNA of the host cell. Still another enzyme has been identified in retroviruses, namely ribonuclease H. This ribonuclease can only hydrolyse RNA which is base-paired to a strand of DNA, an RNA–DNA hybrid, and the RNA-splitting activity is included in the polypeptide chain which is responsible for the transcriptase activity. It appears likely that the enzyme is used in connection with the

replication of retroviruses in order to eliminate RNA–DNA hybrids which arise when retrovirus RNA is transcribed into DNA. RNA viruses with negative-strand genomes which transcribe their mRNA by use of viral enzymes also contain the enzymes needed to add a 'cap' structure to the 5′ end of the mRNA. These enzymes include a guanyl transferase and one or more methyl transferases. Among additional enzyme activities which can be identified in poxviruses can be mentioned a nucleoside triphosphate phosphohydrolase, which has the capacity to hydrolyse nucleoside triphosphates in the presence of nucleic acids. Furthermore, poxvirus contains a deoxyribonuclease activity, a protein kinase which can phosphorylate proteins, and a 'nicking and closing' enzyme. The latter enzyme can introduce breaks in DNA and thus has the capacity to release the tension in superhelical DNA.

Many other enzyme activities have been shown to occur in virions. The envelope of influenza virus as well as most other myxoviruses contain the enzyme neuraminidase which can split off N-acetyl neuraminic acid. The enzyme probably plays an important role in the penetration of these viruses through mucopolysaccharide layers in their natural habitat. Both papovaviruses and adenoviruses have been reported to contain endonuclease activities which can cleave virus-DNA, although without any defined specificity. It is doubtful whether this activity is of viral origin or has any biological significance. More likely it is due to a cellular enzyme which has been included in the virus particles. Many enveloped viruses have been found to contain a number of additional enzymatic activities but, again it is difficult to determine whether one is dealing with authentic viral enzymes or contaminating host cell enzymes. Membrane-containing viruses are very difficult to purify to absolute homogeneity and scepticism concerning findings of enzyme activities in these viruses is justified.

Many viral proteins are subject to cleavage reactions

Many viral proteins are subject to one or more cleavage reactions before they are included in the mature virus particles. One kind of cleavage reaction has been described during infection with RNA viruses with a plus-strand genome. These viruses translate their viral mRNA into a gigantic polypeptide, a *polyprotein*, which is carried through a series of cleavage reactions whereby the individual polypeptides are formed (*see* Chapter 8). In retroviruses the cleavage reactions are carried out by virus-specific enzymes whereas the corresponding reaction in many other cases is performed by cellular enzymes.

Enveloped viruses synthesize polypeptides which include a short, so-called signal peptide. This peptide is found in the N-terminal end of the polypeptide and appears to be responsible for the transport of the polypeptide through cellular membranes before the virus particles are assembled. In connection with this transport, the signal peptide is split off (*see also* Chapter 10).

In many viruses polypeptides are subjected to proteolytic cleavage during the assembly of the virus particles. The functional importance of the phenomenon is only partly known. It has been found that such a cleavage occurs of the fusion (F) polypeptide of paramyxoviruses and in a corresponding fashion in the haemagglutinin polypeptide of orthomyxoviruses. In both cases this cleavage appears to endow the virions with a capacity to deposit their nucleocapsid by membrane fusion into the cytoplasm of infected cells.

Additional components in virions

As mentioned previously, glycosylated proteins occur in many viruses, particularly in those which have an envelope. Glycosylation appears to be a cellular process essentially and virus-specific enzymes do not appear to be involved. It has further been shown that the viral proteins are glycosylated in different ways in different host cells. Thus the carbohydrate chains in togavirus glycoproteins have different compositions depending upon the kind of cells in which the virus has been cultivated. Enveloped viruses in addition contain lipids in their membrane. These lipids derive from the membranes of the host cell and no virus specificity has been demonstrated even though minor differences between the lipid composition of virus and host cells have been found in some systems. The lipid composition of a virus membrane may vary somewhat, depending on the cellular membrane from which the envelope is derived. The lipids found in hepatitis B virus appear to represent a special case. They do not seem to derive from cellular membrane structures but, instead, show similarities to lipids circulating in the blood.

Among additional components to be found in virions are polyamines. In particular, it has been found that herpesviruses contain spermin and spermidin in large quantities, sufficient to neutralize about 50 per cent of the charged groups in herpesvirus DNA. The importance of these polyamines has not as yet been defined.

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4

Viral gene products; their immunological properties and biological activities

Erling Norrby

Many different virus-specific gene products are synthesized in infected cells. The number of these proteins varies from three to several hundred depending upon the amount of genetic information stored in the virus nucleic acid. Some proteins are building stones in virions, *structural proteins*, whereas other proteins, *non-structural proteins*, have an important influence on the metabolism in the infected cell and regulate the different reactions connected with virus multiplication. During the final phase of the infectious process, a large number of virions are released from infected cells and, in addition, varying amounts of non-infectious (defective) virus particles and excess amounts of structural proteins are released. Also, non-structural proteins are released from degenerating cells.

The biological function of different structural components can be studied either by using disintegrated virions or by direct characterization of spontaneously-occurring free structural components. With certain viruses, e.g. adenoviruses, it is easy to isolate structural components, such as capsomers, whereas, with many other viruses, it is difficult to prepare free intact capsomers. Furthermore, it usually is difficult to prepare free envelope components since the hydrophobic proteins which are released from the lipid structure of membranes have a tendency to precipitate. Some virus proteins carry special biological activities, e.g. enzymatic or haemagglutinating activity, which can be used for their identification. Studies of the antigenic and biological properties of viral gene products are performed to characterize the structure and function of different parts of virions, to compare related viruses to each other, and to allow a characterization of virus replication in cells.

Viral antigens

Structural and non-structural virus antigens can be characterized by traditional techniques such as complement fixation and immune diffusion or by more modern and sensitive techniques such as radio-immunoassays (RIA) and enzyme-linked immunosorbent assay (ELISA) (*see* Chapter 20). The majority of virus products have a size and chemical character which make them effective as immunogens. The immunogenicity of components usually is accentuated when they form a part of virions. One prerequisite for the identification of a certain antigen is the availability of a suitable antiserum. Furthermore, it is required that the antigen is present in sufficient quantity. Recently it has become possible to identify separately the

structural antigen(s) with which antibodies present in a serum sample react. For this purpose isotope-labelled virus proteins are used and these proteins are precipitated by antibodies in a radioimmune precipitation assay (RIPA). The complexes of labelled viral antigens and specific antibodies are isolated by adsorption with staphylococcus A protein. Hereafter, the viral polypeptide(s) which are included in the complexes are dissociated with a strong detergent and then fractionated by polyacrylamide gel electrophoresis (*Figure 4.1*).

Analysis of different structural virus products in cells has shown that a certain product occasionally may change its antigenic or biological character during processing in the infected cells. This change may be caused by a post-translational

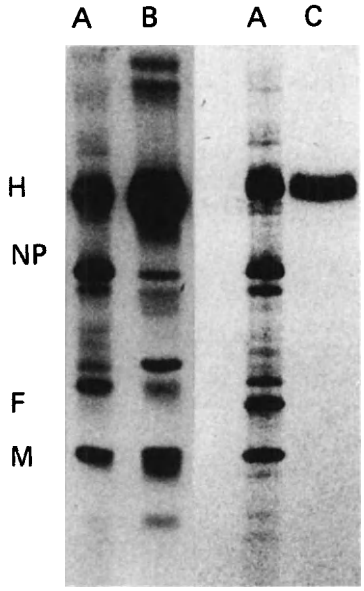

Figure 4.1. Demonstration of the capacity of antibodies to react with different polypeptides in measles virus. Antiserum is mixed with radioactively-labelled virus proteins. The antigen-antibody complexes which are formed after incubation are isolated and the polypeptides which form a part of the complex are identified by electrophoretic separation. Sample (A) shows the polypeptides which are present in the material before precipitation (e.g. H = haemagglutinin, NP = nucleoprotein, F = fusion component, M = matrix protein) and samples (B) and (C), the polypeptides which are precipitated by antisera againt whole virus particles and haemagglutinin, respectively

cleavage of the protein (*see* Chapter 3) or by an association of the protein with other viral structural proteins or the virus nucleic acid. For example, empty poliovirus capsids differ from complete RNA-containing virions concerning the antigenic specificity of the surface of the particles.

In connection with an infection the organism mobilizes a durable and often lifelong immune response against virions. This immune response provides protection against renewed infections with the same virus (*see* Chapter 19). The selective pressure established by the immune reactions has led to the evolution of viruses in two different directions. Some viruses, e.g. influenza virus, have a capacity to modify their surface antigen properties continuously (*see* Chapter 29), whereas in other cases a differentiation against a multitude of different antigenic types forming a virus genus, e.g. rhinoviruses and adenoviruses, occurs (*see* Chapters 25 and 29).

The immunological selective pressure in the first hand influences surface antigens of viruses whereas the central components during evolution may remain unchanged. In a simplified way it can be stated that the antigenic specificity of components in virions increases the closer the surface of the particle in which the component is localized. As examples can be cited influenza A virus and adenovirus (*Figure 4.2*). Influenza A virus has two surface structures, the haemagglutinin and

the neuraminidase. The antigenic character of these components varies independently or in parallel from year to year. The internal structures of the virus, the matrix component, the nucleocapsid and the transcriptase, in contrast, have similar antigenic characteristics in virus strains isolated at different times and in different parts of the world.

The 38 different types of adenoviruses in man show different surface antigens. Also in this case there are two dominating, antigenically-different surface components, the outer part of hexons (non-vertex capsomers) and the distal part of fibres (vertex projections) (*Figure 4.2*). The antigenic properties of these components are characteristic for each type of adenovirus. The two *type-specific antigens* may vary independently of each other between adenovirus types. If an adenovirus has a fibre

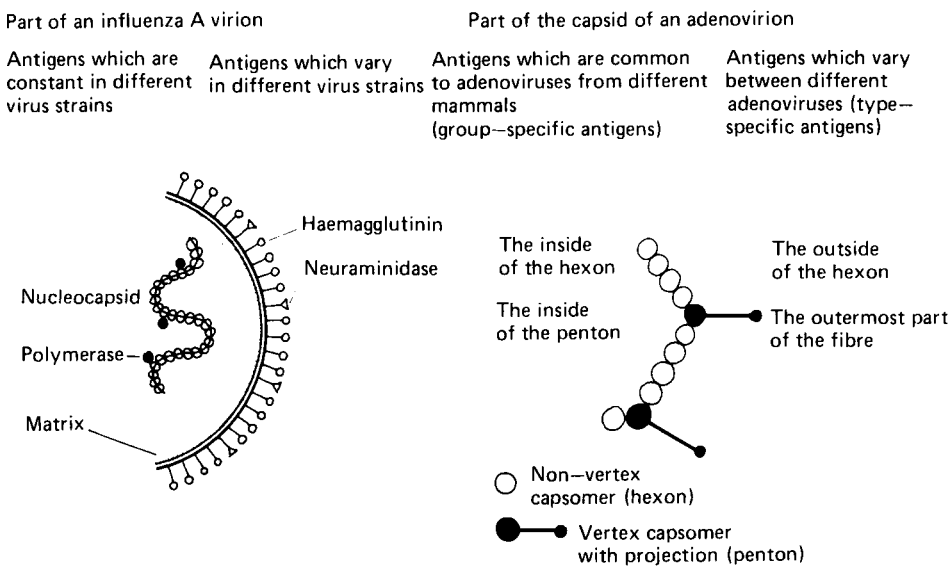

Figure 4.2. Schematic description of antigens with different specificities on the inside and the outside of influenza virions and adenovirions

and a hexon antigen from two different types of adenovirus this results in a capsid mosaic and the virus is characterized as an *intermediate type*. On the inside of the virus capsid there are different antigens, both on hexons and on vertex capsomers which are shared between all types of adenovirus (*group-specific antigens*). Finally, antigens which are structurally located just below the outermost part of hexons and on the outermost part of vertex capsomers, are shared between members of subgroups of adenoviruses. Thus, among adenoviruses, group, subgroup and type-specific antigens can be identified.

Antigens on the surface of a virion can be identified by several different serological techniques. The reaction between virions and antibodies can be visualized by immune electron microscopy (*Figure 4.3*). However, it is more common to identify antibodies reacting with surface antigens by estimating their capacity to interfere with virus replication, *virus neutralization*, or their effect on the biological activities of surface components. Examples of components with such activities are haemagglutinin, haemolysin and neuraminidase (*see below*). Typing

of a virus by these different serological tests does not always give concordant results. This may be due to the fact that the antigen to which neutralizing antibodies attach is different than for, for example, the virus haemagglutinin. This is the case in adenoviruses. Furthermore, a certain surface antigen may occur in many virus types within a genus. This is the case concerning the haemagglutinin in the

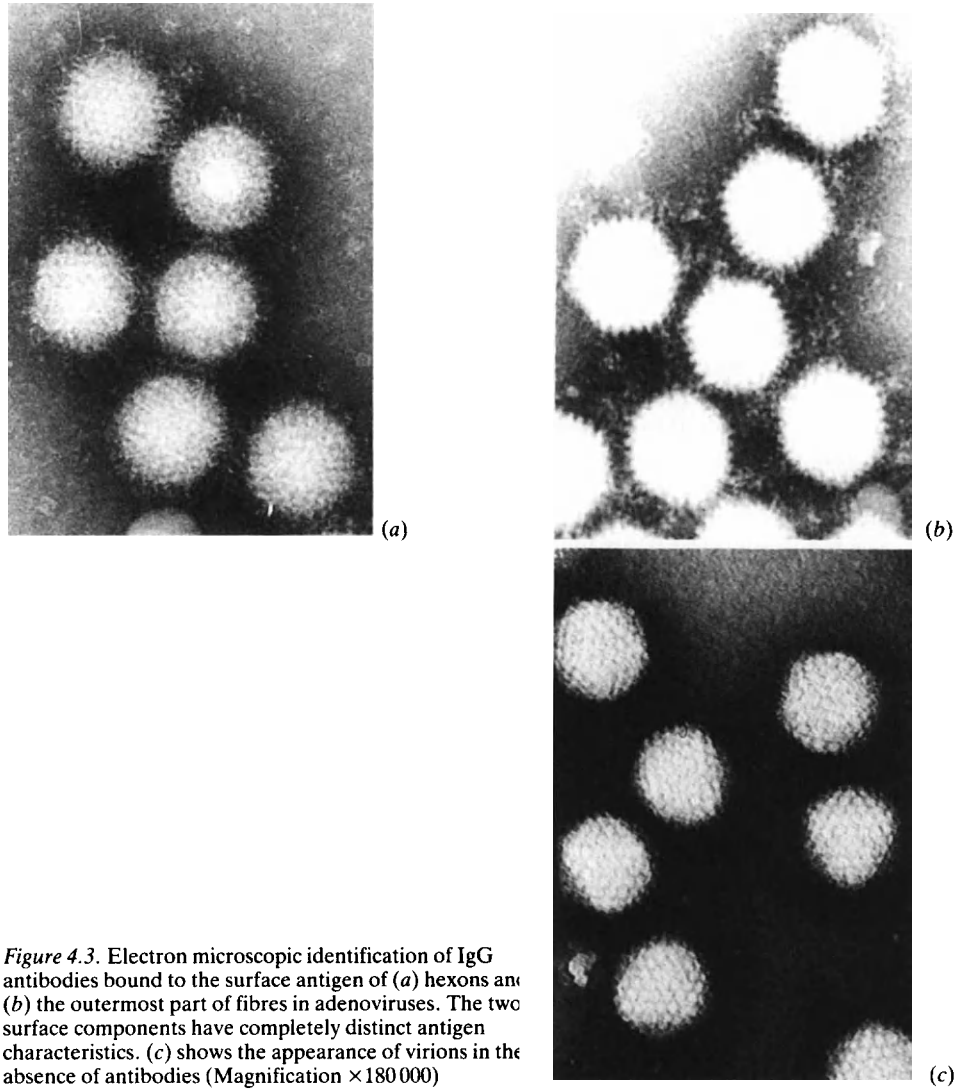

Figure 4.3. Electron microscopic identification of IgG antibodies bound to the surface antigen of (*a*) hexons an((*b*) the outermost part of fibres in adenoviruses. The two surface components have completely distinct antigen characteristics. (*c*) shows the appearance of virions in the absence of antibodies (Magnification ×180 000)

togavirus genera alphaviruses and flaviviruses. The immunological specificity of internal components is usually determined by a conventional immunological test, but more special tests, for example antibody-mediated blocking of a viral transcriptase, can also be used.

The degree of immunological difference between two viruses belonging to the same group which is necessary for distinction to be made between two separate

virus types has been defined pragmatically. Experimental animals are immunized with each one of the two viruses that are to be compared. Both viruses are then tested against homologous and heterologous hyperimmune sera, usually by neutralization tests (*see* the description of this test in Chapters 19 and 20). If the homologous antiserum shows an 8-fold, or larger, neutralizing capacity than heterologous serum (occasionally 16-fold differences are required; neutralization tests are usually performed using a 2-fold dilution step), then this is considered to signify that the two types are distinct. When smaller differences are encountered *virus variants* or strain variations are indicated. The designation *strain* (occasionally isolate) implies that the virus has been isolated on a certain occasion. Thus during an epidemic several strains of the same type of virus may be isolated.

Haemagglutination

It was discovered by accident that some virus products can aggregate red blood cells, *haemagglutination*. In the 1930s the chorioallantoic cavity in embryonated hen's eggs began to be used for propagation of influenza virus. When the allantoic fluid was recovered admixing of blood from disrupted vessels occasionally occurred. It was then found that in materials from infected eggs the erythrocytes were agglutinated, i.e. they formed smaller or larger aggregates. The agglutination of red blood cells was found to be caused by a protein formed during replication of the virus in the chorioallantoic membrane. Since the haemagglutination could be blocked by addition of antibodies against the virus it was demonstrated to be virus-specific.

As was mentioned the haemagglutinating activity can be used in immunological tests to characterize the surface of virions. In addition it has been used as a means of characterizing the mechanism for virus adsorption to cells. This is possible since in most cases it is the same course of events which lies behind haemagglutination by the virus and the first step of a virus infection of nucleated cells, the adsorption (*see* Chapter 7). However, no virus replication can take place in red blood cells. The agglutination of erythrocytes is dependent on the occurrence of specific structures, *receptors*, on the surface of the cell and the presence of structures on the surface of the virions which have an affinity for these receptors. The receptor structures for different viruses have only been characterized to a limited extent. Most but not all types of viruses have been shown to have haemagglutinating activity. Suitable conditions (type of red blood cells, temperature, pH) for demonstration of this capacity vary markedly between different viruses. Within certain virus groups, e.g. adenoviruses, members can be divided into subgroups on the basis of the special conditions which are required for expression of the different forms of haemagglutinating activity. It is possible that the absence of haemagglutinating activity in the case of some viruses is due to the fact that the proper conditions for agglutination have not been defined.

The structural component on the surface of virions which can bind to the receptors on cells varies between different viruses. In non-enveloped viruses it is capsid components and in enveloped viruses it is certain peplomers. In the case of small non-enveloped viruses, intact virions or empty capsids are responsible for haemagglutination, while larger viruses in addition produce different forms of aggregates of capsid structures which can agglutinate red blood cells. The receptor affinity in adenoviruses is carried by the vertex capsomer projection, the fibre. Different forms of adenovirus haemagglutinins are illustrated in *Figure 4.4*.

In enveloped viruses it is the glycoproteins which build up the peplomers that show a receptor affinity. Intact virions and, also, smaller envelope fragments can cause haemagglutination. A splitting of the virus with organic solvents and/or detergents destroys the infectious property of the virus but allows the haemagglutinating activity to be retained or even increased. The haemagglutinin of poxviruses had previously been described to be a structure separate from infectious intracellular particles. However, recently it has been shown that extracellular particles are enclosed by an envelope in which the haemagglutinin is included. A situation analogous to that in the case of other enveloped viruses thus exists.

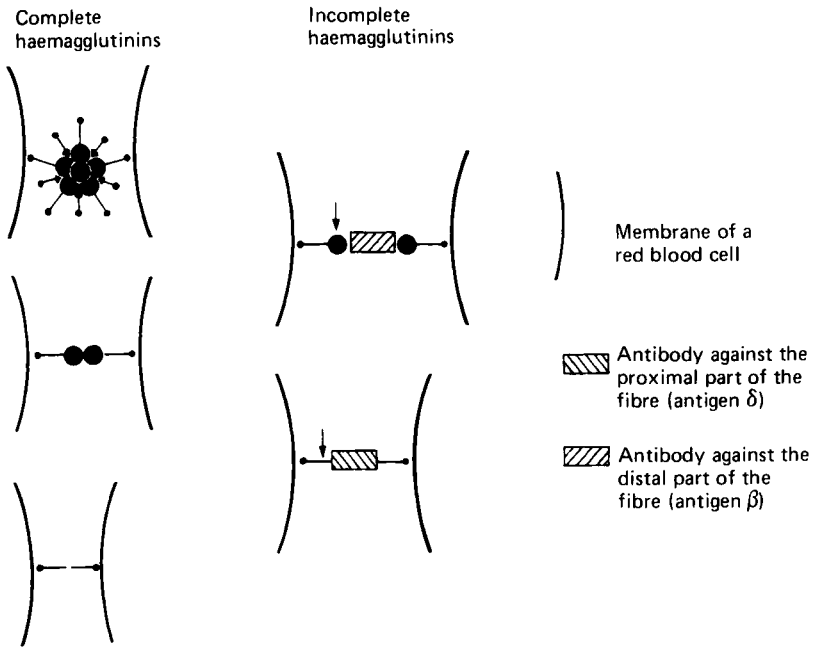

Figure 4.4. Schematic description of different forms of adenovirus haemagglutinins in addition to intact virions and empty capsids, which also can give haemagglutination. Three forms of non-virion complete haemagglutinins have been identified. In addition there are two forms of incomplete haemagglutinins. The latter structures can react with membrane receptors but since they are monovalent the erythrocytes are not agglutinated. Addition of antibodies against vertex capsomers or, alternatively, the proximal part of fibres, however, leads to an aggregation, indirect haemagglutination

The haemagglutinin is inserted into membranes of cells that are infected with enveloped viruses (*see* Chapter 10). Different membranes in the cells can be involved in such a restructuring. In many cases the haemagglutinin is introduced into the cytoplasmic membrane and becomes accessible at the surface of infected cells. As a consequence the infected cell shows a capacity to adsorb red blood cells, *haemadsorption* (*Figure 4.5*).

Haemagglutination by influenzavirus has been characterized in detail. There are two types of peplomers in the virus envelope, one that has affinity for receptors, *haemagglutinin*, and one that shows enzymatic activity, *neuraminidase*. The virus

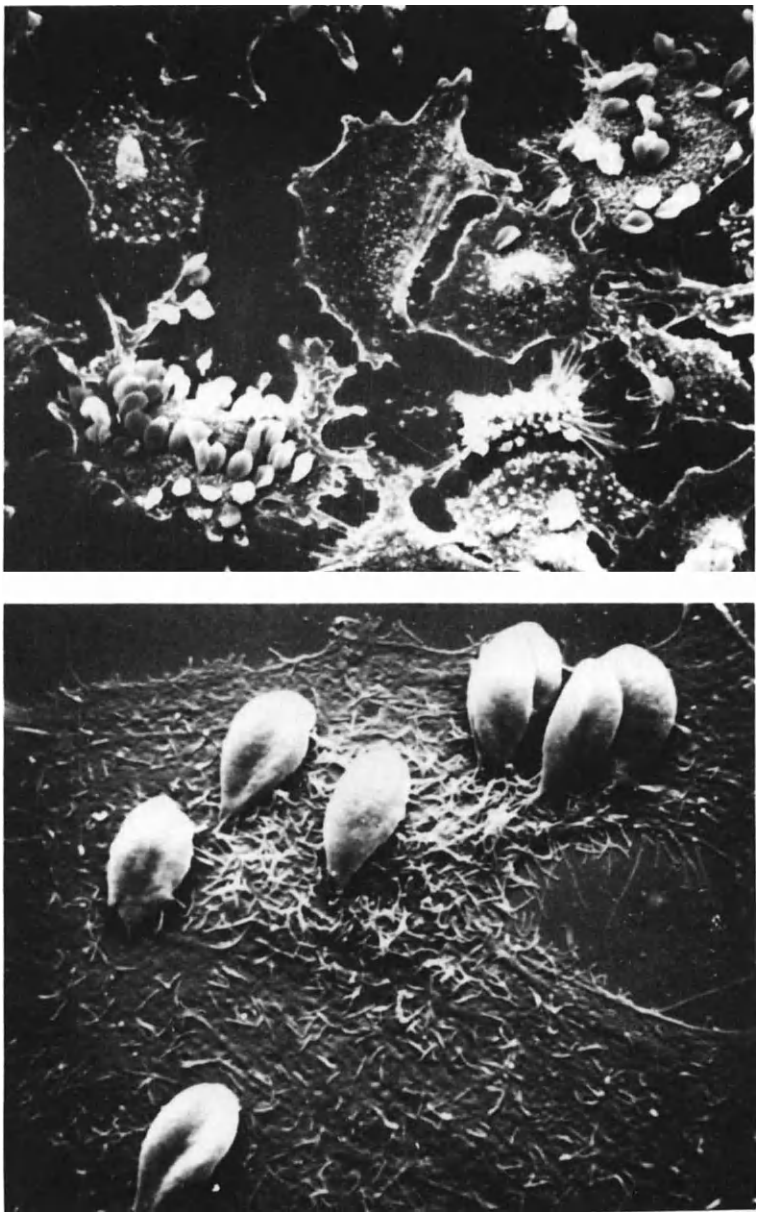

Figure 4.5. Red blood cells adsorbed to measles virus infected cells shown by scanning electron microscopy. The top photograph shows both uninfected and infected cells. The bottom photograph shows an infected cell at a greater magnification. The pronounced formation of villi on the surface of the cell is a characteristic feature of a paramyxovirus infection (Photo reproduced by permission of Dr T. Bächi, Institute for Medical Microbiology, University of Zurich, Switzerland)

particles are anchored to the surface of erythrocytes via the haemagglutinin and an agglutination develops. The receptors to which virus particles become attached have a mucoprotein nature and include a polysaccharide which in its terminal has a sialinic acid connected with a sugar unit via a ketoside bond. The viral neuraminidase splits the mucoprotein and destroys the receptor by releasing N-acetyl neuraminic acid. This breakdown of receptors causes the virus particles again to be released (eluted) from the erythrocyte surface. After incubation for some time at 37°C all receptors are destroyed and haemagglutination ceases. When the mixture of virus and cells is incubated at a lower temperature the haemagglutination is irreversible. However, for many viruses other than influenza the haemagglutination is also irreversible at 37°C.

The significance of neuraminidase activity in orthomyxoviruses and many paramyxoviruses has not been clarified. The enzyme may play a role in facilitating the penetration of virus through the protecting mucosa layer which is present in the respiratory tract. The enzyme may also be important in connection with the release of virus from cells since enzymatic destruction of receptors will prevent the readsorption of the virus to the cell from which it is produced.

Cell fusion and haemolysis

When certain enveloped viruses, e.g. paramyxoviruses and herpesviruses, infect cells in an organ or in a cell culture, multinucleated giant cells may develop (*see* Chapter 11). This phenomenon is caused by the introduction of a certain kind of viral peplomer into the cytoplasmic membrane of infected cells which provides the cells with the capacity to fuse with neighbouring cells. The mechanism behind this membrane fusion has not been defined. A corresponding fusion of membranes can also occur if a large amount of paramyxovirions, for example, is added to a group of uninfected cells. Multinucleated cells can be identified within a few hours after the addition of the virus, i.e. long before maturation of the virus has occurred. This effect is occasionally referred to as *fusion from without* in contrast to the above-mentioned fusion between infected and non-infected cells, which is called *fusion from within*. However, the two situations are different forms of expression of one and the same biological activity. The sequence of events during fusion from without is discussed below, fusion from within is described in Chapter 11.

Fusion from without has mainly been studied by using paramyxoviruses as a model. The fusion occurs without any need for a virus replication in cells. This is apparent both by the relatively rapid development of the process and also by the fact that virus products which lack infectious activity can cause cell fusion. Thus, virions which have been treated with ultraviolet light to destroy their infectious property can fuse cells. There is a collaboration between two different activities in the paramyxovirus envelope which leads to cell fusion. The virions have in their envelope one kind of peplomer, which has haemagglutinating and, in most cases, neuraminidase activity and another which is responsible for the fusion activity. Virions or larger envelope fragments, which are anchored to the cytoplasmic membrane via the haemagglutinin, can be fused into this membrane through the activity of the fusion peplomers. The fusion activity is found only in particles in which the fusion protein has been proteolytically cleaved (*see* Chapter 3). The fusion between the virus envelope and the cell membrane leads to a deposition of

paramyxovirus nucleocapsids in the cytoplasm, i.e. virus penetration (*see* Chapter 7). If the melting in of virus envelope in the cytoplasmic membrane occurs extensively, membrane damage occurs. As a result of the cells' attempt to repair this damage, a fusion with neighbouring cells takes place.

Cell fusion with non-infectious virus has been exploited experimentally in several connections. A fusion between tumour cells, which contain virus nucleic acid but which do not produce virus, and normal cells can activate a virus to form infectious particles. From the point of view of cell biology it is interesting that a fusion between cells from two different species, *heterokaryons*, can be obtained, e.g. between cells from mice and man. The somatic cell hybrids are important tools for cytogenetic studies. A further example of the methodological exploitation of the fusion phenomenon is found with the hybridoma technique. If an animal is immunized and the antibody-forming cells are isolated hereafter, these cells cannot survive for more than a few days in cultivation vessels. However, tumour (myeloma) cells which produce immunoglobulins can divide continuously in artificial media (*see* Chapter 5). The fusion between cells which produce antibodies after immunization and myeloma cells can give hybridomas with properties from both parental cells. Thus, hybridomas can be selected which produce defined and highly specific monoclonal antibodies.

If a virus with a capacity to cause cell fusion reacts with the surface of erythrocytes, this may lead to haemolysis. One prerequisite for this activity, as in the case of cell fusion, is that the particles are adsorbed to the red cell surface via the haemagglutinin. Also in the case of haemolysis the sequence of events starts with a melting in of the virus envelope into the cellular membrane. Viral damage to the membrane is inflicted and the haemoglobin leaks out. Virus-induced haemolysis will not lead to any comprehensive destruction of erythrocytes in an infected individual, since the relative amount of circulating virus in relationship to the number of erythrocytes never becomes so great that a comprehensive haemolysis can be identified.

Enzymatic activities of viral gene products

New virus-specific enzyme activities appear in infected cells. The number of such new enzyme activities is proportional to the size of the virus genome. The enzyme activity usually resides in viral proteins but in some cases active complexes are formed between virus and cell proteins. Some viruses also have the capacity to activate selectively certain cellular enzymes in connection with the infection. Virus-specific enzymes usually belong among the non-structural proteins, which are formed early during infection. However, there are several examples of enzymatic activities carried by virion proteins.

A few decades ago it was believed that virions essentially lacked enzyme activities. It was only the above-mentioned neuraminidase activity of orthomyxoviruses and paramyxoviruses which had been identified. However, today it has been demonstrated that there are several groups of viruses with different kinds of transcriptases (polymerases) included in virions. These enzymes, which alternatively can transcribe DNA to RNA, RNA to DNA or RNA to RNA as well as other viral enzymes of importance for the metabolism of nucleic acids are discussed in Chapters 3 and 24.

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5

Cultivation of viruses

Göran Wadell

Viruses can only replicate in living cells. For studies of the growth of viruses and for the production of virus components it is therefore necessary to have access to cells cultivated in the laboratory. Cell cultures are also of dominating importance for isolation of infectious agents in the diagnosis of virus infections. Certain viruses which are pathogenic for liver and intestinal epithelial cells can only replicate in these highly differentiated cells, and therefore have not as yet been efficiently cultivated *in vitro*. In the early phase of research on viruses their properties could only be studied by the transmission of the virus from infected to non-infected plants or animals. The capacity of the agent to cause disease was a criterion for successful transmission. For the cultivation of a few viruses it is still necessary to use experimental animals. *In vitro* methods for the cultivation of many togaviruses, for example, are not available and the viruses are therefore identified and enriched by infections in mice. During the 1930s methods were developed for the cultivation of, primarily, poxviruses, herpesviruses and certain myxoviruses in embryonated hen's eggs. The introduction of this technique was a major step forward and it is still being used to a certain extent for the quantification of virus infectivity, for vaccine production and for diagnostic purposes.

Propagation of viruses in both live animals and embryonated hen's eggs are included in the concept of *in vivo* systems. The term *in vitro* systems is used to mean the cultivation of viruses in tissue or cell cultures.

During the 1940s well characterized cell culture media were developed and antibiotics became available. Furthermore, it was shown that treatment with trypsin of mechanically torn organ fragments gave a suspension of isolated cells that had the capacity to attach themselves to a firm supporting layer and to start dividing. These observations offered new opportunities for the extensive use of cell culture techniques. A new era in the study of virus infection was initiated in 1949 when Enders, Weller and Robbins showed that poliovirus could be propagated in cultures of epithelial embryonic cells and cause morphological changes in such cultures. Since this observation, the cell culture technique has been increasingly used in virology.

Identification and propagation of viruses in tissue and cell cultures

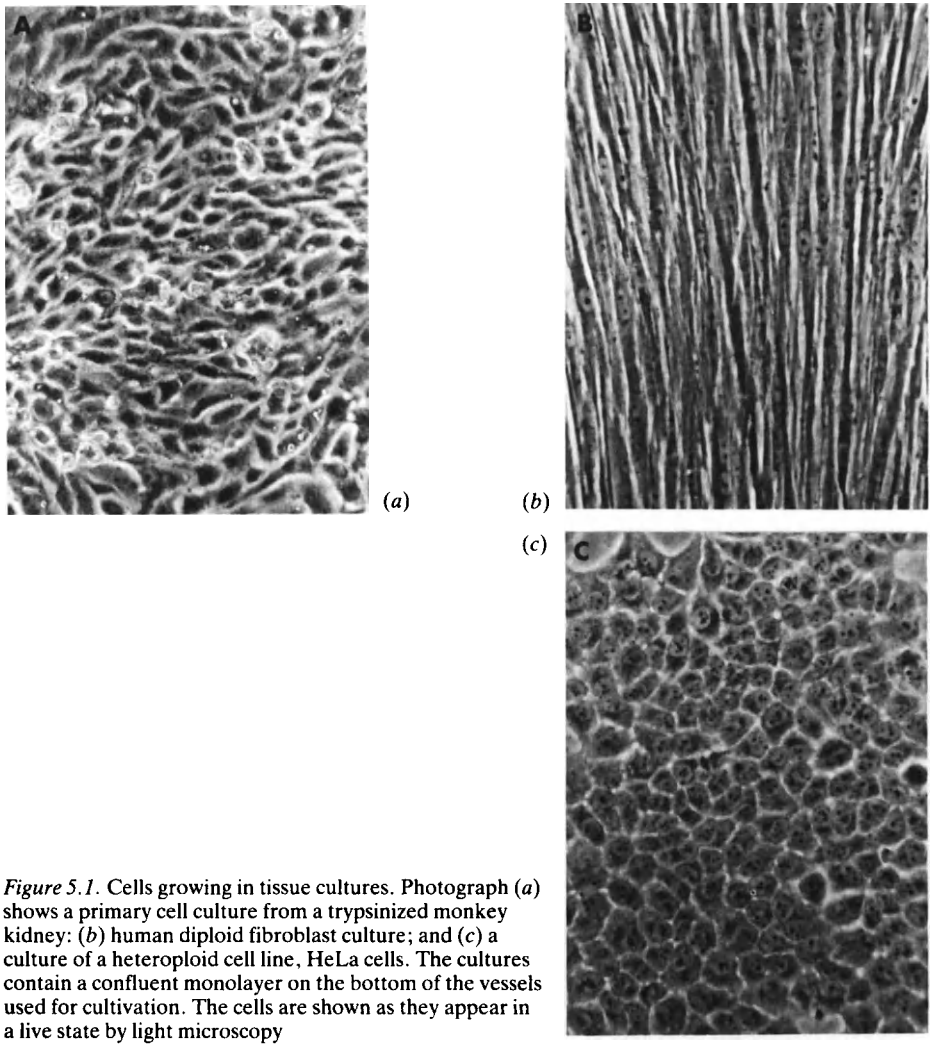
Organ cultures

Organ cultures refer to the thin slices of organs preserved *in vitro* which allow diffusion of nutritional substances or perfused organs. Cells in organ cultures can maintain their high degree of differentiation and specialized functions for some

weeks. The restricted length of life and in particular the difficulties involved in identifying viral changes have led to this type of culture being used only in selected cases. Epithelium from the respiratory and the intestinal tract and fragments from nervous tissue, ovaries, thyroidea, are examples of differentiated tissues that can be maintained in organ cultures. Organ cultures with ciliated respiratory epithelium are used for the cultivation of respiratory viruses (e.g. coronaviruses and certain rhinoviruses), which do not grow in cell cultures (cf. Chapter 20).

Cell cultures

Cell cultures are established by the propagation of dispersed cells. In some cases such cells may grow in suspension, but most often they grow as a monolayer on the supporting material. There are three different kinds of monolayer cultures: primary cell cultures, diploid cell lines and heteroploid, established cell lines (*Figure 5.1*).



(*a*) (*b*) (*c*)

Figure 5.1. Cells growing in tissue cultures. Photograph (*a*) shows a primary cell culture from a trypsinized monkey kidney: (*b*) human diploid fibroblast culture; and (*c*) a culture of a heteroploid cell line, HeLa cells. The cultures contain a confluent monolayer on the bottom of the vessels used for cultivation. The cells are shown as they appear in a live state by light microscopy

Primary cell cultures

Cultures established by cultivation of cells which have been released from organ fragments are called primary cell cultures. A tissue can be homogenized mechanically (explant culture) or by aid of proteolytic enzymes. In the former case groups of cells are obtained, whereas enzyme treatment with, for example, trypsin, provides a monodisperse cell suspension. The cells are suspended in a nutritional solution and transferred to a cultivation vessel. Most live cells attach themselves spontaneously to the bottom of the vessel. Different kinds of cells in a primary culture show a varying capacity to survive. Macrophages, neurons and muscular cells can remain viable for weeks to months, however, without cell division. Epithelial cells can pass through about ten cell divisions *in vitro*, whereas human fibroblasts can divide 50–60 times before the cell line is extinguished. Only cells that are attached to the bottom of the cultivation vessel can divide. This division of cells covers the bottom of the vessel with a cell mosaic and since the division of cells is anchorage-dependent, multiple layers of cells are not formed, the end result being therefore, a *monolayer*. The density of the monolayer varies with different kinds of cells. The phenomenon which leads a cell which is surrounded by other cells to stop dividing is called *contact inhibition*.

A monolayer in a primary culture of kidney tissue, for example, displays a patchy distribution of areas, where similar cells, epithelial cells or fibroblasts, are grouped. Because of the many different cells, a primary culture can be susceptible to infection with a relatively broad spectrum of different viruses. Kidneys from embryos or amnion membranes are frequently used for establishment of human primary cell cultures.

The cells from a primary culture can be increased in number by 'passaging', which means that the cells are transferred from one cultivation vessel to another, where they again are allowed to form a monolayer. The cells are suspended mechanically or by the use of proteolytic enzymes and/or chelate-forming agents, for example EDTA. The latter compound can bind Ca^{++} which is needed for the anchorage of cells to the supporting layer. After distribution of the suspension of cells into three or four new cultivation vessels, cells again attach themselves to the bottom of the vessels and divide until the supporting surface is covered with a layer of cells. Passage of primary cell cultures can be made within one to four weeks after their establishment. With repeated passaging a certain kind of cell eventually becomes dominant.

Diploid cell lines

Repeated passage of cells from fetal lung tissue leads to the dominance of fibroblasts in the culture. The cells retain their diploid character, hence the name 'diploid cell line'. A cell line of this kind is susceptible to a broad spectrum of different kinds of viruses. Diploid cells are used for the cultivation of viruses from patients and also for the production of some live virus vaccines. In order to allow an effective usage of diploid cell lines, the majority of the cells which are obtained with the early passages are stored in a frozen state in liquid nitrogen (−196°C) after the addition of, for example, dimethylsulphoxide (DMSO) to prevent cell damage caused by the freezing. Frozen cells are viable for decades and are used for the establishment of new cultures as the need arises.

Heteroploid (established) cell lines

These cell lines usually derive from tumour cells in a carcinoma or a sarcoma. In spite of the nature of tumour cells it is often difficult to adapt such cells to growth *in vitro*. If cells are successfully established in laboratory vessels they can continue to divide in an unlimited fashion. Cultures can be transferred after a few days and up to a week. Changes in the cell population may occur with the passage of cells. Mutants of cells, which can combine rapid division with the optimal usage of the milieu, become dominant. An established cell line therefore is composed of a limited number of cell clones. The term *clone* is used to describe a population of cells which derives from a single cell and which therefore is genetically homogenous originally. Established cell lines are heteroploid, usually hypotetraploid, and have a reduced capacity to give and respond to signals, which causes contact inhibition. For this reason they can grow to a cell density which is ten times higher than in a culture of diploid cells. Certain established cell lines are not anchorage-dependent for cell division and they can therefore grow in suspension cultures. Established cell lines have a varying susceptibility to infection from different viruses. In each individual case it is therefore necessary to define the cell line that is most suited to the propagation of a certain virus. In some situations the susceptibility of cells to a virus can be increased by treatment of the virus inoculum and/or the host cell with proteolytic enzymes. Established cell lines offer the advantage over other cell lines in that they grow rapidly and that an unlimited number of cells can be obtained.

Among the most commonly used human established cell lines are HeLa cells claimed to be derived from a female cervix carcinoma, HEp-2 cells from a larynx carcinoma, KB cells from a nasopharynx carcinoma and A-549 cells from an 'oat cell' carcinoma. Somewhat unexpectedly it has been found that HEp-2 and KB cell lines do not derive from the assumed original tumours but are clones of HeLa cells. This is because HeLa cells not uncommonly are unintentionally transferred and contaminate other cell cultures, whereafter they can compete with the original cells. BSC-1 and Vero cells which are cell lines derived from green monkey kidney tissue are also used for cultivation of some human viruses. Vero cells have a reduced capacity to synthesize interferon and the cells therefore are suitable for cultivation of viruses which show a high sensitivity to interferon.

Cell lines of human haematopoietic cells

Blood leucocytes and to a certain extent cells from lymph glands in man are relatively easy to obtain. They are propagated in suspension, but can normally survive only for some days in a cell culture. However, cell lines can be established after an infection with Epstein–Barr (EB) virus or by using cells from tumours in the lymphatic system. EB virus infects and can immortalize normal B-cells (*see* Chapters 11 and 31). Haematopoietic cells in culture are divided into lymphoblastoid, lymphoma, myeloma and leukaemia cell lines.

Lymphoblastoid cell lines occasionally can be established to growth *in vitro* from, for example, tonsillar tissue. Cells that divide contain EB virus-DNA, are polyclonal and with few exceptions diploid. Thus the cells mainly have retained their normal properties.

Lymphoma cell lines generally derive from Burkitt's lymphoma cells (BL; *see* Chapters 18 and 31) and to a lesser extent from lymphosarcomas. BL cell lines are readily established to growth *in vitro* and therefore have been the object of

extensive studies. They contain EB virus-DNA, are monoclonal and heteroploid. Furthermore they have the capacity to form colonies in agar and to grow in mice that have a deficient cell bound immunity, so-called 'naked mice' – properties that characterize tumour cells. BL cell lines are used for production of interferon (*see* Chapter 24). Both myeloma and leukaemia cell lines are difficult to establish and as a rule the cultures are overgrown by lymphoblastoid cells.

Microbial contamination in tissue and cell cultures

Cell cultures are cultivated in a nutrition-rich environment and therefore provide excellent conditions for the growth also of microorganisms other than viruses, e.g. bacteria, mycoplasmas and fungi. In order to prevent bacterial infections antibiotics are routinely added in the form of penicillin and streptomycin or, alternatively, gentamicin. Some fungus infections can be prevented by treatment with, for example, amphotericin. Mycoplasma contamination is a major problem in cell cultivation. Mycoplasmas are prokaryotes that grow at or below the cytoplasmic membrane, and they are resistant to many antibiotics. Primary cultures normally are free from mycoplasmas, whereas cell lines commonly are contaminated. This indicates that the mycoplasmas may be transferred from persons handling the cells, or that some ingredient in the medium is a source of contamination. Kanamycin and auromycin can suppress but seldom eliminate a mycoplasma infection. An efficient aseptic technique and avoidance of mouth pipetting are important measures to reduce the occurrence of mycoplasma infection in cultures. Recently it has been reported that it is possible to eliminate a mycoplasma infection by passaging cells in a medium containing specific antiserum against the contaminating mycoplasma. Thus, in this way, valuable cell lines may be rescued and preserved. This might be important, for example, in the production of monoclonal antibodies as the production of hybridoma cells, in particular, is vulnerable to contamination with mycoplasma infections.

Identification and propagation of viruses in embryonated hen's eggs

A technique for the cultivation of viruses in embryonated hen's eggs was introduced in the 1930s and it was used extensively for two decades. Hereafter, different cell cultures replaced the embryonated eggs for most purposes. However, cultivation in eggs is still being used to distinguish pock-forming viruses (e.g. poxviruses and herpesviruses) and for the isolation of influenza A viruses and production of a vaccine against this virus. Different kinds of viruses are propagated in either of the embryonal membranes surrounding the amnion, chorioallantois or yolk sacs (*Figure 5.2*). The chorioallantoic membrane is highly susceptible to infection with poxviruses and it is therefore used for their isolation and quantification (*see Figure 6.1*). During infection newly synthesized virus spreads from the primarily infected cells to neighbouring cells to form the localized pock-like structure. Different membranous sacs are infected by inoculation of virus directly into the fluid in the sac. The amniotic fluid is in contact with the future respiratory tract of the embryo. Influenza A virus which normally initiates infections in the respiratory tract can be demonstrated with high sensitivity after being inoculated into the amnion sac. Vaccines against influenza A virus can be prepared after the inoculation into the allantoic cavity of a virus that has been adapted to grow in this milieu.

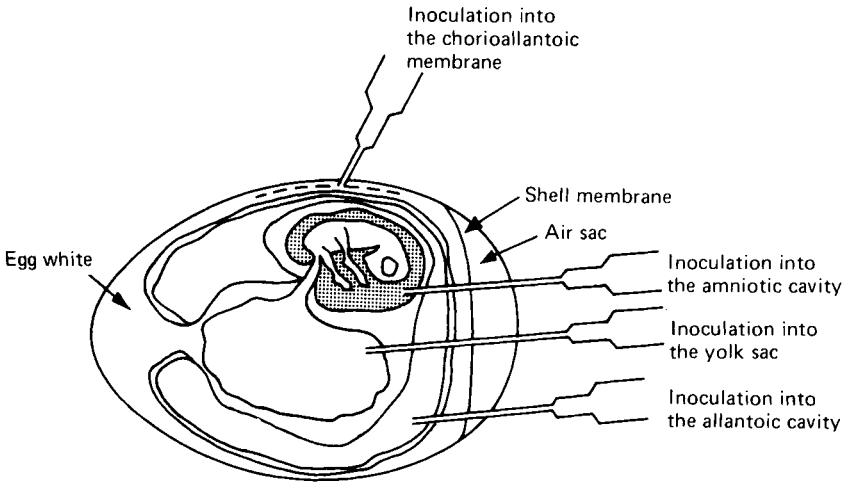


Figure 5.2. Different routes for inoculation of an embryonated hen's egg. The inoculation into the yolk sac is usually made by use of 5 day-old embryo, whereas inoculation into other localities usually is performed with 10–21 day-old embryos

Identification and cultivation of viruses in experimental animals

There is a strong tendency towards reducing the use of experimental animals for preparation of virus materials. However, there are certain disease-related viruses that cannot be cultivated either in cell cultures or in eggs. This is because the cultures which are available do not satisfy the ecological requirements for replication of the virus. Attempts have been made to study non-cultivatable viruses by direct analysis of materials from patients and by an analysis of the immune response (*see* Chapter 20).

Occasionally inoculation of experimental animals provides a more sensitive method for identification of viruses than inoculation of cell cultures. For this reason one tries to isolate, for example, rabies virus, different arboviruses and Coxsackie A viruses by intracerebral inoculation of newborn mice. In order to study the different steps in the disease process and the different defence reactions one is restricted, by necessity, to using experimental animals. Monkeys have been used to analyse the disease process in connection with infections with different hepatitis viruses (cf. Chapter 30) and with certain non-immunogenic atypical infectious agents (cf. Chapter 17). The choice of animal species which is closely related genetically to man is influenced by the fact that certain infectious agents can not replicate in more distantly related species.

Experimental animals are used also for studies of the oncogenic properties of viruses. Mice and hamsters are used primarily. One advantage with the mouse system is the availability of inbred strains. Mice belonging to an inbred strain are genetically identical. Because of this they show a uniform susceptibility to infections and, furthermore, normal and tumour cells can be transplanted from one animal to the other without the development of any rejection reaction.

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6

General views on virus replication

Erling Norrby

As a consequence of the availability of gradually improved methods for morphological and chemical studies the analysis of the relationship between viruses and cells has become increasingly detailed. Two requirements have to be fulfilled to allow optimal studies of the infectious process by cell cultures. One requirement is the availability of methods for quantitative estimation of the synthesis of viral gene products by chemical analysis or by measurements of their biological activity. Studies of biological activity are primarily concerned with the appearance of complete virions, which are identified by different kinds of *infectivity tests*. The other requirement is that all cells in a tissue culture can become infected simultaneously, so that different stages of the infectious process start at approximately the same time in all cells. Under ideal conditions a synchronized, *one-step growth-curve* can be obtained.

Morphological methods for analysis of virus replication

Originally only histological methods were used. By these methods changes in the appearance of cells, occurrence of multinucleated cells, and accumulation of virus products or modified cell products, *inclusions*, in different parts of the cell (cf. Chapter 11) can be shown. By using immune fluorescence methods a further step can be taken beyond that reached by rough morphological analysis. Using specific antibodies against different parts of the virus it is possible to determine where and when different virus components appear in the infected cell. The cells used in this kind of study are fixed in order to allow a penetration of antibodies through cellular membranes. If, instead, unfixed live cells are used it is possible to study antigens which occur at the cell surface and determine where and when these are detectable. Electron microscopy of fixed and sectioned tissue is used for fine morphological studies. Icosahedral and helical nucleocapsids which appear in different parts of the cell can be identified. Furthermore one can demonstrate details in the final phase of morphogenesis of enveloped viruses, i.e. budding from different membrane structures (*see* Chapter 10). Immunological methods can also be used in combination with ultrastructural studies. The use of specific antibodies coupled with the enzyme peroxidase allows the location of certain antigens in the cell to be detected by electron microscopy.

Molecular methods for characterization of virus replication

These methods have been developed extensively during the last two decades. The use of radioactive isotopes frequently coupled to selected precursor substances, e.g. nucleotides and amino acids, has provided new means of analysis. Newly synthesized viral nucleic acids and newly formed virus proteins can be identified. Furthermore one can study how polypeptide products are modified by proteolysis and by glycosylation and in special cases how they are combined with different membrane structures. It is also possible by isotope methods to quantify the appearance of different viral gene products.

Quantitative determination of virus infectivity

The determination of the number of infectious particles which appear inside and outside the infected cells is essential for the understanding of the interplay between virus and cell. Cell cultures used for propagation of viruses usually contain a monolayer of cells covered with a liquid nutritional medium. If a limited amount of virus is inoculated into the culture only a small number of cells are infected. After replication in these cells the virus is released into the medium and the infection becomes general. By light microscopy it is not possible to identify cells which were primarily infected by the virus inoculum. However, this can be achieved by use of the immune fluorescence technique. The number of cells which synthesize virus proteins a short time (3–15 hours) after inoculation is determined. This *fluorescence focus test* demonstrates the number of infectious particles in the material. A more commonly used technique is the *plaque test* which also allows a measurement of the number of infectious units. In this test the liquid nutritional medium is substituted for an agar-containing medium a few hours after inoculation. The latter solidified medium prevents the free spread of newly synthesized virus and from the first infected cells the virus spreads only to neighbouring cells. Distinct focal damage, *plaques*, develops (*Figure 6.1a*). The amount of infectious particles per volume, the *titre*, can be determined by counting the number of plaques which appear after inoculation of the virus material in a suitable dilution. A special situation exists in which certain tumour viruses do not cause cell death but, instead, change the growth characteristics of cells. In this case *foci of transformed cells* appear instead of plaques. Thus it is possible to determine the number of transforming particles in a material. There is still another situation in which the number of infectious units in a material can be quantified. This concerns viruses such as poxvirus, which can grow on the chorioallantoic membrane in embryonated hen's eggs (*see* Chapter 5). Since the outside of this membrane is not covered by any layer of fluid, focal changes corresponding to plaques develop. Each individual alteration of the membrane represents an infectious process initiated by a single particle in the inoculated material (*Figure 6.1.b*).

The number of plaques in a culture normally corresponds to the number of infectious particles in the material inoculated. Diagrammatically, the relationship between the dilution of the material and the number of registered plaques would be linear – a one-hit line. This finding demonstrates that one single infectious particle is sufficient to initiate an infectious process. Not all particles in a given virus material are infectious. Some of the particles lack infectious property because of

some failure in virus synthesis, whereas others may have lost their infectivity by external physical or chemical influences. Occasionally, as little as 1 particle out of 100 000 physical particles are infectious.

In special cases infection from more than one potentially infectious particle is necessary in order to start a virus replication. Examples hereof are adenoassociated virus (a parvovirus) and certain defective retroviruses (*see* Chapters 8 and 9). When the infectious property of these viruses is determined, which can be made only in the presence of a helper virus, a multiple- hit-curve is obtained.

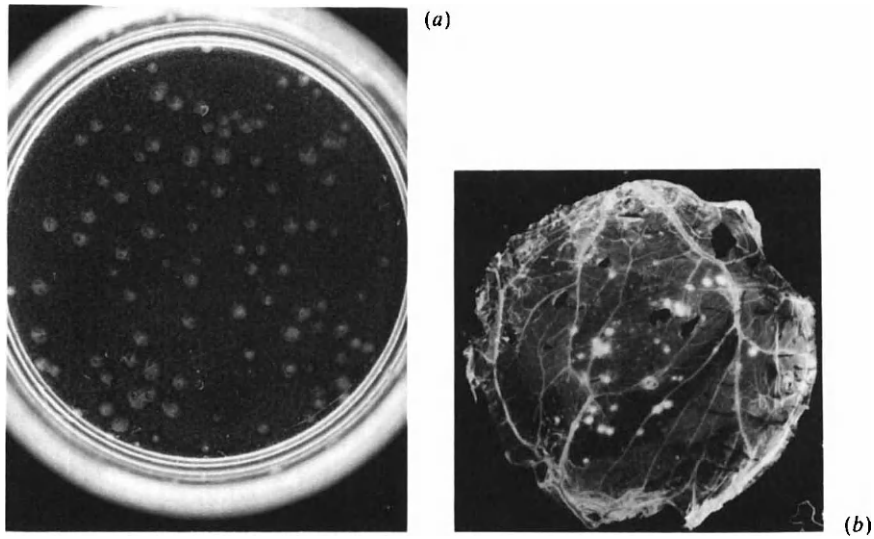

Figure 6.1. (a) Plaques appearing after infection of a heteroploid cell line with vaccinia virus. Each plaque represents some 100 infected cells. The infectious process which has been initiated in isolated cells has been prevented from general spreading by use of a solid agar-containing nutritional medium. (Photo: L. Payne.) *(b)* Chorioallantoic membrane infected with smallpox virus. The membrane has been dissected from the egg and stretched over a glass plate. White dots in addition to the blood vessles can be readily discerned. These dots represent groups of infected cells at the place of the membrane where originally an infection was started by one infectious particle. (Photo: Å. Espmark.)

Occasionally it is technically difficult to obtain a development of distinct plaques since the virus is growing too slowly. In these cases infectivity is determined by another method. A series of dilutions (usually ten-fold) of the material is prepared and a fixed number of tissue cultures are inoculated with material from each dilution. After a few days, up to a week, the cultures are examined by light microscopy to identify cultures which show morphological changes characteristic of the virus. Cultures which show such changes have been hit by one or more infectious particles. Calculation of the highest dilution of material which gives changes of cell morphology in 50 per cent of the cultures, *end point determination*, gives a measure of the amount of infectious particles in the material. The titre of infectious particles is expressed as the inverted value of the end-point dilution. A titre is expressed in ID_{50} (infectious doses). One ID_{50} corresponds to about 0.7 plaques.

Quantitative determination of other biological properties of viral products

These tests include determination of haemagglutination (*see Figure 20.3*), haemolysis, enzyme and antigen activities of virus products. Quantitative determinations are made by use of the same procedure, in principle, as was described above for determination of infectivity titres by end-point estimation. Thus a series of dilutions is set up, usually in 2-fold dilution steps, and the highest dilution which shows, under given conditions, a capacity to, for example, agglutinate or lyse a certain amount of erythrocytes is determined.

Labelling with isotopes coupled to certain precursor substances, e.g. nucleotides or a special monosaccharide, allows comparative quantitative analysis within an experiment. For example, the fraction of the newly synthesized virus DNA that eventually ends up in complete virus particles can be estimated. However, it is not so easy to compare the relative yield of virus particles in different experiments, since it is difficult to standardize the conditions of labelling.

The different phases of virus replication

A number of different stages can be distinguished when a virus replicates in a cell. These stages are most readily studied when conditions of a one-step growth curve are established. This is obtained by inoculation with a minimum of 10 infectious units per cell in a culture. The cells in a culture may be in different phases of the cell cycle and, in the case of certain viruses, this may lead to the infectious process starting at different time points in different cells. Thus several difficulties may be encountered in attempts to obtain an exact synchronization of the infectious process in cells in a culture.

Figure 6.2 illustrates the different phases of virus replication. During the first phase a virion accidentally collides with the surface of a cell and then becomes attached to a certain surface structure on the cytoplasmic membrane, a *receptor*. This step is called the *adsorption phase*. Then the whole virion-or nucleic acid-containing parts of this are taken into the cell. Different viruses exploit different mechanisms for this step, the *penetration phase*. Penetration is considered to be complete when the addition of antibodies against the virus no longer is capable of blocking the continued development of the infectious process. During the subsequent stage no presence of infectious virus can be demonstrated in the infected cell, i.e. the seed virus has established such a relationship with the cell that it has lost any demonstrable infectious property and no new virus has as yet been formed. This stage of virus replication is referred to traditionally as the *eclipse phase*. However, by using modern molecular biological techniques it is now possible to demonstrate that a large number of different changes occur during this phase. Virus gene products influence different cellular processes. The infection may cause blocking to a varying extent of cellular metabolism. In addition, different virus-specific enzymes are being synthesized. As a result suitable conditions for synthesis and accumulation of virus-specific nucleic acids and proteins are provided. In the case of most DNA viruses the nucleic acid replication precedes the synthesis of structural virus proteins. Non-enveloped virions and nucleocapsids can now be formed through a crystallization-like process, the *maturation phase*.

Large numbers of non-enveloped virus particles accumulate in the cell nucleus or in the cytoplasm. These virions are released simultaneously during a short period (1–2 hours) after disruption of the cell, the *release phase*. Maturation of enveloped viruses does not lead to the corresponding accumulation of intracellular virions and a simultaneous release of the particles produced. Formation of enveloped viruses involves an engagement of membrane structures. The nucleocapsids are enclosed by an envelope structure through a budding-off process. The final part of the maturation phase leads to a release of virions, but this occurs continuously during a prolonged time period. The release of virus from cells continues for a few days, up

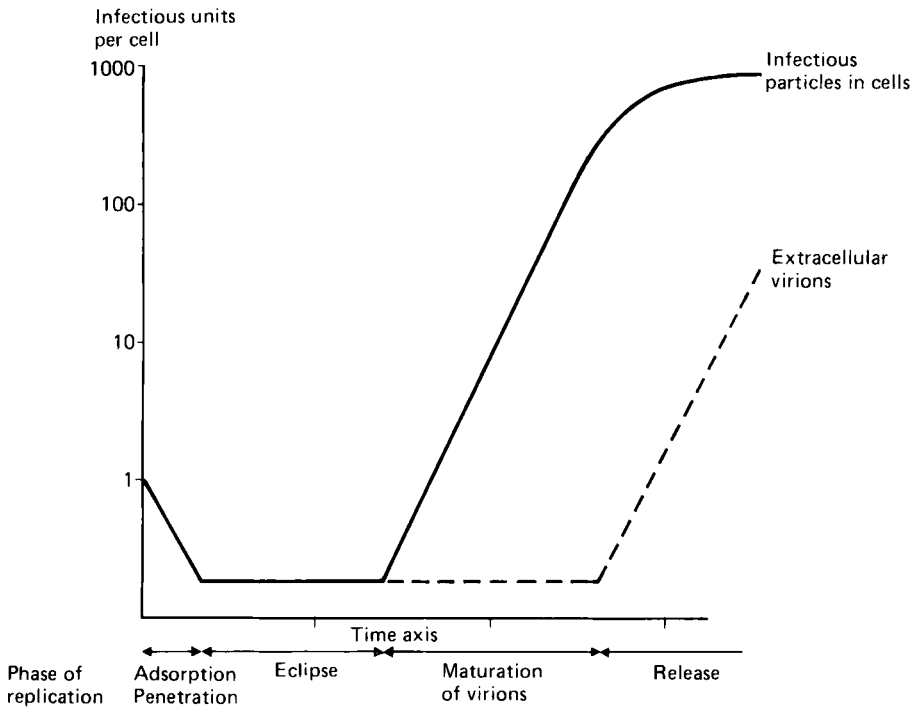

Figure 6.2. Principal stages during virus replication. The cycle of replication of animal viruses covers between 5 and 35 hours. The time difference in appearance of infectious particles inside and outside cells occurs with viruses without envelope, which are released during a short time period with the destruction of cells in the culture. The majority of enveloped viruses mature at different membranes in the cell and are then individually released from the surface of cells by different mechanisms. This release of virus in some cases occurs continuously over many days

to a few weeks, depending on the degree of damage that the virus replication causes on normal cellular functions. It may be mentioned already in this context that the prerequisites for release of enveloped viruses *in vitro* and *in vivo* is somewhat different. In cell cultures the development of the infectious process is a question of interaction between virus and cell, but in the infected organism the occurrence of immune defence reactions causes interference with maturation and release of viruses and/or an elimination of the infected cell.

Blocking of virus replication with metabolic inhibitors

As already mentioned, replication of certain viruses leads to a blocking of normal cellular metabolism. Hereby, the identification of virus-specific events in the infected cell is facilitated. As a means of assistance in studies of virus–cell interactions both in these systems and, by necessity, in connection with analysis of viruses that do not inhibit cellular metabolism, different compounds are used which have the capacity to block certain steps of the metabolism. This artificial blockade can be introduced on different levels, i.e. nucleic acid replication, transcription or translation.

Most RNA viruses do not depend on a functioning DNA in the cell. It is therefore an advantage to study the replication of these viruses in cells treated with the substance actinomycin D to block transcription of DNA to RNA. Many DNA viruses have their synthesis of new proteins divided into an early and a late phase, and the late protein synthesis can only start after replication of virus-DNA. Thus by blocking DNA synthesis with, for example, cytosine arabinoside, a nucleoside analogue, it is possible to study selectively the formation of virus-specific early proteins in such a system. It is also possible to stop, *de novo* synthesis of infectious virus particles in different other stages of the replication cycle. In this case compounds are used which can block protein synthesis, e.g. puromycin, cycloheximide and pactamycin, or glycosylation, e.g. tunicamycin and deoxyglucose. In many studies the method of labelling a special virus product for a short time period and thereafter, in the absence or presence of different metabolic inhibitors, examining the processing of the virus product and its final structural-functional importance (*pulse-chase experiments*) has been found to be particularly useful.

Finally, it should be stated that different kinds of viruses replicate in many different ways. This is because the character of the virus nucleic acid requires the adoption of many different genome strategies and the maturation of enveloped viruses requires the involvement of restructured membranes somewhere in the infected cell. One consequence of the variation between different forms of virus–cell interaction is that the effects of viruses on cells vary extensively (cf. Chapter 11). Infected cells can be destroyed but they may also survive in an altered state. The altered state may represent anything from a chronic infection with continued production of virus particles to the integration of a virus genome in the hereditary material of cells.

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7

Attachment of viruses to cell receptors and penetration of viruses into cells

Lennart Philipson

Adsorption of a virus to the surface of a susceptible cell is a prerequisite for initiation of an infection. The adsorption is the consequence of interactions between specific components on the surface of the virus (virus attachment protein, VAP) and a cellular receptor. The reaction is highly specific. Generally, in the absence of receptors on the cell surface, no infection can take place. Identification of receptors therefore might be important in the understanding of virus pathogenesis. The uptake of a virus into a cell involves as a first step the adsorption of particles and as a second step the penetration in which virus nucleic acid or nucleoprotein is transferred to the cytoplasmic side of the plasma membrane. Electron microscopic studies have shown that virus particles frequently are found in vesicles in the cytoplasm of cells. This mechanism of intake is reminiscent of phagocytosis and has been named *viropexis*. However, the penetration is not terminated until the virus-genetic material has also passed through the membrane of the vesicle. In the case of several DNA viruses, the nucleoprotein must penetrate all the way to the nucleus before any replication can be initiated. The molecular events occur rapidly between adsorption of virus onto the cell surface and the initiation of the virus-directed synthesis of proteins and nucleic acid and they have not been clarified step by step by use of isolated virus and cell components in *in vitro* systems. During the process of adsorption of virus particles, which completely lack capacity for active movement, the virions collide in random fashion with different parts of the plasma membrane. Only once per 10^3–10^4 collisions does this lead to a specific binding between the cellular receptor and the VAP.

The nature of the VAP protein in virions varies with different animal viruses. In enveloped viruses it is represented by the viral glycoprotein which is anchored in the envelope and forms projections from the membrane surface. In non-enveloped viruses projections have been shown only in the large icosahedral adenovirus carrying a fibre structure of each of the 12 vertices and representing, presumably, the VAP protein. With other non-enveloped viruses the VAP protein may be either an individual structural protein on the surface of the virion or a mosaic of several capsid proteins which react with the receptor. The receptor in the cytoplasmic membrane is a surface protein, probably most often a glycoprotein. Different specific receptors for different viruses occur on the surface of cells. In some cases different viruses, for example, coxsackie virus type B3 and adenovirus type 2 use the same type of receptor. The number of receptors in the plasma membrane of a susceptible cell amounts to 10^4–10^6 specific units. The adsorption of a virion to a receptor does not necessarily lead to the initiation of an infection. The initial

binding may be reversible so that the virus again is released from the cell surface, although in many systems an irreversible binding quickly develops when a virus has several VAP proteins that bind to receptors in the plasma membrane.

Virus and receptors

Variations of the VAP protein

Enveloped viruses contain a number of glycoprotein-type projections which are anchored in the membrane. Orthomyxoviruses, paramyxoviruses, rhabdoviruses, togaviruses, as well as coronaviruses and arenaviruses, have such projections. The large poxviruses also have an envelope but peplomers have so far not been identified. The projections are composed of one or two kinds of polypeptides which are glycosylated. The composition of the carbohydrate part varies from virus to virus and also depends on the host cell in which virus replication has occurred.

Two different kinds or projections have been identified in orthomyxoviruses. One kind is represented by the VAP protein (the haemagglutinin) whereas the other kind of projection is represented by an enzyme which has a capacity to cleave the bond between N-acetyl galactosamine and N-acetyl neuraminic acid (NANA or sialic acid) and it therefore acts as neuraminidase. Cell receptors contain sialic acid and the virus enzyme can release the acid by cleaving the receptor. Also paramyxoviruses carry two different kinds of surface projection but now the VAP function and neuraminidase activity reside in the same polypeptide. In most viruses the surface projections are composed of more than one polypeptide as, for example, among togaviruses (Sindbis virus and Semliki forest virus), which have a haemagglutinin formed by three polypeptides.

The VAP protein of non-enveloped viruses has been identified only in some cases. The fibre protein which, like antennae, projects from the icosahedral adenovirus particles, is the virus structure which reacts with the receptors. The fibre of adenoviruses is composed of three identical polypeptides. These form a globular structure in the distal part of the fibre which presumably recognizes the receptor. In order to immobilize a particle of the size of adenovirus it has been assumed that a cooperative irreversible binding of more than one fibre unit to cell receptors is required.

In non-enveloped viruses which lack projections no protein has as yet been defined as being responsible for the VAP function. The surface of picornaviruses appears to represent a polymeric network of proteins and the part that can interact with the receptors may engage several polypeptides in this mosaic.

Cellular receptors

Virus infection in cell cultures and in animals is to a large extent dependent upon the capacity of a virus to adsorb to the target cells, i.e. upon the presence of receptors. In a susceptible animal species the expression of receptors varies in different cell types and even in the same cell type in different stages of differentiation. Receptors for poliovirus only occur in primates. Other picornaviruses, e.g. encephalomyocarditis viruses, appear to be capable of binding to cells from many different species. Species-specific and organ-specific occurrence of receptors has been demonstrated for certain picornaviruses. The coxsackie B virus may cause infection in the nasopharynx and in skeletal and heart muscles in man, independent

of age. In mice, the same virus can only infect newborn but not adult animals. Cellular receptors have been demonstrated in newborn but not in adult mice. Coxsackie A virus receptors, on the other hand, are absent in mouse embryo tissue except for differentiated myoblasts, in which they can be identified before the formation of myotubuli. These observations correlate well with the finding that in mice coxsackie A virus infections attack muscular tissue. With the majority of enveloped viruses, e.g. myxoviruses and rhabdoviruses, receptors can be found in many different animal species. The presence or absence of receptors might explain the capacity of the virus to infect different species (*host range*), and its affinity for different organs in the host organism (*tropism*) (*see* Chapter 13).

The chemical structure of virus receptors has been determined only to a limited extent. As with other cell surface structures there is a continuous turnover of receptors. Receptor material is continuously secreted into the medium of the cell cultures. If intact cells are treated with proteolytic, or in some cases glucosidases, they may lose their virus receptors. After washing and prolonged incubation in a growth medium the receptors are regenerated in a characteristic fashion. This occurs within 2–12 hours. The regeneration of the N-acetyl neuraminic acid-containing receptor for influenza and polyomavirus cannot occur merely by synthesis of the carbohydrate part of the molecule; the protein part must also be synthesized.

It should be noted that the virus-binding receptors probably are important also for normal cellular functions although for most viral receptors these have not been identified. The receptors on the surface of bacteria for certain bacteriophages have been identified recently and appear to be engaged in the uptake and transport of important metabolites, such as maltose and amino acids.

Penetration

When a virus is adsorbed irreversibly to the plasma membrane, parts of the virus or the whole virus is taken into the cytoplasm in order to initiate virus-specific protein and mRNA synthesis. The mechanism of intake varies with different viruses. Enveloped viruses penetrate the cellular membrane either by a fusion process, which means that fusion occurs between the virus envelope and the cell leading to incorporation of virus nucleoprotein, or by a process of phagocytosis. Since large protein structures can be internalized by phagocytosis, it has been proposed that an analogous phenomenon can bring viruses into cells. This has been called 'viropexis'. The mechanism of intake of non-enveloped viruses is even less well characterized.

Infectious nucleic acid

In addition to nucleic acid, the presence of virion-associated RNA polymerases and nucleoproteins may be needed to initiate a virus infection, i.e. replication. As a general principle it can be stated that an isolated nucleic acid is infectious only when extracted from viruses which for their replication do not require a virion-associated RNA polymerase (cf. Chapter 3). The mechanism for cellular uptake of isolated nucleic acid is different from the mechanism responsible for penetration of virus particles. Isolated RNA from picornaviruses, for example, therefore can infect cells from a species which lacks receptors for the virus. These cells allow the

synthesis of complete virus particles but further spread of infection cannot occur owing to the absence of receptors on surrounding non-infected cells. Infectious nucleic acids are demonstrable with RNA from togavirus and DNA from, for example, polyomavirus and SV40 virus. Free nucleic acid has an infectious capacity which is 10^3–10^6 times lower than that of intact virus as calculated per amount of nucleic acid, largely because of the inefficient uptake of nucleic acid.

In the case of large DNA viruses, e.g. herpesviruses and adenoviruses, isolated DNA has a very low infectious capacity owing to the fact that the DNA of these viruses most likely needs to be attached to a protein in order to initiate replication. In adenoviruses it has been shown that the presence of a covalently bound protein at the 5′ terminal of the two DNA strands remarkably increases infectivity. With viruses which have virion-associated polymerases, there are two different situations. In one case, the polymerase is activated by changes induced in the virus envelope during uptake with vaccinia, retrovirus and certain paramyxoviruses, for example. In the other case, proteolytic digestion is required for activation of the polymerase, for example, in reoviruses and related viruses. The different problems concerning virus penetration may best be illustrated by providing details of some selected viruses.

Penetration of poliovirus

Chemical treatment of poliovirus can yield a number of different kinds of subviral particles. By comparing the characteristics of these particles with corresponding structures which can be recovered during the process of virus penetration into cells, certain conclusions can be drawn concerning the mechanism of uptake. The poliovirus capsid is made from four different polypeptides: VP1, VP2, VP3, with a molecular weight of about 25 000–35 000; and VP4, with a molecular weight of about 6000. VP4 is probably located internally in the virion since it cannot be labelled by radioactive isotopes from the outside of the intact virions. After binding of virus to cells at low temperature and a subsequent increase of the temperature of incubation to 37°C, a release of about 50 per cent of virus occurs from the cells. Particles which are released are structurally changed and lack VP4. Furthermore, they do not retain a capacity to readsorb to the cells and probably they have been released together with some membrane components. Antigen analyses of the eluted particles indicate that they have been changed so that they have the same surface properties as heat-treated virus particles. This indicates that an irreversible adsorption to the cytoplasmic membrane leads to a structural change of the virion which causes an opening of the virus capsid thus allowing RNA to penetrate the cell membrane. Recently it was shown that inhibitors of protein synthesis, such as pactamycin, can block the intake of poliovirus, most likely by preventing the release of RNA.

The penetration of adenoviruses

The intake of adenovirus particles into cells requires a long sequence of events before mRNA synthesis can be initiated in the nucleus of the infected cell (*Figure 7.1*). After an irreversible adsorption of virions to receptors, nucleocapsid structures penetrate the plasma membrane and eventually DNA is released into the nucleus of the cell. Regarding most viruses it is still unclear whether they are taken up in vesicles or via direct penetration of the plasma membrane without vesicle

formation. After penetration, intracellular particles which lack fibres and vertex capsomers can be identified chemically. Digestion by nucleases of these subviral particles causes the destruction of about 70 per cent of DNA. In the cytoplasm of cells a nucleoprotein complex of adenovirus which lacks the outer capsid can be demonstrated. Eventually, virus-DNA is delivered to the nucleus of the cell where virus-specific mRNA synthesis takes place. It appears as if virus-specific basic

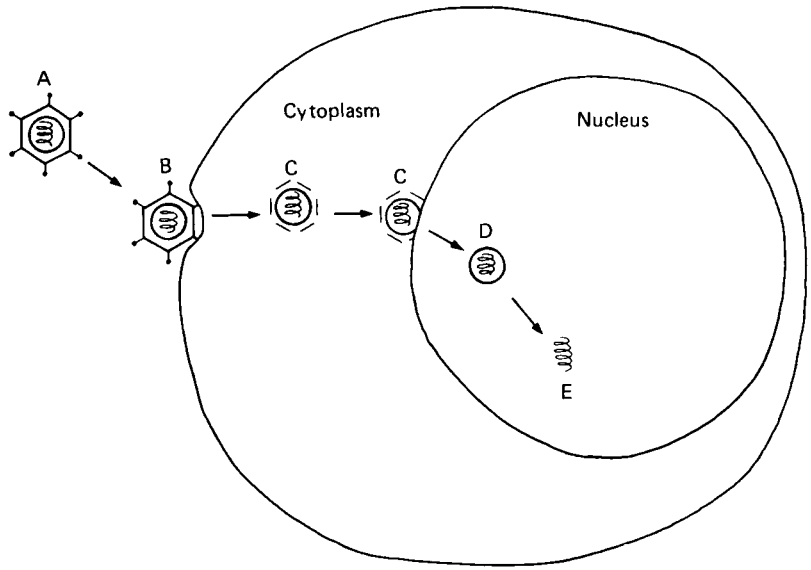

Figure 7.1. A schematic description of the mechanism of uptake of adenoviruses. Intact virus particles (A) are adsorbed to the plasma membrane (B). Inside the cytoplasm particles which lack the peripentonal region (C) are found. The core structure of the virus then is transferred into the nucleus (D). Finally the DNA is released from the core structure (E) and most likely combines with cellular histones

proteins associated with DNA are released and then substituted for cellular histones before the RNA synthesis can be initiated. Most likely the dissociation between nucleoprotein and capsid occurs at the nuclear membrane in proximity to nuclear pores.

The penetration of enveloped viruses by fusion between the virus envelope and vesicles or cytoplasma membrane

The penetration of Semliki forest virus (SFV) has been studied in great detail (*Figure 7.2*). The viral glycoprotein projections form a multivalent complex with the receptors which are transported in the membrane to ultrastructurally defined regions referred to as 'coated pits', whereafter formation of vesicles may take place. This can be observed by electron microscopy. Virus particles can be identified in vesicles and at some later stage the vesicles coalesce with lysosomes by membrane fusion. The infection may be prohibited by treatment of cells with substances (chloroquine and ammonium salts) which accumulate in the lysosome and increase the pH. On the other hand, it has been found that a direct fusion

between SFV and the plasma membrane can be obtained if the pH is less than 6 in the medium. Such a milieu is established when vesicles containing virus particles are fused with lysosomes and the subsequent fusion between the virus envelope and surrounding membrane structures leads to a release of nucleocapsids into the cytoplasm. It is possible that other lipid-containing viruses use a similar mechanism to introduce their nucleocapsid into the cytoplasm of cells. In the case of orthomyxoviruses and paramyxoviruses, for example, mRNA can be formed only by a virion-associated polymerase. The mechanism of intake therefore must allow a

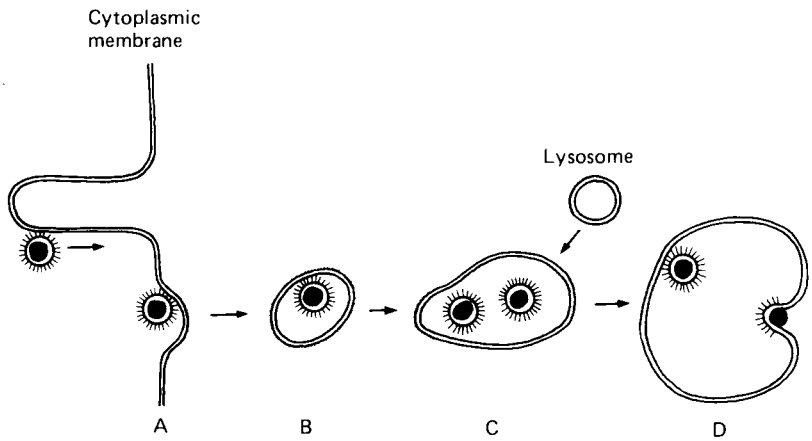

Figure 7.2. Schematic description of the mechanism of uptake of Semliki forest virus (A). The virus recognizes receptors on the cell surface and is transported to certain regions, coated pits, where formation of vesicles can take place (B). Enclosed in cytoplasmic vesicles, the virus is then transported to intermediate vacuoles which then fuse with primary lysosomes (C). As a consequence, the pH in the vacuole is reduced and this causes the virus envelope to fuse with the lysomal membrane and the nucleoprotein is released into the cytoplasm of cells (D). (Reproduced from Helenius *et al.* (1980) by permission of The Rockefeller Press.)

penetration of the nucleoprotein structure. Recent findings indicate that in the case of orthomyxoviruses the mechanism of penetration is similar to that of SFV discussed above. A fusion between vesicles formed by viropexis and lysosomes leads to a reduction of pH which in turn causes fusion between the virus envelope and the surrounding membrane. However, in the case of paramyxoviruses it has long been known that the virus has the capacity to fuse directly with the cytoplasmic membrane and that one of the virus peplomers, the fusion factor, is responsible for this fusion.

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8

Replication, transcription and translation of RNA viruses

Lennart Philipson

The replication of RNA viruses has attracted particular attention since there is no evidence that eukaryotic cells can replicate or transcribe RNA. Thus the mechanisms for transcription and replication of these viruses are unique and they represent a way of expressing genetic information unavailable in the uninfected cell.

After penetration of the virus-genome or virus nucleocapsid to the place for virus reproduction five separate steps in the replication by RNA virus can be distinguished. These steps are:

1. Inhibition of cellular metabolism;
2. Transcription of virus-RNA;
3. Replication of the virus-genome;
4. Morphogenesis or assembly of virions;
5. Release of virions from cells.

Many of these processes require the presence of virus-coded proteins. The different steps may occur simultaneously or in series during the process of infection and their temporal relationship depends upon the properties of the virus. A synchronized productive infection can be used to distinguish the five different steps and to study the biochemical sequence of events involved in each process (*see* Chapter 6). Several differences in replication of various kinds of RNA viruses can be noted. The virus-genome determines the duration of the infectious cycle. Polioviruses, in general, replicate completely within 5–6 hours whereas other RNA viruses, such as orthomyxoviruses and reoviruses, require about 10–24 hours.

Classes of RNA viruses

Different RNA viruses require different procedures for transcription and replication. Since messenger-RNA (mRNA) is required for the translation process at the cellular ribosomes, this molecule has been defined as the positive (plus) strand of RNA. By definition of the structural relationships between viral mRNA and the nucleic acid which represents the viral genome six different classes of viruses can be distinguished (*Figure 8.1*). Classes I and II include viruses which have a double-stranded and single-stranded DNA, respectively.

Among RNA viruses four different classes can be distinguished. Double-stranded RNA viruses, i.e. reoviruses, belong to class III. In these viruses the double-stranded virion-RNA is the template for formation of asymmetrical mRNA. All viruses belonging to class III appear to have a segmented genome, i.e. several pieces of nucleic acid each of which code for one polypeptide. Class IV includes plus-stranded RNA viruses, i.e. the RNA in virions has the same polarity as viral mRNA. Two subclasses of these viruses have been identified. Polioviruses and other picornaviruses belong to class IV A. The mRNA of these viruses has the same length as virion-RNA. This mRNA molecule is translated into one polyprotein which then is cleaved proteolytically to form functional structural and replication proteins. Togaviruses belong to class IVB. These viruses synthesize two

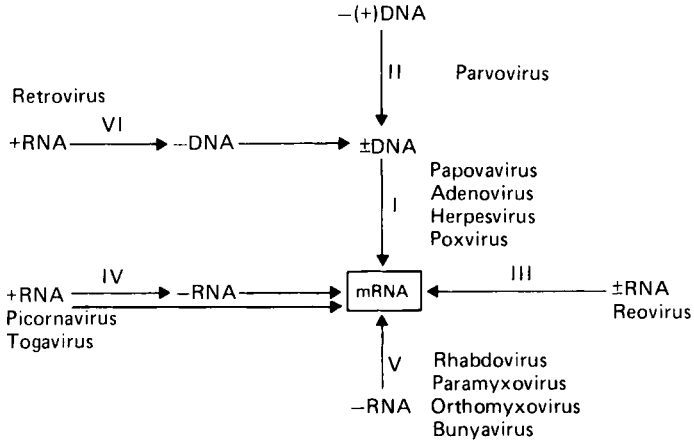

Figure 8.1. Division of animal viruses into classes based on their mechanism of transcription (according to Baltimore 1971). The basic concept behind this division is that virus-mRNA always represents the RNA chain with plus-strand polarity. (Reproduced from Baltimore (1971) by permission of the American Society for Microbiology, Washington, DC, USA.)

different forms of mRNA in cells. One form of mRNA has the same size as virion-RNA whereas the other form of mRNA only amounts to a fraction of the total genome. The latter mRNA is synthesized separately from the 3′ end of minus-strand RNA and codes for structural proteins. Class V viruses are frequently referred to as negative (minus) strand RNA viruses. This class includes rhabdoviruses, orthomyxoviruses, paramyxoviruses, bunyaviruses and arenaviruses. In all these viruses virion-RNA is complementary to mRNA. Thus the virion-RNA does not contain any sequences coding for proteins, but can only serve as a template for the formation of mRNA. Since the cell does not have the capacity to replicate RNA molecules, the virions have to include a polymerase which can transcribe RNA. At least two subclasses of class V viruses can be distinguished. Class VA viruses, which include rhabdoviruses and paramyxoviruses, have one single molecule of virion-RNA and form a series of mRNAs from this template. Each of these mRNAs appear to be monocistronic and thus code for one protein. Class VB, represented by orthomyxoviruses, have a segmented minus-strand RNA and each molecule serves as a template for synthesis of mRNA. It has been assumed also that these mRNAs are monocistronic but recent findings have demonstrated that splicing occurs after transcription of these viruses which allows some of the RNA segments

to direct the synthesis of more than one protein. Finally, class VI includes retroviruses. These viruses are similar to class IV viruses in that virion-RNA corresponds to mRNA, but synthesis of mRNA after infection must take place via a DNA intermediate, which most likely needs to become integrated into the cellular genome. The capacity to synthesize DNA from RNA is absent in the host cells and therefore retroviruses also by necessity contain polymerases in the virions.

Reoviruses

Reoviruses lack an envelope and have an inner core structure and outer capsid. The core structure contains 10 segments of double-stranded RNA and varying amounts of oligonucleotides. The function of the latter is unknown. Ten virus-specific

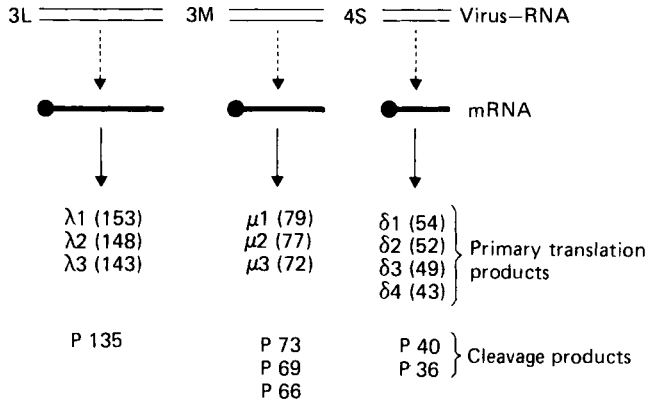

Figure 8.2. Schematic description of the way in which the reovirus genetic information is expressed. mRNA is transcribed from individual double-stranded virus-RNA segments and each single mRNA is translated to a specific polypeptide. L (large) and M (medium) classes of double-stranded genomic RNA include three segments whereas the S (small) class includes four RNA segments. Figures within parentheses denote the molecular weight in thousands for the primary translation products, γ, μ. δ. The molecular weight of the cleavage products are presented without parenthesis

polypeptides are synthesized in the infected cells. Each RNA segment functions as one gene and codes for one polypeptide. All the genes and their corresponding products have been identified (*Figure 8.2*).

Transcription

Since double-stranded RNA cannot serve as mRNA in animal cells, the RNA segments either have to be transcribed or separated in order to make mRNA available. Furthermore the absence of any capacity of cells to transcribe double-stranded RNA necessitates a virus-coded polymerase in virions. Intact viruses do not show any capacity to synthesize mRNA but such a synthesis can be activated after treatment of virions with proteases. The treatment leads to a degradation of

the outer capsid and makes the machinery for mRNA synthesis available. The reovirus polymerase is a stable enzyme and in experimental cell-free systems RNA synthesis can be maintained for several days, facilitating the production of large quantities of reovirus-specific mRNA which can then be used for synthesis of virus-specific proteins. The reovirus-mRNA lacks poly(A) but has a 'cap' at its 5′ end. The minus strand in the double-stranded RNA, however, lacks this structure. It appears as if the primary nucleotide structure at all four ends of each double-stranded RNA is identical in all segments. The whole machinery for synthesis of mRNA is present in virions, including the RNA polymerase activity and the enzyme activity which can add a cap and methylate the 5′ end of mRNA. The core structure has 12 projections which may contain channels through which RNA molecules can leave the structure during the process of synthesis. The release of newly synthesized RNA molecules is an active process which possibly involves a viral adenosine triphosphatase. The structural proteins in the core structure appear to represent the enzymatically active proteins. It has not been possible to obtain the different enzymes in an isolated form and the intact symmetrical core structure therefore seems to be necessary for the transcription.

Replication of virus-RNA

In addition to synthesis of mRNA (*primary transcription*), the virus must be capable of synthesizing strands to allow replication of double-stranded virion-RNA. The mRNA is formed before the minus strands in the infected cell. It seems that the core structure of virions is involved also in the synthesis of minus strands and it is possible that the morphogenesis of the core structure is coordinated with the synthesis of minus strands. In order to form a precursor of the core structure a binding of the 10 minus strands to the proper proteins is required. The replication appears to be a stepwise-developing sequence of events and not to be the result of a concomitant synthesis of minus strands from all mRNA: studies with temperature-sensitive mutants (cf. Chapter 12) have shown that a reassortment of RNA segments readily occurs in connection with the process of morphogenesis.

During the early stages of infection the core structure of reoviruses is released inside the cells and lysosomal enzymes probably contribute to the activation of virus polymerase. The virions are not degraded further than to their core structure and parental core structures may obtain a new outer capsid later during the infection. The phase of primary transcription lasts 3–4 hours, whereafter the synthesis of the double-stranded RNA can be demonstrated. After the synthesis of the double-stranded RNA, the mRNA synthesis again increases in an accelerating fashion. New virus particles can be demonstrated 10 hours after the initiation of infection and the infectious cycle is finished within 16 hours.

Picornaviruses

Poliovirus represents the most carefully studied member of class IVA viruses. Cells which are infected at a high multiplicity will produce about 10^5 virus particles per cell. About 1000 physical particles correspond to one infectious unit in a cell culture. All picornaviruses contain four virus proteins (VP1–4) and in addition a protein which is covalently bound to virion-RNA (Vg). Virion-RNA is a single molecule with a molecular weight of 2.6×10^6. This corresponds to 7500

nucleotides with a coding capacity for about 2500 amino acids. The 5′ terminal of virion-RNA is represented by pUp which is covalently bound to tyrosin in the Vg protein via 5′ phosphate. The mRNA of poliovirus has the same size as virion-RNA but it has a free 5′ end without any protein. At the 3′ end of poliovirus-RNA there are about 75 adenosine nucleotides but this poly(A) is not necessary for the translation of virus-mRNA. It is needed, however, for the replication of RNA which is initiated at the 3′ end of virion-RNA. The entire nucleotide sequence of poliovirus-RNA has recently been established with the aid of recombinant DNA clones, which also appear to be infectious.

Synthesis of virus-RNA

Newly synthesized virus-RNA can be detected as soon as 15–30 minutes after the initiation of infection, but maximal synthesis of RNA occurs firstly 2 hours later. Virus-RNA is synthesized by a structure which is called the replication complex. The whole cycle of replication is localized to the cytoplasm of cells. Thus synthesis of poliovirus can take place in cells from which nuclei have been removed. The replication complex can be separated from virus-specific polyribosomes and there is

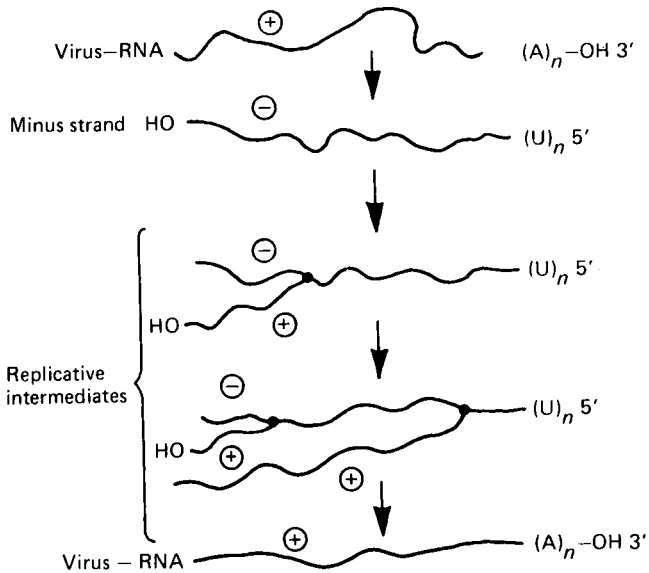

Figure 8.3. Schematic description of the synthesis of poliovirus-RNA. Virion-RNA gives rise to a minus-strand RNA as a first step in replication and plus strands are then synthesized on replicative intermediates. (Modified from Luria *et al.* (1978) by permission of John Wiley and Sons Inc., New York)

no coupling between synthesis of virus-RNA and protein. The replication complex contains an enzymatic activity which cannot be detected in uninfected cells and which has a capacity to synthesize virus-RNA in a cell-free system. Recently it has been shown that the polymerase contains a virus-coded polypeptide and it has been possible to purify and characterize this polymerase.

Virus-specific RNA occurs in cells in three different forms:

1. Single-stranded RNA which has a plus-stranded character and is identical with virion-RNA;
2. Double-stranded RNA which contains complete plus and minus strands of RNA;
3. Replicative intermediates which are formed by a minus strand of RNA and incomplete plus strands of RNA.

Thus replicative intermediates are precursors of both double-stranded RNA and virion-RNA. *Figure 8.3* illustrates schematically the synthesis of poliovirus-RNA. A complete piece of poliovirus RNA is synthesized in one minute, which means that the speed of replication is about 100 nucleotides per second. Since each replicative intermediate contains an average of five nascent plus strands, each cell can synthesize about 3000 copies of RNA per minute. This means that there are about 600 replicative intermediates in each infected cell.

Synthesis of virus proteins

The term *polyprotein* is used to define a polypeptide which after proteolytic cleavage gives rise to one or more proteins. By use of amino acid analogues or other substances which prevent the cleavage of a polyprotein, it was shown that the protein coded for by the complete poliovirus genome has a molecular weight of about 200 000. *Figure 8.4* shows how poliovirus-mRNA directs the synthesis of poliovirus proteins. Three different forms of proteolysis can be distinguished in connection with the formation of poliovirus proteins. The first cleavage of the protein occurs immediately after the ribosomes have formed the peptide bond which is going to be cleaved. By this cleavage three polypeptides are formed from

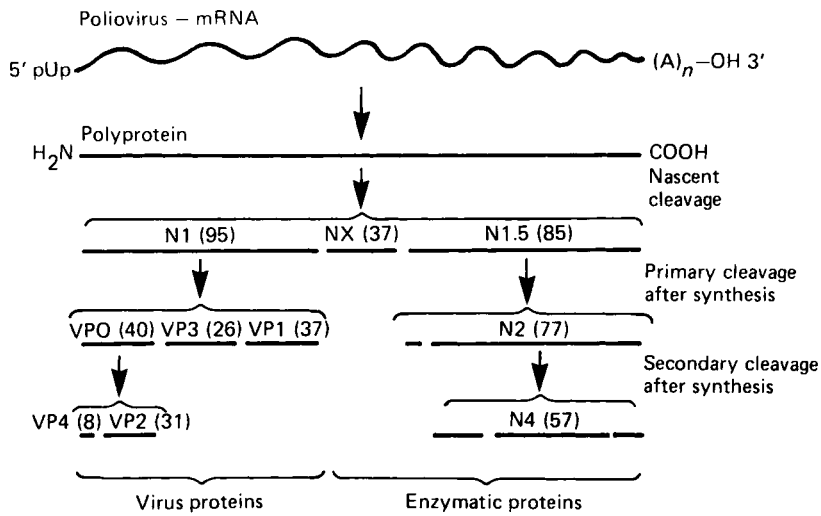

Figure 8.4. Schematic outline of the synthesis of poliovirus proteins. The virion-RNA, which represents one single piece of mRNA, codes for the polyprotein NO. The first cleavage of this protein occurs during synthesis and the products N1, NX and N1.5 are formed. N1 is further cleaved into virus-structural proteins wheras NX and N1.5 are involved in the replication of virus-RNA. The molecular weights in thousands of cleavage products are given in parenthesis. (Modified from Luria *et al.* (1978) by permission of John Wiley and Sons Inc., New York)

the polyprotein. The second form of proteolysis occurs within about 20 minutes after synthesis of the polyprotein. Thus this cleavage occurs after the protein has been finished and released from ribosomes. The third form of cleavage, occurs in connection with maturation of the virion itself and leads to VPO being divided into the peptides VP2 and VP4. Translation of a polyprotein which is cleaved into specific proteins can be used to identify the sequence of genes coding for the different proteins on mRNA. This sequence is illustrated in *Figure 8.4*. The synthesis of a polyprotein was originally identified in poliovirus-infected mammalian cells and later has been shown to occur in animal cells but it has not been encountered in bacteria. A possible explanation for the occurrence of this complicated process for synthesizing of several proteins might be that each mRNA in eukaryotic cells only contains one initiation point for protein synthesis. A similar mechanism has recently been shown to be used in the synthesis of a number of important hormones in the pituitary gland in man and animals.

Togaviruses

Togaviruses are plus-strand RNA viruses which can form two different mRNAs. Sindbis and Semliki forest viruses, which both belong to the alphavirus genus are the most carefully studied members of class IVB. The virion only contains four polypeptides and three of these, named E1, E2 and E3, are glycoproteins localized in the envelope. The fourth protein, C, builds up the nucleocapsid. The virion contains one single piece of RNA with a molecular weight of 4.2×10^6. This RNA is infectious and can serve as mRNA in cell-free translation systems. The 3′ end of the virion-RNA is polyadenylated and the 5′ end has a 'cap'. Thus togavirus-RNA is structurally similar to mRNA in animal cells, with the only difference being that the 'cap' nucleotide, but not neighbouring nucleotides, is methylated.

Synthesis of virus-RNA

Both 42S- and 26S-RNA can serve as mRNA in infected cells. These forms of mRNA occur in virus-specific polyribosomes and can be translated in cell-free systems. The synthesis of both forms of RNA requires the availability of a minus strand in the infected cell. This form of RNA with a size of 42S can be identified in a double-stranded RNA structrue and in replicative intermediates, but no minus-stranded 26S-RNA has been identified. It appears therefore that 26S-mRNA is formed from a minus-strand of 42S-RNA by use of an alternate initiation point for transcription located close to the 3′ end of minus-stranded 42S. The entire sequence of the 26S-mRNA has recently been established.

Synthesis of virus proteins

26S-RNA represents the mRNA which codes for a polyprotein including all structural proteins. By use of temperature-sensitive mutants it has been possible to prove that the polyprotein has a molecular weight of 130 000. The sequence of the cleavage products is C, E3, E2 and E1 (*Figure 8.5*). Also, 42S-mRNA directs the synthesis of a polyprotein which after proteolytic cleavage gives rise to four specific polypeptides. Two of these polypeptides represent the RNA replicase which can synthesize a minus strand from the 42S-RNA in virions. Since the 26S-RNA

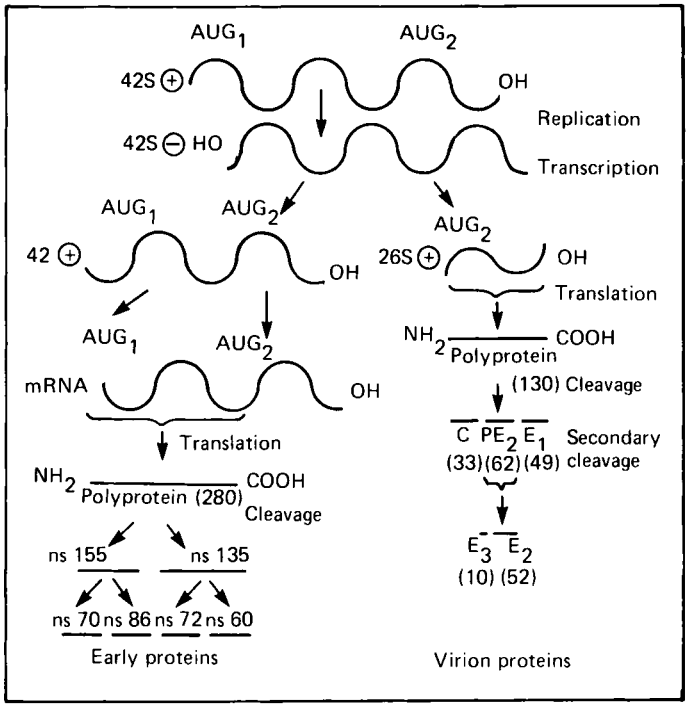

Figure 8.5. Schematic description of the expression of gene functions in togaviruses. Plus-strand virion-RNA forms a complementary minus strand from which 42S- and 26S-virus mRNA are synthesized. Virus-RNA has two initiation codons for protein synthesis of which only the one at the 5′ terminal is used for synthesis of a polyprotein early during the process of infection. The second initiation codon is used by 26S-mRNA which is synthesized separately and represents the 3′ end of virus-RNA. This mRNA is used for synthesis of a polyprotein which is a precursor of the structural virus protein. Figures used for designation of protein products (in the case of structural proteins these figures are given in parenthesis) denote their molecular weight in thousands. ns (non-structural) proteins are not included in the final virion. (Modified from Luria *et al.* (1978) by permission of John Wiley and Sons Inc., New York)

represents the 3′ end of 42S-RNA there must be two initiation codons for protein synthesis in virion-RNA, but only the codon in the 5′ end is used early during the infectious cycle to direct the synthesis of replicase and other early proteins. Thus togaviruses make a clear distinction between early and late functions since 42S-mRNA codes for early, and 26S-mRNA for late functions. In consequence the virus separately can control the amount of mRNA for synthesis of early and late proteins, a system of control which DNA viruses have developed into perfection.

Rhabdoviruses and paramyxoviruses

Members of these families have single-stranded RNA genomes with a minus-strand character, which thus cannot act as mRNA. The virions contain an RNA polymerase which after penetration of the nucleocapsid into cells transcribes virion-RNA to mRNA. This process is called *primary transcription* and can be observed in cell-free systems by using partially degraded virus particles. The

primary transcription leads to the appearance of several mRNAs, each of which codes for one protein. After the formation of viral mRNA they are translated by cellular polyribosomes and the proteins which are formed catalyse the replication of the virus genome, whereafter further viral mRNA can be formed. This mRNA is identical with that formed during primary transcription and the process therefore is referred to as *secondary transcription*. In contrast to the situation with togaviruses, members of class VA make no distinction between proteins that are formed during primary and secondary transcription. The virus most extensively studied in class VA is vesicular stomatitis virus (VSV). The virion is shaped like a bullet and is surrounded by an envelope. In its central part it contains a nucleocapsid which is composed of virus-RNA combined with a nucleocapsid (N) protein. The nucleocapsid is surrounded by a matrix (M) protein which is anchored on the inside of the envelope containing the virus glycoprotein (G). In addition virions contain two proteins in smaller quantities, L (large) and NS (non-structural), which also are included in the nucleocapsid. L represents the virus-coded polymerase.

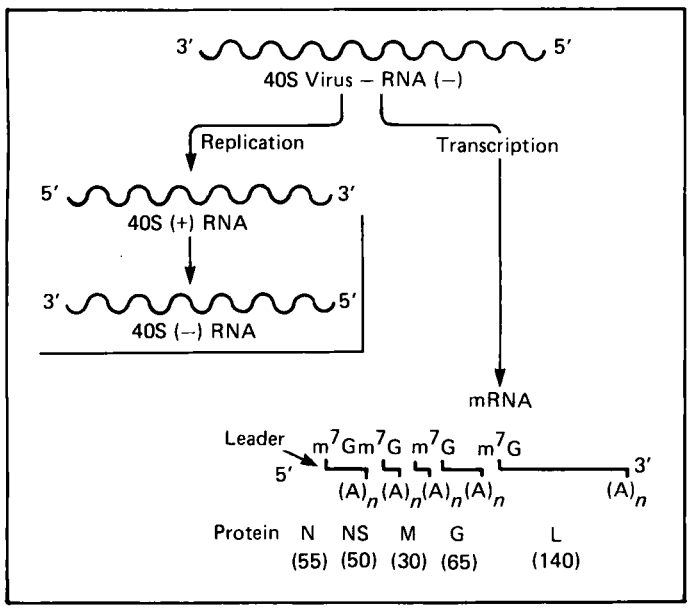

Figure 8.6. Schematic description of the genetic system directing the replication of a rhabdovirus, vesicular stomatitis virus. Replication of RNA and transcription are separate processes. The order in which mRNA is coded along virus-RNA has been identified. During transcription, separate mRNAs are formed for each protein. Molecular weights in thousands of the gene products are given in parenthesis. (Modified from Luria *et al.* (1978) by permission of John Wiley and Sons Inc., New York)

Virus-RNA has a molecular weight of 4×10^6, which corresponds to 12 000 nucleotides and therefore has a coding capacity for a protein with a total molecular weight of 400 000. This amount corresponds to the sum of the molecular weights for the five virion proteins, L 190 000, G 65 000, N 55 000, NS 50 000 and M 30 000. All the three proteins in the VSV nucleocapsid are needed for transcription activity. L and NS proteins are apparently needed for polymerization, but the RNA must be

covered by the N protein in order to serve as a template. In connection with the primary transcription, the polymerase starts at the 3′ end of virion-RNA and transcribes continuously from the template, but the products are five separate mRNA molecules with a 'cap' at the 5′ end and poly(A) at the 3′ end (*Figure 8.6*). No larger precursor-RNA has been identified which means that the polymerase has to stop and reinitiate a new synthesis several times on the same template. By use of coupled cell-free transcription and translation systems, it has been possible to demonstrate that the sequence of the genes in the VSV genome from the 3′ end is N, NS, M, G and L. It has also been possible to identify a leader sequence which is formed before the mRNA for the first gene in the transcript, the N gene, can be synthesized. Several enzymatic activities for 'cap' synthesis, polyadenylation and methylation are involved in the transcription process. All these enzymes are present in purified virions. This means that the L and NS proteins must carry several enzymatic functions. During the cycle of virus replication, a synthesis of new virus-RNA also must occur. The synthesis of virus-RNA requires a template which is as large as virion-RNA and such a molecule can be identified in infected cells in the form of a replicative intermediate. *Figure 8.6* illustrates the occurrence of separate transcription and replication steps during the replication cycle. This arrangement represents a more complicated genome strategy than is the case for class IV viruses.

Paramyxoviruses also belong to class VA but they have two glycoproteins in the envelope instead of one as is the case in VSV.

Orthomyxoviruses

Influenza virus is an extensively studied member of the orthomyxovirus family. It has two glycoproteins located in the virus envelope, the haemagglutinin (HA) and the neuraminidase (NA). A matrix protein (M) on the inside of the envelope has been demonstrated, which surrounds a segmented nucleocapsid. This is composed of eight segments of minus-stranded RNA which all are combined with a nucleoprotein (NP) and three additional proteins (P1, P2 and P3). Since the virus genome has a minus-strand character a polymerase must be present in the virus particle and P1 and P2 together represent this polymerase. The enzyme most likely can only form RNA which is complementary to the minus strands, i.e. it synthesizes a plus-strand RNA which represents mRNA. Seven virus-specific proteins have been identified in virions. In addition there is a non-structural (NS) protein in infected cells and each segment of RNA was therefore originally considered to represent one gene in the influenza genome (*Figure 8.7*). However, recent studies have shown that the mechanism of splicing is used by the genes for both P and M proteins. Thus each of these genes can synthesize more than one protein.

The molecular weight of the 8 RNA segments varies between 3×10^5 and 1×10^6. The sum of the segments of the genome gives a molecular weight of $3.9–4.9 \times 10^6$. It was shown recently that the base sequence of the first 13 nucleotides in the 5′ end is identical for all the 8 individual segments of different influenza virus strains.

Genetic studies of influenza viruses have shown the exceptionally frequent recombination between different strains. This type of recombination involves a phenotypic mixing of individual RNA molecules which thus function as separate genome pieces in the same way as has been shown for class III viruses. In the influenza viruses it is possible to correlate single RNA segments directly with

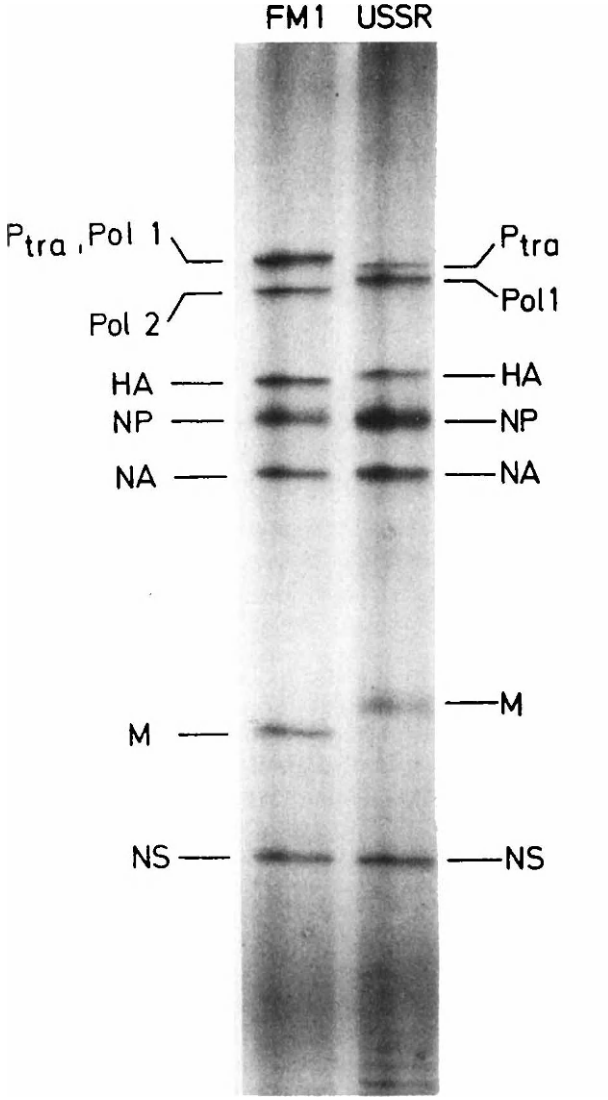

Figure 8.7. Polyacrylamide gel electrophoresis of virion-RNA from different strains of influenza A virus. Eight segments of genome can be distinguished. The two different strains have RNA segments of different sizes. The different products coded by each segment of RNA are illustrated. (Photo reproduced by permission of Dr C. Sholtissek, Institut für Virologie, Justus–Liebig–Universitat, Giessen, West Germany)

specific gene functions. One method of studying these relationships is to characterize the rate of migration of the RNA segments and of viral proteins in polyacrylamide gels. The migration characteristics of different components varies for different influenza virus strains (*Figure 8.7*). After the formation of a recombinant it is possible to identify directly the fragment of RNA that has been exchanged by characterization of the phenotype of the recombinant.

Synthesis of virus-RNA

As a first step after adsorption, penetration and release of nucleoprotein inside the cell, mRNA must be synthesized from each of the eight segments of virion-RNA using the nucleoprotein-associated RNA polymerase (*primary transcription*). It has been shown that these mRNAs have poly(A) at the 3′ end and a 'cap' in their 5′ end. It appears that a certain transcription from cellular DNA is necessary for the synthesis of a functional virus-mRNA and it has recently been demonstrated that host cell mRNAs serve as initiators for virus transcription. Virus-mRNAs are bound to ribosomes and new virus proteins are formed. Shortly thereafter another kind of plus-strand RNA with a length equal to that of minus-strand virion-RNA can be identified. This complementary RNA serves as template for the replication of virion-RNA. Complementary RNA molecules have to be present in equimolar amounts for each RNA segment. In contrast, it can be shown that the amount of mRNA from different segments varies. The occurrence of mRNA correlates well with its capacity to form the individual virus proteins. Thus it appears that the synthesis of viral proteins is controlled by transcription of mRNA from different segments.

Retroviruses

Retroviruses have properties which are characteristic of both RNA and DNA viruses. Virions contain RNA, but their intracellular form is a DNA which is integrated into cellular DNA. After the virus nucleoprotein has been introduced into the cytoplasm of cells, the virion-associated polymerase converts virion-RNA into DNA which then can be integrated among cellular genes. As a consequence, virus-DNA can be transferred from one generation of cells to the other in the form of a stable, integrated molecule of DNA. In contrast to other cytolytic RNA viruses, the retroviruses have a capacity to maintain a productive infection for a long time without killing cells. The central and most important property of retroviruses thus is their capacity to convert RNA into DNA, a process which has been defined as *reverse transcription*. Retroviruses represent the only known example of information flow in the opposite direction to that which is seen in normal cells.

All retrovirus genomes contain between 5000 and 9000 nucleotides and each virion contains two identical RNA molecules which are coupled at their 5′ ends. The RNA is combined with four capsid proteins that are derived by cleavage from the polyprotein *gag* (*g*roup *a*ntigen). The capsid also contains a reverse transcriptase, *pol* (*pol*ymerase) and the envelope of the virus contains one glycoprotein, *env* (*env*elope).

One retrovirus which has been studied extensively is *avian sarcomavirus* (ASV). The ASV genome contains four genes with the following sequence from the 5′ end of RNA (*Figure 8.8a*): gag, pol, env and src (sarcoma). The src gene is responsible for transformation of the host cell (*see* Chapters 11 and 18).

Avian and *murine leukaemia viruses* (ALV and MLV, respectively) have a genome organization which is similar to that of ASV but they lack src (*Figure 8.8b*). Between env and poly(A) in the genome of ALV there is a sequence, named 'c', of about 300 nucleotides which is shared between ASV, ALV and certain other avian retroviruses. This sequence most likely does not code for any protein, since there

are stop codons in all reading frames.The c region probably also occurs in murine retroviruses.

In addition, cells of both birds and mice contain *endogenous retroviruses* which are integrated into their genomes. One difference between endogenous avian retroviruses and ALV is that the c region may be absent in the former.

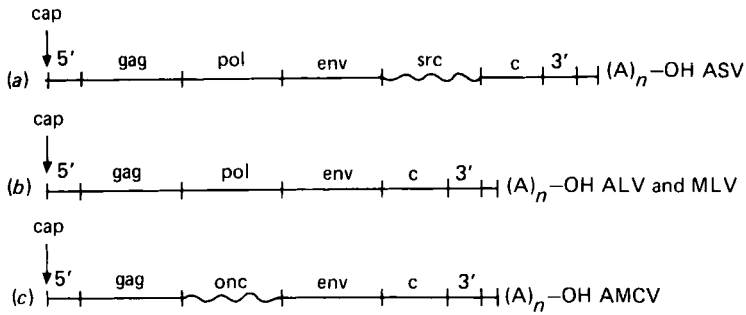

Figure 8.8. Schematic description of the gene structure of some different retroviruses. (*a*) Avian sarcoma virus (ASV). (*b*) Avian and murine leukaemia viruses (ALV and MLV). (*c*) Avian myelocytomatosis virus (AMCV). See text for explanation of the abbreviations

Defective leukaemia viruses, which lack pieces of one or more of the genes needed for virus replication, are common in birds and mice. These defective viruses all require an intact helper virus, such as ALV or MLV, for their replication. One example is avian myelocytomatosis virus (AMCV). The RNA of these viruses (*Figure 8.8c*) lacks pol and parts of gag and env, which are substituted for by sequences of cellular origin.

Replication and gene expression of retroviruses

During replication the reverse transcriptase starts near the 5′ end of the genome with assistance from cellular tRNA which partly is hybridized to virus-RNA. *Figure 8.9* describes the different steps in the replication which eventually lead to a circular retrovirus DNA being integrated into host-cell DNA. The mechanism for integration is unknown and it is not clear if linear or circular DNA are integrated. Synthesis of viral RNA occurs in the cell nucleus with the aid of RNA polymerase II from the host cell and integrated DNA most likely represents the template during this reaction.

Three different kinds of cytoplasmic RNA have been described as products of viral RNA synthesis of ASV. The largest form of RNA contains 9500 nucleotides and codes for all gene products and therefore most likely is identical with virion-RNA. This mRNA directs the synthesis of a polyprotein which after cleavage yields the different gag products. It is also possible that the polymerase is formed from the same mRNA. The second mRNA is 5400 nucleotides long and it codes for env and, in ASV, also for the src and c sequences. However, in ASV only the env part of mRNA can be translated, which leads to the appearance of a glycoprotein with a molecular weight of 79 000–90 000. The smallest form of mRNA contains 3500 nucleotides and codes for src. The leukaemia viruses which lack the src gene obviously cannot generate this mRNA. The product of the

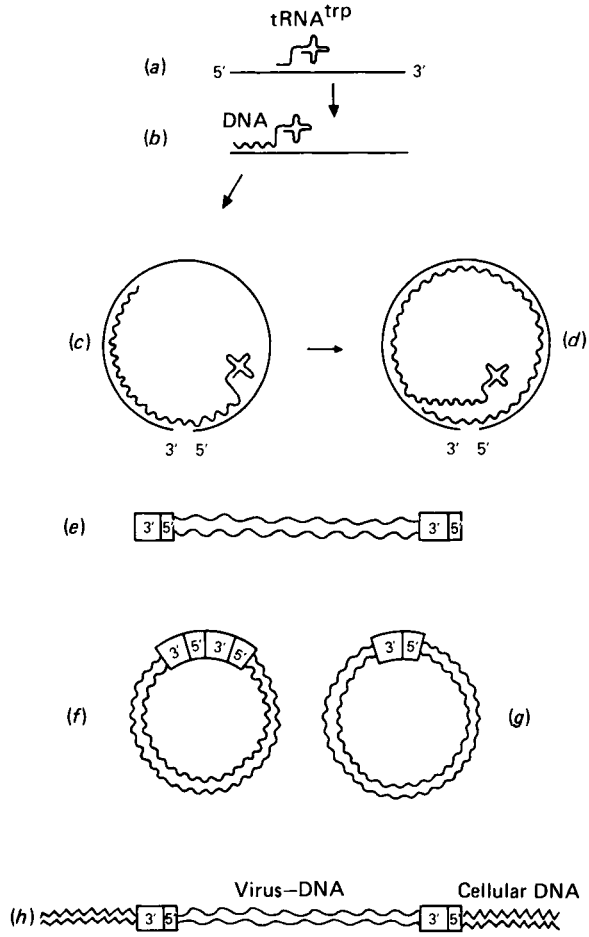

Figure 8.9. Reversed transcription of retrovirus-RNA. A cellular tryptophane tRNA initiator hybridizes to 35S-virion-RNA in avian viruses about 100 nucleotides from the 5′ end (*a*). The first product which is formed is a DNA fragment, which initially is covalently bound to the tRNA initiator and reaches to the 5′ end of the RNA template (*b*). In the subsequent step the transcription is transferred from the 5′ to the 3′ end of virus-RNA because of the occurrence of a short sequence which is repeated in both parts of the genome (*c*). Virus-DNA is then copied and a new transfer from the 5′ to the 3′ end occurs (*d*). The complete double-stranded DNA copy which has repetitive 3′ and 5′ sequences in each end (*e*) can be circularized at the 5′ and the 3′ sequences in two forms retaining the repetition (*f*) and excluding one set of repeated sequences (*g*). Finally, integration of virus-DNA into the cellular genome occurs, probably via the repeated sequences in the end (*h*). (Modified from Luria *et al.* (1978) by permission of John Wiley and Sons Inc., New York)

src-mRNA is a polypeptide with a molecular weight of 60 000 and it carries protein kinase activity. All three forms of mRNA have a non-translated leader sequence of about 100 nucleotides. This sequence has been added by use of a splicing process. Similar to the situation with the eukaryotic mRNA, only one protein is directed by each mRNA molecule.

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9

Replication, transcription and translation of DNA viruses

Ulf Pettersson

The replication cycle of DNA viruses starts with penetration of virions whereafter the DNA is uncoated. Poxviruses replicate in the cytoplasm of cells whereas other DNA viruses are transported to the nucleus where viral-DNA replicates and is transcribed. Transcription of viral DNA starts early and is followed by synthesis of viral products. Frequently competition between virus and cell metabolism develops and in order to improve their possibility for survival, many viruses have the capacity to shut off certain metabolic functions of the host cell. In normal cells DNA is synthesized for only a comparatively short portion of their replication cycle, (the S phase), and for this reason the DNA-synthesizing machinery in a cell normally needs to become activated during infection with a DNA virus. The kind of cell which is infected influences the course of the infection process.

Following the classical description of lytic replication of DNA viruses, this can be divided into two major functional phases. During the first, *early phase*, only a limited number of genes are expressed. These are called early genes. In this phase primarily there occurs the synthesis of polypeptides which have a catalytic role during virus replication. The early phase is concluded when the viral DNA synthesis is initiated, starting the *late phase*. During this late phase the remaining viral genes are transcribed and the protein products which are synthesized represent, primarily, the structural polypeptides of the virus particles. Accumulation of these components leads to maturation of virus particles during which newly synthesized DNA is combined with structural proteins.

In the case of enveloped viruses processes are added which include a restructuring of cellular membrane structures and their addition to nucleocapsids during a budding-off process (cf. Chapter 10).

Parvoviruses

Parvoviruses (class II, cf. Chapter 8) are the least complicated animal viruses and they have a genome with a molecular weight of only 1.5×10^6, representing about 4700 nucleotides. Parvoviruses hold a special position among animal viruses. The genome is represented by a single-stranded DNA. Parvoviruses can be divided into two subgroups, autonomous and defective parvoviruses. As is apparent from these names, the former kind has a capacity for an independent complete replication, whereas the latter kind needs a helper virus for replication. Adenoassociated viruses (AAV) are an example of defective parvoviruses which need adenoviruses

for their replication. Owing to the limited size of their genome, parvoviruses to a large extent are dependent on host-cell functions for their replication. Defective parvoviruses, in addition, need functions that are provided by a helper virus. The absence of certain replicative functions in these uncomplicated viruses is partly exemplified by the fact that only autonomous parvoviruses can replicate in cells which are in the S phase.

DNA structure

Parvoviruses contain single-stranded linear DNA molecules. In the case of defective parvoviruses, the exceptional situation has been encountered where the complementary strands of DNA are packed into separate virus particles. Autonomous parvoviruses are different in that they only pack one of the DNA strands, the one that is complementary to viral mRNA. At the ends of the genome, parvoviruses contain a repeated sequence and in AAV there is in addition an inverted and repeated sequence. One additional interesting property of parvovirus-DNA is that its ends contain a mirror sequence, a palindrome, which makes it possible for DNA to form a hairpin-like structure by internal base-pairing.

Synthesis of virus proteins

At an early stage during infection the virus is transported to the cell nucleus where replication occurs. During virus replication parvovirus-DNA is transcribed so that several different mRNAs are formed for further translation to viral polypeptides. The virus particle contains polypeptides with a molecular weight of 91 000, 72 000 and 60 000. Their combined molecular weight exceeds the coding capacity of the genome and it is therefore necessary that they either share gene sequences or are coded in different reading frames of AAV-DNA. Most likely the smaller polypeptides represent cleavage products of the largest polypeptide. About 90 per cent of the viral genome is transcribed into RNA which, after splicing, forms a number of mRNA molecules with a partly common structure. In addition to the above-mentioned structural polypeptides, infected cells also contain two virus-specific polypeptides with a molecular weight of 25 000 and 16 000. The importance of the latter non-structural proteins has not as yet been determined.

Adenovirus is needed as helper virus for the replication of AAV. Without the presence of this helper virus, no AAV-specific DNA or RNA is formed and no particles are produced. It is still not clear at which stage of the virus replication the helper virus is needed, but one or more of the early adenovirus gene products are required. Replication of AAV can also be supported by herpes simplex virus. However, in this case AAV-specific DNA and RNA are being formed but no infectious particles.

Replication of DNA

An interesting model for replication of AAV-DNA as been proposed. According to this model the hairpin structure which the DNA can form is used. This structure is extended so that a completely double-stranded DNA molecule is formed. The hairpin is then cleaved by an endonuclease after which the whole molecule is again

copied into a double-stranded structure leading to the release of one copy of single-stranded DNA. The procedure may then be repeated which leads to the complementary strands being released one by one from the replicating molecule and being packed into virus particles.

Papovaviruses

The papovavirus family includes viruses which can infect animals and man. In addition to human papilloma virus, a number of other human papovaviruses similar to SV40, including the types JC and BK (*see* Chapter 33), have been isolated. Papovaviruses show marked similarities, both in structure and genetic composition, and they most likely have a common origin. Polyoma and SV40 viruses are the two members which have been studied in most detail and both these viruses have three structural polypeptides, VP1, VP2 and VP3. In addition, cellular histones bound to virus-DNA are present inside the virus particle. On the nucleotide level the polyoma and SV40 genomes show very few similarities in spite of the fact that their genes are organized in an almost identical fashion. Papovaviruses are more complicated than parvoviruses, which is reflected in the difference in patterns of replication. Their genome codes for several catalytic functions in addition to virus structural components, and in contrast to the situation with autonomous parvoviruses, infected cells do not have to be in their S phase to allow replication of papovaviruses. Instead, these viruses have the capacity to induce cells which are in a resting stage to change into S phase. Most papovaviruses can show both complete lytic replication in permissive cells and an abortive cycle of replication in non-permissive cells. Their replication occasionally leads to cell transformation.

Structure of DNA

All papovaviruses contain a genome which is represented by a covalently closed double-stranded circle (class I). The DNA of polyoma and SV40 virus has a molecular weight of about 3.5×10^6 and contains about 5300 base pairs. Members of the papilloma virus genus generally have a somewhat larger genome and different strains display a certain variation in the size of their DNA. Since the DNA of papovaviruses is covalently closed it is also superhelical (*see Figure 3.5*).

Synthesis of viral proteins

SV40 is the papovavirus which has been most extensively studied and it is therefore used as a model in this chapter. However, there is strong evidence that in principle the pattern of replication also holds true for other papovaviruses with the possible exception of the papilloma viruses. After penetration the virus capsid is dissociated and the DNA and associated histones are transported to the cell nucleus, where transcription is initiated 6–8 hours after infection. The replication of SV40 can be divided into an early and a late phase. The demarcation between these phases is the initiation of the synthesis of viral DNA which occurs 15–18 hours after infection. Virus-DNA is transcribed both early and late by use of the host-cell RNA polymerase II. At an early stage of the infection long RNA molecules are synthesized in the nucleus and, after splicing, two different mRNA molecules are formed. These give rise to two related polypeptides which are called tumour

antigens (cf. Chapter 11) and can be detected 8–9 hours after infection. These antigens are composed of two structurally similar polypeptides with molecular weights of 94 000 (T antigen) and 20 000 (t antigen), respectively. During replication a third polypeptide, the middle T antigen is synthesized. The virus proteins which are formed during the early phase prepare the cells for synthesis of viral structural proteins. During the early phase, the cellular DNA synthesis is stimulated which is important to the accumulation of necessary components for replication of viral DNA. The two tumour antigens most likely have a number of replicative functions. It has been shown that the T antigen can bind to virus-DNA at the place where DNA replication is initiated and that this protein plays an important role in the initiation of this synthesis. Two additional SV40-specific proteins can be identified during the early phase of infection, U antigen and TST

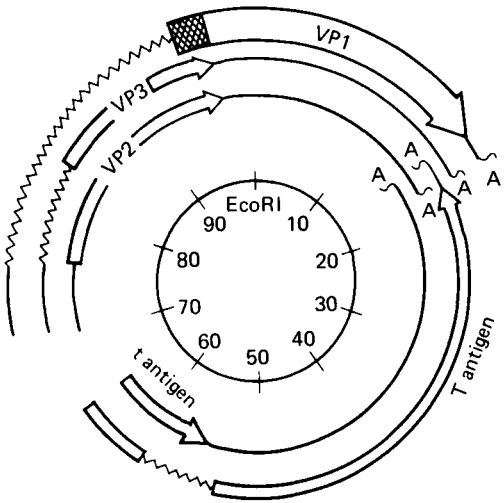

Figure 9.1. The topography of the SV40 genome. The early genes are transcribed from a promotor which is located at position 67 (the SV40 genome is divided into 100 map units. Position 0 on the SV40 circle is the place where the restriction enzyme Eco RI cleaves). Transcription of the early genes occurs counter-clockwise in relationship to the gene map for SV40 and results in two different spliced mRNAs (the empty arrows show the coding sequences) coding for the large (T) and the small (t) tumour antigens. The promotor for late transcription also is located in position 67 but the transcription of the late genes occurs clockwise. Since the transcription always occurs in a 5′ to 3′ direction, early and late genes must be located on different DNA strands. Late mRNA is formed by splicing and code for the capsid proteins VP1, VP2 and VP3, of which VP2 and VP3 contain completely overlapping amino acid sequences. Between positions 94 and 96 there is a highly interesting region (hatched) which in one phase of the sequence codes for the C-terminal end of VP2 and VP3 and in another phase for the N-terminal end of VP1. This exemplifies that one and the same DNA sequence can code for several proteins

(*t*umour *s*pecific *t*ransplantation) antigen (cf. Chapter 11), which are bound to nuclear and cytoplasmic membranes, respectively.

During the later phase of virus replication, a large number of new viral DNA molecules are synthesized and the pattern of transcription is changed drastically. Also, late mRNAs are formed by splicing and they code for the capsid proteins VP1, VP2 and VP3. The two latter polypeptides contain completely overlapping amino acid sequences (*Figure 9.1*).

Replication of DNA

SV40-DNA is a covalently closed molecule and remains in this form during the replication. The DNA synthesis starts at a fixed point, located at position 67 on the gene map of SV40, and from this point DNA grows in both directions via two forks which are present in the replicating molecule. Virus-DNA is synthesized with the aid of cellular DNA polymerase although the viral T antigen plays an important role in the initiation of DNA replication. SV40-DNA is connected with cellular histones, both in virus particles and during replication.

Adenoviruses

Adenoviruses hold an intermediate position among DNA viruses with regard to genetic complexity. This family of viruses offers an excellent model system for studies of virus replication and processing of virus-genetic information.

Structure of DNA

Human adenoviruses contain a linear double-stranded DNA with a molecular weight of $20–23 \times 10^6$, which corresponds to about 36 000 base pairs. The genomes of avian adenoviruses are somewhat larger and have a molecular weight of 30×10^6. Theoretically the adenovirus genome could code for about 30 medium-sized polypeptides, provided only one phase of the nucleotide sequence of DNA is used for coding purposes. The ends of adenovirus-DNA contain inverted repeated sequences which means that single strands of adenovirus-DNA can form circles. A protein is covalently bound to the 5′ end of each polynucleotide chain and the protein in the two ends of the DNA molecules can interact with each other so that the DNA forms circular structures. However, these circles are not covalently closed in the same way as papovavirus-DNA. Many adenoviruses have a base composition which markedly deviates from that of cellular DNA. Thus, for example, adenovirus type 2 DNA contains 57–59 per cent GC base pairs whereas cellular DNA only contains 40–42 per cent GC base pairs. Due to this difference, it is possible to separate viral and cellular DNA by using gradient centrifugation in caesium chloride, which has markedly facilitated studies of the replication of adenovirus-DNA. The content of GC nucleotides in different adenovirus-DNA varies markedly. The cleavage sites on the adenovirus type 2 genome for a large number of restriction enzymes have been described and can be used as a reference point in the characterization of the genome. Traditionally, one speaks about a left and right end of the viral genome. The left end is defined as the one which contains the transforming genes. With DNA oriented in this way, it is possible to define an r-strand (transcribed rightwards) and an l-strand (transcribed leftwards).

Transcription of virus-DNA

The adenovirus genome has a complex structure with early and late genes distributed in a mixed order along the viral DNA (*Figure 9.2*). Both early and late genes have been mapped by use of hybridization with viral mRNA and with restriction enzyme fragments of adenovirus-DNA. It has been shown that adenoviruses have genes on both their DNA strands which are therefore transcribed in different directions.

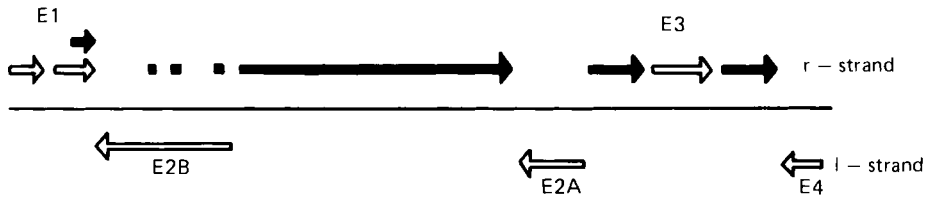

Figure 9.2. Schematic description of the topography of the adenovirus genome. The arrows show the direction of transcription. A large number of different early (filled arrows) and late (empty arrows) genes are distributed along the adenovirus-DNA. Both the r and l strands of DNA are used for coding of both early and late proteins. Five distinct blocks of genes (E1, E2A, E2B, E3 and E4) are transcribed and expressed early during the infection

Mainly five distinctly separated groups of genes which are referred to as E1, E2A, E2B, E3 and E4 are transcribed early during the infection. E1 and E3 are located on the r-strand whereas E2A and E2B and E4 are located on the l-strand.

Later during the infection the remaining parts of the viral genome are used. Some viral DNA is integrated into the host-cell genome during the lytic infection but the importance of this phenomenon has not been determined.

Synthesis of virus proteins

The cycle of replication of adenoviruses lasts about 20 hours and can be divided into an early and late phase. The DNA replication starts about 6 hours after infection. Transcription and replication take place in the nucleus of cells, whereas translation of viral mRNA occurs in the cytoplasm. Virus-DNA which enters the cell is combined with virus-specific basic proteins but a short time after infection it acquires a nucleosome-like structure, probably because of the addition of cellular histones. Transcription of adenovirus-DNA can be initiated as early as one hour after infection and both the early and late transcription is carried out mostly by the host-cell RNA polymerase II. The early transcription leads to the formation of RNA molecules which after splicing mature to a large number of mRNA molecules, many of which share parts of their nucleotide sequences. More than 20 different mRNA molecules have been identified but it remains to be shown whether each of these molecules gives rise to a functional polypeptide chain. Many of the early proteins probably have some catalytic function in connection with virus replication.

One product from the E1 region influences transcription of other early genes and among products from the E2 region, another has a DNA-binding capacity which probably plays an important role in connection with the replication of virus-DNA. The E3 region codes for a glycoprotein which has been identified in the cytoplasmic membrane of the virus-infected cell.

The shift to synthesis of late proteins occurs rapidly. The late genes mainly code for the 10 different structural polypeptides which constitute the virus particles. One exception is a protein with a molecular weight of 100 000. The function of this non-structural protein has not been defined. One additional non-structural virus protein with a molecular weight of 50 000 is synthesized at intermediate times after infection and appears to play a role in the assembly of virus particles. The transcription of late mRNA is distinctly different from the transcription which occurs early during virus replication and is illustrated in *Figure 9.3.*

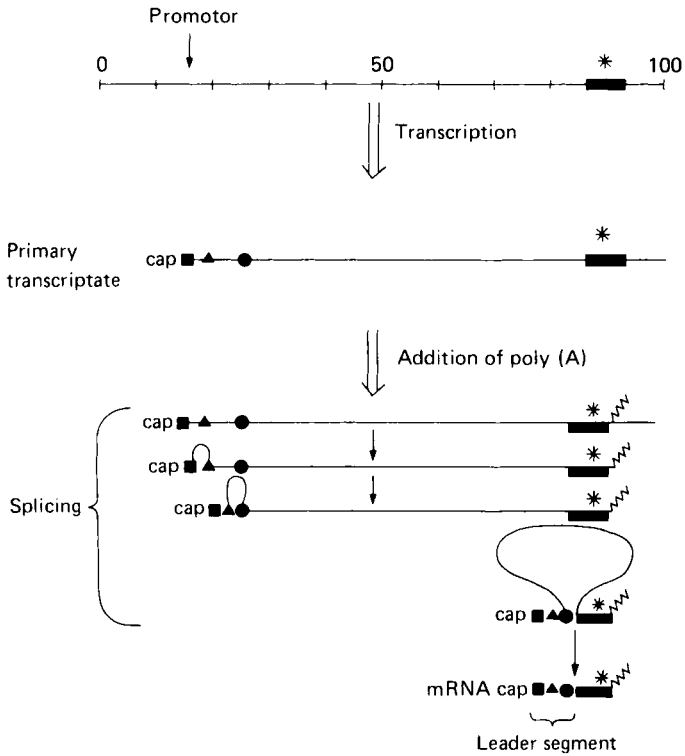

Figure 9.3. Schematic description of the synthesis of late adenovirus-mRNA. Late transcription starts at the promotor which is located at the position 16.3 on the gene map (the adenovirus genome is divided into 100 units and position 0 is located at the end of the linear genome which contains the transforming genes). Long RNA chains are synthesized from this promotor and after splicing they give rise to different mRNA molecules from the primary transcript. In contrast to the conditions early during infection splicing during the late phase leads to mRNA molecules being provided with identical 5′ ends, leader segments. The illustration shows how transcription of a late adenovirus-mRNA corresponding to one gene (*) from the promotor at position 16.3 takes place. A leader segment 203 nucleotides long, which is formed by splicing together sequences from three different regions, is coupled to each mRNA. The probable reason why viral mRNA is provided with a leader sequence is that this sequence serves as a signal through which the mRNA can be effectively translated and compete with cellular mRNA

Replication of DNA

Adenovirus-DNA is replicated as a linear structure. This replication appears to be accomplished with the aid of host-cell DNA polymerase.

It seems likely that several viral components contribute to DNA replication. However, so far, only two proteins have been shown to play a role in this context. One is the DNA-binding protein, the synthesis of which is directed by genes in the E2A region of the viral genome. The second is the terminal protein which is covalently linked to the termini of adenovirus-DNA. Its gene is located in region E2B.

Replication of adenovirus-DNA has been studied both by electron microscopy and by biochemical analyses. According to one model for replication of adenovirus-DNA it starts in either of the two ends of the genome. Initiation of replication is believed to take place with the aid of the protein which is covalently bound to the ends of DNA. Replication of one of the DNA strands leads to a release of the complementary strand. Since adenovirus-DNA contains an inverted repeated terminal sequence, the released single-stranded molecule can form a circular structure. The base-paired structure which is responsible for the formation of the circle is identical with the terminal structure of the original double-stranded DNA molecule and thus can serve as an initiation point for replication of another complementary strand of DNA. This pattern of replication of adenovirus-DNA differs distinctively from that of other systems. In contrast to replication of SV40-DNA, for example, the replication of two strands of adenovirus-DNA occurs independently. A similar pattern of replication has previously been observed in mitochondrial DNA.

Herpesviruses

The genomes of members of the herpesvirus family show a larger variation in both size and structure than that found in the case of other DNA viruses. In contrast to adenoviruses and papovaviruses, which are highly selective with regard to host organism, herpes simplex virus can replicate in a wide spectrum of different types of cells. Herpesviruses have a complicated composition. At least 24 different polypeptides have so far been identified in virions but it is likely that a detailed analysis will reveal the occurrence of additional components. The size of the genome of herpes simplex virus allows it to code for about 100 medium-sized polypeptides. Owing to the complexity of the system and the difficulties of purifying virions in large quantities, the replication of herpesviruses has been less well characterized than is the case with adenoviruses and papovaviruses. However, herpesviruses attract a considerable interest since members of this group are responsible for a spectrum of different infections which are of major medical importance (*see* Chapter 31).

Structure of DNA

Herpes simplex virus-DNA is linear and double-stranded and has a molecular weight of 100×10^6, whereas DNA from certain other members of the group have a markedly higher molecular weight. Because of the size of the DNA, it is difficult to isolate it in an intact form and to subject it to detailed examination. Within the

herpes simplex virus genome several sets of repeated sequences have been identified which give the genome a complicated structure (*Figure 9.4*). The herpes simplex virus-DNA is composed of two different segments, the large segment, L, and the small segment, S. Both the L and the S segments are surrounded by inverted and repeated sequences. The herpesvirus-DNA can code for a large

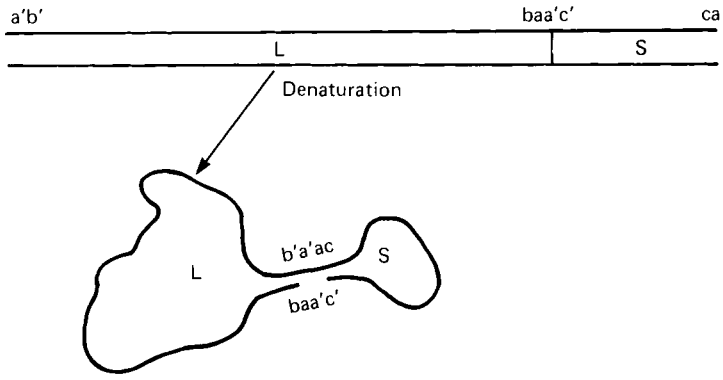

Figure 9.4. Schematic description of the herpes simplex virus genome. The DNA is composed of two different segments; a large (L) and a small (S) segment. Both the L and S segments are surrounded by inverted and repeated sequences. In the case illustrated, the L segment is surrounded by the sequences a', b' and b, a, respectively, whereas the S segment is surrounded by the inverted sequences a', c' and c, a respectively. One consequence of the occurrence of the inverted repeated sequences is that single strands of herpes simplex virus-DNA form a complicated structure composed of two loops. These loops are held together by a double-stranded segment formed by base pairing between the repeated sequences. In the replication of virus-DNA the mutual orientation of the L and S segments can be changed which leads to possibly four different forms of DNA appearing. In addition to the inverted repeated sequences, herpes simplex virus-DNA also contains directly repeated sequences; in the illustration the sequence (a) appears in both ends of the molecule. (Modified from Luria *et al.* (1978) by permission of John Wiley and Sons Inc., New York)

number of different polypeptides and hybridization-analysis between DNA and viral mRNA shows that the larger part of the genome is transcribed during the infection. About 50 different virus-specific polypeptides have been identified and about half of these represent structural proteins which build up the mature particles.

Synthesis of virus proteins

The size of the herpesvirus genome allows it to contain genes for a number of replicative functions in addition to genes for structural components of virions. As a consequence, herpesviruses are less dependent on the physiological state of the host cell than DNA viruses with a smaller genome. After penetration virus-DNA is transported to the nucleus where transcription and replication takes place. The following description of replication of herpes simplex viruses will be used as a model for the replication of herpesviruses in general.

The time course for virus replication varies depending upon the host cell and the multiplicity of the infection. During replication of herpes simplex viruses distinct division into an early and a late phase in relationship to replication of DNA can be

distinguished. In spite of the fact that all genes in the herpesvirus genome can be expressed independently of synthesis of DNA there appears to be a time structure in operation concerning the activity of different groups of genes. If the protein synthesis in the infected cell is blocked temporarily at an early stage by use of specific inhibitors, only one class of viral mRNA is formed, namely mRNA for the α polypeptides. First, after the synthesis of these polypeptides, the synthesis of mRNA for another group of polypeptides, the β polypeptides, can be started. When this occurs, the synthesis of the α polypeptides decreases. The synthesis of β polypeptides in turn leads to the synthesis of yet another class of polypeptides, namely γ polypeptides. Also, the initiation of γ polypeptide synthesis is followed by a gradual decrease in the synthesis of β polypeptides. Thus there exists a complicated interaction between different components synthesized during a herpesvirus infection which can be described as cascade-like control mechanisms. It has not been clarified how these cascade-like mechanisms are established. Some experimental data indicate that the control occurs on a post-transcriptional level, since all RNA sequences can be demonstrated in the cell nucleus before the synthesis of a polypeptides has been initiated. This control can imply that either the transport or the translation of different groups of viral mRNAs are controlled by viral products. In very few cases has the function of the viral polypeptides been identified. All structural polypeptides are included among the γ polypeptides, whereas the α and β groups of polypeptides most likely include proteins with a replicative function. Many viral polypeptides have been found to be modified after their synthesis and several kinds of modification have been identified; certain polypeptides are subjected to proteolytic cleavage whereas others are modified via glycosylation and phosphorylation.

As already mentioned the size of the herpesvirus genome is reflected in the diversity of the replicative functions which can be observed. Several virus-specific enzymes have been identified and among these the thymidine kinase has been the most extensively studied. The gene for this enzyme has been identified on the viral genome. The enzyme differs from cellular thymidine kinases in that it can use both thymidine and deoxycytidine as substrate. The role of this enzyme in replication is not firmly established although probably it is to improve the nucleic acid metabolism in the infected cell. However, mutants of herpesviruses which lack this enzyme but can still replicate normally have been isolated. Thus the presence of this enzyme activity is not an absolute requirement for virus replication. Another enzyme which has been the focus of interest is herpesvirus-specific DNA polymerase which appears during the infection. This enzyme differs from the corresponding cellular enzyme by its sensitivity to certain inhibitors, for example phosphonoformic acid (*see* Chapter 24). Also, a number of other enzymes have been shown to occur in increased concentration during virus replication. Among these belong a ribonucleotide reductase, a DNA-cleaving enzyme, protein kinase and several others. However, in many cases these enzymes probably represent virus-induced host-cell enzymes rather than authentic viral gene products.

Poxviruses

Poxviruses are the most complicated of all known viruses. It is characteristic of these viruses that they are surrounded by both a membrane and an envelope and that their replication occurs in the cytoplasm independently of nuclear functions of

cells. Thus it has been shown that poxviruses can replicate in cells from which nuclei have been removed. Vaccinia virus is used here as a prototype for this group. A poxvirus induces the synthesis of a large number of polypeptides. In the virus particle alone more than 30 different proteins with a combined molecular weight of about 2×10^6 have been identified. Poxviruses contain almost 50 times as much DNA as papovaviruses and because of this complexity only a very limited knowledge of the organization of the genome has accumulated. Like herpesviruses, the poxviruses code for a large number of different replicative functions and many different enzyme activities have been identified during virus replication. Compared to herpesviruses, one additional degree of specialization can be identified in poxviruses. This is the presence in virions of an enzyme activity which directly can transcribe a part of the genome leading to the production of viral proteins which in their turn can initiate virus replication. It is because of this capacity that poxvirus replication has become independent of the cell nucleus.

Structure of DNA

Poxviruses contain one single intact molecule of double-stranded DNA. In the case of vaccinia virus, the DNA has a molecular weight of $122–130 \times 10^6$, but poxviruses with larger genomes have also been encountered. The vaccinia virus genome has an exceptionally low content of GC nucleotides, about 36–37 per cent. It is characteristic of the virus-DNA that the intact molecule cannot be denatured. It was previously thought that this was due to the occurrence of a number of covalent cross-linkings between the two DNA strands. However, later it was shown that the two DNA strands are joined in both ends of the molecule. As a consequence denaturation of poxvirus-DNA leads to the formation of a gigantic covalently closed circle. Since the strands of DNA cannot be removed from each other renaturation occurs rapidly.

Synthesis of virus proteins

In the process of virion penetration through the cellular membrane the envelope is eliminated and hereafter parts of the inner membrane and the capsid are eliminated. The first stage of virus-uncoating occurs within 20 minutes after infection. After this a phase is initiated which eventually leads to the nucleocapsid being dissolved and DNA becoming accessible in the cytoplasm. This latter phase cannot take place if protein synthesis is blocked since it requires the synthesis of one or more virus proteins. The fact that poxviruses replicate in the cytoplasm suggests certain problems as regards both transcription and DNA replication. Normally, no RNA polymerases are accessible in the cytoplasm of the host cell and therefore the incoming virus must carry along a DNA-dependent RNA polymerase in order to start transcription. This enzyme transcribes a set of genes representing about 14 per cent of the whole genome. The early transcription leads to a synthesis of proteins, one function of which is to be responsible for the complete uncoating of the virus particles. When a complete uncoating of DNA has occurred further genes can be transcribed and this leads to viral DNA synthesis being started and, as a further consequence, late transcription being initiated. Newly-synthesized virus-DNA can be demonstrated within 1½ hours of infection and the viral DNA-synthesis reaches a peak ½–1 hour later. The exact mechanism of viral DNA synthesis has not as yet been clarified. The fact that viral DNA is covalently linked

at its end suggests certain problems regarding replication since the DNA strands cannot be unwound. However, in connection with the virus infection an enzyme is synthesized which has the capacity to open and close covalently linked polynucleotide chains.

Many enzymes have been demonstrated as occurring in increased quantities during a vaccinia virus infection. However, it is still unclear which of these enzymes are coded for by the viral genome and which of them represent cellular enzymes used by the replicating virus. The RNA polymerase which occurs in virus particles probably is coded for by the poxvirus genome. In addition a virus-specific DNA polymerase and thymidine kinase have been identified. A poly(A)-polymerase which provides viral mRNAs with poly(A) segments in their 3′ ends has also been identified. Finally, there are also enzymatic activities which provide viral mRNA with a 'cap' structure. The synthesis of all these enzymes is necessary since replication of poxviruses occurs in the cytoplasm where normally these enzyme activities do not occur. Most likely a considerable number of other virus-specific enzymes participate in the virus replication. Among the proteins which are synthesized later during infection, different structural components occur, some of which carry the enzymatic activities demonstrable in virus particles.

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10

The morphogenesis of virions

Erling Norrby

The maturation and release of non-enveloped viruses

Virions with the capsid as their outmost layer, mature through a crystallization-like process during which all structural components are assembled in a strictly organized fashion. All particles produced are released simultaneously with disruption of the cell. The architecture of the capsid to a major extent is determined by the structural properties of the capsomers, i.e. the form and the chemical properties of the capsomers determine how they are phased together to form the capsid. The details of the sequence of events during virion assembly are only partly known. Concerning poliovirus (*see* Chapter 8) it has been shown that the structural proteins are aggregated into a procapsid. Hereafter virus-RNA is introduced into the procapsid leading to the formation of a provirion. After proteolytic cleavage of structural proteins the final maturation into virions takes place. In an analogous way structural proteins and nucleic acid forming adenovirions are put together. Firstly, empty viral capsids are assembled. These capsids have approximately the same composition as the final virion with the exception that they contain a special maturation protein and lack DNA. In one end of the DNA molecule there is a special nucleotide sequence ('the packing sequence') which allows viral DNA to penetrate the empty capsid and become enclosed in its interior. In the final step of maturation certain polypeptides are cleaved and DNA is surrounded by virus-specific basic protein.

The reason why the infected cell eventually breaks up is not known and it may possibly vary for different viruses. It is apparent that the extensive exploitation of the metabolic machinery of the cells during virus replication may lead to derangement of organized functions. It has been shown that certain non-enveloped viruses, e.g. poliovirus, cause a depolymerization of cellular actin. This destruction of the cytoskeleton of the cells must lead to an increased sensitivity both to inner and outer influences. One additional factor of importance in this context may be virus-induced membrane changes. All non-enveloped viruses with the exception of the smallest viruses, picorna and parvoviruses, induce such changes. This change may have secondary effects leading to permeability disturbances, for example, and thus influence the coordinated functions of the cells.

The morphogenesis of enveloped viruses

The virus envelope is formed by restructuring the membranes of infected cells. As mentioned in Chapter 3, the virus envelope contains virus-specific proteins, whereas carbohydrates and lipids derive from the cell. Virions acquire their

envelope through a budding-off process and virus particles are released continuously during a shorter or longer period of time. The special features of this maturation process are discussed in this chapter. Firstly, some general structural and functional properties of membrane structures are described and then the membrane morphogenesis for different families of enveloped viruses is described.

General aspects on membrane structures

The outside and inside of a membrane structure is determined by the localization and orientation of the proteins which it contains and the occurrence of glycosylation of proteins and lipids (*Figure 10.1*). The central structure in cellular membranes – and thus also in virus envelopes – is a bimolecular layer of phospholipids oriented so that they turn their hydrophobic part towards the centrum of the

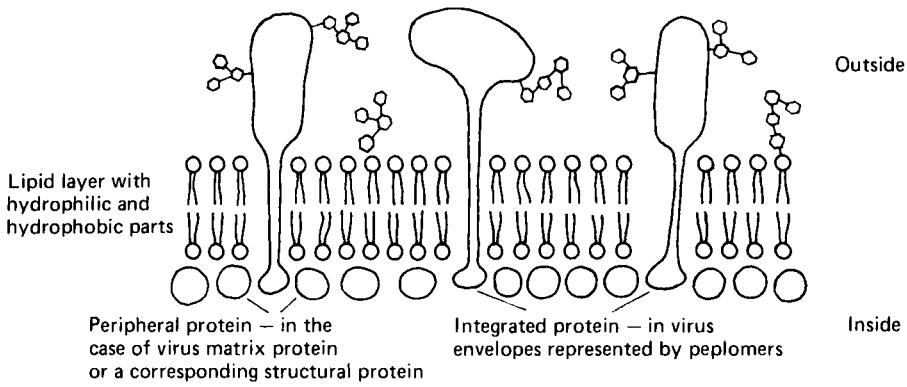

Figure 10.1. Schematic description of the structure of a membrane. The asymmetry of a membrane is expressed in different ways. Carbohydrates are coupled only to the integrated proteins, which are exposed on the outside of the membrane. In contrast, peripheral proteins occur on the inside of the membrane and have a matrix function

membrane and their hydrophilic part towards the periphery. One distinguishes between peripheral and integrated proteins, which are located on either side of the membrane and pass through the membrane and are accessible on both sides, respectively. The asymmetry of the membrane is manifested by the fact that carbohydrate chains are coupled only to proteins and lipids on the outside and that peripheral proteins only occur on the inside. In the virus envelope the peplomers represent integrated proteins, whereas the matrix protein represents the peripheral protein. Not all enveloped viruses have a matrix protein. In the smallest enveloped viruses, the togaviruses, the capsid protein performs the function of the matrix protein. Detailed studies of selected togaviruses have shown that a number of structural components in the capsid corresponds to the number of peplomers. The cellular membrane has a complicated composition and contains a large number of different proteins. In contrast, there are virus envelopes, which contain only two proteins, one of which has a matrix function and the other is the glycoprotein in peplomers. For this reason model studies of enveloped viruses have been performed to elicit more general information about the principal composition of membranes and the mechanisms of formation.

Formation of the envelope of RNA viruses

All enveloped RNA viruses cause the appearance of nucleocapsids in the cytoplasm of cells during their replication. The final maturation occurs through a budding-off process either directly against the external surface of the cell or into vesicular structures in the cytoplasm. The molecular events concerned with the formation of the envelope have been studied in detail in the case of certain togaviruses and rhabdoviruses. In the following description the maturation of rhabdoviruses is used as an example, although there are reasons to believe that analogous processes lie behind the formation of envelopes of all RNA viruses.

One rhabdovirus named 'vesicular stomatitis virus' (VSV), which can give vesicular changes in the mouth of cattle, is formed by a budding-off process of elongated virions at the cytoplasmic membrane. The virion is composed of five proteins of which three are included in or associated with the nucleocapsid, one represents the matrix protein of the envelope and one is the glycoprotein, which builds up the peplomers. In a cell, protein synthesis can occur either by use of free ribosomes in the cytoplasm or by membrane-bound ribosomes. The latter ribosomes are used for formation of integrated membrane proteins and of proteins, which will be secreted. In the case of VSV, this means that the glycoprotein is

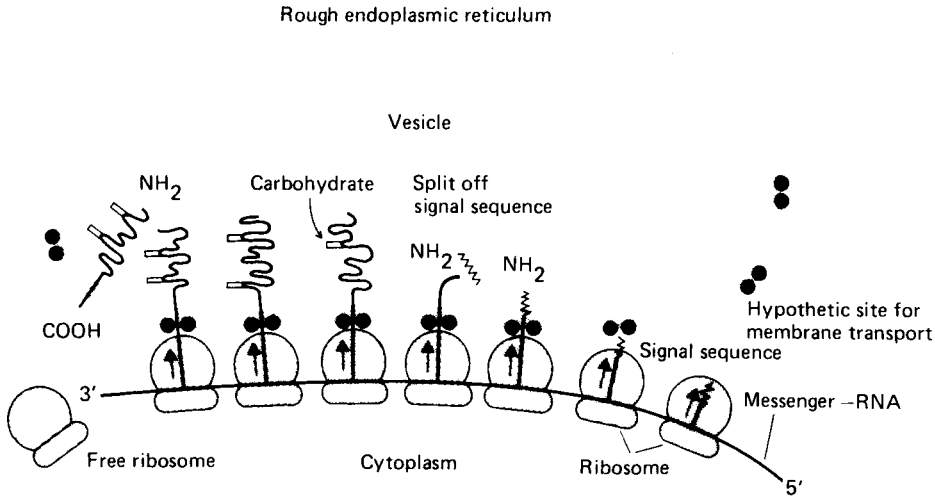

Figure 10.2. Different steps in the synthesis of an integrated protein. The first part of the polypeptide which is synthesized, the signal sequence, is coupled to the membrane around a vesicle. The polypeptide is fed into the cavity of this vesicle. Hereafter the signal sequence is split off. The final protein remains attached to the membrane by its carboxyl-end.

synthesized on ribosomes attached to the endoplasmic reticulum, whereas the other four proteins are formed on free ribosomes. Different stages in the synthesis of the glycoprotein are visualized in *Figure 10.2*.

The synthesis starts on free ribosomes but these are coupled at a very early stage to the endoplasmic reticulum. The charged protein, which is being synthesized, is pushed through the hydrophobic central part of membranes, a process which

appears to be made practical by use of membrane transport spots. The first part of the NH_2-terminal end of the protein appears to have a transport-directing function. This part is called the *signal sequence* and once inside the vesicle in the endoplasmatic reticulum it is split off. Finally, there is a transfer of ready-made carbohydrate chains from cellular glycolipids to two places in the protein, where arginine is present, and the protein eventually obtains its final configuration. It remains connected to the membrane via its COOH end.

The carbohydrate chains, which are coupled to the protein, contain mannose and N-acetylglucosamine. However, in the final virion the carbohydrate chains have a different composition. This is because vesicles with viral glycoproteins are not transported directly from the endoplasmatic reticulum to the cytoplasmic membrane but that firstly they are combined with vesicles from the Golgi apparatus. In these combined vesicles carbohydrate chains which have been attached in the endoplasmatic reticulum are trimmed and supplementary carbohydrate chains are added. These additional chains contain further N-acetylglucosamine but also galactose, sialic acid and fucose. The vesicles with the glycoproteins in this final form are eventually transported to the cytoplasmic membrane and inserted by a fusion process. In connection with these processes a strict membrane orientation is retained so that the glycosylated surface is turned towards a vesicle or against the surroundings of the cell. During all these steps of maturation the virus makes use of the normal processes of the cellular machinery. A final maturation of virions occurs by means of a budding-off from the cellular membrane (exemplified in *Figure 10.3* with maturation of RS virus). The relationship between the coiled nucleocapsid and the matrix protein and between the latter protein and the integrated peplomer proteins probably is strictly regulated. Cellular proteins are not included in the final virus envelope. Since proteins can move freely in membrane structures it is likely that the cellular proteins are excluded from the virus envelope in the final shaping of the virion.

Studies of paramyxoviruses, which also bud from the cytoplasmic membrane, have given additional insight into the formation and composition of the envelope. Paramyxoviruses, with the exception of morbilliviruses, contain the enzyme neuraminidase in their envelope. As a consequence all neuraminic acid-containing structures not only in the virus envelope but also in the cytoplasmic membrane of the infected cells are eliminated. An interesting consequence of this is the fact that the infected cell loses the receptors, which are needed for adsorption of virions. This in turn means that newly synthesized virions cannot become readsorbed. Membrane changes, which influence the occurrence of receptors, may be a common phenomenon.

The virus envelope has the same lipid composition as the cellular membrane from which it derives. The presence of the enzyme neuraminidase in myxoviruses, however, excludes neuraminic acid containing structure from the virus envelope. It therefore has a lipid composition somewhat different from that of the cytoplasmic membrane.

One of the two kinds of peplomers which occurs in the paramyxovirus envelope is responsible for the fusion activity of the virus. It is to be expected that this biological activity might disturb the regulated fusion processes, which lie behind the combination of the microsomes with Golgi vesicles and after further transport for insertion of envelope structures into the cytoplasmic membrane. It has been shown, however, that the glycoprotein responsible for the fusion activity only displays this activity after proteolytic cleavage. It is therefore attractive to consider the

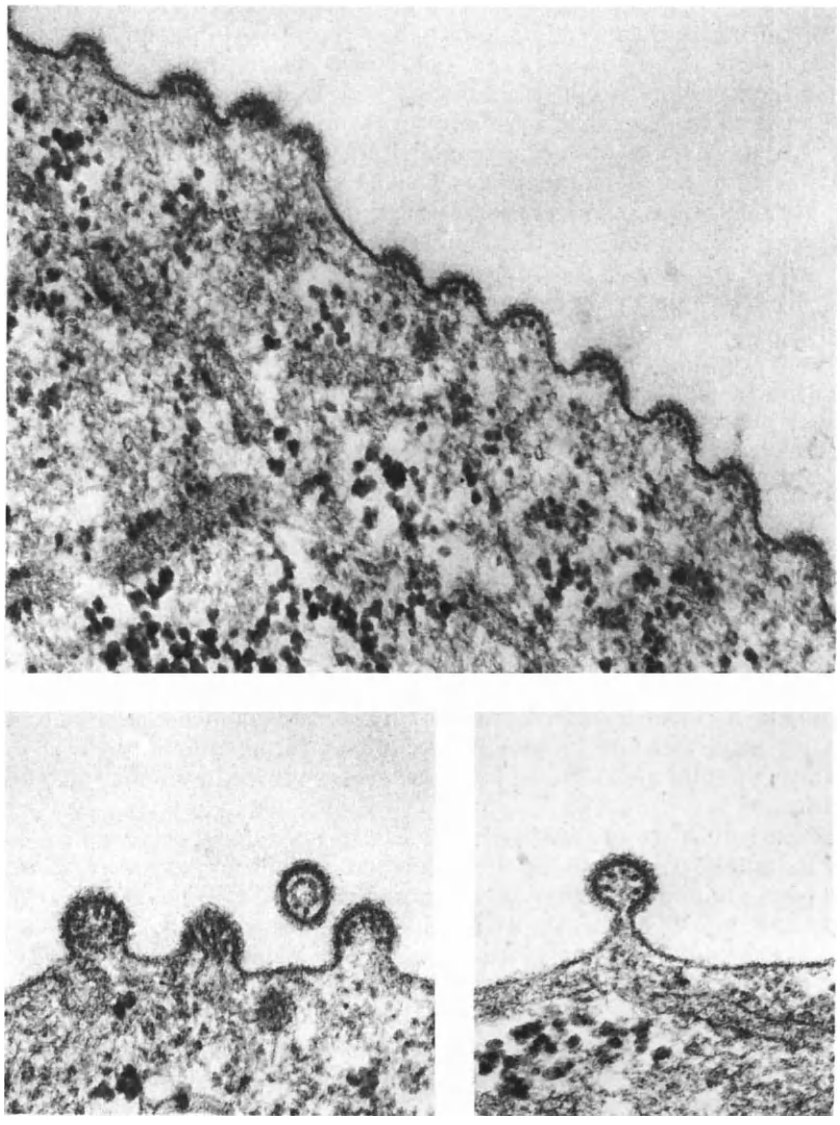

Figure 10.3. Formation of RS virus by budding from the cytoplasmic membrane. Different stages in the budding process are illustrated. Note that the cross-sectioned nucleocapsids are arranged symmetrically under the cytoplasmic membrane and that the peplomers only extend from the part of the cytoplasmic membrane under which nucleocapsids are localized (Magnification: ×~98 500)

possibility that this cleavage occurs late in the morphogenetic process leading to the peplomers only acquiring fusion capacity in connection with their final localization into the cytoplasmic membrane.

When antibodies against the envelope components of a virus are added to an infected cell all peplomers which are located in the cytoplasmic membrane accumulate at one cell pool, 'capping' (*see* Chapter 13). This phenomenon occurs

because peplomers, like other integrated proteins, can be moved laterally in the membrane and, through their linkage via bivalent antibodies, they accumulate in a restricted area in the membrane. However, it is not only integrated membrane proteins which are redistributed in the cell in connection with capping. Structural components also on the inside of the membrane and in the cytoplasm, e.g. nucleocapsids, are redistributed and accumulate under the cytoplasmic membrane where the virus peplomers have become localized. Thus there exists in addition to the transmembranous communication a further connection to structures in the cytoplasm. Possibly, the contractile cellular constituent, actin, is important for this communication. This cellular protein is included in paramyxovirions and rhabdovirions, as mentioned previously.

Many of the enveloped RNA viruses, which are synthesized in the cytoplasm have a haemagglutinating capacity (*see* Chapter 4). The virus-haemagglutinin is included among the glycoproteins which are built into the cytoplasmic membrane. As a result, the infected cell acquires a capacity to adsorb red blood cells to its surface, *haemadsorption*. This phenomenon can be used to demonstrate the presence of a virus in a cell culture with an infection not displaying cytopathic effects.

The total number of infectious particles produced by a single cell varies markedly for different enveloped RNA viruses depending on the properties of both the virus and the infected cell. In some cases more than 10 000 infectious particles can be released from a single cell before it is impoverished and dies. In other instances the infection does less harm to the cells. Virions may be produced at a slower rate but, since the cell can survive the infection, the total number of virions that can be produced in principle is unlimited.

Formation of the envelope of DNA viruses which have a nucleocapsid that matures in the nucleus

Members of the herpesvirus group synthesize structural proteins in the cytoplasm of infected cells. Many of these proteins are transported to the cell nucleus and some of them are combined to form the nucleocapsids of the virus. It is not known by which routes of transport these proteins arrive in the nucleus. Herpesvirus glycoproteins are formed on ribosomes combined with membranes in the endoplasmic reticulum. There is some evidence that herpesviruses can modify pre-existing transferases or possibly even code for their own glycosyl transferases. Certain structural features of the herpes simplex virus glycoproteins indicate that a more direct influence of the virus on the glycosylation process may occur. Glycoproteins which are being synthesized eventually appear in the cytoplasmic membrane, but they can also by unknown processes be built into the inner of the two nuclear membranes. Presumably these processes include repeated fusion-vesicle formation steps whereby the barrier, which the outer nuclear membrane represents, can be forced. As with other instances of virus envelope morphogenesis, it has been assumed that the virus utilizes normal cellular functions. Also, the normal cell must have the means to transport proteins, which belong in the nucleus or in nuclear membranes, from their location of synthesis in the cytoplasm.

When mature nucleocapsids occur in the nucleus and peplomers have been located on the outside of the inner nuclear membrane and matrix antigen on the inside, virions can be formed through a budding-off process (*Figure 10.4*). The

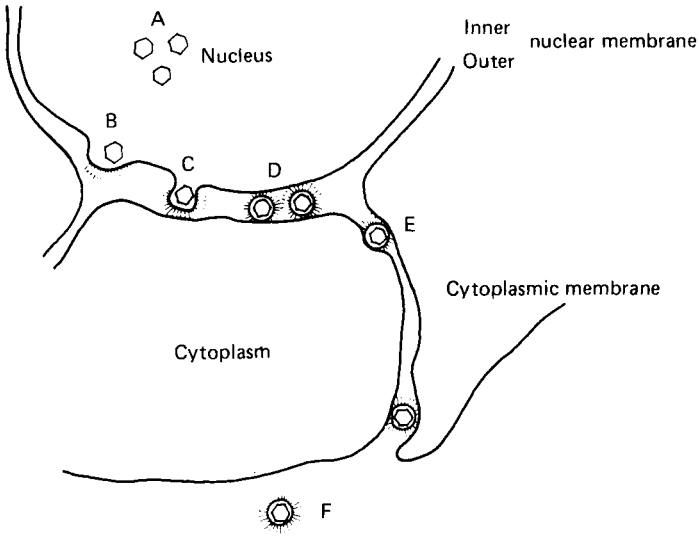

Figure 10.4. Schematic description of the morphogenesis of herpesviruses. The nucleocapsid matures in the nucleus (A). Via a budding-off process from the inner nuclear membrane (B), mature virions (C) are formed, which accumulate in the space between the two nuclear membranes (D). These particles are transported to the outside of the cell (E, F) via a channel system. The glycoproteins which are present on the outside of the envelope also appear in the cytoplasmic membrane in spite of the fact that this membrane is not utilized during virus morphogenesis

mature virions are enclosed between the inner and outer nuclear membrane. Their transport to the outside of cells probably occurs via channels that open at the cell surface. About 10 000 infectious particles can be produced by a single cell.

Formation of the envelope of DNA viruses which have a nucleocapsid that matures in the cytoplasm

The mechanism for poxvirus envelope formation has only been clarified during recent years. Particles with a complicated structure are formed in the cytoplasm of infected cells. The centre of these particles contain the nucleocapsid and two lateral bodies enclosed by a membrane, which is synthesized *de novo* in the cytoplasm of infected cells (*Figure 10.5*). This is the only example of a membrane structure belonging to a virus which is formed without restructuring of a pre-existing cellular membrane. This virus membrane distinguishes itself concerning its lipid composition from various normal cellular membranes. The membrane-enclosed intracytoplasmic particles have infectious property and it was thought for a long time that they represented the final structure in virus morphogenesis. However, it has become apparent that particles which are spontaneously released from infected cells are enclosed by one additional membrane structure (*Figure 10.6*). This membrane in all respects can be compared with the virus envelopes discussed above. It will therefore be referred to as an *envelope*, whereas the membrane structure which is synthesized inside the cytoplasm is called an *inner membrane structure*.

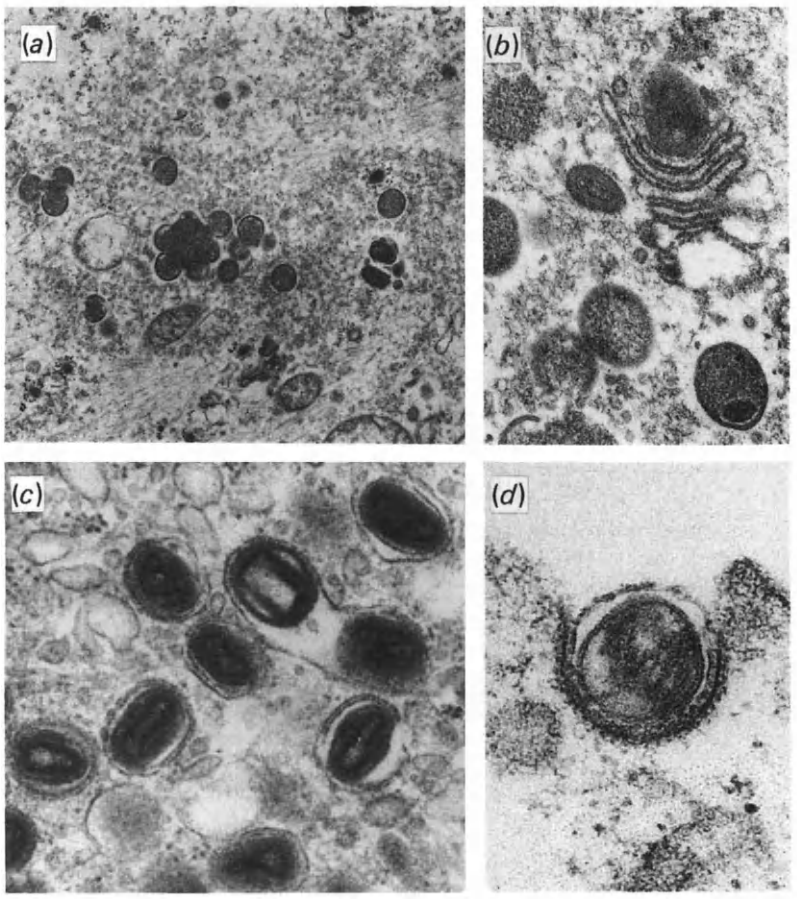

Figure 10.5. Poxvirus morphogenesis. A membrane is formed by *de novo* synthesis in the cytoplasm and encloses the nucleoprotein and the lateral bodies of the virion (*a*). This intracytoplasmic membrane-enclosed particle combines with the membrane system in the Golgi apparatus (*b*). By a process of invagination, particles are then surrounded by a double-sac structure (*c*). Release of mature virions occurs by a fusion between the outermost sac of this structure with the cytoplasmic membrane (*d*). The inner sac structure becomes the envelope of the final particle (Magnification: (*a*) × 13 500, (*b*) ×44 000, (*c*) ×36 000, (*d*) ×61 000. Photo: K. Kristensson and L. Payne)

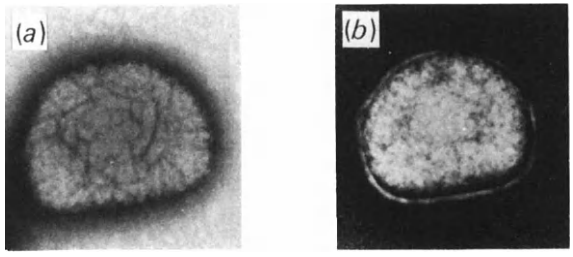

Figure 10.6. Electron-microscopic pictures of a non-enveloped poxvirus particle. (*a*) extracted from infected cells and an enveloped extracellular particle. (*b*) the complete virion (Magnification: (*a*) ×90 000 and (*b*) ×84 000. (Photo: L. Payne)

Poxviruses acquire their envelope through a process which engages membranes in the Golgi apparatus (*Figure 10.5b–d*). Membrane-enwrapped particles in the cytoplasm are enclosed by a vesicle of the Golgi apparatus and hereby become surrounded by an additional double-membrane sac structure. Virus-specific glycoproteins occur on the outside of the inner membrane and on the inside of the outer membrane. The structure with both these membranes is transported to the cytoplasmic membrane and the outermost membrane sac is fused to it. Hereby a particle which retains one envelope is released. During the fusion process, virus-specific haemagglutinin is inserted into the cytoplasmic membrane and may play a role in the endowment of the infected cell with haemadsorbing capacity.

About 10 000 infectious particles can be formed by a single cell. However, the majority of these particles remain intracellularly in a cell culture system and only a limited number of extracellular enveloped particles are produced. It is possible that *in vivo* conditions for release of enveloped poxviruses may be more effective.

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11

Virus-induced changes of cell structures and functions

Erik Lycke and Erling Norrby

Generally the occurrence of virus-binding receptors on the plasma membrane of the cell is a prerequisite for establishment of the virus infection. The many different consequences for the cell to which the virus infection may lead are summarized in *Table 11.1*. Depending upon whether new infectious virions are formed or not, it is possible to distinguish between *productive* and *non-productive* infections. A productive infection in a cell culture system may be of varying intensity and alternatively lead to (1) destruction of cells, (2) an equilibrium of destruction of infected cells and growth and division of non-infected cells or (3) survival of infected cells. Two different circumstances may cause a state of chronic infection of the cells. In one case the occurrence of certain properties of host cells hampers virus replication, in the other the virus has no or only a very limited cytocidal effect. The latter situation may be caused by the virus being itself very mildly cytopathogenic or by some mistake having occurred during virus replication. Mutants of the virus, in particular those with *temperature-sensitive* (ts) characteristics (cf. Chapter 12), may emerge and become dominant in the infection. Another phenomenon is the appearance of genomes of subnormal size as a result of errors of replication. These subgenomic structures may interfere with the replication of complete genomes and they are then referred to as *defective interfering* (DI) *particles*. The defects in virus replication may become so pronounced that no infectious virus is found, i.e. the infection has become non-productive and therefore, by definition, it can only be maintained by continued division of cells. One additional example of a non-productive infection is the situation when the virus-genome is integrated into the cellular genome (or persists in an episomal form) with a reduced degree of expression of the former. This *latent* infection (virogeny) in some cases leads to cell *transformation*.

Furthermore the concept *permissive* is used to denote cells which allow complete replication of virus in contrast to *non-permissive*, which indicates cells that allow virus adsorption and penetration but block complete replication of virus. In some special cases the blocking of virus replication in non-permissive cells can be abolished by the simultaneous infection with another virus. In other cases infection of non-permissive cells may lead to cell transformation.

Cytopathic changes

Figure 11.1 illustrates the different changes which may develop in virus-infected cells. Viruses which cause cell destruction are usually referred to as *cytocidal* or

cytopathic. The cytopathogenic effect of a virus is called the *cpe* and cell destruction is referred to as *cytolysis*.

In spite of the fact that virus cpe has been studied for many years the mechanism behind the cytocidal activities of a virus is not fully understood. As a rule it can be stated that infection with non-enveloped viruses usually gives a more rapid cell death than infections with enveloped viruses which mature through a continuous budding-off process. Since virus replication in many cell systems leads to an almost

TABLE 11.1. Different effects of virus replication in cell cultures

Virus-cell interaction	*Effect on cells*
Productive replication of virus leading to damage of the cellular metabolism	Cellular destruction – *cytocidal effects*
Productive but moderately intensive replication of virus (e.g. restricted by production of interferon) leading to damage on the cellular metabolism	Restricted cytocidal effects which are compensated for by division of non-infected cells (*'steady state' infection*)
Productive replication of virus under conditions which allow survival of the infected cells. A mild infection may be caused by the fact the virus itself is only weakly cytopathogenic or may depend on the occurrence of mistakes in virus replication (e.g. the appearance of temperature-sensitive mutants or the generation of defective interfering particles). In the latter situation a development into a non-productive infection may occur	*Chronic infection* (carrier state)
Non-productive infection in a permissive cell. Integration of the virus-genome into cellular DNA or persistence of the genome in an episomal form	(a) *Latent infection* with possible changes of cellular functions. Possibilities exist for activation leading to a complete replication of virus (*virogeny* analogous to lysogeny in bacteria) (b) *Transformation* with a defective virus-genome
Infection in a non-permissive cell: (a) Supplementation of the milieu of replication by products from a helper virus, e.g. human adenovirus and SV40 in monkey cells.	Cytocidal effects
(b) Integration of the virus-genome into cellular DNA	Transformation with a complete or defective genome

complete blocking of the normal cell metabolism it has been tempting to interpret cellular degeneration as a phenomenon connected with a reduced cellular activity. The cell has been assumed to die because of starvation caused by the virus infection taking over the cellular energy resources. In some virus-cell systems it has been found that cellular polysomes are disorganized during the early phase of infection. This may lead to a release of ribosomes which become accessible for synthesis of virus-specific proteins. The discovery that virus cpe need not necessarily be correlated with virus replication has shown that its background causes are probably complex.

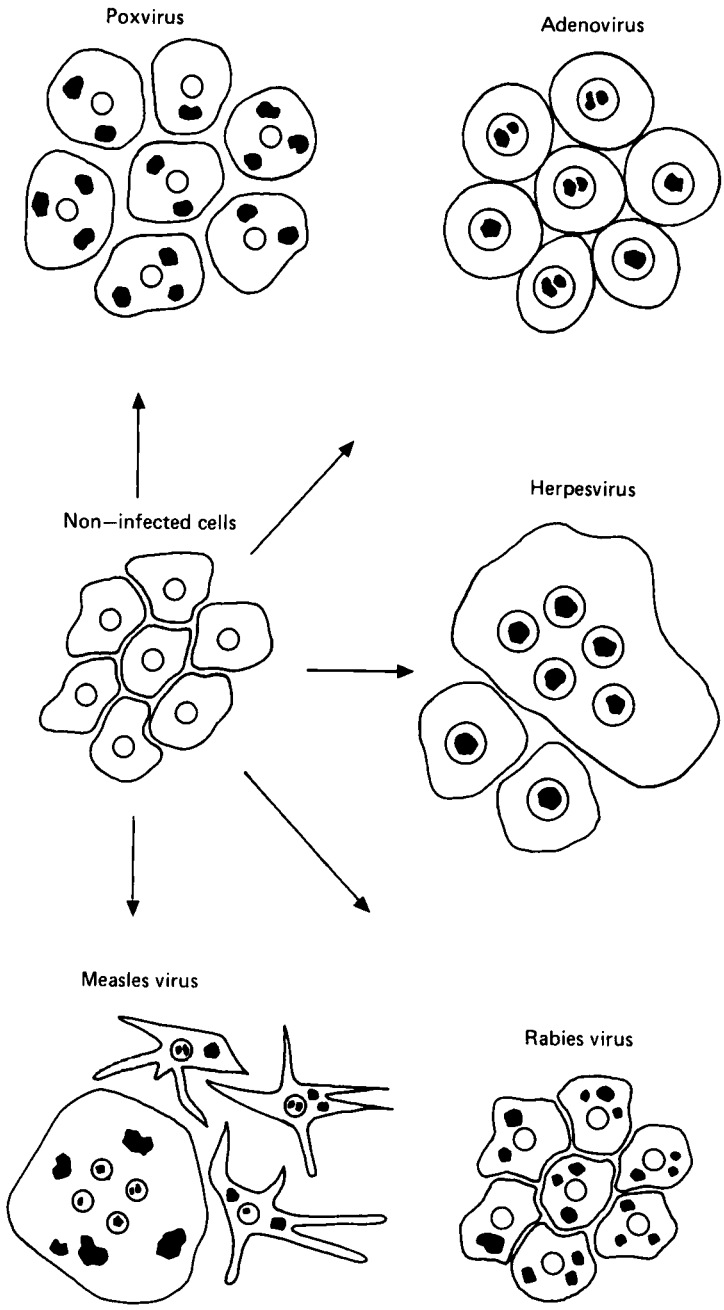

Figure 11.1. Schematic description of cytopathogenic changes caused by infection with different kinds of viruses. Darkly-stained areas represent inclusions. These may occur in the cytoplasm and/or in the nucleus of cells and they contain virus-specific products or modified cellular products. The shape of the nucleus and the cytoplasm can be changed during the infection. Furthermore, infections with herpesviruses and measles virus, for example, may cause a fusion of cells into multinucleated giant cells – syncytia

Vaccinia virus which is treated with ultraviolet light cannot replicate but it still causes cpe when inoculated into cell cultures. Poliovirus infections can cause cell destruction even when protein synthesis is blocked. Frequently the cytocidal properties of a virus appear to be correlated with the amount of virus that is produced in the cell system. The more extensive the destruction in the cell culture the more virus is formed. However, this rule cannot be generalized. In particular there are variations in virus-cell systems in which virions are formed by budding from the plasma membrane. A virus which in one type of cell can replicate effectively without any significant cpe may cause extensive cell destruction in another kind of cell in spite of a poor virus replication. These relationships have been studied in particular in paramyxovirus-infected cells. It has been assumed that the formation of virus-matrix protein may be the rate-limiting factor during virus release and that the capacity to synthesize the matrix protein may vary in different cells.

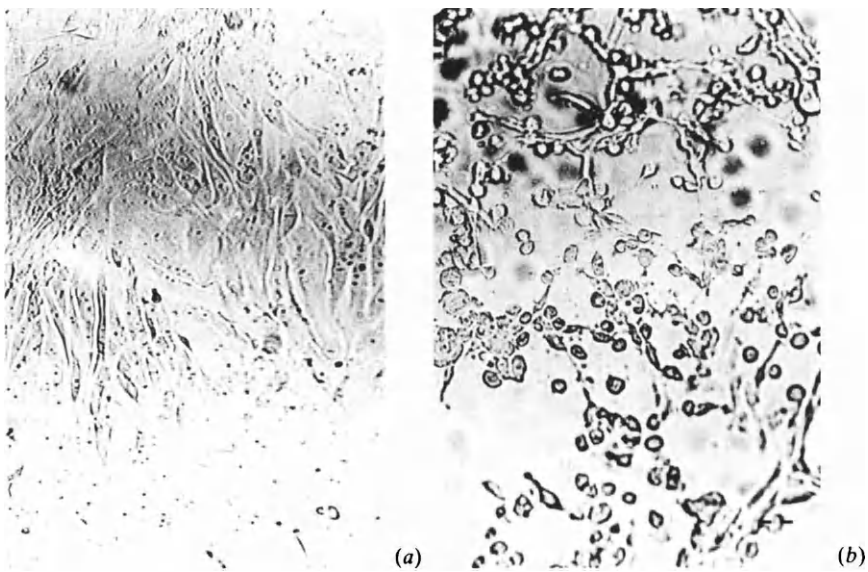

Figure 11.2. Primary monkey kidney cells in culture. The bottom of the bottle used for cultivation is covered by a monolayer of cells. (*a*) shows uninfected cells and (*b*) cells infected with an enterovirus. The preparations have been photographed unstained directly in the light microscope

In vaccinia virus-infected cell cultures two different phases of morphological change can be observed, one early phase which relates to the first 4 hours after the inoculation of culture, and a late phase which occurs 12–24 hours later in connection with the maturation and release of newly formed virus. Puromycin, an inhibitor of protein synthesis, reduces synthesis of early viral gene products and also the early-appearing cytocidal effects of the virus infection. It appears, therefore, as if the swelling and rounding-off of cells which is seen during the early phase of a vaccinia virus infection is dependent on the formation of one or more specific cell-toxic virus proteins. Vaccinia virus late cpe, in contrast, appears to correlate with the intracellular release of lysosomal cellular enzymes. In agreement with this, membrane-stabilizing compounds have been found to reduce the

breakdown of cells. It is therefore likely that certain viral gene products may have a direct toxic effect, for example by blocking an important enzyme reaction, and that the virus infection may cause defects in cellular organelles, for example, by the activation of lysosomes leading to autolysis.

Histologically, virus cpe is characterized by changes in the morphology of cells; anomalies with long thin extensions or rounded buds of cytoplasm are observed. The nucleus may show pyknosis and karyorrhexis and the cells may break down.

Commonly, cells infected with cytocidal viruses, as a first sign of cpe, swell and become rounded and when viewed with light microscopy show a strong refraction of light (*Figure 11.2*). A leakage of ions from the cells shows that the plasma membrane has become abnormally permeable.

Inclusions in virus-infected cells

After histological staining of virus-infected cells, areas of varying size which are stained differently than their surrounding cellular substance are found. These *inclusions* (*Figure 11.3*) have been described by morphologists long before the

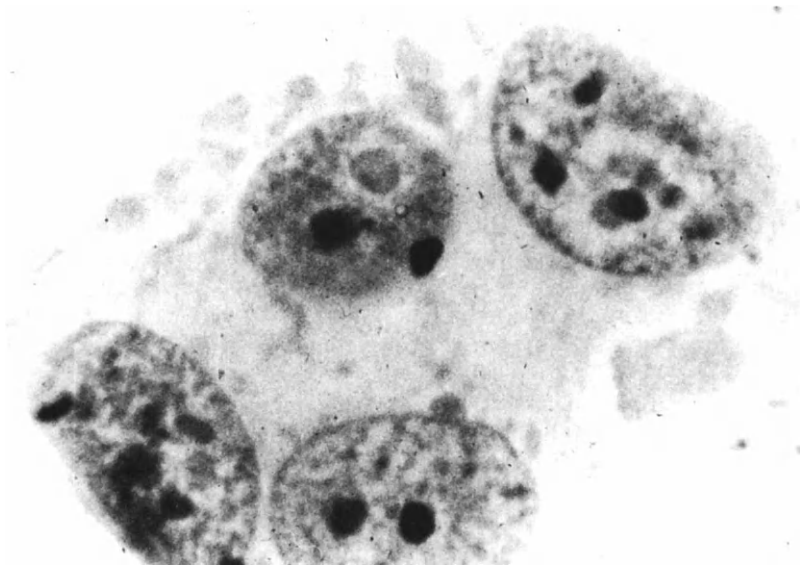

Figure 11.3. Infection with measles virus in a human heteroploid cell line grown in tissue culture. Nuclei from four cells share a common cytoplasm. The upper middle nucleus contains a distinct inclusion and there are a number of inclusions in the cytoplasm. The cells have been fixed and stained with haematoxylin-eosin

identification of viruses as infectious agents and they have previously played a major role in the histopathological diagnosis of virus infections. The inclusions may be acidophilic or basophilic and they occur in the nucleus and/or the cytoplasm of cells (*Table 11.2*). The inclusions represent a local accumulation in cells of virions or virus-structural components (*Figure 11.4*) or metabolites.

The effect of integration of viral antigens in cellular membranes

Several observations show that the integration of virus-induced proteins in the plasma membrane of infected cells causes many different changes of cellular functions. When the viral glycoproteins have been synthesized in the endoplasmatic reticulum they are transported to the plasma membrane. In this position they can be identified by use of immunological techniques, e.g. immunofluorescence.

TABLE 11.2. Examples of inclusions appearing during infections with some different viruses

Virus	*Localization in the cell*	*Staining properties*	*Possible diagnostic importance*
Poxvirus	Cytoplasm	Acidophilic	Differential diagnoses by histo-
Herpesvirus	Nucleus	Acidophilic	pathology between smallpox and varicella
Rabies virus	Cytoplasm	Acidophilic	Indication of a rabies infection in a diseased animal
Adenovirus	Nucleus	Basophilic	–
Morbilli virus	Nucleus and cytoplasm	Acidophilic	–

Virus-induced glycoproteins which are produced by paramyxoviruses and herpesviruses cause *cell fusion* (cf. Chapter 4). Infected cells containing fusion factor in their plasma membrane may fuse with neighbouring non-infected cells – fusion from within. In this way *polykaryotic giant cells* are formed (cf. *Figure 11.3*). Some paramyxoviruses have such a large amount of fusion factor in their envelope that they can bring about a fusion between uninfected cells under conditions where the cells are exposed to sufficiently high concentrations of enveloped virus particles – fusion from without (cf. Chapter 4). This fusion appears within 1–3 hours after exposure without any replication of virus. Both kinds of fusion reactions are dependent on a preceding reaction between the virus adsorption protein (haemagglutinin) and cellular receptors, allowing the fusion-inducing glycoprotein to get in close contact with the plasma membrane. The immediate cause of the fusion has not been defined.

The introduction of viral proteins into the cytoplasmic membrane can endow the cells with new properties in addition to the changed immunological surface properties. Myxovirus-infected cells can adsorb red blood cells (haemadsorption) and show neuraminidase activity, depending on the presence of virus-haemagglutinin and neuramindase, respectively, in the plasma membrane.

Paramyxovirus-infected cells in addition express haemolytic and, as was already mentioned, fusion-inducing properties, and herpesvirus-infected cells acquire a capacity to bind immunoglobulins by the appearance of Fc-receptors on the cellular membrane.

Effects of viruses on functions of highly specialized cells

The possible effect of viruses on functions of highly specialized cells has not been extensively analysed. There are certain problems in studying these phenomena since cells grown in cultures frequently lose more specialized functions. However, by use of organ cultures it has been shown that rhinovirus infections cause a loss of

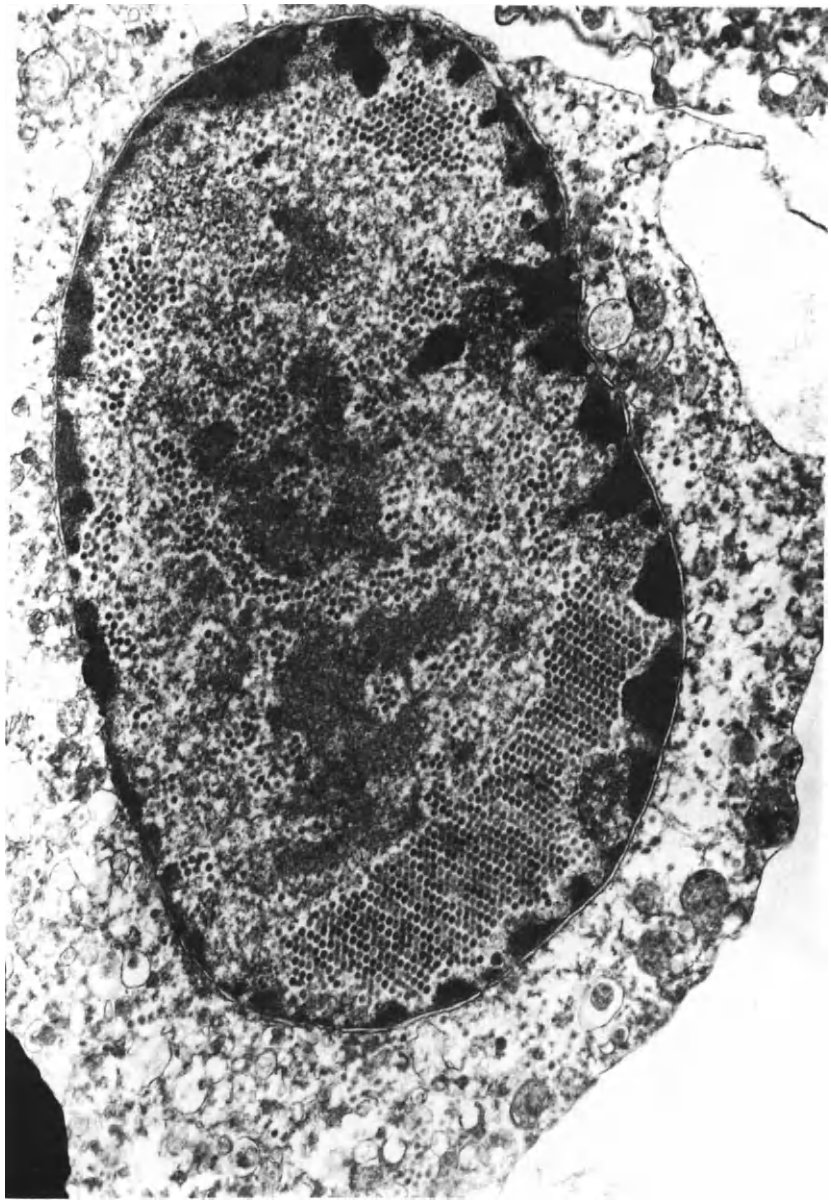

Figure 11.4. Electron-microscopic picture of a cell infected with adenovirus. The photograph has been taken at a late stage of virus replication. Large amounts of complete virions have accumulated in the nucleus. Note that these particles are arranged symmetrically. In addition to the virions in the nucleus, excess virus-building units can be found. The chromatin of the nucleus has been pushed towards the periphery and appears in a marginated position.(Magnification: ×~16 500. Photo reproduced by permission of Dr R. Marusyk, Department of Medical Bacteriology, University of Alberta, Canada)

cilia activity in the respiratory epithelium. The cilias are discharged from the epithelial cells but the cells do not die. It has further been shown that certain enzyme activities can disappear from virus-infected cells although the cells continue to grow and divide. The concentration of adenylate cyclase and acetylcholinesterase is drastically reduced in cultures of neuroblastoma cells which carry infection with rabies or measles virus. Apparently, these enzyme activities are not required for the survival of the neuroblastoma cells in a tissue culture. The loss of such properties have been described as 'loss of luxury functions of cells'.

Chromosomal changes

Replication of a virus in cells can lead to morphological changes in chromosomal structures. In connection with infections with measles and yellow fever virus an increased frequency of *chromosomal breakages* has been observed. Frequently these breakages or gaps in the different chromosomes are randomly distributed. In most cases they appear to be secondary to non-specific cellular degeneration. Cellular nucleases may have been released from lysosomes. It is not clear if these chromosomal changes have any consequences for cellular functions in the long term. It is likely that cells which show these kinds of changes have a reduced viability. However, it might be mentioned that a rubellavirus infection leads to inhibition of mitosis in certain cultivated epithelial human cells. Cell cultures with a persistent rubellavirus infection show chromosomal breakages in 20–25 per cent of the cell population. These observations are of particular interest against the background of the fetal damage which may be caused by a rubellavirus infection.

Another type of alteration called *chromosomal pulverization* has been found in cells infected with, for example, measles and herpesviruses. The origin of this phenomenon is a fusion between two or more cells which are in different stages of mitosis. The influence of the cytoplasm from one cell can bring about too early a chromosomal condensation in another cell, leading to the appearance of chromosomal fragments. This phenomenon presumably has no biological or medical importance.

Chromosomal changes in virus-transformed cells occasionally have a more specific character, as is discussed below.

Virus-induced cell transformation

Several different viruses have a capacity to change markedly the growth characteristics of cells in culture, *transformation*. The major change concerns the increased capacity of mitosis displayed by transformed cells. The limitation in number of possible mitoses and generations of the cell population, which is a characteristic of non-transformed cells, is lacking in transformed cells. As a result of transformation the cells acquire a capacity to divide, presumably unrestrictedly. They have become immortalized (cf. Chapter 5). The phenomenon of transformation forms the basis for the capacity of cells to grow as a tumour in a host organism (cf. Chapter 18). However, the defence mechanisms of host organisms *in vivo* prohibit or reduce the possibilities for transformed cells to divide unrestrictedly. Virus-induced cell transformation has attracted considerable interest since it has both cell-biological and oncological importance.

Properties of transformed cells

Fibroblasts and epithelial cells can only grow when they are anchored to a firm supporting layer. In the case of non-transformed cells, both movement and mitosis are regulated primarily by the *contact inhibition*. The molecular basis for contact inhibition is essentially unknown. It has been assumed that cells in a tissue culture stop in a G1 phase when they have reached a certain critical cell density. In contrast, transformed cells often have a modified morphology and appear to have a reduced contact inhibition. Therefore they grow in a more irregular fashion and to a relatively higher cell density. They may form more than one layer in the culture but all cells always retain contact at some point with the walls of the supporting vessel.

During later years the term *topo-inhibition* has been used instead of contact inhibition. This change emphasizes that it might be the lack of space in the culture rather than the contact between cells which influences the cell mitotic activity. However, it is a fact that properties characteristic of a particular cell influence the density of cells in cultures. Cells of different origin grow to different cell densities in cultures.

The capacity of transformed cells to exhibit an unlimited cell division can be registered also in ways other than by measuring their capacity to be 'passaged' repeatedly *in vitro*. The transformed cells can grow into colonies if they are mixed into soft agar, whereas non-transformed cells do not divide under these conditions. Similarly, transformed but not normal cells grow into a tumour in naked mice which have a defect in their cellular immunity. Naked mice lack defence not only against transformed mouse cells but also against transformed cells from other species.

In connection with transformation marked chromosomal changes are regularly observed. Exceptionally, a virus may give a morphological transformation without changing the diploid set-up of chromosomes. This special form of transformation occasionally is reversible. The normal course of events with transformation is that the chromosome number is doubled and afterwards chromosomes are gradually lost so that the cell becomes hypotetraploid. In some cases, a more regularized and restricted appearance of the changes in the cellular chromosomes has been observed. Adenovirus type 12, which belongs among the oncogenic types of adenoviruses, causes breakages in chromosomes 1 and 17 in human cells. Epstein–Barr virus (a herpesvirus) causes infections which lead to an immortalization of B lymphocytes and, in connection with the clonal selection which gives a basis for development of a Burkitt lymphoma, a translocation from chromosome 8 to chromosome 14 is observed.

Transformed cells not only show new growth properties and morphology but they also have a markedly changed metabolism. In addition a dedifferentiation may also be observed. This is reflected, for example, in the observable changes in the surface properties of cells, including the appearance of embryonic (oncofetal) antigens.

The occurrence of virus-specific antigens in transformed cells

Viral transformation means the introduction of viral functions into the altered cell. This is shown by the appearance of virus-specific antigens, which can be of two different kinds. One kind, the *tumour-specific transplantation (TST) antigen*, is present in the cytoplasmic membrane and contributes besides the oncofetal

antigen(s) in giving the transformed cells new surface-antigen properties. In spite of its name, the degree of specificity of TST antigens in different systems has not been defined. Thus it is not known to what extent virus-coded proteins contribute to the change in surface-antigen properties of transformed cells. An alternative name, *tumour-associated transplantation (TAT) antigen*, is therefore occasionally used. The second kind of tumour-associated antigen appears intracellularly either in the nucleus or in the cytoplasm depending upon the virus that is responsible for the transformation. The antigen is a virus-specified product — *tumour (T) antigen* in the case of certain DNA viruses (cf. Chapter 18).

Transformation mechanisms of different tumour viruses

Both RNA and DNA viruses can cause transformation. The sequence of events connected with a virus-induced cell transformation has not been clarified in spite of intensive studies. However, there appear to be differences between the processes of transformation with different viruses.

Retroviruses

These RNA viruses occur everywhere in nature. Frequently the viruses appear to live in harmony with their host cells. Certain observations indicate that they may function as genetic control elements and perhaps have an important role in connection with embryonic differentiation. Molecular events concerning replication of retroviruses are described in Chapter 8. The relationship between the virus and the host cell is intimate. For a long time it was unclear how an RNA virus could bring about permanent changes in the cellular genome.

The discovery in 1970 of the enzyme reverse transcriptase in retroviruses made it clear that the retroviruses are dependent for their transcription on an integrated DNA copy of virus, *a provirus*. The existence of retroviruses in the form of a DNA copy was supported by the observation that DNA extracted from a transformed cell can induce a transformation of normal cells, a phenomenon called *transfection*. However, in later experiments using the transfection technique it has been shown that transforming genes can be identified also in normal cells. Thus this technique cannot provide any conclusive evidence for the possible integration of virus-specific transforming genes into cellular DNA.

The transforming capacity of one group of RNA tumour viruses has its explanation in the capacity of these viruses to transfer cellular sequences from one cell to another. These gene sequences are referred to as the *oncogenes*. For example, certain avian retroviruses induce sarcomas and transform fibroblasts in tissue culture. The viruses carry a sarcoma (src) gene. It has been shown that the gene is present also in normal cells and that cellular nucleotide sequences similar to src have been preserved during the evolution. The fact that these sequences have been retained over wide phylogenetic distances indicates that they play an important role in normal cellular functions. Other retroviruses in spite of inducing leukaemia in poultry and mice apparently lack transforming genes and, as a consequence, do not transform *in vitro*. The mechanism by which they induce leukaemia in the animal is yet unsolved. The retrovirus infection does not have to be harmful for the cell although it leads to the formation of new retrovirus particles. Retroviruses replicate normally in the absence of the oncogenic src gene. However if the infecting retrovirus carries the src gene the effect might be transformation of

the cell and formation of tumour in the infected animal. Furthermore, when infection with a virus lacking the src gene leads to integration of the viral DNA, the event may by itself cause a mutation of the cellular genome and result in transformation. Both the src gene and the mutation of the cell-genome leading to transformation is considered to induce the transformation via phosphorylation of the amino acid, tyrosine. The phosphorylated tyrosine apparently is an essential regulator of cell growth.

RNA virus-induced tumours carry virus-specific immunological markers. On the cell surface TST antigen is present, but its relationship to virus-coded glycoproteins is unclear and in the cytoplasm virus-specific structural proteins are found.

Papovaviruses

This family of DNA viruses includes a number of oncogenic members. Because of their relatively simple structures, transformation of cells by papovaviruses has been studied in detail.

Papovaviruses can transform permissive as well as non-permissive cells, which in the case of SV40 virus is represented by monkey and hamster cells, respectively. Normally, the virus gives a lytic replication in monkey cells but occasionally a transformation may occur. In this case only defective virus is responsible for the transformation. The situation in hamster cells is different. The frequency of transformation is low but, since the cell is non-permissive, transformation may be achieved also by complete virus. Cocultivation of transformed hamster cells and cells permissive for SV40 virus may induce completely replicating virus.

The virus-genome is integrated into cellular DNA in transformed cells. Only a part of the virus-genome becomes expressed. By use of immunological techniques intranuclear T antigen, which can be of different kinds (cf. Chapter 9), can be demonstrated. T antigen is an early protein with as yet undefined functions. It may be noted that in lytic infection the synthesis of cellular DNA is stimulated, which is reflected by the occurrence of an increased number of mitoses. In addition to T antigen the transformed cells carry TST antigen on their surface.

Adenoviruses

Several different adenoviruses occur in man and animals. Human adenoviruses replicate lytically in human cells. Transformation can only be demonstrated after infection of rodent cells, which are non-permissive. Different adenoviruses have a different capacity to give transformation. During transformation a defective adenovirus genome is integrated into cellular DNA. By using fragments of virus-DNA, it has been shown that 7 per cent of the left end of the gene (cf. Chapter 9) suffices to give complete transformation and to allow a tumour growth *in vivo*. The left end of the virus-genome contains the E1 region which codes for different early proteins. The critical transforming product has not as yet been defined.

The tumour cells contain adenovirus-specific T antigen in the cell nucleus and TST antigen in the cytoplasmic membrane.

Herpesviruses

Many different human herpesviruses have been shown to be capable of transforming cells. Herpes simplex virus regularly gives cytolytic infections and is only able to

transform after partial inactivation of the genome, by treatment with ultraviolet light, for example. Transformation of rodent cells has been demonstrated but the underlying mechanism is difficult to study in cell cultures and much remains to be learnt about this system.

Of special interest to man are infections with EB virus. These are discussed in Chapters 18 and 31. In this context it will only be noted that EB virus has B lymphocytes as a selective target cell. Cord-blood lymphocytes can be immortalized by infection with EB virus and propagated continuously. In an analogous way, B lymphocytes from persons who have had an infection with EB virus may acquire properties for growth *in vitro*. These cells carry the EB virus-genome primarily in an episomal form. The genome is only partially expressed. In the cell nucleus the EBNA (Epstein–Barr nucleus associated) antigen analogous to the T antigen induced by papovaviruses and adenoviruses can be found. In a low frequency the virus-genome in B cells is activated and structural antigens can be formed. Consequently, the cell dies although complete virions are not formed.

Interferon

It has been long known that the replication of one virus can have a negative influence on, interfere with, the simultaneous replication of a second virus. This *interference* can be explained in many different ways, for example, the blocking of shared receptors and competition for metabolites. In 1956 Isaacs and Lindenmann showed that the phenomenon of interference can be caused by a cell-specific product which could be induced by a virus infection. The product was called *interferon*. It was discovered later that interferon is not one substance, but instead occurs in many different forms. Different interferons are produced by cells of different character and origin. It has further been found that interferon not only has antiviral effects but also can influence cellular functions and immunological reactions. The production of interferons in cells, their physicochemical properties and their effects in cells, as well as their importance in the defence against virus infections, is described in Chapter 19 and their potential usefulness as antiviral compounds is discussed in Chapter 24.

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12

Virus genetics

Lennart Philipson

During the last decade, the methods for studying the genetics of animal cells, prokaryotes and viruses have undergone a revolutionary change. Traditionally, mutations were induced and phenotype changes were observed. A rough mapping of the position of the mutation was obtained by determining the frequency of recombination between mutants leading to the reappearance of the original wild type. Recently, biochemical techniques have been introduced. These include cleaving of DNA by restriction enzymes, utilization of hybrid-DNA technology and determination of the sequence of nucleotides. Using the information obtained it is now possible to introduce point-specific mutations. Eventually this will give an insight into the control mechanism for the expression of genetic material. The modern molecular techniques also allow studies of the genetic material of RNA viruses. By use of reverse transcriptase in retroviruses, it is now possible to obtain DNA copies of RNA viruses. Recently it was shown that isolated DNA copies of the whole poliovirus-RNA genome were infectious.

Mutations

The term 'mutation' is used to denote permanent changes in type, number or sequence of nucleotides in nucleic acids. There are effective reparation systems in cells to correct the mistakes which are made during nucleic acid replication. In spite of this, mutations occur under normal conditions to a certain extent. Viruses do not have the same careful control system against replication errors as the cellular genome. It is therefore easier to demonstrate isolated mutations in viruses than in animal cells. Also, since animal cells are diploid, i.e. they have two copies of the same gene, whereas viruses are haploid, it is much more difficult to demonstrate phenotypic changes resulting from mutations in the genome of a cell.

Chemical and physical mutagenesis

A number of chemical agents can induce mutations, in particular substances which can modify or attach to nucleic acids.

In order to express their mutagenic action, certain chemical compounds which attach to nucleic acids require a replication of the nucleic acid. Other classes of chemical mutagens can act only if one of the bases is substituted for a base analogue during replication. Using 5-bromouracil or 5-fluorouracil, it is possible to introduce guanosine instead of adenosine to the base analogue in the replicating DNA strand.

High energy radiation also can influence nucleic acids. Both x-ray radiation and ultraviolet radiation have a mutagenic effect. It has been discussed whether x-rays cause mutations by introducing damage to bases or by causing double-strand breakages. The specific effects of u.v. radition have been partly clarified. Under the influence of u.v. radiation, neighbouring thymidines form dimer structures in the DNA. Both bacteria and animal cells normally have reparation enzymes available which can eliminate thymidine dimers and therefore mend the defect in DNA.

The effect of mutations on the phenotype

In the case of viruses, mutations may lead to different phenotypic changes. Mutations which affect the third base in a code word may be completely silent and have no influence on virus replication. This is the case if the mutation hits a triplet which codes for an amino acid for which there are several code words varying only in the third base. In other cases the mutation may lead to the protein containing a different amino acid in a single position, a phenomen which does not necessarily imply that the configuration or the stability of the protein is changed. Thus in this case also the mutation may be phenotypically silent. If the mutation has changed an amino acid in a structurally important part of the protein, normal functions may be lost. The mutation may as a consequence have a lethal effect. However, in occasional cases the modified protein configuration will lead to the protein remaining stable and retaining its function at lower temperatures, but developing a configurational change at higher temperatures which leads to it being readily broken down by cellular proteases. In these rare cases a *temperature-sensitive* (ts) mutant has been established. Such ts mutations frequently affect single bases and usually have a substitution character. When a deletion or an addition of a nucleotide base has occurred, the mutant that arises is often lethal since usually the reading frame becomes incorrect.

Certain mutations lead to a code word for an amino acid possibly being changed to a code word that signals a termination in the translation mechanism. Code words for such nonsense mutations as expressed in mRNA are UAG, UAA and UGA. If the mutation leads to the appearance of a termination codon, the protein which is synthesized will be smaller than normal. However, mRNA which contains such altered sequences can be expressed to its normal length in cell-free translation systems if a special form of tRNA, suppressor-tRNA, is added. This tRNA can introduce an amino acid at the place of the nonsense codon. Suppression mutations have been of great help in the identification of genes in bacteria and phages, since certain bacteria have a suppressor-tRNA which can compensate for the nonsense mutations. By comparing the protein synthesis in suppressor-negative and suppressor-positive cells it has been possible to identify directly the modified gene product.

Concerning animal cells, it has not as yet been possible to isolate cell lines which contain suppressor-tRNA and suppressor mutants therefore have to be analysed using *in vitro* translation systems. Both in the case of adenovirus and herpesvirus genes, it has been possible to demonstrate suppressor mutants by this technique. *In vitro* translation of mRNA from these mutants can synthesize a normal protein in the presence of suppressor-tRNA from yeast. Thus the protein altered by the mutation can be identified directly. Temperature-sensitive mutants and suppressor mutants are called *conditional lethal mutants* because the mutations are lethal only under certain conditions.

All cell-dependent mutants referred to as host-range mutants belong also in the category of conditional lethal mutants. These virus mutants can replicate normally in certain cells but not in other cells. They frequently show a normal phenotype in a cell which is transformed by a DNA fragment containing the gene which is influenced by the mutation. This occurs via complementation between the virus and the cellular genome or via recombination between the two genomes. These kind of mutants have primarily been used to map the early genes in transforming animal DNA viruses, such as polyoma viruses and adenoviruses. Since the transformed cells contain the part of the virus-genome which is needed for transformation, a virus which has been mutated in this gene cannot induce productive infection in normal cells but may do so in transformed permissive cells. A prerequisite for the usefulness of this technique is that the genes which are required for transformation are also needed for a productive replication of virus. For adenoviruses and polyoma viruses it has been shown that the early transforming genes exert control over the formation of early gene products. On the other hand it appears that the transforming genes, oncogenes, which occur in retroviruses are not required for replication of these viruses. In contrast, a presence of helper viruses is needed for replication of defective leukaemia viruses which are responsible for transformation.

Selection of mutants

In order to identify virus mutants which either have arisen spontaneously or after induction with mutagens, selective methods favouring the growth of the mutant have to be available.

Polioviruses, which normally can replicate only in primate cells, can be made to replicate in mice or rat cells after repeated passaging in these cells. The virus variants with the increased host range appear to have the same general structure as wild-type poliovirus, but a more refined analysis of the nucleic acid shows that the genome has been modified in several places. This phenomenon is called *adaptation* of virus and implies a selection of mutants which have a capacity to grow also in the new host cell. The technique has been used for isolation of attenuated viruses which have a reduced capacity to cause disease and therefore can be used for preparation of a live virus vaccine. Attenuated poliovirus strains which are currently used in live vaccines have been isolated by this technique.

New variants of a virus may arise in the infected organism owing to immunological selection. By using antibodies against a virus it is possible to subject the virus to a selective pressure. All virus particles which react with the antibodies will be eliminated and only particles which display considerable antigenic changes on their surface can remain as infectious agents. The selection primarily involves the genes which code for the external virus proteins. One example of this kind of natural selection is represented by the drift in antigenic character of orthomyxoviruses (*see* Chapter 26). New forms of orthomyxoviruses appear with modified haemagglutinin and/or neuraminidase proteins. A similar drift may possibly occur among non-enveloped viruses and may be the reason for the accumulation of a large number of different types of picornaviruses. Considering the possibilities for immunological selection it is surprising that many viruses display a remarkably high antigen stability. Measles virus isolated from different parts of the world displays the same major immunological surface properties.

Recombination

When two mutated virus-genomes infect the same cell it is possible to recover a wild-type virus provided mutations do not affect the same gene and are located at a certain distance from each other. This process, *genetic recombination*, can imply a single reciprocal crossing between the two genomes. It is also possible that the recombination is non-reciprocal if a single-stranded DNA fragment is transferred to the mutated fragment. The same result is obtained by a double reciprocal crossing. The frequency of recombination increases the further the two mutated genes are located from each other on the genome. It appears as if all circular virus-DNA genomes have a greater difficulty in recombining than large linear genomes. Recombination occurs with extremely low frequency in the use of SV40 virus but it is common in the case of adenoviruses and herpesviruses. By use of ts mutants of adenoviruses, it has been possible to demonstrate a frequency of recombination to wild type in about 15–20 per cent of the progeny. A recombination creates both wild-type recombinants and with a corresponding frequency also creates recombinants which carry both mutated genes in the case of single crossings. However, only the wild type virus can give rise to a productive infection under non-permissive conditions. The recombination frequently can be used to determine the relative distance between two genes on a virus genome.

By simultaneous use of genetic recombination and restriction-enzyme analysis it is possible to identify exactly at which place on the genome the crossing and recombination have occurred. If ts mutants from adenovirus type 2 and adenovirus type 5 concomitantly infect cells under conditions of restriction, e.g. high temperature, recombination can occur since these viruses are closely related. Since the restriction-enzyme maps for both these viruses have been defined and are known to differ, it is possible to demonstrate exactly at which place the crossing between the two virus-genomes has occurred and thus reveal the specific locality of, for example, a ts mutant. By using restriction-enzyme fragmentation of virus-DNA, this technique can be carried one step further. After infection with a mutant virus-specific DNA fragments can be introduced into infected cells. The fragment which is needed for synthesis of wild-type virus can be identified (marker rescue). A simple form of genetic recombination which originally was discovered in bacteriophages but has also been identified in animal viruses, is a process called *multiplicity reactivation*. When virions are exposed to high doses of u.v. radiation their infectivity is destroyed owing to the appearance of repeated mutations in the form of thymidine dimers in the genome. If inactivated u.v. radiated virus is used for a high multiplicity infection, i.e. involving the inoculation of many particles per cell, wild-type virus appears because of recombination between the mutated genomes.

One additional mechanism for genetic recombination, which has been observed in animal viruses, involves a redistribution of genetic material, *reassortment*. This occurs with viruses which have segmented genomes, for example reoviruses and orthomyxoviruses. After simultaneous infection with two virus strains that differ in certain marker, a high percentage yield of viruses containing a mixture of the segmented genome from both parents can be identified. It appears that the progeny virus selects its genome segments from a common pool that occurs in the infected cells and, furthermore, that single virus particles can contain only one copy of each RNA segment. It has further been found that each virion can only contain one set of fragments, probably because the packing process for the genome segments is a carefully controlled process.

Among RNA viruses which do not have a segmented genome, no genetic recombination has been demonstrated except in one case. Picornaviruses have been found to be capable of giving rise to recombinants and it has been possible to establish a gene map for this virus and to show that that map corresponds to the one obtained by chemical analyses. The mechanism for the crossing-over of RNA in a double-stranded or single-stranded structure is not understood, but the recombination probably occurs owing to a shift of template during the early phase of synthesis of complementary minus strands. This mechanism requires that the virus polymerase can start on one RNA and then change the replication template. The frequency of recombination is low and maximally reaches 2.2 per cent in the case of distantly located markers. Finally, retroviruses, which have an intermediate DNA structure capable of integrating into the cellular genome, also seem to be able to recombine. This recombination occurs at the DNA level and exchange of sequences between endogenous and infecting retroviruses has frequently been observed.

Phenotypic mixing and complementation

From double infections with mutants performed for the purpose of examining recombination mechanisms, other forms of interaction between viral gene products have been identified. Viruses which replicate in the same compartment in the cytoplasm or in the nucleus have a greater chance of establishing interaction between gene products than viruses which replicate in different places in a cell. Viruses which bud from the plasma membrane can cause the appearance of mixed products in connection with the budding-off process.

Phenotypic mixing of virus components

When two related viruses, A and B, are used for a simultaneous infection, the progeny includes virus particles which can be neutralized by antibodies against either virus A or virus B. However, a small fraction of the virions formed can be neutralized by antibodies against both virus A and virus B. This is because of the occurrence of phenotypic mixing of capsomers in the capsid, or alternatively, peplomers in the envelope, i.e. the formation of phenotypic mosaic virus. Among the virus particles which are neutralized by antibodies against either virus A or virus B, there are also particles which, with a subsequent multiplication, cause the production of virus B and virus A, respectively. In these cases, the nucleic acid of one virus has been enclosed in a capsid or an envelope of the other virus, *transcapsidation* or *pseudotype formation*. However, phenotypic mixing between viruses does not only occur with closely related viruses.

In the case of enveloped viruses that bud from the plasma membrane, a mixing may occur between different kinds of viruses. Thus, for example, vesicular stomatitis virus (VSV), can mix phenotypically with several other enveloped viruses. The nucleocapsid from paramyxoviruses, retroviruses or herpesviruses, can become enclosed in a VSV envelope and form pseudotypes and mosaic viruses. In the same way, the VSV capsid may become enclosed in a retrovirus or paramyxovirus envelope (*Figure 12.1*). The biological importance of pseudotype

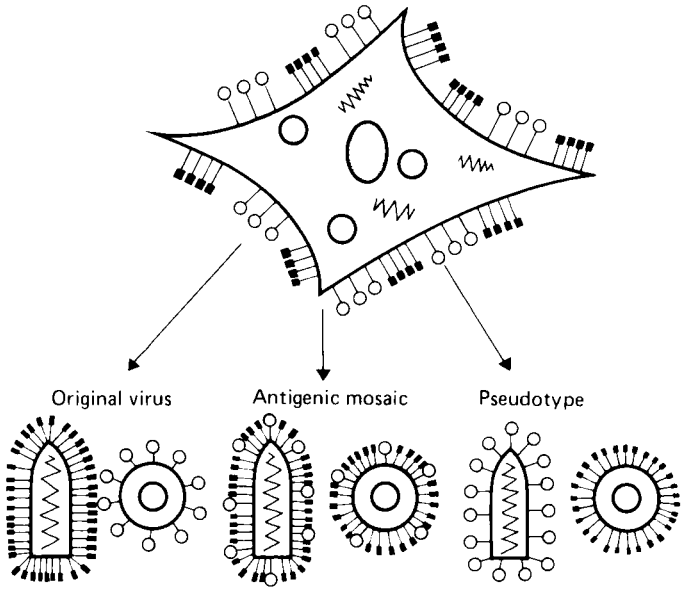

Figure 12.1. Schematic figure illustrating different forms of phenotypic mixing between vesicular stomatitis virus and an RNA tumour virus. When these two viruses concomitantly infect a cell this leads to a production of both the original viruses, virus particles which have an antigenic mosaic in their envelope and finally pseudotype virus which has a VSV genome in a retrovirus envelope and vice versa

formation is unclear, but when a retrovirus-genome has been enclosed in a VSV envelope the normally restrictive host-cell spectrum for the tumour virus is increased. Pseudotype formation therefore can be used as a tool for identification of obscure tumour viruses.

Complementation

An intermolecular recombination is not always required for a mutant to cause a lytic infection. Certain host range mutants can replicate in cells where parts of the virus-genome are established and expressed in the form of gene products. In spite of the fact that no recombination takes place between the mutant and the virus genes which are pre-existent in the cell, infection with the mutant may lead to production of complete virus. Thus the missing gene function in the mutant is complemented with the aid of a gene product from the integrated virus-genomes. A prerequisite for complementation is that a certain virus-coded function can become expressed and that the gene product makes it possible for the mutated genome to replicate. This kind of function is also called *trans-stimulation*, i.e. a gene does not have to be recombined into the DNA to exert its complementary function. In cases where recombination is required the process is called *cis-stimulation*.

A map depicting whether defects of different mutants concern the same or different gene products is drawn up by complementation studies. For classification, groups of mutants are sorted into *complementation groups*. The number of such groups depends upon the complexity of the genome. This kind of analysis gives an

idea about the number of gene products but it does not provide any information about the topographical location of genes in the genome.

A complementation between two genomes can also be a necessity for replication of certain viruses. The incomplete parvoviruses do not have a complete replication mechanism of their own, but they thrive on another virus as regards genome replication and transcription of gene products. Adenoassociated virus (AAV) which has a single-stranded DNA genome is a parvovirus which requires adenovirus for a productive infection. The product which is required in translation from adenovirus has not been identified but probably is represented by early gene products whose function can be found in adenovirus-transformed cells and in adenoviruses which have been deleted in the parts of their genome which direct the synthesis of late proteins. Certain adenoviruses can not replicate in monkey cells probably because late transcription or translation does not function in these cells. A simultaneous infection of adenovirus and SV40 virus allows a complete lytic infection of adenovirus at the expense of replication of SV40-DNA in the monkey cells. The function which is provided by SV40 is determined by early genes since SV40-transformed monkey cells can provide the help required. Complete adenovirus virions dominate among the particles formed but there is also a fraction of particles which are composed of an adenovirus capsid surrounding a hybrid between SV40 and adenovirus-genomes. In most cases virions containing the hybrid DNA cannot replicate in a cell unless this cell at the same time is infected with adenovirus containing intact DNA.

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13

General aspects of pathogenesis of virus infections

Erik Lycke

Far from all virus infections cause gross morphological changes of the infected cells or inhibition of cellular growth. Neither have the virus infections of multicellular organisms, man or animal to be accompanied by symptoms of disease. It is rather the opposite which is more frequent. Some virus infections only rarely cause clinically detectable disease. In fact it is sometimes reactions elicited by the immune defence and not the virus infection itself which provoke symptoms associated with the virus infection.

As a rule the emergence of disease, *the pathogenesis*, can be most concisely followed in virus infections where there is a direct connection between the appearance of infection and symptoms of disease. However, the medical importance of the common *asymptomatic* or *subclinical virus infections* should not be neglected. Subclinical infections are important not only for spread of the infection and for the establishment and maintenance of immunity but also there are reasons to suspect that subclinical infections may be involved as causes or triggers of slowly-propagating pathophysiological processes. In turn, these may lay the ground for degenerative and chronic illnesses. Only many years after the onset may the changes caused by the infection have reached the level of development making them clinically overt.

Virus infections may be caused by *exogeneous* or *endogeneous viruses*. As exogeneous we consider viruses which have reached us from without, i.e. from man, animal or other parts of our environment. Endogeneous viruses are assimilated and carried with the cell, e.g. integrated in the genome of the cell, and thus transferred as hereditary factors. Cells carrying endogeneous viruses most often do not produce virions, but under certain conditions an endogeneous virus can be activated and its activation then leads to production of infectious virus. The spread of virus infections from one individual to another can follow two different directions, a *horizontal* or a *vertical* spread. The latter term is used for an infection which is transmitted by sperm, ova or an infection of the pregnant woman transmitted to the embryo while the fetal membranes are still intact, *a congenital infection*. Perinatal infections can be considered as vertically transmitted if the infection occurs during delivery of the child. Infections which are acquired postnatally are considered to be horizontally transmitted even when the infection is then transmitted from the mother to her child.

At the initiation of the infection a number of cells of an organ are infected, a *primary focus* of the infection is developed. The size of the lesion and its localization will determine if, and which, functional changes follow. The primary

infection can itself be extensive and yield local symptoms, e.g. the nasal discharge of a common cold. The symptoms of illness will be most pronounced if the infection affects a system of organs where tissue damage will cause serious deterioration of vitality for the whole organism. From the primary focus the infection may further be spread to other parts of the body. Although the infection thus will be *generalized*, symptoms of disease may originate from one organ only, the *target organ*. This can be exemplified by the symptomatology of paralytic poliomyelitis. Poliovirus can be isolated from several different tissues and virus is excreted in large amounts with the stools of the patient. The paralysis which is the dominant symptom of the illness is caused by the death of poliovirus-infected motor neurons. Thus the nervous system can be considered as the target organ of the infection.

Many virus infections interfere with the metabolism of infected cells. Viral proteins or metabolites from virus-infected cells can, in addition, cause cellular changes, for example, by blocking cellular enzymes or by disorganizing cellular membranes. In the latter case, cellular lysosomal enzymes may be set free in the cells and cause autolysis.

When cells in a cell culture which is infected with virus degenerate, the cell death is generally described as being a result of *virus cytocidal properties* (*see* Chapter 11). *In vivo* cytopathogenic properties of the virus might produce tissue lesions. The efficacy of the immune defence will determine if the infection will be established, and the presence of intact barrier systems might limit the spread of the infection within the body. However, immune reactions elicited by the infection can also have a direct negative influence, as will be described. Tissue lesions can result from immune reactions directed against virus antigens on the plasma membranes of infected cells.

The *dose of the infecting virus* and the *rate by which the virus is multiplying* both influence the course of the infection. The dose of infecting virus will determine how many cells are initially infected and the amount of virus produced before the immune defence of the body is able to intervene will influence the number of cells secondarily infected. It is easy to conceive that cell death might be reflected in symptoms of disease but virus infections might also in other ways be associated with the apperance of illness. In experimental animals virus-induced cell transformation may cause formation of tumours. Functions of highly differentiated cells, such as the ability of cells to react on various signal-substances like hormones or the capacity of cells to secrete hormones and other membrane-activating substances, may be disturbed. In turn, these cell changes might be sufficient to evoke symptoms of disease.

Pathological changes as results of virus infections should be considered as being caused by multifactorial influences depending upon the properties of both virus and host. Viruses causing infections with symptoms of disease are referred to as *pathogenic* while an *apathogenic* virus is associated with inapparent or subclinical infections. As mentioned, most viruses will cause subclinical infections. Virus infections and strains of viruses are also described as more or less *virulent* depending on whether they are associated with a severe or a more harmless form of disease. If the number of deaths during an epidemic exceeds, in all age groups, what is normally seen the epidemic is probably caused by a very virulent virus strain. Should a high mortality always be noted among, for example, people of old age and small children the virus infection might be characterized as more pathogenic for these two groups of individuals, both of which might be sensitive to infectious diseases. The degree of severity of the disease can be described as a

function of both pathogenicity and virulence factors, reflecting properties of the host as well as of the infecting virus.

The incubation period, the time between the initiation of infection and the appearance of the first symptoms (*Table 13.1*) may be shorter or longer depending upon various factors, such as whether symptoms derive from the primary focus of infection or from infection in target organs in a generalized disease, the virulence of the virus, the mode of spread of the infection within the body, the efficacy of the rapid immune defence and the size and localization of the primary focus of infection. Respiratory virus infections, in which virus rapidly reaches the target organ during breathing, often have short incubation times while very long incubation periods are associated with infections propagating slowly in large target organs like the liver and the CNS. The incubation period of the spongiform encephalopathies can extend over several years (cf. Chapter 17).

TABLE 13.1. Incubation times of some common virus infections

Influenza	1–3 days
Herpes simplex stomatitis	5–10 days
Polio	1–2 weeks
Measles	1.5–2 weeks
Chickenpox	2–3 weeks
Mumps	3–3.5 weeks
Rubella	3–3.5 weeks
Hepatitis A	4–5 weeks
Mononucleosis	4–6 weeks
Rabies	0.5–4 months
Hepatitis B	2.5–4 months

Often *fever* may be one of the first symptoms of infection and the course of the infection is reflected in the fever curve. A virus infection which becomes generalized may demonstrate a fever curve with two peaks and a fever-free interval extending from a few hours to a couple of days between the peaks. Sometimes the second peak of fever coincides with the onset of the immune response and therefore might reflect reactions evoked by immunologically-induced cytotoxic reactions directed against the virus-infected cells.

Modes of transmission of virus diseases

Size as well as physical stability of virions and their envelope and capsid structures play important roles in virus transmission. Many virus infections are spread by *aerosols* and, possibly, by airborne dust. Small liquid droplets and dust particles can stay airborne for several hours. The precipitation rate is proportional to the size of the particles. Viral infectivity can be well maintained in organic material; for example, it has been demonstrated that dried bloodstains or epidermal cells in dust can harbour infectious viruses. As viruses can be confined also to very small droplets or dust particles it is possible for viruses to reach the lower respiratory tract (*Figure 13.1*). Patients with lower-respiratory-tract infections seem to create aerosols with very small droplets, while coughing and sneezing patients with upper-respiratory-tract infections yield aerosols of relatively larger droplets. The latter will precipitate mainly in the upper respiratory tract while smaller droplets may penetrate the lower parts of the respiratory tract.

Enveloped virions are as a rule more sensitive to environmental effects. As an intact envelope is a prerequisite for maintained infectivity the instability of the envelope will be a factor limiting the possibilities of transmission of an infection. Herpesvirus infections are effectively transmitted by virus-containing secretions and discharges, and the primary infection occurs usually on mucous membranes of oral or genital tracts or the eyes.

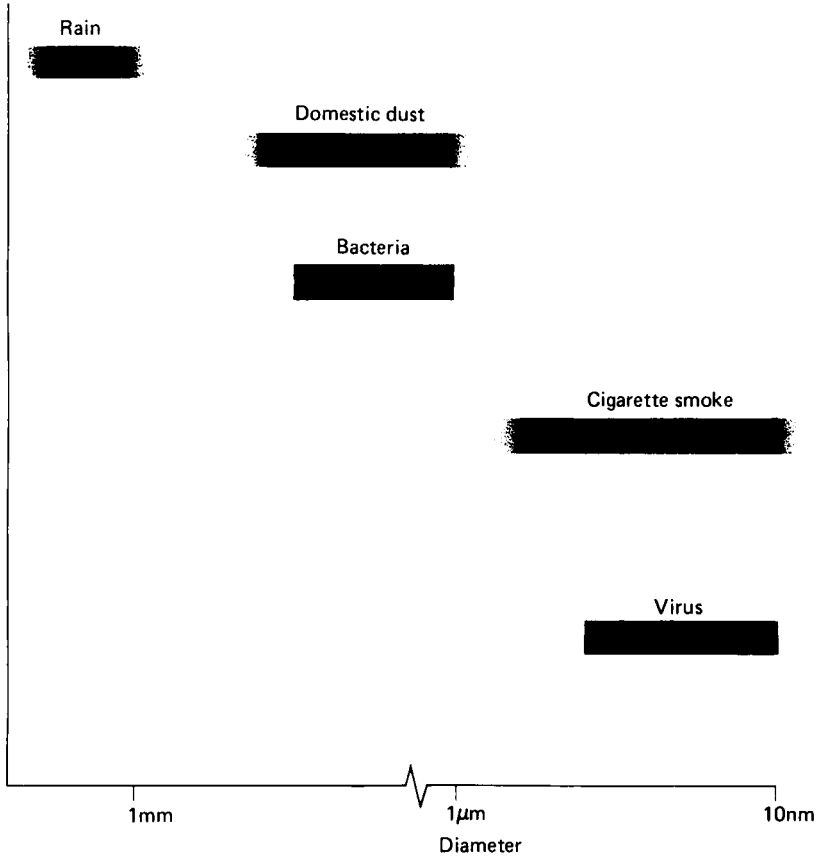

Figure 13.1. Sizes of virus particles in relation to size ranges of bacteria, droplets and airborne dust particles

Non-enveloped viruses are more resistant to chemical and physical influences. Transmission of infection by contaminated water, food or via the hands to the mouth are therefore common routes of infection for enteroviruses, adenoviruses and rotaviruses. The primary infection will be localized to tissues of the oral or intestinal tracts. Undoubtedly, respiratory tract infections caused by non-enveloped viruses can also be transmitted by direct contact from one individual to another.

The spread of many togaviruses is mediated by *vectors* (mosquitos, flies and ticks). Togaviruses replicate in the arthropod and are transmitted with arthropod bites when the vector is feeding on an animal or man. This mode of transmission increases the possibilities for the rapid spread of virus to organs well supplied with blood such as the liver, spleen and the meninges.

Most viruses replicate well at temperatures around 37°C. Some viruses may require a lower temperature for optimal replication, however. Rhinoviruses have ranges for optimal growth at temperatures around 33°C (and replicate less well at 37°C). Here is perhaps one of the reasons why rhinovirus infections (the common colds) are localized to the nasal mucous membranes and restricted to the upper respiratory tract.

A survey demonstrating different modes of transmission of different virus infections is given in *Table 13.2*. Again it should be recalled that the properties of the virus alone do not determine the mode of transmission of infection. It is often

TABLE 13.2. Modes of transmission of some common viruses*

Aerosol	*Infected secretion*	*Faecal-oral route*	*Inoculation*
Adenovirus	Cytomegalovirus	Adenovirus	Arboviruses
Enterovirus	Epstein–Barr virus	Enterovirus	Cytomegalovirus
Measles virus	Herpes simplex virus	Rotavirus	Hepatitis B virus
Parainfluenza virus	Hepatitis B virus	Hepatitis A virus	Rabies virus
Rubellavirus			
Smallpox virus			
Varicella zoster virus			

* The table presents some examples. Virus considered to be transmitted by aerosols may often be transmitted also by direct contact with infected saliva or secretions and vice versa. Viruses transmitted by an oral route may be transmitted by contaminated water or food supplies. The term *inoculation* covers also viruses spread by bites and transfusions.

difficult to reveal if it is the virus-genome, the host, or the environment which has had a dominating influence in the evolution of the epidemiology. Illustrative examples are given by the adenoviruses. These viruses are spread in many different ways. The virus can be transmitted by aerosol and the infection then often appears as a respiratory-tract infection. As a waterborne infection, adenoviruses can cause infections of the conjunctivae and throat and outbreaks have been observed among swimmers visiting public swimming pools. Without marked symptoms in other organs, adenoviruses can also cause symptoms of acute gastroenteritis. In the latter case the virus might have been transmitted by contaminated hands, for example. Thus, there exist several environmental conditions influencing the spread of the adenoviruses. However, it is also possible to observe that there are certain genetic differences between adenovirus strains isolated from respiratory-tract infections and virus strains isolated from cases of acute gastroenteritis (*see* Chapter 28). Within the same virus family, genetic differences in virus type can obviously influence the target organ destination of the various adenoviruses.

The importance of cellular and viral receptor functions

The plasma membrane of cells susceptible to a virus infection carries structures functioning as receptors for the virus. The receptors are essential for the adsorption of the virion to the plasma membrane and thus for the initiation of the infection (*see* Chapter 7). Cellular receptors do not only recognize VAP common within a family of viruses. It has been observed that viruses of different families can use one and the same cellular receptor. On the other hand, two types of the same virus can utilize different cell receptors. To what extent occurrence or lack of virus-specific cellular receptors influences the pathogenesis of the virus infections is as yet not

fully known. Experimental data indicate that affinity of viruses for certain tissues and organs and the spread of virus within the body, as well as the demonstrable genetically-controlled resistance against some virus infections, can depend upon presence or absence of cellular virus-binding receptors.

The classic example of the importance of cellular receptors for restriction of a virus infection is the polio virus infection. Only man and other primates are naturally susceptible to polio virus infection. Cells from non-primates – although they might have capacity to synthesize polio virus after an artificial inoculation of polio virus RNA – are resistant to infection. They lack cellular receptors for adsorption of the virus. In man the gene for the polio virus receptor is localized on chromosome 19. The *host range* of polio virus infections can therefore be considered relatively narrow while rabies, for example, infecting a large number of species, has a broad host range.

Many viruses utilize receptors which are present on cells of many different species. Influenza virus binds to structures with terminal sialic acids whether or not the sialic acid is situated on a glycoprotein or a ganglioside. Consequently, the number of species which can offer cells with virus-binding capacity for influenza virus is relatively large (*see* Chapter 26). Semliki forest virus (SFV), an arbovirus belonging to the togavirus family, binds to some histocompatibility antigens, HLA-A and HLA-B antigens on human cells and H-2K and H-2B transplantation antigens of the mouse. Nevertheless, these histocompatibility antigens are not identical with the receptors for SFV, since some cell-lines lacking the transplantation antigens will also be infected with the virus. Thus binding of virus to cells does not always indicate presence of viral receptors. For most viruses the chemical nature of the cellular receptors is as yet unknown.

The VAP-receptor compatibility does not alone influence the establishment of infection. Several functions, both immunological and non-immunological, might in addition contribute to resistance against virus infection. The observation that one and the same virus infection regularly can induce symptoms from the same target organ is often referred to as a sign of *tropism* of the virus infection. Some herpesviruses and rabies viruses demonstrate affinity for the tissues of the nervous system, for example; the liver is the target organ of hepatitis A and B viruses, etc. This affinity of a virus infection for a particular target organ has been considered by some authors as suggesting that there are different qualities and quantities of virus-binding receptors on plasma membranes of cells of different organs. If, for example, receptors for herpes simplex, varicella-zoster and rabies viruses were abundant in nerve cell terminals and synapses, this would increase the possibilities for establishment of these infections in the nervous system. The capacity of the virus to infect certain organ systems (i.e. to be *neurotropic, viscerotropic* etc.) might perhaps to some extent be explained by receptor specificities and differences in densities of receptors in various organs. However, as will be illustrated below, the tropism of a virus infection apparently reflects a summation of influences of which receptor compatibility is one only, and many factors at cell and organ levels are important for manifestation of the tropism of virus infections.

The spread of virus infections within the body

Viruses released from infected cells are spread to susceptible neighbouring cells over the intercellular space. Spread of viruses from the primary focus of infection may occur also when antibody formation has started if virions or infectious viral

nucleic acids can pass from one cell to another within cell bridges. Paramyxoviruses and some of the herpesviruses can infect by causing cell fusion and formation of multinuclear giant cells (*see* Chapter 11).

From the primary focus of infection the spread of viruses can be mediated by blood, lymph, secretions and the axoplasmic flow of the nerves (*Figure 13.2*). Virions or virus-infected white blood cells can enter the circulation of the lymph and the blood, *viraemia*, thereby inducing rapid spread of infection throughout the body. Viraemia is usually encountered during a relatively short period in the early phase of the infection but it can also be observed in the agony when the immune defence no longer has capacity to control the infection.

With the release of virus from infected leucocytes the viraemia can be maintained. If, in addition, immunocompetent cells die as a result of the cytocidal effects of the virus, the immune defence will be weakened. With infected blood

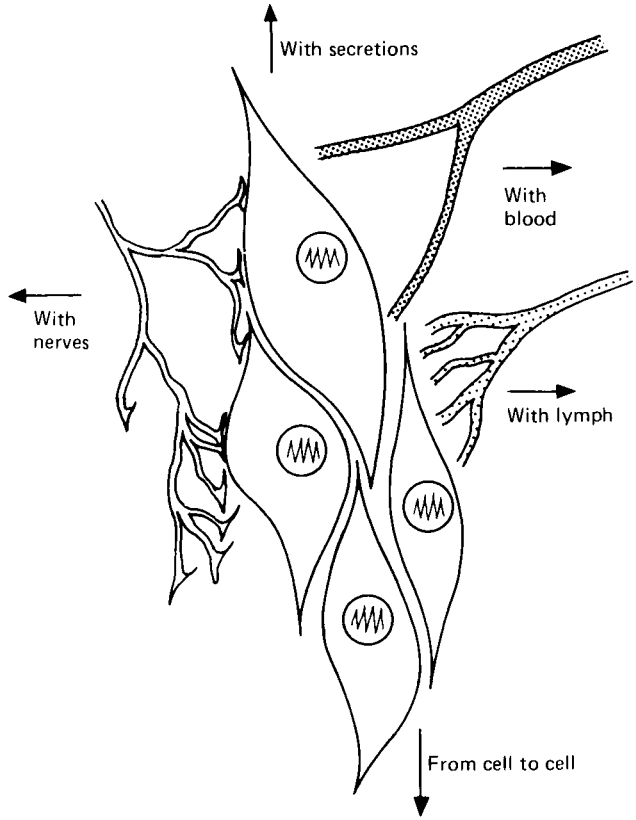

Figure 13.2. The various ways in which viruses may spread from the site of primary infection

cells, virus can be passed extravascularly and the infection propagate through mucous membranes and barrier systems. Infected white blood cells are taken up by lymphoreticular cells of the spleen, liver and the lymph nodes and in the plexus choroideus. The reduction in the number of circulating white blood cells thus achieved seems to reduce the viraemia but, secondarily, will contribute to maintain

the infection when virus is released from the reticuloendothelial cells of organs with blood-rich sinuses. Viraemia usually disappears when the antibody formation starts and cell-mediated immune reactions are activated.

There exists also a *neural spread* of virus infections. Herpes and rabies viruses are transported within the axons and viruses can be transported by the anterograde as well as the retrograde axonal flows, i.e. from, as well as towards, the CNS. It has been shown experimentally that polio virus can also be transported in nerve axons. Most observations would indicate however that spread of polio virus to the CNS is dependent upon viraemia and that the viraemia probably is pathogenetically the more important way for the propagation of the polio virus infection.

The importance of barrier systems in limiting the spread of the infection

Functional and structural barrier systems of the body restrict the spread of an infection. Intact surfaces of skin and mucous membranes always provide protection against spread to underlying tissues although the bactericidal components secreted by the sweat and sebaceous glands of the skin probably have a weak virus-inactivating capacity. However, the skin and the mucous membranes of the mouth and genital tracts are surfaces which are often exposed to infections and often have minor lesions. An injured area of the skin can be an entrance for the infection. Individuals who by their profession are exposed to human blood (e.g. some hospital personnel, laboratory technicians or dentists) might be at risk of contracting hepatitis B virus infection. *Milkers' nodules* are caused by a paravaccinia infection inducing granulomas and hyperkeratosis on hands and fingers of farmers and others working with infected cattle. Virus is spread from infected udders to skin lesions on the hands. Warts may easily be transmitted to skin lesions on the hands and feet of non-immune children. Cytomegalovirus, herpes simplex virus and hepatitis B virus can all be sexually transmitted, probably via lesions in mucous membranes of the genital tracts.

The ciliated cells of the respiratory tracts are considered to have an important cleansing function since they remove particles, cell debris and microorganisms with the mucus. When mucus appearing in the pharynx is swallowed, the infectious agents in the mucus are exposed and inactivated by the acid gastric juice. The barrier function of ciliated cells can be damaged temporarily when the respiratory epithelium is exposed to virus infections. Rhinovirus infections, for example, seem to affect the ciliated cells and cause destruction (*Figure 13.3*).

Moreover, the mucous layer of the respiratory tract may protect by reducing the possibilities for the viruses to reach cells susceptible to the infection. The mucus contains not only antibodies but also glycoproteins with a capacity to bind to some viruses and interfere with adsorption of the viruses. Soon after the infection, NK cells, secretory IgA and other components of the immune defence, will appear in mucus on membranous areas. The combination of locally recruited immunocompetent cells, antibodies and the cleaning by the ciliated cells, makes the mucous membranes of the respiratory tracts an efficient barrier system which is difficult to penetrate.

The blood–brain barrier, the placenta and the organs of lymphoreticular cells, are other examples of barriers capable of restricting infections. However, the cells of the barrier system can themselves be susceptible to virus infections and thus

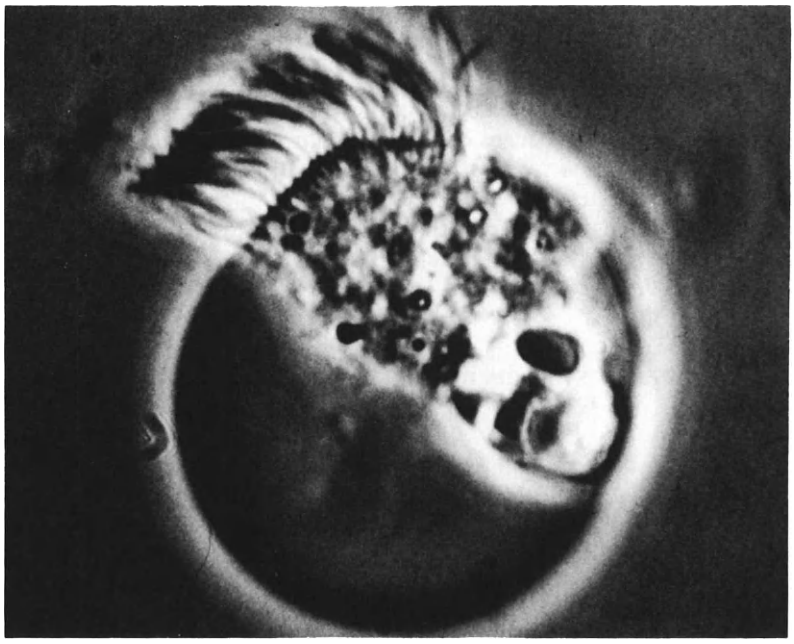

Figure 13.3. Infections with rhinovirus and some other respiratory viruses may cause destruction of ciliated epithelium (Photo: Bertil Hoorn)

function as target organs. Meningitis is probably such an example. The efficiency of the barriers can be counteracted by migration of white blood cells which by moving through the capillary walls may transfer the infection through the barrier. Congenital infections often originate from a local focus in the placenta and are transmitted by virus-infected leucocytes.

Finally, the barrier function exerted by the virus-inactivating capacity of the gastric juice and the bile should be mentioned. Enveloped viruses are rapidly inactivated at a low pH. Of the picornaviruses, rhinoviruses, having a capsid structure which is unstable in an acid milieu (*see* Chapter 24), are probably unable to pass the stomach maintaining their infectivity, whereas enteroviruses, the other large subgroup of picornaviruses, with capsids more resistant to acid hydrolysis, seem able to infect even after passing the acid gastric juice.

Influence of immune reactions on the pathogenesis

As already mentioned immune reactions might influence the pathogenesis of virus infections. In *Table 13.3* a number of immune reactions are listed which may result in cellular damage and thus in the development of disease (*see also* Chapter 19). Some of these immunopathological reactions are not yet completely understood.

Virus infections can sometimes be complicated by a prolonged period of viraemia and the release of large amounts of viral proteins into the blood. *Immune-complexes* between viral antigens and circulating antibodies against these antigens might be formed. Normally such complexes are eliminated by a complement-activated phagocytosis but if the complexes formed are of a certain critical size they

TABLE 13.3. Immunopathological reactions induced by virus infections

Reaction	*Immunopathology*	*Symptoms*
Circulating antigens react with antibodies	Immune-complexes Deposit of complexes Complement activation Cytotoxicity	Arthritis, glomerulonephritis, vasculitis, hepatitis
Binding of antibodies to viral antigens on the surface of infected cells	Release of immune-complexes by capping. Cytotoxic reactions against infected lymphocytes and other cells	Lymphopenia, immunodepression, organ symptoms such as bronchiolitis
Binding of immunoglobulin to Fc receptors	Blocking of antigens on surfaces of infected cells. Steric hindering of cytotoxic reactions (?)	Contributing to establishment of latent infections
T-, K- and NK-cell reactions	Cytotoxic reactions against infected cells	Exanthema, encephalopathy, immunosuppression, autoimmune reactions (?)

may be trapped in one of the physiological filters of the body, such as the glomeruli of the kidneys or the plexus choroideus. If immune-complexes are deposited they may cause tissue damage when the complement is activated. The post-infectious arthritis following rubellavirus infection, glomerulonephritis and vasculitis, as well as the immune-complexes observed in arterioli, joints and kidneys in association with hepatitis B virus infections, might reflect immune-complex formation triggered by virus infections.

Binding of antibodies to viral antigens exposed on surfaces of infected cells has been shown experimentally to produce *capping*, i.e. an enrichment of antigen-antibody complexes over one pole of the plasma membrane of an infected cell. Capping is followed by release of complexes from infected cells. In this way antigen-antibody complexes can be shed into the blood. Infected cells which release antigen-antibody complexes will, at least temporarily, be devoid of viral antigens on their plasma membrane. Cytotoxic immune reactions directed against these antigens will thus be delayed and elimination of infected cells by cytotoxic reactions will be less efficient. Indirectly, this can favour development of persistent virus infections. Moreover, the phenomenon may contribute to the selection of virus variants which induce short-term antigenic changes in membranes of infected cells. Capping has been demonstrated experimentally in measles, mumps, herpes simplex and retrovirus infections. Infections with the herpes simplex – and Epstein–Barr – viruses will induce Fc-receptors on the cell surface. The receptors are able to react with the Fc region of the immunoglobulins. As specific antibodies directed against virus-antigens will bind to the antigens via the Fab part of the antibody molecule, antibodies might theoretically be linked to the cells by both the Fc and the Fab regions. Perhaps the destruction of infected cells by T cells and macrophages can be sterically hindered. Some authors have speculated on the possibility that such a hindering of cytotoxic immune defence reactions could contribute to the development of latent herpesvirus infections.

Cytotoxic immune reactions are important not only for their capacity to eliminate virus-infected cells but also because of the tissue damage associated with the destruction of cells. The cytotoxic immune reactions might thus result in development of disease. Virus infections affect the number of white blood cells, including the lymphocyte populations. A reduction in the number of immunocompetent cells will appear as an immunosuppression. This suppression may be manifested in various ways. Latent infection, for example, might be activated and become generalized. The healing of virus-infected tissues might be retarded and cause persistency of infection (*see* Chapter 16). Disappearance of delayed hypersensitivity reactions might occur. It is well known that in the early phase of measles infection the cutaneous hypersensitivity reaction to tuberculin may disappear. Only when the number of lymphocytes has been restored and the T-cell functions have been normalized will the various functions of the cell-mediated immunity return, and the patient again show hypersensitivity reactions against various allergens including tuberculin.

Infections caused by respiratory syncytial (RS) virus often demonstrate symptoms from bronchioli, and children with this type of lower-respiratory-tract infection suffer from bronchiolitis and pneumonia. The bronchiolitis has been considered to be the result of an allergic type of reaction against RS-virus-modified antigens of infected cells of the bronchioli. It is probable that side-effects observed in association with vaccination against measles using an inactivated virus vaccine might have had an analogous pathogenetic background. Individuals vaccinated with inactivated measles virus vaccines became only partially immunized and were victims to immunopathological reactions when they, after vaccination, were exposed to a wild-type measles virus infection. Finally, the rashes observed in association with measles and rubellavirus infections are considered to be manifestations of immunologically-induced reactions (*see* Chapter 14).

The possible association between virus infection, immunological cytotoxic reaction and the development of autoimmune disease should be noted. Helper T cells stimulated by viral antigens, and B cells forming antibodies against antigen complexes containing components of viral as well as host-origin, are potential triggers of autoimmune cytolysis directed against homologous host antigens of normal non-infected tissues. It is known that some virus infections demonstrate incorporation of host-cell proteins in virions when viruses are budding-out from the plasma membrane. Thus, for example, actin is found in virions of measles virus and γ-glutamyltranspeptidase can be demonstrated as a component of the envelope of murine mammary tumourvirus. A transient IgM-antiactin response is observed in measles. Furthermore, the tissue damage produced might itself result in a leakage from infected cells of normal cellular constituents and antigens, a possible stimulation of autoimmunity.

Influence of constitution, age, sex and genetic background

The physical constitution of the patient will always influence the course of infection. As described in Chapter 19, some virus infections are controlled mainly via humoral immune-defence mechanisms, others mainly by the cell-mediated immunity. Antibody-deficiency syndromes can sometimes imply increased sensitivity to virus infections but defects in the cell-mediated immunity, particularly, are accompanied by pronounced effects.

When the cell-mediated immunity is impaired as a result of treatment with immunosuppressive drugs, or because of genetic defects or other complications, infections which are normally harmless might become pernicious. Vaccinations with live virus vaccines can become life-threatening infections. Latent virus infections may be reactivated.

It is well known that a virus infection such as measles is a serious disease for children in developing countries. The number of deaths associated with measles in such countries exceeds by several times the mortality figures reported from industrialized countries. The many conditions contributing to the impairment of resistance against infections are the probable causes. Undernutrition, particularly protein deficiency, affects the somatic development in general and is probably harmful to the cellular immune defence. Repeated infections with protozoa and parasites, such as malaria and trypanomiasis, might in addition have immunodepressive influences. Sanitary and epidemiological conditions in developing countries cause numerous infections during early childhood.

Two ages of life show a particular sensitivity to infection – very early childhood and old age. For both periods this increased sensitivity might, at least partly, reflect a reduced immunological capability. Many virus infections demonstrate high attack rates among young individuals and in experimental animals several possible control mechanisms have been described. In mice the relatively larger resistance to virus infections in the adult animal in comparison to that of the young seems to depend upon the resistance of the macrophages. In the mouse the resistance of macrophages to herpes simplex infections is developed during the first month of life. Reduced production of interferon is another factor rendering the infant mouse more susceptible to infection. With increasing age a reduced number of virus-specific cellular receptors have been recorded. With increasing age an increased amount of virus-blocking substances in mucus and serum has been observed. Apparently, many factors may be responsible for age-dependent resistance to virus infections.

Hormonal changes are also reflected in the varying susceptibility to virus infections. Observations that steroid-treated patients and pregnant women are at risk from virus infections are of great medical importance. Treatment of patients with corticosteroids should be avoided in pox- or herpesvirus-infected patients as such treatment has been associated with severe side-effects and fulminant infections. Patients demonstrating signs of systemic immunosuppressive treatments are particularly at risk. On the other hand, corticosteroids can normally be given to patients who already have protection from a secondary immune response, for example, patients with herpes zoster. The latter patients generally are able to control their infection immunologically before negative side-effects from the corticosteroid treatment interferes.

A serious course of disease may occasionally be encountered in pregnant women who have contracted an acute virus infection, for example hepatitis or poliomyelitis. Also an increased frequency of subclinical infections, such as, for example, cytomegalovirus infections, are demonstrable. The physiological immunosuppression of the pregnancy and a cytomegalovirus-induced immunosuppression may plausibly influence and enhance the shedding of cytomegalovirus at the end of the pregnancy.

Immunological reactivity and virus-binding receptors on cells are genetically controlled and differences in resistance to some virus infections can, at least partly, be described in genetic terms. There exists substantial knowledge about the genetic

background of the susceptibility of mice to virus-induced leukaemia and other tumours as well as about the association between tumour induction and immunological reactivity. The resistance of inbred strains of mice to certain togavirus infections has also been genetically characterized. In the mouse, resistance against some togaviruses is based on several, different but co-reacting control systems. In resistant mouse strains an *interfering defect non-infective virus* is produced in addition to infective virus, while in the susceptible mouse only complete infective and disease-producing virus is formed. In the resistant animal the infection is inhibited by production of interferon, induced by the defect virus, and the animals appear to be non-susceptible. Furthermore, one mechanism of resistance is illustrated by infections caused by the mouse hepatitis virus, a virus which is adsorbed to cells from resistant as well as susceptible mouse strains. A blocking of the penetration of virus is observed in the resistant mouse strains and no transcription of the virus-genome takes place. The two examples presented demonstrate that genetically-controlled differences in susceptibility to infection can be regulated by several, and different, biomechanisms.

Genetically-determined susceptibility to infections and the causes of differences in resistance to infections have as yet been little studied in man. A number of epidemiological, clinical and cell biological observations will all emphasize, however, that genetic differences are of importance regarding both resistance against and susceptibility to virus infections in man. Interesting observations have been made during recent years concerning, for example, patterns of histocompatibility antigens in relation to varying sensitivity to infections.

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14

Pathogenesis of acute virus infections

Erik Lycke

Virus infections may induce acute febrile illnesses as well as persistent and chronic diseases. The acute infections often have a rapid onset, a relatively short duration and a sudden disappearance of symptoms. They may be restricted locally but can also be generalized with the symptoms of the target organ characterizing the clinical picture. A description of the pathogenesis of acute virus infections might therefore emphasize the various tropisms of virus infections but will, in addition, demonstrate the weaknesses of the concept of tropisms. Different viruses may cause very similar symptoms although quite different pathogenetic mechanisms might be operating. The description of the virus diseases and their pathogenesis will be supplemented in the following chapters by biomedical and clinical details about infections caused by the various virus families and, in Chapter 34, by descriptions of the viral syndromes.

Respiratory infections

Virus infections of the lungs and other parts of the respiratory tracts are established as a result of virus transmitted via aerosols, by direct contact, or by viraemia. In most cases, when infections first appear in the respiratory tract, the virus is introduced with the inhaled air or via the nasal or oral routes by, for example, contaminated hands. Viraemic spread of virus to the lungs might indicate a late and more advanced stage of an infection. In contrast to infections entering the body through breathing, viruses spread by viraemia induce infection and changes in the vascular endothelium, and spread of the infection to the respiratory cells occurs secondarily.

The background to the varying localization of infections within the respiratory organs is not known, although it is reasonable to assume that not only disease factors are operating, but certain controlling factors also. Rhinoviruses and the parainfluenza type 1 and 2 viruses show affinity for the respiratory ciliated epithelial cells of the nasal and laryngeal tracts, while the parainfluenza type 3 and RS viruses cause lower-respiratory-tract infections affecting the epithelium of the bronchioli, bronchi and alveoli. The borderlines are not strict, however, and most respiratory viruses may be associated with upper-respiratory-tract infections and even rhinovirus infections might occasionally develop into a bronchopneumonia.

Virus infections which affect the ciliated epithelial cells cause a reversible destruction of the ciliary function and an inflammatory reaction with increased vascular permeability but no cellular desquamation. The increased permeability

will enhance the antibody-mediated immune defence of locally secreted IgA by leakage out of IgM and IgG antibodies. Infections involving the surface layer of mucous membranes seldom induce a durable immunity. Reinfections are common and important for maintenance of immunity.

Tissue damage induced by virus infections in the lower respiratory tract is probably often elicited by the immune reactions directed against viral antigens of infected cells. Influenza pneumonia, a dangerous disease in the very old, is associated with a high mortality rate. At autopsy the lung tissue demonstrates haemorrhagic inflammation and exudates. Bacterial infection is a frequent complication of influenza pneumonia.

Bronchopneumonias caused by adenovirus infections are often found by x-ray to be localized to the hilus area with enlarged lymph nodes, suggesting that the infection might have been propagated via the trachea and bronchi. Another, more diffusely extended, form of adenovirus pneumonitis has been observed in small children. Most of the lung parenchyma reveals pathological changes and a fatal outcome is not uncommon. Immunopathological reactions are considered to be important for the pathogenesis in these cases.

Both adenovirus and influenza virus infections of the lower respiratory tract can be associated with the spread of the infections to the pleurae causing exudation. Pleurisy is, however, a symptom more commonly associated with epidemic pleurodynia (epidemic myalgia or Bornholm disease) and is then the result of a coxsackie B virus infection. The slight pleurisy demonstrable in some patients in outbreaks of coxsackie B virus infections is normally of short duration. Probably the pleurisy reflects a systemic spread of the infection representing one symptom of a coxsackie B virus-induced syndrome.

Infections of visceral organs

Gastroenteritis

Hitherto it has been extremely difficult to elaborate cell culture systems for isolation of the viruses which replicate *in vivo* in human enterocytes and cause symptoms of gastroenteritis. In fact these viruses are often referred to as 'non-cultivable'.

The rotaviruses are the most common aetiology to diarrhoea in small children (*see* Chapter 34). Virus is spread by the faecal-oral route and, perhaps, as an airborne infection as well. The enterocytes of the intestinal villi are the targets of the infection yielding a transudate and diarrhoea. The amounts of virus shed into the stool is impressive and 10^{10} virus particles per gram faeces have been recorded. To overcome the difficulties with production of human rotavirus diagnostic antigens, rotaviruses of cultivable bovine and non-cultivable human strains have been recombined and cultivable hybrid strains with antigenic properties of human rotavirus have been produced. Recently cell strains susceptible to infection with human rotaviruses have been reported. Possibly these cells can be used for production of diagnostic antigens as well.

Enteroviruses are not replicated primarily by the intestinal cells in spite of their name. They multiply in tonsils and lymphoid tissues of the intestinal tract and although large amounts of virus are excreted with the faeces, diarrhoea is not a consistent symptom of enterovirus infections.

Hepatitis

The viral hepatitis will be more thoroughly discussed in Chapter 30. In the present chapter we shall comment on some differences in the pathogenesis of hepatitis A and B virus infections. Hepatitis A virus has been classified as a member of the picornavirus family. It is probable also that the pathogenetic pattern of the hepatitis A infection is similar to that observed with the enteroviruses. The infection is transmitted by a faecal-oral route and virus replicates in lymphoid tissues. The symptoms of hepatitis dominating the clinical picture are probably caused by the viral cytocidal effects induced. Subclinical infections are undoubtedly common. Signs of infection disappear when specific antibodies are formed.

Hepatitis B virus enters the body via injections or transfusions with virus containing blood, the use of virus-contaminated syringes and instruments. Hepatitis B can also be acquired sexually as transmitted diseases, or professionally by surgeons and dentists in contact with virus-containing blood or blood products. Viral antigens and antibodies are often concurrently demonstrable. Formation of immune-complexes is probably important for the development of extrahepatic complications. Development of hepatitis appears to be associated with immune reactions. Persistent forms of infection are recognized in about 10 per cent of the clinical cases.

Infections of the pancreas

In recent years results of experimental research have supported the hypothesis that virus infections might be aetiologically important in the development of the juvenile form of diabetes. This assumption is based on experimental studies of animals, mainly studies with mice.

The encephalomyocarditis virus of the picornavirus family will induce a pancreatitis by being selectively cytocidal against the pancreatic β cells. This specific affinity for the insulin-producing cells may cause an infection with sequelae which in several respects mimic human diabetes. The pathogenesis is influenced also by the genetic constitution of the host. The susceptibility of the β cells for the virus infection is controlled by an autosomal recessive gene regulating the occurrence of receptors for the virus on the plasma membrane of the β cells. In other studies β cell cytocidal mutants of coxsackie virus B4 and reovirus type 3 have been isolated by culturing virus on mouse β-cell cultures. These mutants have been able to induce a diabetes-like disease when inoculated into young mice.

Coxsackie viruses and reoviruses are two candidates to be considered as possible triggers of juvenile diabetes in man. Serological and clinical investigations have, in addition, focused attention on mumps and rubellaviruses. In fact cases have been reported where clinical and laboratory findings strongly suggest that symptoms of diabetes might appear as late sequelae in mumps virus infection. Perhaps there is more than one virus infection potentially responsible, should the genetic backgrounds of host and virus create a situation compatible with development of the disease.

Myocarditis and pericarditis

It is conceivable that virus infections reaching the CNS or the lungs and accompanied by high fever will be deteriorative to heart function and circulation. Myocardiopathy with virus infections of the myocardium and pericardium is seen in

tropical and subtropical regions to be associated with some arbovirus infections and haemorrhagic fevers. In temperate-climate zones cases of myocarditis and pericarditis are observed in outbreaks of enterovirus infections, in particular those caused by coxsackie B viruses. Of medical importance are the myocardiopathies associated with perinatal coxsackie B virus infections. Pericarditis is the more common symptom in adults while myocarditis is more often seen in infants.

Histopathologically the virus myocarditis is characterized by interstitial infiltration of monocytes. Areas of degenerating muscle fibres are seen and they are probably caused by the cytocidal effects of the infection. Experiments with animals have demonstrated that heart muscle cells have receptors for the coxsackie viruses. From the pericardial fluid from cases with pericarditis, infective virus can be isolated as a sign of virus replication in pericardial cells.

Orchitis, oöphoritis

That the anatomy of the infected organ may also influence the pathogenesis is illustrated by mumps virus infection of the gonads. As mentioned above, mumps virus demonstrates affinity for many glandular tissues, the parotid, submandibular, sublingual and pancreatic glands, in addition to the gonads. The infection of testicles and ovaries will cause inflammatory reactions with an interstitial oedema. In the ovary the oedema seems to occur without persistent tissue damage while the testis with its surrounding inner firm capsule is vulnerable to an increased inner pressure. In individual cases the oedema of the testis may be followed by sterility if the infection affects both testes.

Infections of the skin and mucous membranes

Some different skin lesions and the possible pathogenetic mechanisms involved are listed in *Table 14.1*. The table does not give a complete survey but presents some illustrative examples. Virus-induced changes can appear both as an *exanthema* and an *enanthema*, i.e. skin eruptions as well as efflorescences on a mucous surface. Often both kinds of changes appear simultaneously. The appearance of the exanthema – from the early signs of inflammatory reactions, the maculopapular rash, to vesicles, pustules and crusts – is more or less characteristic of the different

TABLE 14.1. Pathogenesis of some virus-induced exanthemas

Virus	*Efflorescences*	*Pathogenesis*
Pox- and herpesviruses	Maculopapular or vesicular rashes, crusts	Cytocidal virus infections; infective virus can be isolated
Enteroviruses	Maculopapular, sometimes a vesicular rash	"
Measles and rubella viruses	Maculopapular rash	T-cell dependent hypersensitivity reactions; infective virus can usually not be isolated

infections. The fluid of the vesicle is clear and often contains infective virus. The fluid of the pustule is turbid as it is rich in inflammatory cells. Presence of antibodies in pustular fluid reduces the possibilities for virus isolation. When the healing process has started, vesicles and pustules become covered by crusts. In some infections the progress of the exanthema is retarded in the maculopapular stage and vesicles are hardly ever seen. Secondary bacterial infections may be encountered in ulcerated vesicles. In addition to the infections listed in *Table 14.1*, many other virus infections (adeno-, myxo-, entero-, reo- and togaviruses) may be associated with a rash, often of short duration and resembling that of measles or rubella in appearance.

The classic clinical picture of the now- eradicated smallpox infection (*see* Chapter 32) often started with changes of the mucous membranes of the mouth and pharynx and a maculopapular exanthema which after a few days developed into the

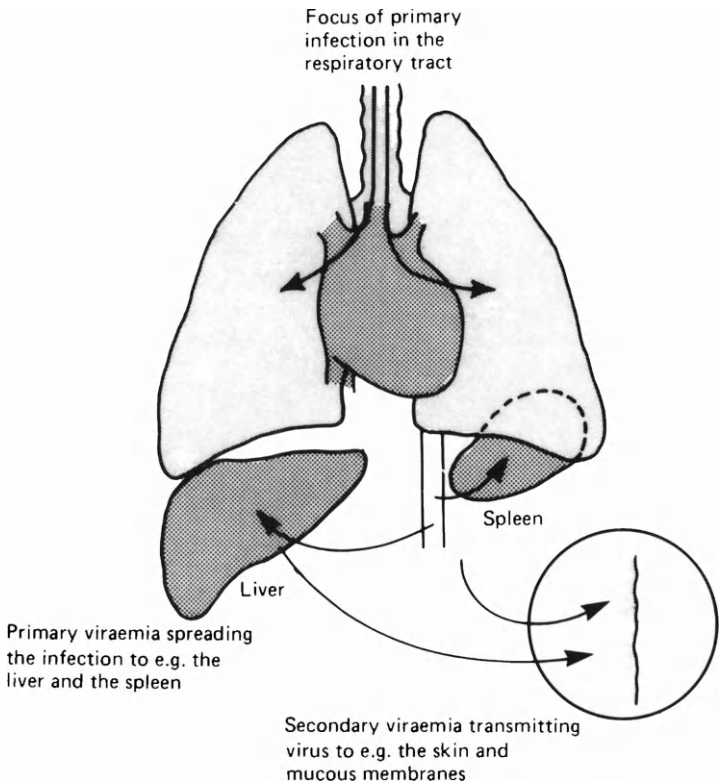

Figure 14.1. Viraemic spread of smallpox virus

characteristic pocks. Virus was spread by viraemia from the primary focus of infection in the respiratory tract to organs of the reticuloendothelial system (*Figure 14.1*). Replication of virus in the liver, spleen, bone marrow, etc., yielded a second massive viraemic phase which carried the virus to the skin. A similar process probably occurs in chickenpox (*see* Chapter 31). However, smallpox virus is considerably more virulent than varicella-zoster virus. The multiplication of virus is faster and the large amount of virus shed into the blood will give an almost

synchronous appearance of the pocks whereas varicella-zoster pocks will be more irregularly developed with efflorescences in all stages of development.

The vesicles of herpes zoster are restricted to a dermatome as the infection is the result of a reactivated latent varicella-zoster virus infection of a sensory ganglion and the newly formed virus is transported to the skin with the anterograde axonal flow. Morphologically, the skin lesions of varicella and herpes zoster are identical.

Also the spread of recurrent herpes simplex virus infections of the skin and mucous membranes is dependent upon axonal transport of virus from a latent reactivatable infection of ganglionic nerve cells. The skin area implicated in recurrent herpes simplex virus infection is as a rule smaller than in herpes zoster. Perhaps less virus is produced or a lower number of neurons infected. The fact that

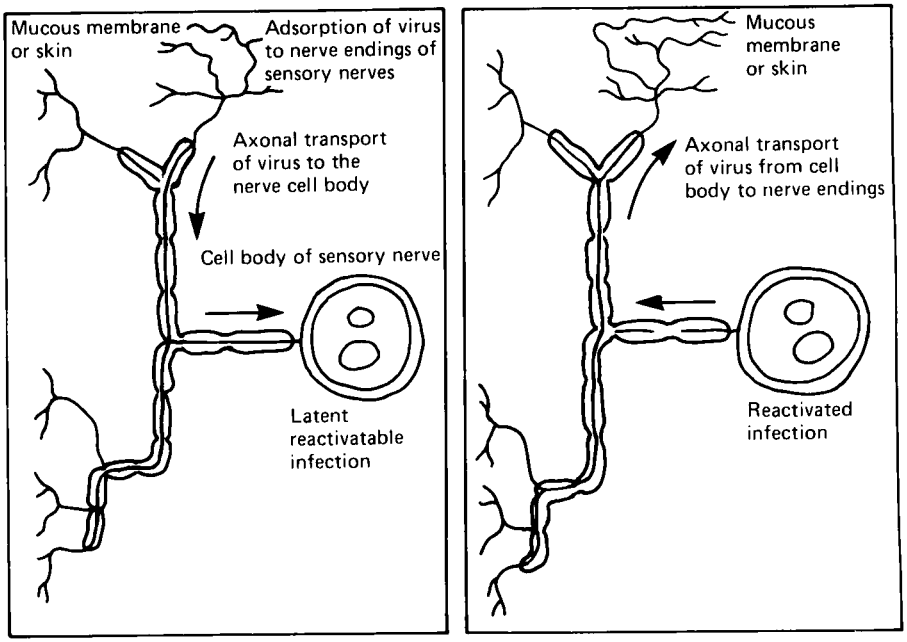

Figure 14.2. Neural spread of herpes simplex virus from skin and mucous membranes to nerve cell body (left part of illustration). Virus is adsorbed to nerve endings and transported with the axonal flow. A latent non-productive infection is established. When the infection is reactivated,newly formed virus is axonally transported towards the skin (right side of illustration)

the recurrent infection often appears in the same area of the skin has been interpreted as indicating that the infection is emanating from cells of the same ganglion (*Figure 14.2*).

Normally, infective virus can be isolated from skin lesions caused by pox- and herpesviruses, especially in early stages of the infection before antibodies are present in the vesicle fluid. Exanthema is a result of the viral cytocidal effects. Degenerating cells are seen in particular in the stratum spinosum of the skin. An intracellular oedema will disrupt the plasma membrane of infected cells and one or more liquid vesicles are formed close to each other, the intersepta originating from the plasma membranes. The horny layer of epidermis, loosened from the underlying tissue – an acantholysis – will constitute the roof of the vesicles. The healing

processes are initiated when specific antibodies and immunocompetent cells are present locally in the exanthema.

Enteroviruses may cause infections associated with exanthemas, herpangina and hand-foot-and-mouth syndromes. From these syndromes, but also from the more uncharacteristic maculopapular exanthemas in patients affected by outbreaks of coxsackie virus and echovirus, infectious virus can be isolated. The rash is considered to be the result of cytocidal effects of the enterovirus infection.

The exanthemas of measles and rubellavirus infections have another pathogenesis. The maculopapular rash of measles appears at approximately the same time as measles antibodies are detectable in serum, about two weeks after the initiation of the infection. The viraemia leads to infection of the endothelial cells of the capillaries and these cells demonstrate viral antigens in the plasma membranes. Both humoral and cell-mediated immune reactions are evoked, directed against the viral antigens, and the rash is elicited by a T-cell-dependent, delayed hypersensitivity reaction. Microbleedings are induced. Patients with a T-cell deficiency will accordingly not demonstrate the rash.

A general capillary fragility with extravascular outflow of erythrocytes in microfoci gives the exanthema a red colour. Bleeding of areas of varying sizes of infected skin and mucous membranes is not an unusual symptom in virus infections. The reasons may be effects on the blood-producing organs and on the formation of components necessary for blood coagulation. The congenital rubella infection is associated with thrombocytopenia and a purpura. The haemorrhagic fevers, dengue and yellow fever, may yield severe generalized bleeding of mucous membranes. In these infections, as well as in the pox- and herpesvirus infections, the tissue damage can itself be so extensive that bleeding is caused.

Warts and condylomas are hyperplastic proliferations and are histopathologically described as hyperkeratosis and papillomatosis, respectively. In both cases the proliferative changes are restricted and only in very rare exceptions will these benign cell proliferations be transformed into malignant tumours. However, the so-called flat condylomata are often associated with presence of atypic cells in the tissue. Virus as a cause of tumour formation is dealt with in Chapter 18.

Infections of the nervous system

Acute virus infections of the nervous system and the meninges are clinically observed as (1) meningitis, (2) encephalitis or encephalomyelitis and (3) infections of the peripheral nervous system (PNS), but may probably also be the causes of (4) imperfect development of the nervous system in the unborn and growing individual. Chronic infections of the central nervous system and the encephalopathies are discussed in Chapters 16 and 17.

The viral meningitides seldom appear as single symptoms and a thorough clinical investigation will also reveal involvement of the brain, a meningoencephalitis. Encephalitis with no signs of a concurrent meningitis might have reached the CNS by neuronal pathways and sometimes both CNS and PNS are affected. Medically, infections of the nervous system are among the most important virus infections. The mortality rate of some of the encephalomyelitides is high and neuron damage may cause irreparable loss of mental and physical functions in patients surviving the acute stage of the infection.

Meningitis

During the viraemic phase, virus may be deposited in the meninges and infect endothelial cells of the capillaries. In the evoked inflammatory reaction numerous monocytes will appear in the cerebrospinal fluid. The function of the blood–brain barrier may be impaired by cytocidal viral effects, but also by cytotoxic immune reactions. When immune-complexes with virus maintaining its infectious properties are deposited in the plexus choroideus, macrophages may be infected and the infection carried extravascularly by infected phagocytizing cells, thus transferring the infection to the brain tissues.

Meningitis is a common symptom in many enterovirus infections. An increased number of monocytes in the cerebrospinal fluid is frequently seen in mumps, including the uncomplicated cases, suggesting that involvement of the meninges can occur without marked symptoms.

Encephalomyelitis

Table 14.2 gives a survey of the pathology and pathogenetic mechanisms for some CNS infections. Viral CNS infections are associated with proliferation of glial cells and perivascular infiltration of inflammatory cells, in particular monocytic cells. The inflammatory reactions are accompanied by intercellular and intracellular oedemas. Sometimes it is the raised intracranial pressure which is the most dangerous for the patient.

TABLE 14.2 Pathogenesis of some acute virus infections of the CNS

Virus	*Pathology*	*Pathogenesis*
Toga- (arbo-) viruses	Meningoencephalitis	Vectorborne, usually endemic, infections. Virus reaches CNS after systemic dissemination by viraemia. Infective virus is demonstrable in the CNS
Herpes simplex virus	Encephalitis, meningoencephalitis	Neural spread of virus, often from a reactivated latent infection of a sensory ganglion. Infective virus is demonstrable in biopsies from infected CNS; in cases of meningitis, virus is isolated from the cerebrospinal fluid
Measles virus	Encephalitis, postinfectious encephalitis	The encephalitis is a complication of an acute measles. The postinfectious encephalitis is a result of an immunopathological reaction. Infective virus is generally not demonstrable
Poliovirus	Meningoencephalomyelitis	Usually viraemic spread to CNS. Virus destruction of neurons causes paralytic disease. Infective virus may be isolated from faeces, saliva, blood, CNS
Enteroviruses, mumps virus	Meningoencephalitis	Viraemic spread to meninges and other parts of the body. Enteroviruses are often isolated from faeces, saliva, as well as cerebrospinal fluid; mumps virus from saliva and frequently also from cerebrospinal fluid

The cytological changes induced by virus infections in cells of the CNS are not different from those seen in virus-infected cells in general. Cytoplasmic and/or nuclear inclusions can be demonstrated histologically (*see* Chapter 11) and demonstration of viral inclusion bodies in cases of suspected rabies or herpes simplex encephalitis can still be of some diagnostic significance.

Cytocidal effects of the virus infection are responsible for death of neurons with paralysis or other neurological symptoms as a consequence. Some virus infections regularly involve specific anatomic entities of the CNS and the monitoring of the infection to these areas has led to much speculation. Herpes simplex type 1 virus infections are generally localized to the temporal lobes, rabies to structures of the limbic system, e.g. hippocampus, polio virus infections to the motor neurons of the spinal cord and hemispheres, etc. There seem to be several pathogenetic factors working together (routes of infection, presence of cellular receptors, mode of spread of infection within the body, etc.).

In general, virus infection of the CNS will be associated with more widely spread damages than corresponding PNS infections. One plausible reason might be the susceptibility of the glial cells for virus infections: another that the immune defence requires antibody response within the blood-barrier including migration of immunocompetent cells to the CNS.

In experimental animals some acute virus infections are associated with demyelination which probably is caused by infection of Schwann's cells and oligodendroglia. As one oligodendroglial cell myelinates several nerve cells an infection which is directed against oligodendroglia can exert extended impairment of the myelin. Demyelination may also be induced by cytotoxic immune reactions triggered by virus infections. The postinfectious encephalomyelitis after measles, rubella and varicella, and postvaccinial encephalitis are diseases considered to have an immunopathological pathogenesis. These types of encephalomyelitis are clinically manifested at the end of the acute infection or shortly after the end of the acute stage. Infective virus is then not demonstrable. It has been assumed that the infection has contributed to the exposure of modified cellular antigens and that the T-cell-mediated regulation of autoantibody-producing B lymphocytes is affected.

Sometimes, changes in behaviour and personality are the earliest detectable symptoms of a viral encephalitis. Such behavioural changes have been observed especially in patients with rabies or herpes simplex virus infections of the CNS. Experiments with laboratory animals have suggested that dysfunctions in neurotransmission and effects of the virus infections on the turnover of transmitter substances may be pathogenetically involved.

Retardation of CNS development and hydrocephalus

Many different factors contribute to making the immature individual sensitive to virus infections (cf. Chapter 15). The nervous system of the fetus and the newborn may be more susceptible to viral infections since the perineurium which in the adult encloses the nerves and prohibits diffusion of macromolecules into the nerve is incomplete. Hence, also, invasion of viruses into the CNS is facilitated. Experimental studies in animals have demonstrated that many infections, for example, the parvovirus infection of the newborn cat and hamster, will cause a retardation in the development of the cerebellum. The measles and mumps virus infection of the newborn hamster can lead to stenosis of the aqueduct and development of hydrocephalus. Thus many animal models suggest a pathogenetic

association between virus infection of the fetus and the newborn, and pathological changes of the nervous system. It is still not certain, however, how medically important the virus infections are regarding later established CNS dysfunctions. Rubellavirus, but possibly also cytomegalovirus infections, can induce a physiological retardation, for example, micro-ophthalmia, and for several years the association between mental retardation and cytomegalovirus infections has been studied. It is interesting that in a few cases lymphocytochoriomeningitis virus infections in pregnant women have been associated with hydrocephalus in the newborn infants. Lymphocytochoriomeningitis virus will induce hydrocephalus in animal models. The medical importance of the virus is as yet unknown.

Infection of peripheral nerves

Multiple peripheral neuritis (Guillain–Barré syndrome) has been observed in patients with herpesvirus infections but also in some vaccinees injected with influenza vaccines and after infection in patients with epidemic influenza. Varicella, Epstein–Barr, and herpes simplex viruses have been discussed in the context of the aetiology of Bell's palsy, usually a one-sided paralysis of the fifth cranial nerve.

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The pathogenesis of congenital infections

Marianne Forsgren

During the last four decades there has been a marked increase in the awareness that certain virus infections in pregnant women may have severe consequences for the embryo or the newborn child. However, only a small proportion of all forms of fetal damage has been attributed to virus infections. In cases when the virus which is responsible has been identified it is possible to introduce preventive measures. A prerequisite for prophylaxis is knowledge about the pathogenesis of disease.

Certain problems can be studied by animal experimentation but the conclusions drawn from animal models cannot unreservedly be applied to man since, for example, the structure and function of the placenta show marked variations between different species. The majority of new knowledge therefore has to be acquired by studies of autopsy materials from abortions and stillborn children,

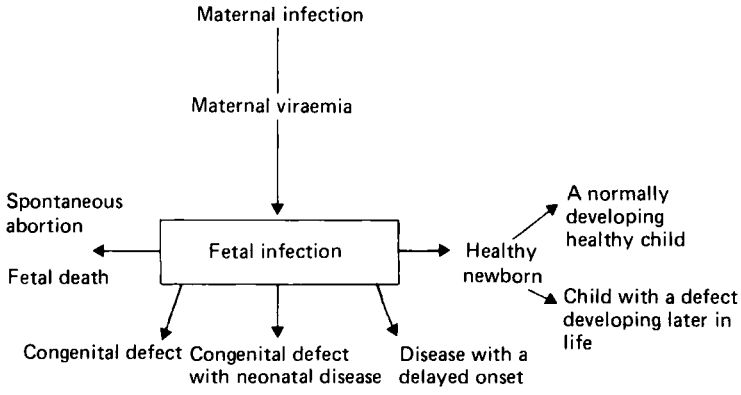


Figure 15.1. Different effects of a fetal infection

clinical observation of damaged children, and comprehensive prospective epidemiological analysis. Many infections occur without any symptoms either in the mother or the child and thus virological laboratory diagnosis plays an important part in this kind of study.

The results from different clinical virological investigation have shown that most maternal virus infections do not have any harmful influence on the fetus. However, in certain cases, a virus infection may have a damaging effect which varies from mild to severe and from shortlasting to persistent (*Figure 15.1*). The damage may

be caused by a direct attack by the virus on the fetus or by an indirect influence on the placenta or the function of the uterus. The outcome is dependent on the reactions of the mother and the fetus to the infection, which in turn are influenced by such factors as the stage of development of the fetus, genetic disposition and the nutritional conditions of mother and fetus.

Different ways by which a virus may cause fetal damage

A large number of different virus infections can be transmitted to the fetus. This may occur at a very early stage of the embryonic development and the egg cell or the germinative epithelium may already be infected. In laboratory animals it has been discovered that lymphocytic choriomeningitis virus, murine leukaemia virus and certain other viruses, which cause infections without any cytotoxic effects on cells, can be transmitted in this way (vertically) through many generations. These *vertical infections* need not have any harmful influence on the normal development of the fetus but, postnatally, immune pathological disease or leukaemia may develop. No corresponding mechanisms of transmission have been identified in man. Other viruses can infect the egg cell or the early embryo from a focus of infection in the uterus or be transmitted by seminal fluid. Such a transmission probably can occur in man also; for example cytomegalovirus (CMV) has been isolated from seminal fluid. However, no direct observations are available.

TABLE 15.1. Probable routes of transmission of virus to fetus/child. Intra partum = during delivery, after rupture of the fetal membranes; post partum = after delivery, during the first weeks of life.

Type of virus	*Transplacental*	*Intra partum*	*Post partum*
Cytomegalo	++	++	++
Rubella	++	−	(+)
Herpes simplex	+	++	+
Varicella	+	−	(+)
Coxsackie B	+	+	++
Hepatitis B	−?	++	+

Route of transmission: ++ common; + rare; − without importance

Once the fetal membranes have developed, they appear in most cases to provide an efficient protection against infectious agents such as the viruses which may occur in the cervix or vagina. Thus virus infections usually are not transmitted to the fetus as long as the membranes remain intact, but they can reach the fetus via the placenta, *transplacental transmission*. Many different viruses have been found to be capable of passing the placental barrier and infecting the fetus; in man rubella and CMV are the two most important viruses. Other viruses which in occasional cases may cause a transplacental fetal infection are herpes simplex, varicella-zoster, variola, vaccinia, measles and enteroviruses.

After rupture of the fetal membranes new ways for transmission of the infection become available. Virus present in the cervix and vagina may spread into the uterine cavity and cause a fetal infection. During delivery the child is exposed to infectious agents which may occur further down in the genital tract, in the blood of the mother and in faeces (*intra partum transmitted infections*). After delivery (*post*

partum), during the newborn period, possibilities for transmission of infections from other individuals in contact with the child are added. Also breast milk may transmit a viral infection. This primarily concerns CMV. The child may also become infected by various different medical interventions (*iatrogenic infections*). Thus for example CMV may be transmitted with blood transfusion. The routes of infection for the most important of the viruses which are known to cause congenital infections are summarized in *Table 15.1*.

Infections which occur before the rupture of the fetal membranes will be referred to as *congenital* whereas other infections which occur intra or post partum will be referred to as *neonatal*. The latter infections are discussed further in Chapter 34. The perinatal infections deserve particular attention since viruses, which later on in life only rarely cause severe disease, may cause serious infection during the newborn period. In connection with perinatal infections organs which already are developed may be damaged. In severe cases this may lead to definite degenerative changes but in milder cases a normal organ function may be restored.

Spread of virus in congenital infections

The most important route of transmission is a transplacental infection by the virus. One prerequisite is that the virus can reach the placenta, i.e. that the infection includes a haematogenous spread of the virus, *viraemia*. Thus the fetus is not threatened by maternal infections with viruses which only replicate locally in the respiratory tract or in the gastrointestinal tract. With a generalized infection involving viraemia the degree of severity of the infection in the mother does not have a decisive influence on the extent of viraemia and spread of virus to the fetus. In fact, the maternal infection may be completely subclinical, for example in the case of CMV and in some cases of rubella. Viraemia usually can develop only with the primary infection. Convincing evidence has shown that this is the rule in rubella. Congenital rubella has not been observed in more than one sibling with the exception of twins. Consequently there are good opportunities of protecting women susceptible to rubella by vaccinating before child-bearing age and thereby preventing fetal damage. An immunity in the mother against, for example, measles and varicella, also would protect the fetus against these infections. In CMV infections the conditions are somewhat different. CMV can be transmitted to the fetus not only during a primary infection but also in connection with a reactivated infection. Thus congenital CMV infections have been observed in siblings and also children from mothers who already had CMV before the pregnancy. Reactivation of CMV during pregnancy is a common event and most likely it is related to the physiological immunosuppression which develops in pregnant women. The transmission of the infection to the fetus is probably dependent on the fact that the reactivated infection occurs in the form of a low grade viraemia of virus in nucleated cells in the circulation. However, at the same time the immune response in the mother during the activated infection may modify the fetal infection. Most evidence therefore indicates that a primary infection is more dangerous than a reactivated infection.

When a virus has reached the placenta it has to spread further in order to establish an infection in the fetus. This may occur in different ways. One mechanism of transmission which most likely is of importance is the replication of the virus in the placental cells with a seeding of virus into the fetal circulation.

Viruses which cause fetal infections have a capacity to infect placental cells of both maternal and fetal origin. This infection can be demonstrated by virus isolation, identification of viral inclusions or viral antigens in the trophoblastic cells and histopathological changes. Rubellavirus,CMV and herpes simplex virus can cause placental infections in man.

The placentitis caused by rubella during early pregnancy is characterized by an attack on the epithelium in the chorion villi and on the endothelial cells in the placental capillaries, which leads to focal necroses. The endothelial cells are discharged into the lumen of the fetal blood vessels and thus may cause the appearance of infected emboli. These may spread via the fetal circulation to different organs in the fetus. The changes may become severe and impair the functions of the placenta which may contribute to fetal damage or even to fetal death. However, virus has been isolated from placental tissue which appears normal. In placentitis caused by CMV, focal inflammatory changes are seen in the chorion villi with infiltration of plasma cells. With herpesvirus infections, focal necroses in the trophoblast epithelium can be found, usually without any inflammatory reaction.

As was mentioned, the virus does not always pass over to the fetus in cases when the placenta is infected. The mechanisms which regulate the immune defence in the placenta are inadequately understood, but it is possible that a capacity of placental cells to produce interferon may be important regarding the restriction of infections. The frequency with which the infections are transmitted to the fetus varies with the time point for the maternal infection and the character of the virus. With most rubellavirus maternal infections during the first trimester the fetus also becomes infected but infections during the second, and the third, trimester less often lead to an infection of the fetus. In contrast, primary CMV infections can be transmitted during the whole pregnancy and this seems to occur in about half of all cases. Primary herpes simplex or varicella virus infections occurring during early pregnancy only rarely are spread transplacentally. However, during a late phase of pregnancy, varicella virus infections of the fetus are more common.

It takes varying times for a virus to pass the placenta. The fetus may develop symptoms of infection at the same time as the mother or immediately after a certain incubation time as, for example, in varicella during the later part of pregnancy. If a pregnant woman contracts varicella a few days before delivery the child is taken ill immediately after birth. The incubation time is about ten days compared with the normal incubation time of two to three weeks. Partly because of the uncertainty concerning the time point for the fetal infection the risk of damage is calculated from the time point during pregnancy at which the woman is taken ill. The length of pregnancy is calculated from the first day of the last normal menstruation.

Direct effects of fetal infections

When a virus infection occurs in a fetus the course of events is dependent upon the properties of the virus and of the fetus. Because of the occurrence of an intensive cell division and poorly developed defence mechanisms, a fetus offers an excellent milieu for virus replication. If the fetus is hit by an infection with a virulent virus which gives cytocidal effects, this frequently leads to fetal death and subsequent abortion. This is the case with some herpesvirus infections in animals (cattle, horse, swine) and it probably contributes to the increased risk of abortion with a primary

genital herpes simplex virus infection during early human pregnancy. In contrast, if a fetus is infected with a virus that has milder effects on the cells, the survival of the fetus is less threatened but, instead, growth retardation of varying degrees of severity may develop. Rubellavirus and CMV have this effect on fetuses in man. The harming effect of these viruses is reinforced by their capacity to establish a chronic infection in the fetal cells. One additional factor of considerable importance concerning damage of organs is the state of fetal development at the time of infection. If the fetus is infected during the embryonal period, when cell division is particularly intensive and different organs are beginning their development, a disturbance of organogenesis may occur and lead to malformation. Examples are the damage of heart, eyes and hearing organs in connection with rubella during the first ten weeks of pregnancy (*Figure 15.2*) and disturbance of the growth and

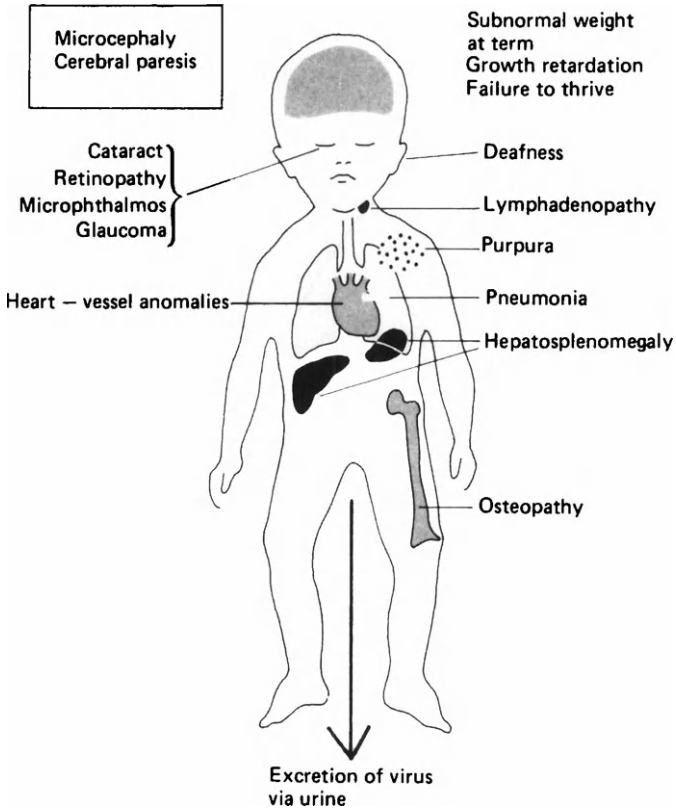


Figure 15.2. Organ damage which occurs in connection with congenital rubella

development of the brain by early infection with CMV. This damage is the result of a direct influence of virus on cells. Cell death, chromosomal damage and retarded cell division can be demonstrated.

During this period the immune defence mechanisms in the fetus are immature and, in addition, other factors which may restrict the infection, for example production of interferon, are not as effective as in the mature individual. Furthermore, a protection by maternal antibodies does not occur in infections

during early pregnancy (*Figure 15.3*). All this contributes to the fact that rubella and CMV infections can become chronic. Even later on, when the immune defence mechanisms of the fetus mature and antibodies, particularly the IgM class, against the virus can be formed and an increasing proportion of IgG antibodies are transferred from the mother, no effect on the intracellular infection can be obtained. Consequently the child is born with a chronic infection (*Figures 15.2* and *15.3*). The excretion of virus ceases first after some months to years. The risks of damage to the fetus is increased by the persistence of the infection. Thus organs which start to develop after the initial phase of the infection may also incur damage. In a more mature fetus the inflammatory reaction directed against the generalized

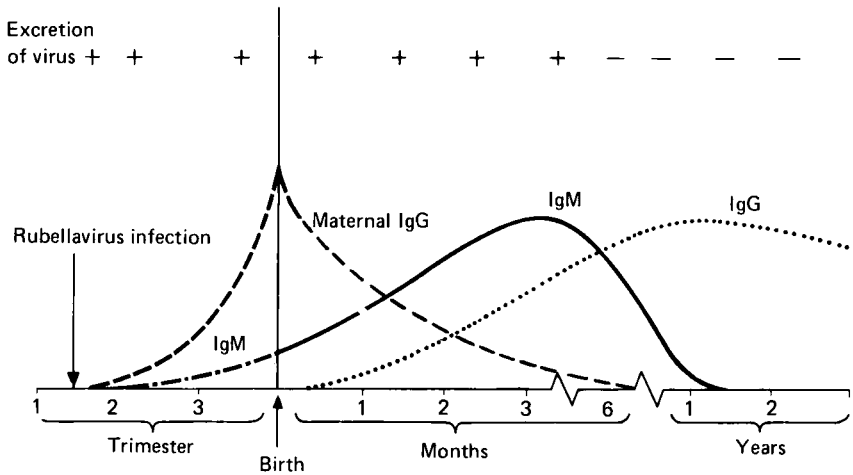


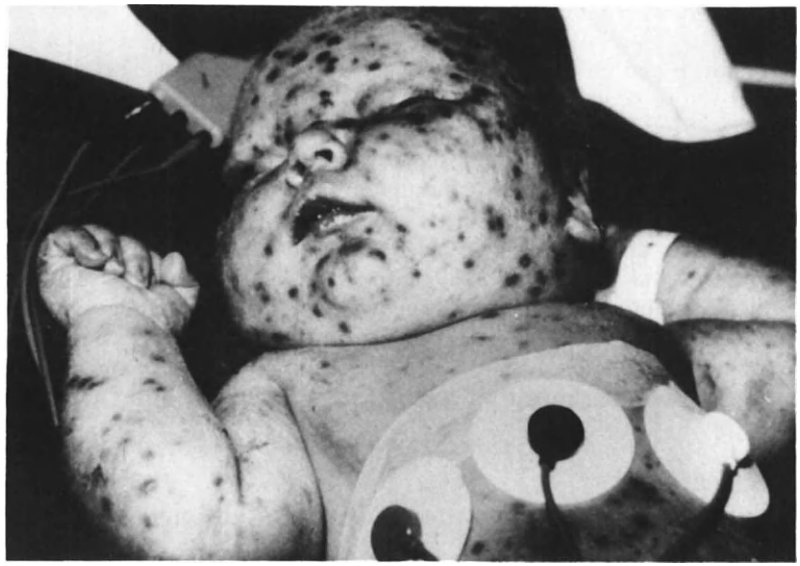
Figure 15.3. Schematic illustration of excretion of virus and development of antibody responses in congenital rubella

infection may contribute to the damage. This can explain many symptoms which may appear in children with congenital rubella or CMV infection (*see Figures 15.2* and *15.4*); hepatosplenomegaly, icterus, thrombocytopenic purpura, anaemia, pneumonia, chorioretinitis, osteitis and meningoencephalitis. Paraventricular calcification in the brain is a sign of a severe early infection in this area. After the embryonal period the effect of a rubella infection is much less devastating. The organs are already formed and they continue to grow in size, but the cell division is less intensive than earlier. Furthermore the immune defence mechanisms of the fetus are developed. After the first trimester, fetal damage in connection with a rubella infection is markedly reduced and after the sixteenth week of pregnancy it is very small (*see also* Chapter 26). Damage which occurs during this later period primarily concerns the hearing organs. During the latter half of pregnancy, rubella infections rarely become chronic. The child may have serological signs of an intrauterine infection but no virus is excreted at birth.

In contrast, a CMV infection persists long after birth whatever the time during pregnancy at which the infection has occurred in the fetus. The risk period is not known, but it is probably longer than that with rubella (*see also* Chapter 31). Of decisive importance for prognosis is the possible occurrence of encephalitis and the intensity of such infection.

The severity of the symptoms varies from a fulminant disease to a single mild symptom. Many children with congenital rubella and most children with CMV infections are in fact completely without symptoms at birth. However, damage to hearing capacity may be discovered at a later stage.

The congenital chronic rubellavirus infection occasionally may manifest itself as a late-onset disease. Signs of fetal damage as a rule are present at birth but symptoms are added at the age of 3–12 months. Such additional symptoms are chronic exanthema and interstitial pneumonia. It has been speculated that immune



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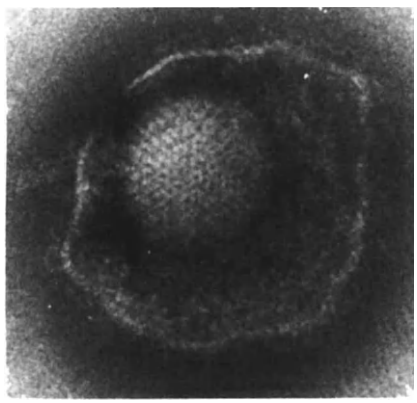

Figure 15.4. (*a*) A newborn child with a severe congenital cytomegalovirus infection; 'Cytomegalic inclusion disease (CID)'. (*b*) CMV identified in a urine sample from the child by the use of electron microscopy (Magnification ×182 000. Photo: Kerstin Brebäck)

complex formation may be a possible cause of these late complications. Recently, activation of a latent rubellavirus infection in the brain was described in 11–12 year-old children with a congenital rubella (*see* Chapter 16). Thus rubella virus-infected cell clones may remain for a long time in the organism.

The chronic fetal infections are those that have been studied most extensively. They represent the most common congenital infections and excellent possibilities for laboratory diagnosis are offered by the fact that the virus remains in the

organism. Also, in the case of other viruses, there are individual reports of damage caused by direct infection of the fetus. This concerns vaccinia, variola, herpes simplex and varicella-zoster virus infections during early pregnancy. Varicella occurring a short time before delivery may cause a severe infection if the antibody production of the mother has not started before the birth of the child.

Evidence for the possible occurrence of fetal damage with measles is somewhat contradictory. Prospective materials collected in industralized countries, however, do not indicate any marked risk for defects other than a spontaneous abortion. Measles prophylaxis in the form of immunglobulin to susceptible women who have been exposed to the virus, or measles vaccination before pregnancy, is recommended.

Occasionally, children can be born with a coxsackie virus infection and display symptoms similar to those seen in connection with a neonatal infection (Chapter 34). The importance of enterovirus infections early during a pregnancy has not as yet been clarified. However, it is suspected that certain forms of brain damage and malformation in the urogenital organs may be related to coxsackie virus infections. Enteroviruses do not have the capacity to persist until birth and later laboratory diagnoses therefore are difficult. A possible occurrence of fetal damage in connection with mumps has been discussed extensively, but so far prospective studies do not indicate an increased risk of damage. The persistent form of hepatitis B, which occurs in 10–12 per cent of the population in some developing countries, normally does not transmit to a fetus, but perinatal infections are common and frequently lead to the establishment of persistent infections in the newborn children.

Indirect fetal-damaging effects in connection with infections of pregnant women

The majority of all virus infections are rarely or never transmitted to the fetus. However, infections which are restricted to maternal tissues may also influence the fetus through disturbances in the infected woman, for example fever, nutritional-electrolytic disturbances and reduced oxygen tension. It is not known in detail how these different factors act, but the possible effects obviously are related to the degree of severity of the maternal infection. Viral infections occurring in pregnant women may be more severe than in non-pregnant women. This most likely is due to a certain degree of physiological immune suppression during pregnancy. During the influenza epidemic 1918–1920 (the Spanish grippe) the mortality of pregnant women was high, particularly if pneumonia developed. During later influenza epidemics, no corresponding increase in mortality has been found, but, particularly towards the end of a pregnancy, influenza may be complicated by pneumonia. In connection with influenza as well as measles and mumps, there is an increased risk of spontaneous abortion and premature delivery, respectively. Furthermore, there are some reports on the occurrence of an increased but still small number of malformations in the central nervous system after some influenza epidemics. However, no definite causal relationships have been demonstrated.

Acute hepatitis is a major problem in the developing countries and it represents one important cause of death among pregnant women. In industrialized countries acute hepatitis A and B run a milder course and occur with much lower frequency. The same holds true for poliomyelitis which frequently leads to paralysis if it

attacks pregnant women. Spontaneous abortion and premature delivery occur with increased frequency in connection with both poliomyelitis and acute hepatitis.

In conclusion, it should be emphasized that our knowledge about the importance of virus infections during pregnancy is still incomplete. Rubellavirus and CMV infections dominate the discussion, but the effects of these viruses also are far from completely elucidated. Long-term effects as well as the influence of genetic factors on fetal infections are topics which are attracting an increasing amount of attention. Certain data indicate that a genetic disposition for development of hearing damage may contribute to the establishment of hearing defects in children with congenital rubella.

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16

The pathogenesis of persistent infections

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For a long time the main focus of attention in viral diseases has been directed towards acute infections. The connection between cases of disease caused by a certain infectious agent shows very clearly in the pattern of person-to-person spread of the agent such as that seen during an epidemic of influenza. Acute viral diseases are characterized by a fixed shorter or longer incubation time and by the development of symptoms usually following a characteristic pattern. Through mobilization of the defence mechanisms of the body the virus and virus-infected cells are eliminated and after a phase of reparation normal organ functions are usually restored. During the last few decades, studies of viral diseases to an increasing extent have come to be focused on the different conditions where an infection has not been effectively eliminated from the body. Different persistent infections can depend on both the special conditions in the host organism and on the special features of the virus which become expressed in its interaction with cells.

In Chapter 11 it was pointed out that viruses in cell culture systems frequently give cytocidal infections but that under certain circumstances persistent infections of different character can be established. In a similar way persistent infections may also arise in the infected organisms. In the intact host, however, there are two important conditions in addition to those connected with the cell culture system.

The cell culture systems which are used for propagation of viruses have certain limitations. In many cases they do not provide a cellular milieu corresponding to that of special organs in the multicellular organisms, which is required for the growth of a specialized virus. A virus of this category can only replicate in selected localities in the organism, for example in the intestinal or respiratory epithelium. After spreading to other parts of the body the virus may encounter cells that do not allow complete replication of the virus, non-permissive cells. By definition such cells do not allow a complete virus replication but it is possible that the virus can remain in a form which may affect normal cellular functions. Analogous phenomena may also concern viruses which are cytocidal in cell culture systems. These viruses may destroy many kinds of cells in the body, but possibly there are other kinds of cells which, on account of their metabolic specialization or other conditions, provide a non-permissive milieu. A virus may remain in the latter cells and possibly induce functional disturbances. Ecological conditions within the body thus are of considerable importance for the interaction between a virus and cells.

The second condition which is of decisive importance for the possible development of an acute into a persistent infection is the immune response. Different situations can be distinguished. If the infected individual has a defective humoral or

cell-bound immune response a normally acutely developing infection with a cytocidal virus may turn into a serious persistent infection (cf. Chapter 19). Examples of this are infections with poliomyelitis and measles viruses, respectively.

Infections of cell cultures with non-cytocidal viruses may lead to establishment of persistent infections but in the infected organism the conditions are different. The immune defence attacks cells carrying new surface antigens due to the presence of the non-cytocidal infection. A dangerous situation may arise if there is an infection with non-cytocidal virus in the presence of an inefficient immune defence. Persistent infections of a serious nature may then arise. Examples of this are prenatal infections with rubellavirus and cytomegalovirus. Occasionally a third situation which is a variant of the previous one may develop. An infection with moderately or weakly cytocidal viruses may under normal conditions, as a consequence of immune defence reactions, cause marked organ damage. If the immune defence functions poorly the infection may instead acquire a subclinical nature – i.e. in the absence of an effective immunological 'cleaning-up' action no symptoms may develop, but as a consequence of this the infection may persist. One example of this is persistent infection with hepatitis B virus.

Thus persistent infections can have their origins in many different mechanisms. It is impossible to make an exact distinction between the different categories since there are gradual differences between some of them. The following categories of persistent infections are used in this chapter.

(1) Progressive persistent infections as a consequence of a defective immune defence. This group includes generalized disease in (a) newborn individuals with a physiologically-immature immune defence mechanism, (b) individuals with congenital immune defects and (c) persons with acquired immune defects or patients who are undergoing immunosuppressive treatment. These situations are discussed further in Chapters 14 and 33.

(2) Infections caused by a spread of virus to the intrauterine milieu. Some of the infections become chronic and they are discussed in Chapter 15.

(3) Persistent subclinical infections due to a deficient or ineffective immune defence leading to a development of an immune pathological disease at some later stage. In these conditions there is a continuous production of virus and viral antigens in the body. Infections of this kind are discussed partly in this chapter and partly in Chapter 19.

(4) Persistent latent infections after a clinical or subclinical primary infection with single or repeated separate episodes of recurrent disease. Infectious virus cannot be detected in the intervals between the different disease periods. This form of reactivatable persistent infection is referred to as latent.

(5) Slow virus infections. This term is used for infections which persist after a subclinical or clinical primary infection and, after a long incubation period, cause a slowly developing disease frequently with a fatal outcome. The disease development is similar to that seen in connection with an acute disease but the course of events is extended over a long period, varying from months to years instead of days to weeks. This kind of infection primarily concerns the central nervous system and is caused by conventional viruses such as measles and rubellaviruses but also by a

group of atypical infectious agents. Because of the very special nature of this kind of infectious agent it is dealt with in a separate section (Chapter 17).

(6) Virus infections leading to the development of tumours are an additional example of persistent infections. Of decisive importance in connection with tumour development is the loss of the cell's capacity to repond to growth-regulating controls and also the failure of the immune defence to control the transformed cells. Tumours caused by viruses are discussed in Chapter 18.

Finally it should be mentioned that there is a possibility that persistent non-cytocidal virus infections may cause subtle changes in cellular functions. Discrete infections in the central nervous system might disturb the mechanism for the spread of transmitter substances leading to different kinds of disease. In a similar way the function of cells with an endocrine activity might be disturbed. The possible occurrence of diseases of this kind, however, is still speculative.

Chronic subclinical infections with immune pathological reactions

The prerequisite for this group of infections is that the virus establishes a chronic subclinical infection because of a failing or ineffective immune defence. The medical importance of this kind of infection regarding disease in humans has not yet been confirmed. Therefore this kind of infection will be exemplified by three different diseases occurring in animals.

Lymphocytic choriomeningitis

Lymphocytic choriomeningitis is caused by an arenavirus with the name *lymphocytic choriomeningitis (LCM) virus*. When this virus infects adult mice it causes a serious disease, frequently with a fatal outcome. If however the animals are immunosuppressed it is found, paradoxically, that the symptoms may be eliminated in spite of an abundant occurrence of infected cells. Thus the immune defence apparently in this case, as with several other virus diseases, is considerably important in that it contributes to the appearance of symptoms. If the virus is inoculated into very young mice the course of the infection is markedly different. As with the situation in adult animals the virus replicates effectively but there are no symptoms. This is because the young animals have not as yet developed a capacity to mobilize immune defence reactions. If immune lymphocytes from adult animals are transferred to infected newborn animals symptoms may arise. The physiologically poor immune defence in the young animals prevents the infection from being eliminated from the body. The animals become carriers of an LCM virus infection for the rest of their life. Late in life, animals infected as newborns can develope immune complex disease causing, as one consequence, kidney damage.

Aleution disease

Aleution disease occurs in mink, especially the variant blue mink. The disease is caused by a parvovirus and the infection is usually initiated early in life. In many,

perhaps all, cases the virus transmission occurs prenatally. It becomes chronic in spite of the fact that it elicits a pronounced immune response. Paradoxically, the immune globulins which are produced lack the capacity to neutralize the infectious property of the virus. Instead, infectious complexes of virus and antibodies circulate in the blood. As a consequence of the powerful but fruitless activity of the immune response, a generalized increase in the number of plasma cells, the enlargement of liver and spleen, and a pronounced hypergammaglobulinaemia can be seen. The generation of large quantities of circulating immune complexes cause e.g. kidney damage since the complexes accumulate in the basal membrane in glomeruli. Immunosuppression prevents the development of symptoms.

Visna

Visna is a disease which occurs in sheep. The infection is caused by a retrovirus (genus lentivirus) which, however, has not been shown to have any capacity to transform cells. Originally, visna was described as a slow virus infection (*Figure 16.1*). The incubation time after inoculation of sheep varies between a few months to many years. During this time the virus replicates in several different organs. The prolonged course does not depend on a slow replication of the virus. The virus first replicates in the reticuloendothelial system and later on also in the central nervous system. A specific immune response is mobilized but this occurs slowly and it does not seem to have a capacity to prevent further replication of virus. Comparative studies of virus isolates at different time points after inoculation show that there is a continuing change in the character of the virus surface antigen in the infected animal. As a consequence, neutralizing antibodies which are synthesized during the early part of the course of infection cannot influence the replication of the variant of the virus which occurs at a later stage. These changes in the properties of the virus surface antigen may be an important factor in the persistence of the virus. Another important fact is the capacity of visnavirus to produce a DNA copy of virion-RNA by use of its reverse transcriptase. This DNA copy may become integrated into the cellular genome. The development of symptoms of disease from a visnavirus infection occurs late and is caused by a demyelination in the central nervous system. These changes do not appear to depend on a direct attack by the virus but, instead, autoimmune reactions seem to play a role. The development of symptoms can be slowed down by immunosuppressive treatment.

There are certain similarities between persistent hepatitis B virus infections in man (*see* Chapter 29) and the different conditions discussed above. In the presence of a weak immune response the symptoms of liver affection in connection with a hepatitis B virus infection are mild but at the same time the risk of development of a persistent infection is increased.

The persistent infection may be of two different kinds, one of which is contagious and the other which is non-infectious. With an infection of the first kind, complete infectious virus is produced and there is a risk of the development of progressive liver damage. A spread of the infection to persons in the vicinity may occur. With the non-infectious kind of persistent hepatitis B virus infections, only a defective virus is produced. It has been discussed whether possibly the persistent hepatitis B virus infections may cause a development of circulating immune-complexes which might cause organ damage. However, there is no clearcut evidence that deposition of immune-complexes can lead to organ damage in these cases.

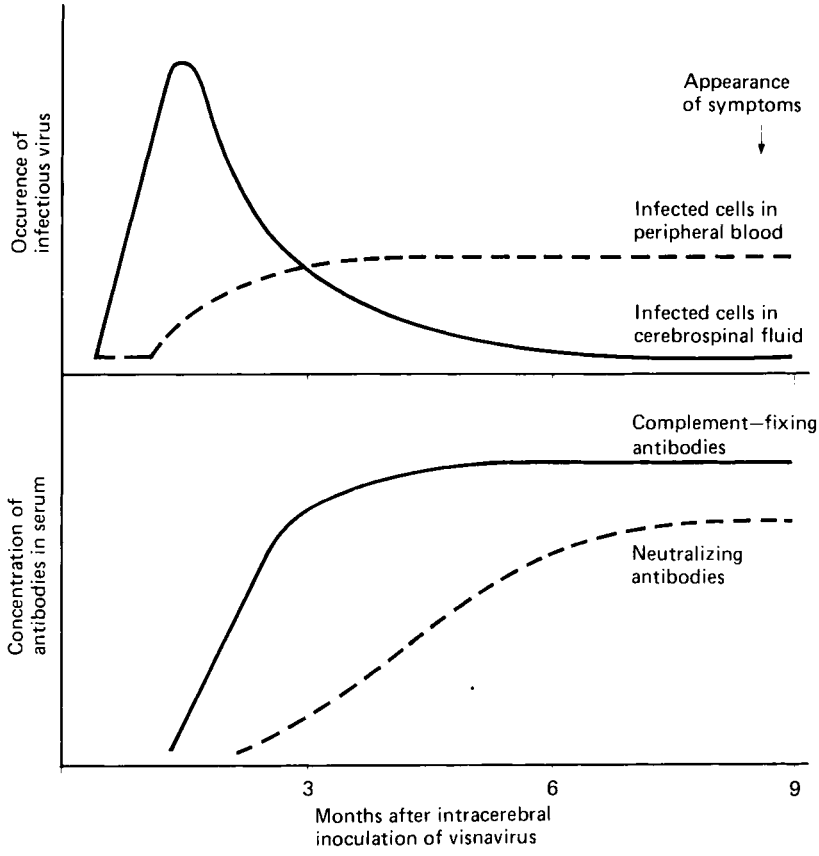

Figure 16.1. Course of infection after the intracerebral inoculation of visnavirus into sheep. Infected cells can be demonstrated firstly in cerebrospinal fluid and thereafter in peripheral blood (leucocytes). The humoral immune defence develops slowly and complement-fixing antibodies appear before neutralizing antibodies. Symptoms of disease at the earliest appear nine months after inoculation. In many cases the incubation time extends over years

Latent infections

Several types of herpesvirus can give persistent infections which represent classic examples of so-called latent infections. It is characteristic of these infections that there is an alternation between quiet intervals during which infectious virus cannot be demonstrated and episodes of emerging disease. These bouts of disease are caused by activated *endogenous virus*.

Herpes simplex

There are two different types of herpes simplex virus. Type 1 virus causes infections primarily in the mouth and facial region whereas type 2 mainly gives genital infections (cf. Chapter 31).

The first contact with herpes simplex virus type 1 usually occurs in childhood and in about 1 per cent of the cases it leads to a stomatitis, but the overall majority of infections in children are subclinical. In some individuals the virus infection reappears, usually at intervals of months or years. The appearance of vesicular changes in the skin in certain regions around the mouth is characteristic. Corresponding locally-reappearing infections can also be seen in the eye region or, in the case of type 2, in the genital tract. A number of stimuli, for example intensive exposure to sun, fever (fever blisters), menstruation and damage to the facial nerves, can induce the re-emerging facial infections. The mechanism of activation of the dormant viruses is not known. However, it has been established that during the quiet phase the virus does not remain in the skin or mucosal area which is affected but instead is localized in sensory cells in the trigeminal or sacral ganglia. This has been illustrated by the fact that with certain surgical interventions or radiating treatments which concern the trigeminal ganglion, a herpes simplex virus infection can be activated and further that the presence of virus in ganglia of accidentally deceased, healthy individuals can be shown. Most of the individuals from which virus can be isolated have never had any problems with recurrent herpes simplex infections. The occurrence of dormant herpes simplex virus infections in sensory ganglia thus is a common phenomenon and may possibly concern all individuals who have had a primary infection. The reason why only some individuals contract recurrent infections is not known. Variation in the immune defence does not seem to play a role. At least it does not seem as if the presence of high concentrations of circulating antibodies prevents repeated attacks.

The virus cannot be demonstrated in ganglia tissue by use of direct methods such as electron microscopy and immunofluorescence. Only after dissociated ganglia tissue has been cultivated for some days or weeks in cell cultures can viral antigens and infectious virus be identified. This indicates that the virus remains dormant, possibly in the form of virus-DNA integrated into the cellular genome (this condition occasionally has been referred to as *virogeny* in analogy with the condition, lysogeny, in the case of bacteriophages) or free as an episome rather than in the form of a balanced productive infection which involves a very limited number of cells. The presence of virus in a more retracted form may also explain why the immune defence mechanisms cannot eliminate infected cells.

Herpes simplex virus reaches the sensory cells in ganglia via a retrograde (centripetal) dissemination of virus in nerve fibres from the place of the primary infection in the mouth or eye or genital tract mucosal membranes. After activation of the dormant virus there appears to be a short phase of local virus replication in the ganglion and thereafter an anterograde (centrifugal) neurogenic spread of virus occurs. This takes place inside axons and possibly in the form of infectious subviral products. Within a few days a local virus replication is established in the epidermis, which leads to formation of vesicles. The infection does not spread from the place in the epidermis where it is initiated because of the effective containment that is provided by the immune defence. If, however, there should be a dysfunction in the immune defence system, an activated latent herpesvirus infection might disseminate in the body (*see also* Chapter 31).

Herpes zoster (shingles)

Herpes zoster infections are caused by varicella virus. The first contact with this virus usually leads to a development of a classic varicella (*see* Chapter 31). From

places in the epidermis where a local replication of virus occurs, it can spread via retrograde dissemination and establish itself in sensory ganglia in the dorsal roots of the spinal cord or in the cranial nerves. It is not known how frequently this may occur but probably it is a common event. In occasional cases dormant varicella virus can be activated but the mechanism of activation is unknown. In the same way

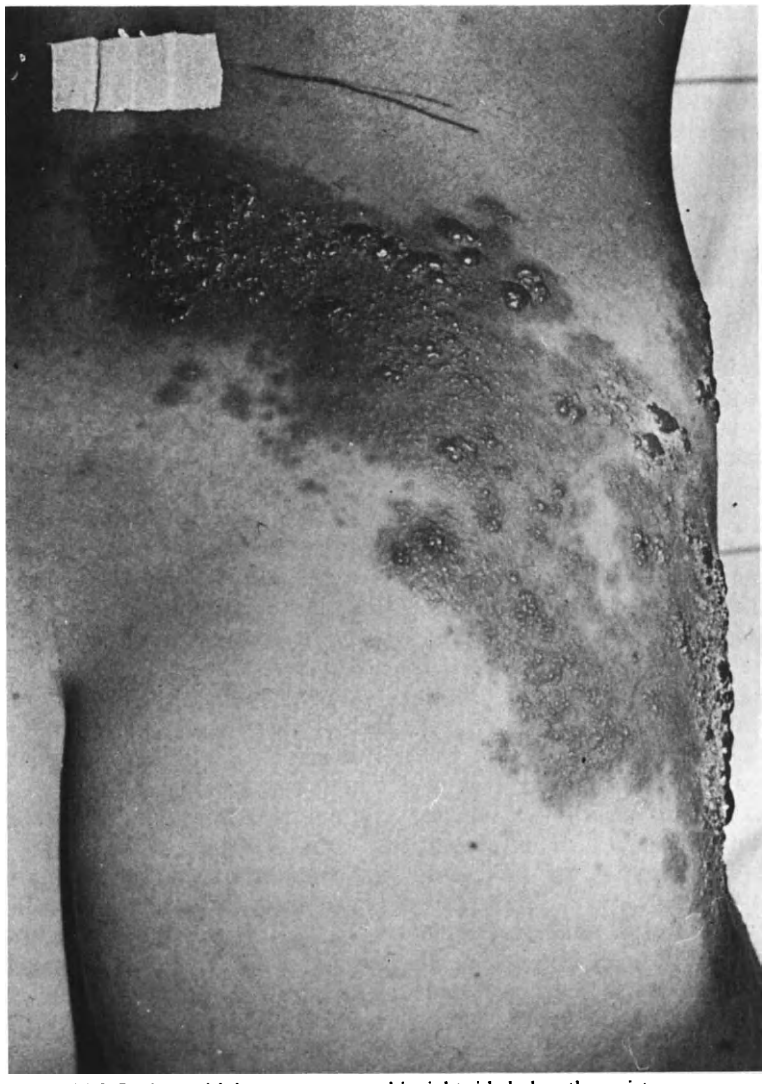

Figure 16.2. Patient with herpes zoster on his right side below the waist (Photo: G. Sterner)

as described above, the virus may replicate in the ganglion where it has been activated and hereafter spread centrifugally in nerve fibres. Local epidermal infections, as a consequence, appear over the whole area (dermatome) which is in contact with nerve fibres from the infected ganglion (*Figure 16.2*). The infection remains restricted to this area because of the occurrence of an effective immune

response. Herpes zoster thus is a clinical manifestation in an immune individual of an activated endogenous varicella virus which has a unique capacity for intracellular spreading. A patient with herpes zoster can spread varicella to non-immune individuals in the vicinity. It has been suggested that occasionally the appearance of cases of herpes zoster can be seen in individuals who have been in contact with a case of varicella. However, there is no logical reason to expect this to reflect virus transmission, since the herpes zoster infection has an endogenous origin.

Persistent infections with other herpesviruses

Herpesviruses other than herpes simplex and varicella-zoster virus can cause persistent infections. However, these do not have the same character as the latent infections in sensory ganglia described above. Endogenous cytomegalovirus infections can become activated in connection with immunosuppressive treatments. This indicates that the virus infection has remained in the body in groups of cells in which the infection is kept under control by the immune defence. The cells which harbour this low grade infection have not been identified.

Another herpesvirus, Epstein–Barr virus (EBV), has a special capacity to infect B lymphocytes. In connection with a primary infection, mononucleosis (glandular fever) may develop (*see* Chapter 31). After an infection with EBV, the virus often remains in a non-productive form in some B cells. Certain viral antigens can be demonstrated in these cells but a complete virus replication does not occur. It has not been demonstrated whether an activated dormant EBV virus infection can cause disease in man. The persistent infection in B cells provide these cells with a capacity to replicate under *in vitro* conditions (immortalization). The possible importance of EBV in the development of special tumours is discussed in Chapter 18.

Slow virus infections caused by conventional viruses

Three different infections in man belong in this category. All these infections concern the central nervous system and they all represent uncommon late complications to infections with the generally occurring (*ubiquitous*) viruses: measles virus, rubellavirus and an SV40-like papovavirus.

Subacute sclerosing panencephalitis (SSPE)

This encephalitis engages both the white and the grey substance. The course of the disease is slowly progressive and the patient usually dies within 6–12 months after the debut of the disease.

In connection with a regular measles virus infection, a complicating encephalitis is encountered at a frequency of 1 case per 1000–2000 infected individuals. However, electroencephalographic changes are seen very frequently in patients with measles. This implies that measles virus has a marked tendency to infect cells in the central nervous system. In rare cases, approximately 1 per 100 000–300 000 infected individuals, virus remaining in the brain is activated 5–7 years later and causes SSPE. Half of all patients who develop SSPE have had their measles infection before the age of 2 years. The disease is three times more common in boys than in girls. The debut in the majority of cases occurs between the age of 2 and 20

years. It is not known in which form the virus remains in the brain tissue and furthermore the mechanism by which the dormant infection is activated has not been defined. Cells with intranuclear inclusions can be identified in the brain tissue of patients with SSPE. These inclusions contain coils of nucleocapsids (*Figure 16.3*). Measles virus antigen can be demonstrated in cells with inclusions and also in other cells but no infectious virus can be isolated. The reason for this is that the infection is caused by a defective virus and recent data indicate that the persistent virus has a poor capacity to synthesize a functional matrix protein. Consequently the virus can not mature in the normal way by budding from the cytoplasmic membrane. In spite of this the defective virus infection can spread by direct cell-to-cell contact. It is possible that the slow development of the disease is because of the dependence upon this mechanism of spread.

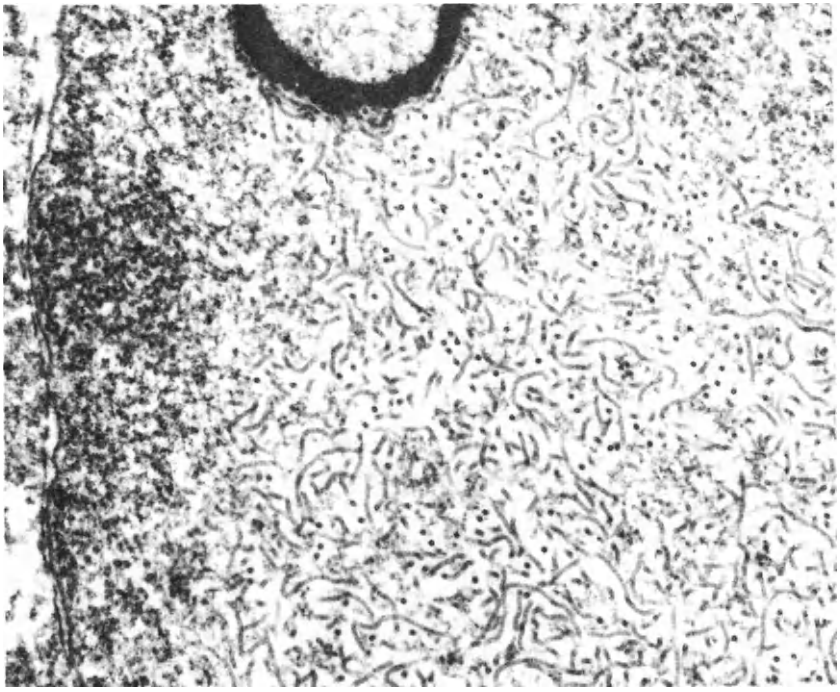

Figure 16.3. Electron-microscopic picture of part of a cell in brain tissue from a patient with SSPE. The nuclear membrane is seen on the left side of the picture. Within the nucleus there are large amounts of tubular structures in longitudinal as well as cross-sections. These structures represent measles virus nucleocapsids (Magnification: ~×36 200. Photo reproduced by permission of Dr K. Johnson, University of Maryland School of Medicine, Baltimore, Maryland, USA)

In explant cultures of brain tissue from patients with SSPE a defective virus infection can be demonstrated. Cultivation of the explanted cells, together with cells which allow complete replication of measles virus, occasionally leads to isolation of a virus which can replicate completely. The properties of the isolated viruses are indistinguishable from those of regular wild measles virus.

The synthesis of large quantities of measles antigen in the brain causes a local hyperimmunization. Increased concentrations of IgG can be demonstrated in the

cerebrospinal fluid and by use of electrophoresis it can be shown that this IgG has an oligoclonal character. The occurrence of a pronounced increase of the content of measles antibodies in serum and the demonstration of readily identifiable antibodies in cerebrospinal fluid are important for the establishment of the diagnosis. Apparently, the persistent virus infection can develop in the presence of the intense humoral immune response and, as shown separately, a normal cell-bound immunity. Possibly the defective virus has a low capacity to insert virus-structural components into the cytoplasmic membrane hereby avoiding the immune surveillance mechanisms. The frequency of SSPE appears to be reduced at least ten-fold when the occurrence of wild virus infections are prevented by using a live measles vaccine. This probably depends on the vaccine virus having a much lower tendency to infect the central nervous system.

Progressive rubella panencephalitis (PRP)

This unusual disease shows many similarities to SSPE, but it is caused by rubella-virus. In contrast to the situation with the persistent measles virus infection, no intranuclear inclusions can be demonstrated. The debut occurs between the age of 5 and 20 years and the majority of cases have occurred in individuals with a congenital rubella infection. Virus has been isolated in a single case and in some other cases viral antigen has been demonstrated in brain tissue. The occurrence of large amounts of antigen in the brain tissue causes a local synthesis of oligoclonal rubellavirus-specific IgG in analogy with the situation in patients with SSPE. PRP, in further analogy with SSPE, is a fatal disease and the time course of the disease usually is less than one year although cases of longer duration have been encountered.

Progressive multifocal leucoencephalopathy (PML)

PML occurs primarily in patients with some underlying disease in the lymphatic system, most often Hodgkin's disease. The underlying disease gives an immunosuppression which allows the activation of a dormant virus. In a few cases PML has been seen in patients who have received immunosuppressive treatment. The virus (JC,BK) which is activated belongs to the papovavirus group and it is similar to SV40. Two different strains of virus have been isolated. It was argued for some time that PML might be a late complication to an infection with SV40 virus which patients had received via an injection of polio vaccine contaminated with this virus. However, this was later proved not to be the case. The virus which causes PML appears to be of human origin and studies of the occurrence of antibodies show that infections with this virus are relatively common. These infections do not appear to give any symptoms but they provide possibilities for the establishment of a persistent infection in the body.

In connection with a disease-related or a treatment-induced immunosuppression the virus can become activated. It attacks primarily oligodendroglial cells, in which virus particles accumulate in the nucleus and cause large inclusions (*Figure 16.4*). Destruction of oligodendroglial cells by the replication of virus causes a loss of function leading to demyelination. Since each oligodendroglial cell is responsible for the myelination of about 50 neurons, destruction of only a limited number of glial cells will lead to comprehensive damage.

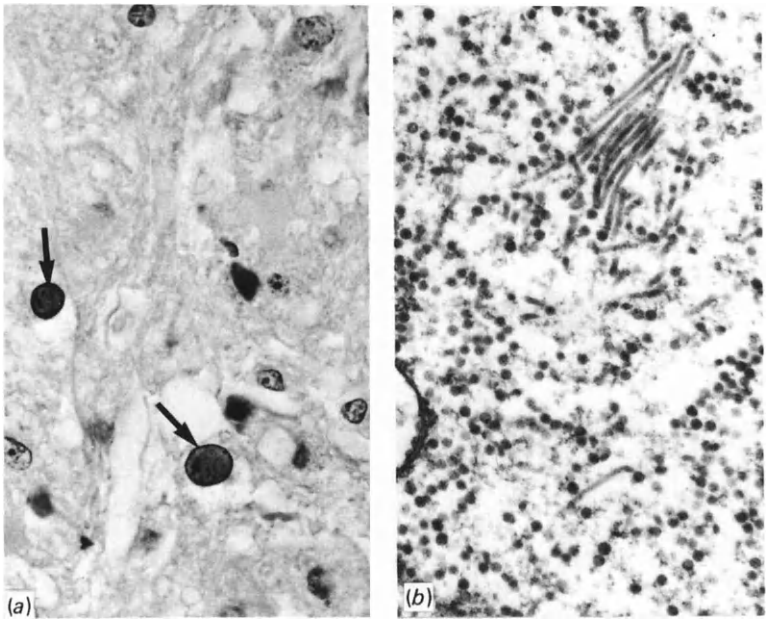

Figure 16.4. Histologically stained brain tissue from a patient with PML. (*a*) The arrows indicate nuclei of two oligodendroglial cells which each contain a large inclusion. These inclusions contain large amounts of papovavirus particles which are seen in the electron micrograph (*b*) (Magnification: (*a*) ×385, (*b*) ×21 000. Photo reproduced by permission of Dr K. Johnson, University of Maryland School of Medicine, Baltimore, Maryland, USA)

PML is an uncommon disease which debutes at the age of 30–70 years. It usually has a fatal outcome within about a year. The diagnostic identification of this infection can be complicated since the virus is difficult to isolate and the immune reactions usually are poor on account of the underlying disease. In some cases the amount of virus present in the brain tissue allows a direct identification of virions by use of electron microscopy and the possibility exists for using immune electron microscopy to determine the type of virus.

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17

Slow infections caused by non-immunogenic infectious agents

Erling Norrby

As mentioned in Chapter 16, slow virus infections can be caused not only by conventional viruses, for example measles virus, but also by certain atypical infectious agents. The majority of the features characteristic of the classic infectious processes are absent in the degenerative disease of the central nervous system which can be caused by this kind of atypical infectious agent. Thus there is no inflammatory reaction; hence the term *encephalopathy* is used instead of encephalitis.

Different forms of infectious encephalopathy

Four different diseases in animals and man are included in infectious encephalopathies. These are scrapie in sheep, encephalopathy in mink, and kuru and Jakob–Creutzfeld disease in man. The human diseases are uncommon but principally important. The description of their pathogenesis has introduced completely new concepts into the field of infectious diseases. The possibility that other non-inflammatory degenerative diseases may also have an infectious aetiology must be considered. It has been difficult to characterize the atypical infectious agents since they cannot be studied in cell cultures. All investigations have to be performed with infected animals and these develop the disease only after relatively long incubation periods. Since, in addition, the atypical infectious agents lack antigenic activity, their capacity to cause infection is the only biological activity which can be used for identification.

Scrapie

Scrapie was originally considered to be a genetic disease. However, during the 1930s it could be shown that the disease was transmissible by experimental infection of healthy animals with brain extracts from diseased animals. The incubation period in infected animals varies between 9 months and 4 years. From the time of appearance of the first symptoms there is a relentless development towards extensive brain degeneration which eventually causes the death of the animal. As was mentioned above, there is no inflammatory reaction in the brain tissue of the infected animal and furthermore there are no other signs indicating that the organism mobilizes any defence against the infectious agent.

It has been possible to transmit the disease to mice and other rodents. The incubation period in these animals is 4–13 months. This relatively shorter incubation period offers improved possibilities for experimental studies but experiments with scrapie-infected mice also require a considerable patience. The development of infection after inoculation of mice is illustrated in *Figure 17.1*. The infectious process can be followed by measuring the amount of infectious agent in different tissues by the further passaging of tissue homogenates into other mice. Immediately after inoculation, there is a phase during which no infectious agent can be detected.

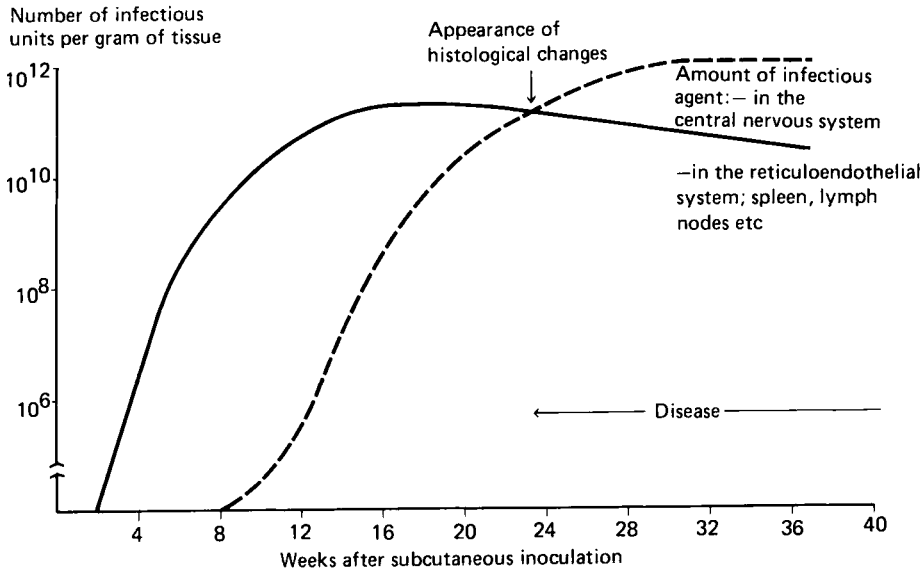

Figure 17.1. Development of infection after subcutaneous inoculation of mice with the scrapie agent

After this, the agent appears in increasing concentrations in the reticuloendothelial system and after another month it can be recovered from the central nervous system. There is a gradual increase in the amount of infectious agent in the brain tissue and eventually as much as 10^{8}–10^{12} infectious units per gram of tissue can be demonstrated. Changes in the brain tissue, which parallel the development of symptoms, appear relatively late. Among tissue changes can be mentioned vacuolization of cells (spongiform degeneration) and occasional amyloid changes. This amyloid does not seem to be composed of immunoglobulin light chains and thus does not indicate the occurrence of any immune response. The development of disease varies in different experimental systems depending upon the mouse strain used and the strain of agent inoculated. This emphasizes the importance of the genetic constitution of the host and furthermore the fact that different variants of the infectious agent may have different pathogenic potential.

Mink encephalopathy

Mink encephalopathy and scrapie are caused by the same infectious agent. The introduction of this agent into minks occurred when the animals were fed with sheep-meat contaminated with the scrapie agent. After introduction, the infectious

agent appears to be capable of spreading effectively among groups of minks and the only possibility of eliminating the disease is a complete slaughtering of infected animals. A similar method was previously used successfully for eradicating scrapie among sheep in Iceland.

Kuru

Kuru is a remarkable degenerative disease in the central nervous system which occurs amongst the Fore tribe in New Guinea. In the language of this people 'kuru' means to tremble and tremor is an important symptom of the disease. The infectious aetiology of this disease and the routes for spreading of the infectious agent has been elucidated by Gajdusek. The disease has an unique epidemiology. In the 1950s it occurred primarily among children and adult women (*Figure 17.2a* and *b*). The attack rate was high and since the disease in all cases progresses to death of the patient within about 6 months from the first appearance of symptoms there developed a relative excess of men in the community.

The transmission of the infectious agent was shown to occur in connection with the ritual cannibalism which was practised among the Fore people. As a funeral

Figure 17.2(a). Boy with kuru in the final stage of the disease (Photo reproduced by permission of Dr C. G. Gajdusek (1977). Department of Health, Education and Welfare, National Institutes of Health, Bethesda, Maryland, USA)

Figure 17.2(b). Woman with kuru. The patient in the picture still has mild symptoms and can stand upright by use of two sticks. Note that the woman is pregnant and most likely will give birth to her child before she dies with the disease. No child who has been born by a woman with kuru has developed the disease and it therefore appears that the infection does not spread beyond the placenta. (Photo reproduced by permission of Dr C. G. Gajdusek, Department of Health, Education and Welfare, National Institutes of Health, Bethesda, Maryland, USA)

ritual the diseased individual was consumed by his relatives and during the preparation of the meal children and women came into contact with infected blood. In the year 1957 the ritual cannibalism ceased and no person born after this year has developed kuru (*Figure 17.3*). However, the disease still occurs which implies that the incubation period in man may be as long as 25 years or more. Within another generation the disease will be completely extinguished. Vertical transmission of the infectious agent does not appear to occur. Children born of women developing kuru have not contracted the disease.

The infectious agent has been transmitted to experimental animals. By inoculation of brain tissue from patients with kuru the disease has been reproduced first in chimpanzees, later in different kinds of monkeys, and some isolates give disease in mice and other rodents. In experimental systems the incubation period has a minimal duration of 7–8 months. The development of symptoms is similar to that in man and in the brain tissue of diseased animals degenerative non-inflammatory changes can be identified. There are no signs of any mobilization of either immune or interferon defence mechanisms.

Jakob–Creutzfeld disease

Jakob–Creutzfeld disease occurs worldwide and it is considered to be the industrialized world equivalent to kuru. The disease has a course similar to that of kuru and the histopathological changes in the brain have a similar character. The disease makes its appearance at an average age of 57 years (variation 35–75 years) and the patient dies within 4–12 months from atrophy of the brain. The disease occasionally is described as presenile dementia, i.e. an aging of the brain before the stage of physiological aging. The incidence of the disease is 1–2 cases per 1 million people. In certain countries such as Israel and Czechoslovakia an accumulation of cases in time and space has been found. About 15 per cent of all cases occur within certain families.

Jakob–Creutzfeld disease has been transmitted to chimpanzees and other experimental animals by using infected brain tissue from man. Kuru and Jakob –Creutzfeld disease both in man and animals cannot be distinguished on the basis of the clinical features of the disease or the histopathological changes in the brain. The possibility of transmission to experimental animals is equally good when material from patients with the non-familiar or the familiar form of Jakob –Creutzfeld disease is used.

Biological and physical-chemical properties of atypical infectious agents

The infectious agents which cause the diseases discussed above have not been purified and thus have not been characterized chemically or morphologically. Therefore their nature is still enigmatic. However, by studying the infectious agent identifiable in organ extracts it has been possible to show that the agent has remarkable properties. In spite of comprehensive analysis it has not been possible to detect any antigenic activity. Furthermore the agents have been found to have a very high resistance against both chemical and physical treatments. It is completely resistant to enzymes which degrade nucleic acids and also to treatment with β-propiolactone and formalin. Consequently the infectious agent may be isolated, for example, from formalin-fixed brain tissue which has been stored for many

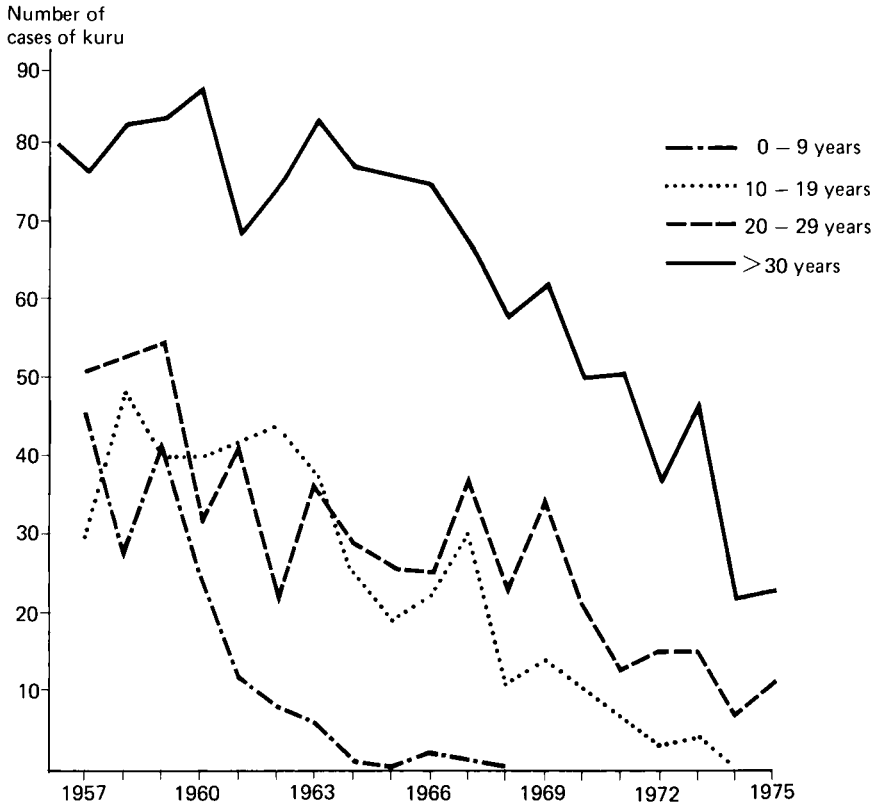

Figure 17.3. The epidemiology of the kuru disease in the Fore people in New Guinea. Note that no person born after 1957 has developed the disease

years. Heating to 100°C does not destroy all infectious activity. Treatment with u.v. light or ionizing radiation leads to destruction of the infectious property only when very high doses are used. This means that the infectious agent does not contain nucleic acid in any amount corresponding to that found even in the smallest conventional viruses. If nucleic acid is a part of the atypical infectious agents – some scientists have even questioned this – its molecular weight should not exceed 150 000.

How do atypical infectious agents spread?

The ways in which atypical infectious agents spread have not yet been defined to a major extent. *Figure 17.4* presents different partly substantiated, partly speculative modes of transmission and considers the possibility that all four diseases discussed in this chapter may be caused by an infectious agent with a common origin.

The spread of scrapie infection in a group of sheep has been considered to result from the behaviour of consuming placental tissues after the birth of lambs. The transmission of scrapie to mink also appears to be explained. In contrast there is no

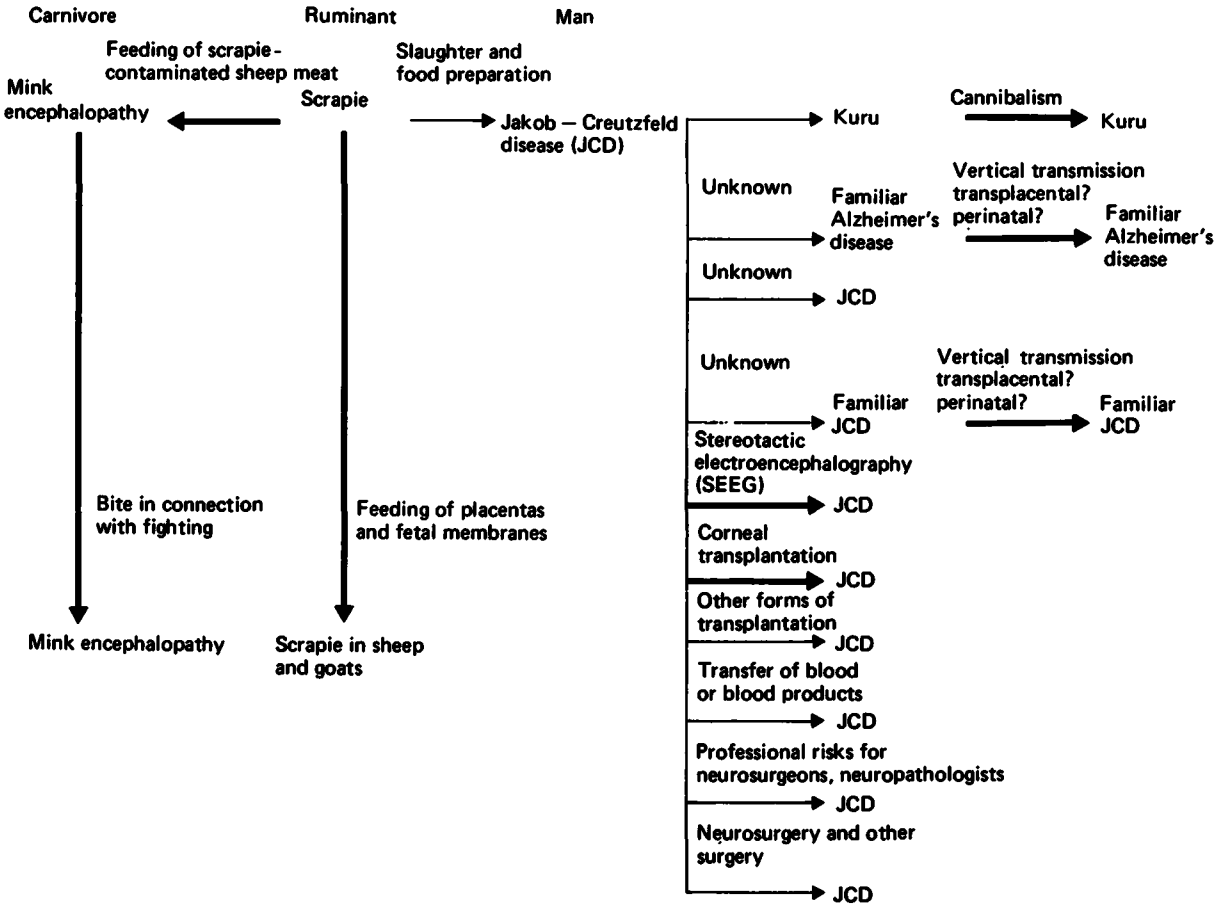

Figure 17.4. Possible routes of transmission of atypical infectious agents which can cause spongiform encephalopathies. Speculative connections are indicated by thin arrows and substantiated or probable routes of transmission by thick arrows. (Reproduced by permission of Dr C. G. Gajdusek, Department of Health, Education and Welfare, National Institutes of Health, Bethesda, Maryland, USA)

evidence that the scrapie infectious agent can cause disease in man. Thus the possible relationship depicted in *Figure 17.4* is hypothetical. It should be noted that the extreme stability of these agents implies that they may retain their infectious property during most of the conditions which are used for preparation of food. Jakob–Creutzfeld disease occurs also in countries where no cases of scrapie have been found.

Concerning man it is possible that kuru represents an epidemic accumulation of cases of Jakob–Creutzfeld disease as a consequence of the practising of ritual cannibalism. In industrialized countries also a transmission of tissue occurs in connection with organ transplantations. In one case Jakob–Creutzfeld disease was transmitted in connection with a corneal transplantation. Other medical interventions also may cause risks of transmission of the atypical infectious agents. One such transmission accidently occurred to two persons of the age of 20 years in connection with stereotactic electroencephalography. The incubation times were 16 and 19 months. The electrode which was used for the measurements had previously been used in the examination of a patient who later developed Jakob–Creutzfeld disease. The technique which was used for sterilization did not suffice to inactivate the agent. This experience, together with the observation that some cases of the disease have occurred among persons (neurosurgeons, neuropathologists) most likely to have been contaminated in connection with professional activities, has raised the question about the risk of transmitting atypical infectious agents with different medical procedures. In this connection it should be noticed firstly that it is possible to destroy the infectious property of these agents by, for example, autoclaving or treatment with strongly oxidizing solutions, organic solvents or strong detergents. Secondly, it is apparent that transmission of the infection only occurs through direct contact. In connection with post mortem dissection, for example, it is important therefore to avoid transmission via blood contamination by adopting the same rules which are used to prevent spread of hepatitis B virus infections. Finally, it should be emphasized again that Jakob–Creutzfeld disease is a very uncommon disease. The possibility that other similarly non-inflammatory and possibly familiar diseases may also have an infectious origin is however worth further consideration.

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(*See also* bibliographic references in Chapter 16)

18

Viruses and tumours

Eva-Maria Fenyö and Britta Wahren

Viruses have been considered as one potential cause of tumour diseases since the beginning of this century. In 1908 Ellerman and Bang showed that erythromyeloblastosis in fowls can be transmitted by cell-free filtrates. In the following years, Rous demonstrated a viral aetiology for various forms of solid tumour growth in fowl. However, the full impact of Rous' discovery was not appreciated until the discovery of a murine leukaemia virus by Gross in 1951. From that time on the avian and murine virus-induced tumours have come to represent very important models for studies of the relationship between viruses and tumours.

Many different viruses can change permanently the growth characteristics of cells *in vitro*, i.e. cause transformation (cf. Chapter 11). Properties of both the virus and the cell decide whether transformation will occur. In this connection the importance of integration of the whole or part of the tumour virus-genome into host-cell DNA should be emphasized. It is generally assumed that *in vitro* transformation of cells and the development of tumours in the animal host are based on similar phenomena. However, in the living organism there are a number of different conditions that need to be fulfilled before a tumour can develop. The present chapter describes the importance of viruses in the appearance of tumours in animals and in man.

The tumour cell

Tumour cells can be distinguished from normal cells by their morphology and altered growth pattern. These properties are genetically determined. The tumour cells may differ from normal cells in the size and appearance of their nuclear and cytoplasmic structures. In tissue culture, tumour cells often form multiple cell layers with irregular orientation of individual cells. From the point of view of the living organism, tumours may be *benign* or *malignant*. Whereas malignant tumours show a varying degree of invasive growth and spread to distant organs (i.e. metastases), benign tumours lack both the capacity for comprehensive invasive growth and also for distant spread.

Transformed cells show changes on their surface. Some of the normally occurring proteins and carbohydrates may be lost, new surface components may be added. Losses or additions can be detected with the help of immunological techniques. Fibronectin is one major glycoprotein of the normal cell surface that may change in distribution or disappear after viral transformation. The intracellular transport of

certain metabolites may be modified. These changes may be accompanied by physical and chemical changes, such as alteration in surface charge and cell agglutinability by lectins that measure specific carbohydrate-binding properties.

Normal cells can be converted into tumour cells in one step by the attack of strongly mutagenic agents or tumour viruses. In other cases the process may develop stepwise. A *carcinogen* (the *initiator*) will act as a mutagen whereas another substance (the *promotor*) or a cocarcinogen will complete the transformation leading to the appearance of the tumour cells. A cocarcinogen has a weak or no cancer-causing effect by itself but it can enhance the effect of carcinogens.

Viruses as carcinogens

Certain DNA and RNA viruses are able to induce tumours in animals; they are said to be *oncogenic* (*Table 18.1*). RNA tumour viruses all belong to the retrovirus group, whereas oncogenic DNA viruses have members among papovaviruses, adenoviruses, herpesviruses and poxviruses. Some viruses have a direct oncogenic effect whereas others function as cocarcinogens. Susceptibility to the effects of a certain carcinogen in an individual depends upon genetic predisposition, immunological factors, hormonal milieu, age, or various combinations of these different factors.

TABLE 18.1. Naturally occurring animal tumours caused by viruses

Tumours	*Nucleic acid*	*Virus family*	*Type of virus*
Avian leukaemia	RNA	Retrovirus	Avian leucosis virus
Lymphatic leukaemia in mice			e.g. Gross virus
Leukaemia and lymphoma in cats			Feline leukaemia virus
Leukaemia in cattle			Bovine leukaemia virus
Breast cancer in mouse			Mouse mammary-tumour virus
Papillomas in different species	DNA	Papovavirus	Papilloma viruses
Adenocarcinomas in the kidneys of frogs		Herpesvirus	Lucké frog virus
Neurolymphomatosis in chickens			Marek's disease virus
Myxomatosis, fibromas in rabbits		Poxvirus	Myxomatosis virus
Epitheliomas in rhesus monkeys			Yaba virus

When a virus transforms a cell the whole or part of the virus-genetic material becomes integrated into the chromosomal DNA of the cell. The inserted virus-genetic material then replicates together with the DNA of the cell. The continued presence of the virus-genome or parts thereof is required for maintenance of a stable transformation. In abortive transformation loss of the integrated genome occurs, and the cells may resume normal morphology, growth and metabolism. This is called *reversion*. Integration of the entire viral genome may under special conditions provide possibilities for activation and replication of complete virus. Defective virus (*see* Chapter 12) also may cause cell transformation if the transforming genes (usually comprising a small part of the virus-genome) are integrated. Replication of the virus is then only possible with a helper virus or after recombination with another virus.

Genetic predisposition

Certain animal species, for example mice and hamsters, are especially susceptible to tumour induction by viruses. Mice can be inbred to give special strains that develop, for example, leukaemia with a high frequency. Such mouse strains possess a viral genome integrated in their germ line.

In man also genetic factors increase the risk of acquiring cancer. Patients with hereditary adenomatosis show an increased risk of colon cancer, mongoloid children with an additional chromosome 21 have a raised frequency of leukaemia, patients with ataxia telangiectasia develop lymphomas and patients with xeroderma pigmentosum develop skin cancers. Even though viruses may not play any role as causative agents in these diseases, cells from such patients are much more susceptible to *in vitro* transformation by oncogenic viruses.

Environmental factors

The incidence of human tumours is presently increasing as a whole. When a population moved from a country with, for example, a low incidence of breast cancer, to another country with a high incidence, the higher incidence was slowly acquired. This and similar cases point to a strong environmental influence. Increase in the average human life span may increase (a) the risk of exposure to carcinogens, (b) the accumulated doses of ionizing radiation and chemical carcinogens and (c) the risk of cancer due to impaired cellular repair mechanisms with increasing age. In addition, modern society may generate new carcinogenic agents. All these factors may also promote tumour appearance after infection with an oncogenic virus.

Traces of viruses in tumour cells

The occurrence of viral genomes integrated into the genetic material of tumour cells can be demonstrated in many different ways.

Viral nucleic acids Virus-specific DNA can be demonstrated by hybridization with purified viral DNA. In a similar way RNA can be detected. The presence of virus-specific mRNA in tumour cells provides additional evidence for the presence of virus-DNA or RNA.

Infectious virus After fusion or cocultivation of transformed cells with normal permissive cells, mature infectious particles occasionally can be demonstrated. One prerequisite for this is that the transformation has occurred with an intact virus-genome. In connection with transfection (*see* Chapter 11) with DNA from transformed to normal cells, infectious virus may occasionally be isolated.

Virus-induced proteins When cells are infected and transformed, new virus-induced antigens may appear in the nuclei, in the cytoplasm and on the cell surface. Virus-coded proteins are present in the nucleus, such as the T antigen of papovaviruses. T antigens represent early proteins, transcribed from a part of the viral genome which may be active even in the absence of viral replication. T antigens are induced both in lytic and transforming infection.

Group-specific and/or type-specific virion antigens can often be demonstrated in certain retrovirus and herpesvirus systems. In the cytoplasm of RNA tumour virus transformed cells polypeptides may appear that carry antigens related to some of the viral structural proteins and the *oncogene*. Since the oncogenes of RNA tumour

viruses originate from normal cells, their products cannot be regarded as strictly virus-specific. Indeed, some of the oncogene products are found in normal cells. Whether the antigens observed in RNA tumour cells are the result of qualitative or quantitative changes remains to be clarified. Finally, the immunological reaction of the host against the cell surface may mediate a rejection of the tumour cells. For this reason, antigens involved in this process have been called *tumour-specific transplantation* (TST) antigens.

Immune response to tumours

Animal tumours have been used by immunologists in studies of cellular and humoral immune reactions. It was observed that young mice exposed to oncogenic viruses regularly develop tumours, whereas adult mice mount an immune response against the virus. Tumours induced by the same virus appear to carry a common antigen. Such antigens were first shown for polyomavirus-induced mouse tumours and have subsequently been identified in many DNA and RNA virus-induced tumours. Because the antigens are the primary targets for the rejection response of the host they were designated tumour-specific transplantation (TST) antigens.

Antigens on the surface of tumour cells were also demonstrated by reactions with antibodies. Cytotoxic lymphocytes, either naturally occurring or induced by immunization with specific antigens, were shown to kill tumour cells *in vitro*. In many cases it is still not known whether the rejection reaction is mediated by the antibodies and/or by the cytotoxic lymphocytes.

Membrane antigens were thought to be important not only as footprints of viral genomes, but also as structures involved in growth control. Contact-dependent signals and hormones that stimulate or restrict cell proliferation may act through membrane receptors. TST antigens have been shown to be unusually stable and to survive selection in, for example, preimmunized mice. It was assumed that the stability of TST reflects a membrane change essential for neoplastic behaviour.

The most important candidate for a transformation protein that may also function as a transplantation antigen has been found in the SV40 system. The early region of SV40 genome, that is involved in the initiation and maintenance of transformation, codes for two proteins. One of these is a 94 000 protein, called large T antigen that induces a cellular response during rejection of tumours. Injection of the purified protein into mice will induce cytotoxic lymphocytes which are able to kill the tumour cells *in vitro*. This approach shows how knowledge about the genome organization of a virus may help to define rejection antigens in tumour cells.

Immune responses to feline leukaemia and sarcoma virus-induced tumours have been studied in detail in the natural host species. All the viral gene products are immunogenic in the cat but the disease is only prevented by immune reactions to the product of the oncogene. Humoral immunity seems to play a major role in elimination of these tumour cells, since binding of specific antibodies and complement to antigens on the cell surface lyse the target cells. Whether immune response against a specific transformation product will be the sole mechanism preventing disease is too early to say. Immune reactions against viral structural products as well as derepressed cell surface antigens (like embryonal antigens) may also take place and cooperate with immune reactions against the specific transformation product. Because of the uncertainty regarding the role of surface antigens in rejection reactions the term *TAT* (*tumour-associated transplantation*) antigen is frequently used instead of TST.

Tumour viruses in animals

Retroviruses are present in normal cells

Retroviruses are enveloped RNA-containing viruses that transcribe their RNA into proviral DNA (described in detail in Chapter 8). The DNA provirus inserts itself (*integrates*) into the chromosomal DNA of the host cell. When integrated, viral genetic material is transmitted vertically from parent to progeny, along with other cellular genes. When packaged into infectious particles transmission occurs horizontally. In this latter form, four morphologically distinct types of retroviruses, types A, B, C and D, have been described (*Figure 18.1*). The type C viruses can be considered either as viruses that are able to replicate as part of a cell's genetic machinery or, alternatively, as unusual sets of cellular genes that may escape from

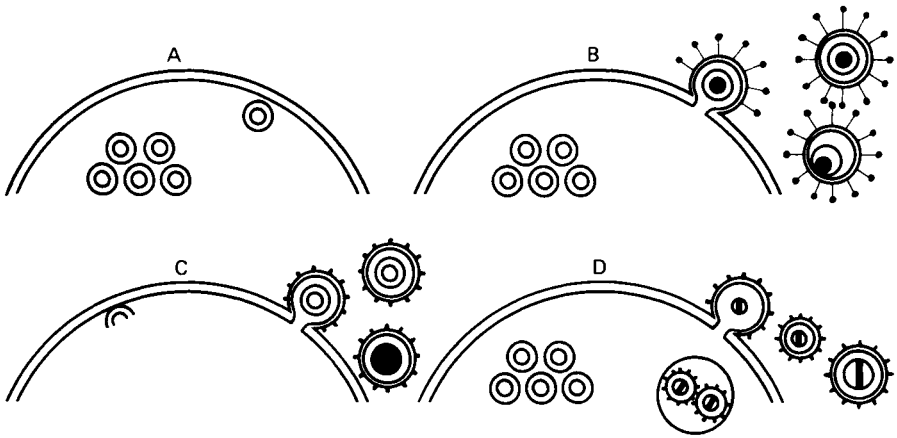

Figure 18.1. Schematic description of the appearance of different kinds of retrovirus. One kind of immature intracellular precursor are called A particles and they represent a ring-shaped nucleocapsid structure. B, C and D particles are different kinds of virions which all mature by budding from the cytoplasmic membrane. The B particles have an envelope with long projections. The nucleocapsid either has a central location in the virions or is more eccentrically located. The C particles have an envelope with short projections and a centrally located nucleocapsid with a somewhat varying appearance in different particles. The D particles are pleomorphic and have a morphologically unique nucleocapsid. The virions can be observed both extracellularly and in cytoplasmic vacuoles

the host's cell-genome. When they escape the viral genome they may be reinserted in other parts of the same cellular DNA, in other cells of the body, in other animals of the same species, or even in other species. Insertion into the same cell leads to the occurrence of multiple viral copies at different sites of cell-DNA. Insertion into the germ cells results in the transmission of viral genes from parent to offspring. But even after integration into the germ line such genes would have the unusual capacity to come out, transfer themselves, and perhaps other cellular genes, to new cells and new species.

Endogenous viruses are thus basically cellular genes present in multiple copies that have the ability to give rise to infectious and potentially pathogenic virus particles. The nucleic acid probes generated from the virus particles have been useful for studies of the evolutionary relationship between retroviruses, as well as

the relationship between their animal hosts. For example, the primate endogenous viruses have been divided into classes on the basis of their nucleic acid homology with baboon endogenous virus (BaEV). Cocultivation of baboon cells with other mammalian cells (also human cells) yields BaEV. Using this nucleic acid as a probe, it was found that the degree of homology between BaEV and the cell-DNA from different primates corresponded to the traditionally defined evolutionary tree (*Figure 18.2*). The results have even suggested that the geographic origin of the development of *Homo sapiens* was in Asia rather than Africa.

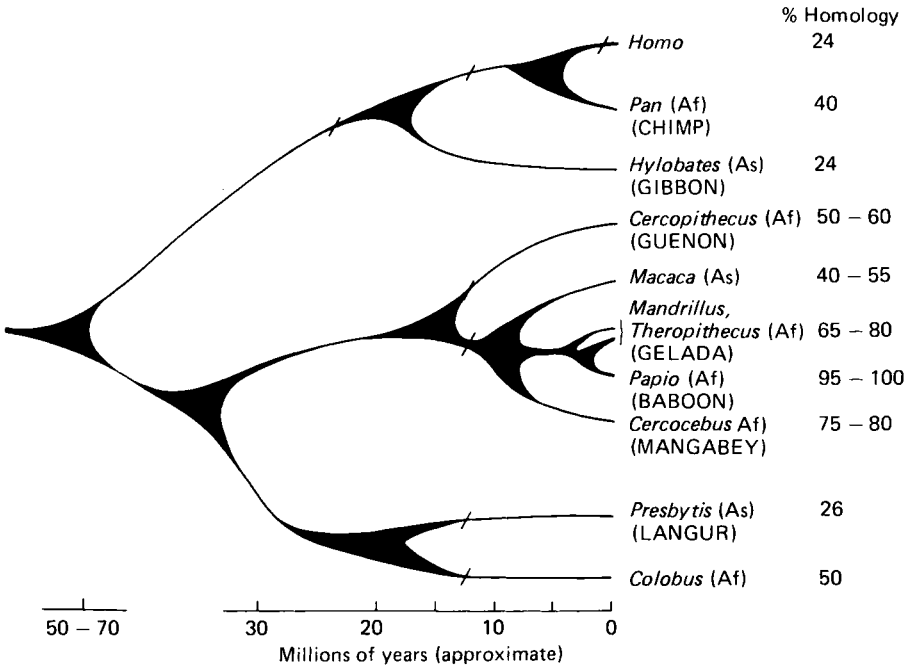

Figure 18.2. The relationship between man and African (Af) and Asian (As) monkeys. The similarity between cell-DNA and DNA of an endogenous baboon virus, BaEV, is expressed as a percentage of nucleic acid homology. (Modified from Donehower *et al.* (1977). *Journal of Virology*, **21**, 932, by permission of the American Society for Microbiology, Washington DC, USA)

Endogenous viruses from cells of most vertebrates can occasionally be induced to form mature virus particles by cocultivation, irradiation, or treatment with halogenated nucleotides. These viruses are not oncogenic, as a rule. Even if human DNA contains sequences distantly related to BaEV, normal human cells do not express known type C viral gene products. Neither has an endogenous retrovirus been activated under stringent conditions from human cells. The type C particles occasionally seen in full term placental tissue remain enigmatic.

Tumours caused by RNA viruses

Many unrelated groups of retroviruses exist in nature. All groups have non-pathogenic as well as pathogenic members. Retroviruses induce a variety of cancers that occur after short or long latent periods. Viruses that cause disease after short latent periods are referred to as *acute viruses* whereas viruses that cause disease after long latent periods are called *non-acute viruses*. Acute viruses have oncogenes

that enable them to transform cells *in vitro*. The oncogenes consist of host information that has been inserted into the viral genome (*Table 18.2*). These insertions have occurred, presumably by recombination of a non-acute virus with host-genetic information, in such a way that the inserted material can be expressed and transmitted as a virus. Thus, immediate disease results from a host gene that becomes overexpressed due to its location within the viral genome. Examples of such diseases are sarcoma, carcinoma and various forms of leukaemia and lymphoma.

TABLE 18.2. Examples of cellular genes that are retroviral oncogenes

*Cellular gene**	*Species of origin*	*Virus that carries the oncogene*	*Associated disease*
c-fes	Cat	Feline sarcoma virus	Sarcomas
c-mos	Mouse	Murine sarcoma virus	Sarcomas
c-sis	Woolly monkey	Simian sarcoma virus	Sarcomas
c-src	Chicken	Rous sarcoma virus	Sarcomas
c-erb	Chicken	Avian erythroblastosis virus	Erythroid leukaemia
c-myc	Chicken	Avian myelocytomatosis virus	Carcinomas Blood-cell disorders Lymphomas
c-abl	Mouse	Abelson leukaemia virus	Lymphomas
c-ras	Rat	Harvey sarcoma virus	Sarcomas

* All of these genes were originally detected in the genome of an acute virus. Retroviral oncogenes have the same three-letter designations as their cellular homologues but with no prefix or the prefix v-

Viruses causing late disease have all the viral genes necessary for replication but apparently lack genetic information for transformation. Still they cause leukaemia, lymphoma and mammary carcinoma, which arise after a long latency. During the long latency, virus integrates at several different sites in the genome of infected cells. Disease induction, however, only occurs when proviruses are integrated adjacent to a specific site. This specific site is one of the cellular homologues of viral oncogenes and varies with the type of malignancy. The provirus appears to govern the potentially-oncogenic cell genes with overexpression as a result. Thus, non-acute disease like acute disease appears to result from viral encoded overexpression of host-genes. The rapid onset of specific disease after infection with an acute virus is explained by the linkage of the viral genes with potentially oncogenic cellular genes in the viral genome. No such linkage is found in non-acute viruses. In the latter case, disease occurs only if a provirus fortuitously is integrated adjacent to a potentially oncogenic host-gene in a position that leads to overexpression of this gene. The fact that new isolates of acute viruses frequently have oncogenes that have already been identified suggests that a limited number (some 16 to date) of cellular genes have the potential to recombine with retroviruses in such a way that they act as oncogenes. These genes appear to be highly conserved among vertebrates, since they occur in species as unrelated as chicken and man. They are able to transform target cells from a wide variety of species. The ability of viral oncogenes to alter normal cell growth suggests that their cellular homologues control normal growth and development. The high degree of conservation of these genes suggests that they regulate extremely basic cellular processes.

The studies of protein products encoded by oncogenes have begun recently. Best studied is the product of *src* gene, pp60*src* (*see below* and *Table 18.2*). The viral and cellular forms of pp60*src* have similar sizes (60 000), peptide maps and antigenic properties. They are connected with the plasma membrane of the cell, are phosphorylated and display protein kinase activity, phosphorylating tyrosine in protein substrates. Phosphorylation of tyrosine was a hitherto unknown reaction that now appears to be an important regulatory mechanism in normal as well as transformed cells. It is unknown in what way overexpression of oncogenes alters the phosphorylation processes and whether these alterations are fundamental for malignant transformation.

Avian leukaemia and sarcoma

Avian retroviruses induce acute as well as non-acute disease. In the case of acute disease the host response occurs immediately after the virus infection. The oncogene of the virus determines the type of target cell affected (cf. *Table 18.2*). For example, Rous sarcomavirus that contains the *src* gene transforms fibroblasts *in vitro* and causes sarcomas when inoculated into chickens. The *erb* gene of avian erythroblastosis virus causes abnormal growth of erythroblasts and fibroblasts and induces erythroblastoid leukaemia. Both viruses cause disease within 3 weeks of inoculation in 100 per cent of animals. A third example is avian myelocytomatosis virus that carries the *myc* gene and causes abnormal growth of endothelial cells, blood cells and fibroblasts. There is a 100 per cent incidence of carcinoma and/or blood cell disease within seven weeks of infection.

In contrast to the acute disease, non-acute disease appears to result from rare and delayed responses of a host to an infection. Not all infected animals develop disease and the type of disease is not strictly determined by the virus. For example, RAV-60 virus induces B-cell lymphoma in 50 per cent of an infected chicken population but, in addition, it induces a low incidence of a variety of other diseases such as fibrosarcomas, osteopetrosis and anaemias. Most of the lymphomas occur between five and nine months after infection, whereas the other diseases occur after about one year.

Murine leukaemia and sarcoma

Conforming with avian viruses, two groups of mouse C-type RNA tumour viruses can be distinguished: acute and non-acute viruses. All acute viruses are replication-defective due to the insertion of the cellular sequences at the expense of viral structural genes. Tumours develop within a few weeks after virus inoculation and include sarcomas, B- or O-cell leukaemias and erythroleukaemia. The cellular insert in sarcomaviruses has originated from either rat or mouse cells depending on whether recombination occurred during rat or mouse passage of the virus (cf. *Table 18.2*). Examples of oncogenes are *ras* (from rat) and *mos* (from mouse). The *abl* gene of Abelson leukaemia virus causes abnormal growth of fibroblasts and macrophages and induces B- or O-cell leukaemias *in vivo*.

A large group of non-acute viruses is found among the mouse leukaemia viruses. The members of this group can replicate and, similar to their avian counterparts, carry no oncogene. Virus has to be inoculated into newborns. In mice that carry the leukaemia virus integrated in the germ line, virus has to be activated early in life. Clinically overt disease, usually T-cell leukaemia, is preceded by a longlasting high

titre viraemia. Several mechanisms have been considered in leukaemia induction; chronic immune stimulation by the virus load, generation of leukaemogenic virus recombinants during preleukaemia and a point mutation in a critical region of the virus-genome. Whether mouse leukaemia viruses activate specific cellular oncogenes like avian viruses remains to be seen.

Feline leukaemia and sarcoma

Sarcomaviruses cause undifferentiated fibrosarcomas under natural circumstances, whereas leukaemia virus causes various forms of lymphoma and leukaemia. Most of the lymphoid malignancies are T-cell tumours. Aplastic anaemia and glomerulonephritis develop in a low percentage of cats. Under natural conditions the feline leukaemia virus is transmitted in a horizontal fashion. The route of virus excretion is saliva. Most free-roaming pet cats become transiently infected with leukaemia virus but only a minority becomes persistently viraemic. Persistent viraemia is a prerequisite for the development of leukaemia or lymphoma. At the molecular and genetic level the leukaemia and sarcomaviruses of the cat show a remarkable resemblance to C-type retroviruses of mice and subhuman primates. It is particularly interesting that the relationship between feline leukaemia virus and its natural host, the cat, shows many similarities to the relationship of human virus candidates and Burkitt's lymphoma, nasopharyngeal carcinoma, hepatoma and cervical cancer (*see below*).

Leukaemia and sarcoma in primates

The defective transforming simian sarcomavirus (SSV) and its non-defective helper virus (SSAV) were isolated from a fibrosarcoma of a pet woolly monkey. Together with the gibbon ape leukaemia virus (GaLV) that shows many similarities to SSV/SSAV, these are the only retroviruses known to cause malignant diseases in primates. The gibbon ape leukaemia virus induces haematopoietic malignancies *in vivo* and has a growth-stimulating effect on lymphocytic cells *in vitro*. It is present in many colonies of captive gibbons and spreads horizontally or congenitally among the animals. The transforming gene of the simian sarcomavirus (*sis*) is derived from a set of conserved cellular DNA sequences, similar to other viral oncogenes. The *sis* gene originated from a woolly monkey once naturally infected with gibbon ape leukaemia virus.

Murine mammary-tumour virus

The murine mammary-tumour virus was identified by Bittner in 1935 and thus one of the first oncogenic viruses discovered. It is a retrovirus with type-B morphology (*Figure 18.1*) and induces carcinoma of the mammary gland. Like type-C viruses, mammary tumour virus-related sequences are present in the normal cell-DNA of many mouse strains. Virus is transmitted to the offspring via germ cells and in high-tumour-incidence strains via milk. The development of the tumour and replication of the virus are subject both to genetic control and hormonal influence. Mouse strains can be inbred to show a high as well as a low preponderance to develop tumours. Castrated male mice develop mammary cancer with the same high incidence as females provided that they are inoculated with virus as newborns and treated with oestrogens.

Tumours caused by non-enveloped DNA viruses

Papovaviruses

Papillomas are benign epithelial tumours in man (*see below*), monkey, cattle, horse, dog, rabbit and many other species. Each species harbours its own papillomavirus types. The papillomaviruses replicate poorly in tissue culture and studies are therefore confined to biopsy material. Viral particles are found in cells of the epidermis, increasing in frequency in the outer keratinized cells of the skin. The genomes of most papillomaviruses appear to share a common region. There is no evidence that the virus-DNA should be integrated in the host-cell DNA. The mature virus is transmitted by direct contact and infection occurs at places where the skin barrier is defective. Infectious virus can be found in large amounts in papillomas of wild cottontail rabbits, but not in papillomas of the domestic rabbit. In the latter case, virus seems to occur in a latent form since infectious DNA can be recovered. The papillomas of domestic rabbits frequently progress to epidermal cancer. The process of progression can be accelerated by treatment with polycyclic carbon compounds such as methylcholantrene and tar.

Simian virus 40 (SV40) causes fibrosarcomas and gliomas when inoculated into newborn hamsters, rats and other rodents. Adult animals with a mature immune defence mechanism do not develop tumours, presumably due to immune reactions that eliminate TST antigen-positive cells. With very high viral doses tumours of haematopoietic organs may occur even in adult animals. SV40 virus is not pathogenic in its natural host, the rhesus monkey. Similarly, polyomavirus does not under normal conditions induce tumours in its natural host, the mouse, When the virus is inoculated into newborn mice multiple tumours (polyomas) may arise in different organs including salivary glands, thymus, mammary glands and ovaries.

Two human papovaviruses, BK and JC, are related to SV40. They both induce tumours in newborn Syrian hamsters and transform several cultured animal cells. JC, which is a neurotropic virus, in addition has the potential to induce brain tumours in adult owl and squirrel monkeys. Both cause the formation of tumour-like colonies from human fibroblasts *in vitro*. These cells multiply rapidly, contain T antigen but have a finite life. In the natural human host, these viruses are ubiquitous but usually non-pathogenic.

Adenoviruses

Oncogenicity has been shown for nine different types of human adenoviruses. In particular, types 12, 18 and 31, induce sarcomas upon inoculation of newborn rodents. In these tumours virus-DNA and T antigens, but not virus particles, can be detected, similar to the situation in SV40 and polyomavirus-induced tumours.

Tumours caused by enveloped DNA viruses

The herpesvirus and poxvirus families have members that cause tumours under natural as well as experimental conditions. A partial or complete maturation of virus can be seen in some tumours, indicating that the whole virus-genome may be present in the tumour cell.

Lucké virus

This virus belongs to the herpesvirus group and causes adenocarcinomas in the kidneys of the leopard frog (*Rana pipiens*) that lives in north and central North America. During the summer virus replication cannot be demonstrated in the tumours but during the cold winter season (+5°C and lower) inclusion bodies and nucleocapsids appear. Thus the genetic information for virus production is continuously present in the tumour cells. Cell-free tumour extracts from animals living in the cold can induce tumours in healthy frogs whereas extracts of tumours taken during the warm season are ineffective. Virus replication and, as a consequence, the epidemiology of the tumour, thus is markedly influenced by the temperature of the surroundings, and allows survival of both the virus and the host organism. Infectious virus is transmitted during breeding that takes place immediately after the period of hibernation.

Marek's disease virus (MDV)

Another member of the herpesvirus group causes Marek's disease in poultry. This is a generalized lymphomatosis with lymphocytic invasion of nerve trunks and paralysis. The disease is highly contagious and has a high mortality. Previously, the disease caused large economic losses, particularly in the USA, where the yearly costs amounted to 200 million dollars. The virus replicates in the feather follicle epithelium of infected chickens and is transmitted by air. The most likely primary infection site is in the respiratory tract. Cell-to-cell transfer of infection and transport of infected cells through the circulation may be the cause of the spread of the virus to remote tissues. The target cells for transformation are T cells. The lymphatic tumour cells contain many copies of virus-DNA but synthesis of nucleocapsids occurs only occasionally. A tumour-associated surface antigen can be detected in all lymphoid cells transformed by MDV even in the absence of viral structural proteins. A cellular immune response to the TST antigen seems to play the major role in protection against the disease. Virus-neutralizing antibodies appear in high titres in diseased chickens, presumably due to a persistent virus infection. They do not protect against tumour formation. Chickens with maternal antibody are only partially protected against the disease.

A live vaccine has been prepared from a related herpesvirus of turkeys. This virus replicates in chickens but is not pathogenic. After vaccination the animals develop viraemia and a mild and self-limiting lymphoid proliferation. The vaccinated chickens mount a cell-mediated immune reaction that destroys target cells with the MDV-specific surface antigens. Marek's disease is the first cancer of any species to be effectively prevented by vaccination.

Herpesviruses in monkeys

Several oncogenic herpesviruses occur in monkeys. Herpesvirus saimiri has been the most extensively studied. In the natural host, the squirrel monkey, virus is transmitted horizontally. The primary infection occurs at the age of 0.5–2 years. Following infection, the animals have circulating antibodies against the virus and carry the viral genome in what appears to be normal lymphocytes. The virus is not pathogenic for its natural host.

When the virus is experimentally inoculated into other monkeys (marmoset, owl, spider and green monkeys) lymphomas and lymphocytic leukaemia develop. T cells represent the target for transformation. Infectious virus can be detected in tumour cells only after cocultivation with other cells.

Human herpesviruses can induce tumours in experimental animals

Several herpesviruses that are pathogenic in man have been shown to cause tumours in experimental animals under certain conditions. For example, herpes simplex viruses and cytomegalovirus, which were rendered replication-defective by treatment with ultraviolet light, can transform hamster embryo cells *in vitro*. Inoculation of the transformed cells into hamsters gives rise to transplantable tumours in a small proportion of cases. Herpes simplex viral genomes can be detected in such tissues, but not as yet cytomegalovirus-DNA. One explanation for this difficulty is that the transforming gene may only represent a minor fraction of the total virus-genome. On the other hand, virus-antigen can be demonstrated on the plasma membrane of tumour cells. Two fragments of HSV type 2 that confer transformation have been isolated by restriction-enzyme cleavage and cloning. Similarly a DNA fragment of HSV type 1 adjacent to but not identical with the thymidine kinase gene gives transformation of hamster cells.

Epstein–Barr virus induces B-cell lymphomas in marmosets and owl monkeys. Cell lines that contain both the EBV genome and EBV antigens can be established from the lymphomas.

Poxvirus tumours

Certain poxviruses may cause rapid, tumour-like proliferation of tissues that often regress spontaneously. One kind of poxvirus causes fibromas in wild rabbits in South America. Inoculation of the same virus into domestic European rabbits or their wild progeny leads to development of a fatal, highly contagious, mucous-forming mixed tumour, myxomatosis. This disease has been used in the field to eradicate wild rabbits in Australia. Yaba virus, a poxvirus of rhesus monkeys, causes benign epithelial tumours in its natural host. The tumours regress spontaneously.

Human tumour viruses

Papilloma viruses

Human papillomaviruses (HPV) cause benign warts in cells of the epidermis or the mucous membranes. Human papillomaviruses are highly host-specific and they cannot cause papillomas in other species. At least 8 different, serologically unrelated HPV exist. Three types are found in common warts (*verruca vulgaris*) and two in flat warts. Genital warts (*condyloma acuminatum*) may infrequently progress to form squamous cell carcinomas of the penis, vulva or anus.

Juvenile larynx papillomas also contain papillomaviruses. The larynx papillomas usually show a spontaneous regression. Only occasionally and after long latency periods of 5–40 years do they become invasive cancers. However, after radiation of papillomas, malignant tumours have been observed.

An uncommon form of virus-rich papillomas, *epidermodysplasia verruciformis*, is caused by human papillomaviruses types 5 and 8. A genetic disposition regularly lies behind this disease. The warts frequently develop into malignant tumours, especially on sun-exposed parts of the body. Virus particles can no longer be discovered in the carcinoma cells. This cancer thus provides an example of the synergistic or cocarcinogenic effect of a virus and an environmental factor, most likely the ultraviolet radiation of sunlight.

Human tumour virus candidates

Retroviruses

Whereas retroviruses have been isolated from many animal species and several have been found to be the aetiological agents of naturally occurring leukaemias, lymphomas and sarcomas, the intensive search for retroviruses in human tissues has often gone astray. Morphology, nucleic acid homology, reverse transcriptase and antigenic relatedness were the criteria used for identification of human virus isolates – many of which were subsequently shown to be animal viruses acquired during laboratory procedures. More recently, several laboratories have isolated retroviruses related to those of gibbons and baboons from human leukaemia and lymphoma cells (but also from normal embryonic fibroblasts). These viruses may be signs of (1) *in vivo* infection with the exogenous primate virus SSV/GaLV, (2) activation of the endogenous viral sequences that give rise to infections and, perhaps transforming viruses by recombination, or (3) laboratory contamination.

Recent virological, serological and epidemiological evidence strongly indicates that particularly malignant types of cutaneous T-cell lymphomas (*mycosis fungoides*) and leukaemias (Sézary syndrome) are caused by a retrovirus. The virus, called HTLV (human T lymphoma virus), is distinct from known animal retroviruses as shown by analysis of proteins and nucleic acids. It can infect and transform umbilical cord leucocytes to cell lines producing retrovirus particles. The disease, T-cell leukaemia, shows a geographical clustering in Japan. Sera taken from all the leukaemia patients and from 25 per cent of healthy adults in that area react with HTLV.

Tumour cells from patients with breast cancer occasionally contain virus-like particles with biochemical characteristics similar to those of mouse mammary-tumour virus. The similarities concern the size and density of particles, the occurrence of an envelope and a nucleocapsid with RNA and reverse transcriptase. A partial nucleotide homology has been detected between RNA from the mouse mammary-tumour virus and DNA of human breast cancer cells. However, the significance of these particles is entirely unknown.

Herpesviruses: Epstein–Barr virus (EBV)

EBV is the aetiological agent of infectious mononucleosis. It is the virus that is most strongly related to human malignant tumours (*see also* Chapter 31). EBV was first detected by electron microscopy in cultured cells from Burkitt's lymphoma (BL). BL is a malignant lymphoma that occurs in children in certain tropical areas of East Africa and New Guinea. The EBV genome and EBV nuclear antigen (EBNA) have been demonstrated in fresh biopsy specimens of BL. EBNA is analogous to the T antigens induced by papova- and adenoviruses. Virus products

are usually not observed directly in the biopsy, but become detectable in the lymphoblastoid cell lines established from the tumours. The only cells that permit infection *in vivo* are B lymphocytes and certain endothelial cells. Cells which are infected with EBV either start to produce infectious virus in connection with a lytic infection or become transformed. The virus-genome in transformed cells is covalently bound to cell-DNA or occurs in the cytoplasm in the form of an episome represented by circular DNA. *In vitro*, too, B cells are easily transformed by EBV to form immortalized cell lines that replicate continuously in culture. As a rule only EBNA is expressed.

Burkitt's lymphoma most likely is not caused only by an EBV infection. In addition, one or more cocarcinogenic factors such as genetic predisposition, stimulation of the reticuloendothelial system or immune suppression have to be present. Holoendemic malaria (monthly exposures to the parasite) may be one factor of importance.

EBV has been implicated also in the pathogenesis of lymphoproliferative diseases in immunosuppressed recipients of organ transplants. In patients with an X-linked genetic defect, EBV may cause a severe lymphoproliferative disease.

Nasopharyngeal carcinoma is a common tumour in African and Chinese people. The tumour cells are of epithelial origin and contain EBV-DNA and EBNA in their nuclei. Although the complete virus is not produced, early antigens can be detected frequently. The patients have high titres of IgG and IgA antibodies against EBV capsid antigen. It has been shown that patients with the highest IgA titres have the poorest prognosis. The reason for this is not clear, but it may be due to a blocking of tumour cells by IgA for cytotoxic IgG antibodies. It seems likely that an EBV infection is related to the appearance of nasopharyngeal cancer, but the pathogenesis of the disease has not yet been defined.

Herpesviruses: herpes simplex virus (HSV)

Cervical carcinoma is one of the most common forms of tumour in women. Precancerous changes and cancer *in situ* commonly occur in patients who also have an HSV type-2 infection. Exfoliated tumour cells have been shown to contain herpesvirus antigens and messenger-RNA for the virus has also been described. The virus can occasionally be isolated from explants of tumour cells, but the HSV-DNA cannot be identified regularly.

Seroepidemiological studies have shown that women with invasive cancer of the cervix have neutralizing antibodies against HSV type 2 in a higher frequency and in higher titres than healthy women of the same age and with a corresponding socioeconomic status. However, infections in the genital tract with HSV type 2 are very common and obviously may occur in the tumour cells without any relation to the tumour induction.

Hepatitis B virus

In Africa, Asia and the Mediterranean countries it has been observed that patients with chronic hepatitis B (HB) virus infections frequently develop primary liver cancer, hepatoma. In families with one hepatoma case other members are frequently found to be carriers of HB virus infections, since HB surface antigen (HBsAg) and anti-HBs can be detected in their serum (*see* Chapter 30). However, in countries with a high hepatoma incidence, HB viral infections and liver cirrhosis

are also very common diseases. It is therefore difficult to define an aetiological relationship between HB virus and hepatoma. HB virus has not been found to have any oncogenic effect in animal systems.

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19

Mechanisms of defence against virus infections

Örjan Strannegård

The defence against viruses in the body is based on several different antigen-specific as well as non-specific reactions. These reactions are dependent on age, genetic and hormonal factors, nutritional conditions, phagocytosis and inflammatory reactions. The immunologically-specific defence mechanisms include the *antibody-mediated, humoral immunity*, which mainly is dependent on B cells, and the *cell-mediated immunity*, primarily mediated by T cells. The relative importance of these forms of immunity varies for different virus diseases. With certain exceptions, the cell-mediated immunity is relatively more important for the defence against viruses than for the defence against bacteria. This is illustrated by the fact that, whereas B-cell defects in humans frequently are complicated by bacterial infections, defects in the T lymphocytes primarily are characterized by an increased severity of virus infections.

The immunological defence against virus infections fulfils two functions: to eliminate ongoing virus infections and to prevent reinfections or at least limit their consequences. To handle the first task the immune defence mechanisms include a capacity to *neutralize* extracellular virus and to eliminate virus-infected cells. In order to prevent reinfections, the immune defence restricts the local development of an infection and blocks a viraemic spread from an already established local infection. In order to solve both these problems an interplay between non-specific and specific immune reactions is required. The inflammatory response is an indication of the mobilization of the immune defence mechanisms. This response is characterized by hyperaemia and an influx of inflammatory cells which, in the case of virus infections, primarily are mononuclear, i.e. they mainly include lymphocytes and macrophages.

Non-specific immunity

In order for a virus to reach susceptible cells and to establish an infection it is required to transgress several anatomical and physiological *barriers* (*see also* Chapter 13). Among these may be mentioned the chemical barriers represented by lactic acid in perspiration, fatty acids which are excreted by glands of the skin, the layer of mucus on many membranes and hydrochloric acid and bile products in the alimentary canal. Different viruses show varying sensitivity to inactivation with chemical substances produced by the body. One example of this is the fact that

rhinoviruses are much more readily inactivated by hydrochloric acid than enteroviruses. Consequently, enteroviruses can pass readily into the intestinal tract whereas rhinoviruses usually are inactivated before they reach this compartment. If a virus comes into contact with cellular membranes this need not necessarily imply that an infection is established since not all cells have receptors on which the virus can anchor.

The age of the individual is of considerable importance for the non-specific defence mechanisms against virus infections. As a rule, generalized virus infections are more severe during the perinatal period, moderately severe during the first years of infancy, milder in children than in adults, and again more severe in aged individuals. However, there are several exceptions to this rule. The mechanisms for the age-dependent variation in susceptibility to virus infections are complicated. They include specific immunological and also non-specific defence mechanisms.

Among the non-specific defence mechanisms the *increase in temperature* plays an important role. Already normal body temperature implies a protection against the dissemination of rhinovirus infections. This is due to the fact that rhinoviruses have an optimum temperature for growth at 33°C, and since this is the approximate temperature in mucosal membranes in the upper respiratory tract the infection usually stays localized to this compartment. Replication of most viruses is markedly restricted when the temperature rises to 39°C or higher. In some cases there is a relationship between the virulence of the virus and its capacity to replicate at an increased temperature. One example of this is virulent polio virus which can replicate at 40°C, whereas, by contrast, temperature-sensitive mutants of this virus are relatively avirulent. The mechanisms behind the hampering effect of a temperature rise on virus replication are manifold. The increase in temperature leads to an increased metabolism and action of interferon and stimulates the specific immunological defence and phagocytosis.

Nutritional conditions are of considerable importance in the defence against virus infections. The best known example of this is the high mortality in measles (20 per cent or more) in certain areas in Africa where children are markedly undernourished. Poor nutritional conditions influence the cell-mediated immunity to a larger extent than the humoral immunity. Therefore these conditions have a more pronounced influence on the course of virus diseases than on most other infectious diseases, since the former mainly are controlled by cellular immunity. In undernourished children a vicious circle may develop. For instance, in measles undernourishment leads to reduced protection which results in the development of a more severe form of the disease including replication of virus in the intestinal tract, which in turn complicates the uptake of nutritional substances.

Hormones have several effects on the defence against virus infections. Corticosteroids are anti-inflammatory and have a restraining effect on the immune defence and interferon production. It can be shown experimentally that treatment with cortisone markedly increases the susceptibility of monkeys to polio virus infections and in man severe forms of vaccinia and varicella virus infections have been observed in patients treated with corticosteroids. The change in the hormonal balance in pregnancy may explain why certain virus infections, for example smallpox, hepatitis, influenza and polio, run a more serious course in pregnant women.

Elimination of a virus by *phagocytosis* can be considered a non-specific immunological defence mechanism even though specific mechanisms, for example the aggregation of virus particles by specific antibodies, may enhance the phagocytosis.

In addition, phagocytosis may be facilitated by the attachment of cytophilic antibodies via their Fc part to the surface of macrophages and the attachment of virus via these antibodies. Granulocytes appear to play a minor role in the defence against virus infections. In contrast, tissue-bound macrophages and their circulating equivalent, the monocytes, are of decisive importance. In connection with virus infections, the macrophages are activated by the influence of T-lymphocyte products, immune-complexes and the complement factor C3b. These activated macrophages can be recognized by the fact that they have an increased metabolic activity and an increased content of lysosomal enzymes. Thus they are suited for phagocytosis and the elimination of virus particles. However, in some cases, the virus is not inactivated after phagocytosis and instead the virus replicates in the macrophages. In this case the spread of virus throughout the body is enhanced. Virus replication in macrophages is observed particularly in lethal and persistent infections.

The most important system among non-specific defence mechanisms against virus infections is *interferon production*. The interferons, of which there are at least three different categories in man (IFN-α, IFN-β, and IFN-γ), can restrict virus replication. The formation of IFN-γ is induced by immunologically specific reactions, although the effect of interferon itself by definition is non-specific. During a virus infection the interferon defence develops very rapidly and is active before the immunological defence mechanisms have started to function. A schematic comparison between interferon and the immune defence mechanism can be made as follows: interferon exerts its effect inside cells whereas the immune defence only can work in the extracellular milieu; the effect of interferon is short-lived whereas the immune defence often provides longlasting protection; interferon effects are non-specific whereas in contrast the immune reactions essentially are specific. Further information on interferon is given in Chapters 11 and 24.

Humoral immunity

A generalized primary virus infection provides a maximal antigen stimulation in the body and leads to the formation of antibodies which are of importance both for the elimination of the ongoing infection and for protection against reinfection. The relative importance of antibodies for the abrogation of an ongoing infection varies between different kinds of virus infections. Thus, for example, antibodies are of decisive importance for the elimination of picornavirus infections, whereas the handling of most other virus infections is dependent on a functioning cell-mediated immunity. One reason why humoral immunity is of great importance in picornavirus infections is the fact that these viruses cannot spread from cell to cell without being exposed to the extracellular milieu, where they may be neutralized by antibodies. In addition, the cell-mediated immunity cannot exert any influence on picornavirus-infected cells, since no new antigens are inserted into the membrane of these cells.

Antibodies do not penetrate the cytoplasmic membrane and thus cannot influence intracellular virus directly. This may explain why certain viruses, for example, herpes simplex and varicella-zoster virus, can occur in a latent form and become reactivated in spite of the continued circulation in the body of antibodies against these viruses.

The protection provided by antibodies against virus infections frequently lasts a life-time. The protection is immunologically specific, which means that viruses which show antigenic variations (influenza virus) or which occur in a large number of different types (for example, rhinovirus) may cause repeated infections. Antibodies in the first place act by blocking the infectious property of the virus by *virus neutralization* mechanisms. Neutralization of the virus may be exerted by at least two different mechanisms: the blocking of virus replication by inhibition of adsorption, penetration or uncoating, and *virolysis*, a reaction leading to the destruction of envelope components with the aid of complement factors.

The virus neutralization mechanisms have been carefully studied *in vitro*. The first step in this reaction is a reversible association between antibodies and virus particles. In the second step, neutralization of the virus is brought about by structural changes in both the antibodies and the virions. Even at this stage the reaction usually is not irreversible and it is possible to reactivate virus by dissociation of virus-antibody complexes under conditions of reduced pH or increased salt concentration. The neutralizing activity of antibodies is dependent on their avidity. Antibodies that appear at an early stage of an infection have a lower avidity and are dissociated from the virus more readily than antibodies that are produced during later stages of the infection. Consequently, the neutralization in an *in vitro* system frequently is incomplete when antibodies from early stages of the infection are used. Under *in vivo* conditions, incompletely neutralized virus may circulate and form infectious immune-complexes which may have relevance for the pathogenesis of virus diseases.

In order to obtain a complete neutralization of virus, it is usually essential that many antibody molecules cover the virion. In addition, it is required that certain critical regions (epitopes) on the virions are covered by antibodies. In some cases there is only one such region, for example on certain bacteriophages, but usually there are several critical regions. Many viruses have multiple haemagglutinin units, which are important for virus attachment to cellular membranes. Antibodies are capable of neutralizing influenza virus only when the majority of these haemagglutinin components are covered by antibodies. If antibodies are to be capable of acting on the level of adsorption it is essential that they are not only bound to certain critical regions but also are so located on the virus surface that adsorption is prevented when the virus and the cell collide. Adsorption may also be hindered sterically by the attachment of factors other than virus-specific antibodies to critical regions on the surface of a virion. Examples of such so-called accessory factors which give *neutralization enhancement* are rheumatoid factors (IgM antibodies against IgG), and complement factors. Both these factors can attach to virion-antibody complexes and accomplish an enhancement of neutralization.

In connection with neutralization of an enveloped virus, activated complement may bring about *virolysis*, i.e. the development of small distinct holes in the virus envelope, which results in loss of virus infectivity. Thus complement can aid in the neutralization of viruses in two ways, either by a mechanism involving steric hindrance of absorption or by effects resulting in virolysis.

In order to obtain neutralization of a virus it is not always required that the adsorption of virions is blocked. In fact virus–antibody complexes frequently are taken up by cells and the neutralization is then an intracellular event. The mechanism behind this form of neutralization is unknown, but it can be shown by electron microscopy that the uncoating of virus particles is incomplete if antibodies are attached to the surface of virions.

The role of antibodies in the defence against virus infections is not restricted to neutralization of virus infectivity. Additional effects of antibodies are (a) *opsonization*, i.e. the facilitation of phagocytosis by the attachment of antibodies to virions; (b) *lysis of infected cells* by activation of the complement system (this mechanism is effective only when antibodies can be bound to virus antigens or virus–cell antigen complexes on the cell surface); and (c) *collaboration with the cell-mediated immune defence* in connection with lysis of infected cells. If K cells and macrophages are to exhibit their cytotoxic effects it is required that they are attached to the Fc part of antibodies, which in turn are attached to viral antigens on the cell surface (*antibody-dependent cellular cytotoxicity, ADCC*).

After antibodies are bound to viral antigens on the cell surface a *capping* phenomenon may appear, which means that virus antigen–antibody complexes located in the cytoplasmic membrane are moved towards one pole of the cell and therefore accumulate locally on the cell surface. These antigen complexes are shed to a major extent from the cell surface which hereby becomes free of membrane-bound virus antigens. The capping phenomenon represents a mechanism by which a cell can escape the attack of cytotoxic antibodies. Thus capping may play a role in situations where persistent virus infections develop in the presence of large quantities of circulating antibodies.

During a virus infection antibodies belonging to the immunoglobulin classes IgM, IgG and IgA and, at least in some cases, also IgE and IgD, are produced. The importance of the two last immunoglobulin classes in the defence against virus infections is unknown whereas, in contrast, antibodies of the IgM, IgG and IgA classes have important and partly different functions. IgM antibodies, which appear at an early stage of a virus infection (*Figure 19.1*) and which usually are undetectable a few weeks later, are very effective in virolysis and agglutination of virus. IgG antibodies are produced continuously for years after the infection, and immunoglobulins of this class carry the main responsibility for immunity against reinfections. IgA antibodies regularly appear in serum after infection but the duration of this antibody response and the relative importance of this kind of immunoglobulin in the defence against virus infections have not been clarified.

The dominating immunoglobulin in secretions is secretory IgA, which is composed of two molecules of serum IgA joined together by a small peptide chain. Secretory IgA is synthesized locally in mucous membranes. The production of secretory IgA is particularly important for the defence against viruses which attack the respiratory tract and the gastrointestinal tract. After a virus infection, IgA antibodies can be detected for a relatively long period in secretions. Following vaccination with live polio vaccine it has been possible to detect antibodies for two years after the immunization. The absence of a secretory IgA response after parenteral immunization with inactivated polio vaccine has been considered a disadvantage in comparison with the satisfactory IgA response that is observed after oral immunization with live vaccine. However, it should be emphasized that after parenteral immunization 'spill-over effects' can be observed which means that a small fraction (0.2–0.5 per cent) of circulating antibodies will appear on mucous membranes and that, therefore, in the presence of high concentrations of serum IgG antibodies, a local immunity is established. The immunological memory of IgA-producing cells appears to be less effective than the memory of IgG-producing cells, and IgA responses for protection against reinfection are therefore probably less important than those of IgG.

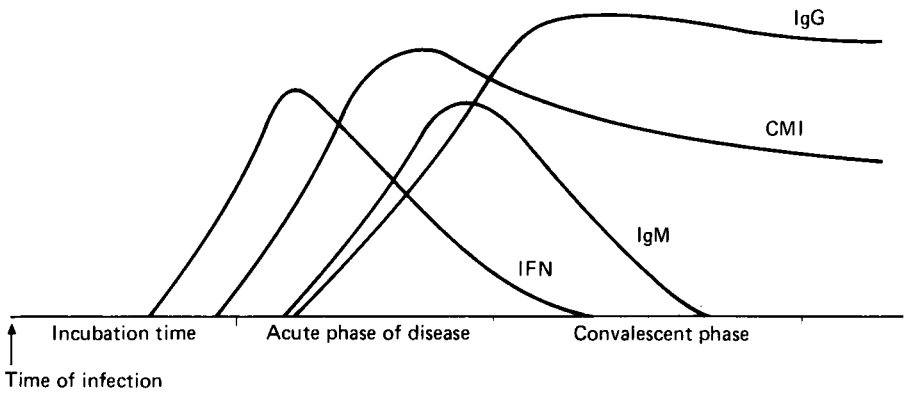

Figure 19.1. Schematic description of the appearance of the interferon (IFN) response, cell-mediated immunity (CMI) and IgM and IgG antibody responses during a virus infection

Cell-mediated immunity

Antibodies are vitally important for the neutralization of extracellular virus. However, antibodies do not penetrate the cytoplasmic membrane and therefore cannot react with and neutralize intracellular virus. Antibodies, in cooperation with complement, can lyse cells by binding to viral antigens which are present in the cytoplasmic membrane of the infected cells. This mechanism for the elimination of virus-infected cells does not appear to be sufficiently effective however, and as a rule virus-infected cells are lysed by mechanisms for which the cell-mediated immunity is responsible.

The cell-mediated immunity develops after reaction of thymus-derived cells (T cells) with the virus. After contact with viral antigens, usually mediated by macrophages, the T cells proliferate. Consequently a clone of inducer T cells is established which are capable of reacting in an immunologically specific fashion with the inducing antigen. The induced T cells have a capacity to assist B cells in the production of specific antibodies and to activate *cytotoxic* T cells and macrophages. An antigen stimulation also causes a production of suppressor cells, which have a regulatory effect on both antibody production and on cell-mediated immunity. The T cells influence a number of other lymphoid cells by producing soluble substances referred to as *lymphokines*. Examples of lymphokines important for the defence against virus infections are the migration-inhibitory factor (MIF), probably identical with the macrophage-activating factor (MAF), chemotactic factors, and immune interferon (IFN-γ).

The cell-mediated immunity develops at an early stage during a virus infection and can be demonstrated in conjunction with the development of symptoms, i.e. it appears later than the interferon response but earlier than the humoral immunity (*see Figure 19.1*). One sign of the development of cell-mediated immunity is the occurrence in several different types of virus infection of a delayed hypersensitivity which can be demonstrated by intradermal injection of viral antigens. An infection causes a rapid development of memory T cells and reinfection leads to a rapid activation of the specific cell-mediated immunity.

The mechanisms of cell-mediated immunity are complicated and include cooperation between several different kinds of cells (*Figure 19.2*). The following mechanisms can be distinguished.

(1) *Cytotoxic T cells* react with membrane-bound viral antigens and can lyse the infected cells by excretion of a cytotoxic factor. This cytotoxic reaction is dependent on both receptor structures on T cells, which react with viral antigens, and structures which react with the histocompatibility antigens of the cell. Thus the reaction shows *histocompatibility restriction* which means that cytotoxic T cells from a virus-infected individual can kill homologous infected cells but not virus-infected cells from an individual who has different transplantation antigens. The importance of this phenomenon has not been clarified.
(2) The influence of chemotactic factors attracts *macrophages* to the place of reaction between T cells and viral antigens. These macrophages remain locally under the influence of MIF and are activated by MAF. The macrophages can phagocytize and destroy virus and virus–antibody complexes. The phagocytosis is enhanced if the virus particles are opsonized by antibodies. Since macrophages have receptors for antibodies on their surface, they can first attract antibodies and then virus which facilitates phagocytosis.
(3) T cells – probably after interaction with accessory cells – produce *immune interferon* which can prevent the spread of an infection to neighbouring non-infected cells. The interferon induces a state of resistance in these cells against virus infection.
(4) *K (killer) cells*, which share certain characteristics with both T cells and monocytes/macrophages, have receptors for the Fc part of antibodies on their surface. These K cells have a cytotoxic effect on cells when they are armed with antibodies which in turn react specifically with membrane-bound viral antigens. Thus the K-cell cytotoxicity represents still another mechanism through which virus-infected cells can be lysed and intracellular virus may be released. Antibody-dependent cellular cytotoxicity (ADCC) may also be exerted by macrophages.
(5) *NK (natural killer) cells*, which probably are variants of K cells, can lyse cells directly and do not require an intermediate link in the form of antibodies. NK cells are activated by interferon and since virus-infected cells excrete interferon there will be an increased activity of NK cells at the locality of a virus infection. The importance of NK cells for the defence against virus infections has still not been clarified, but it is probably of importance that the activity of circulating NK cells increases markedly during the early stages of virus infections and is correlated to the concentration of serum interferon.

Immunopathological effects of virus infections

Many viruses can infect macrophages and lymphocytes. The latter kind of cells usually are relatively insusceptible to virus infections when they are in a resting stage, but they can produce virus when they are activated by an antigen or a mitogen. Depending on the kind of cells that are infected by a virus, different forms of disturbances in the immune defence system may develop. It has been shown that

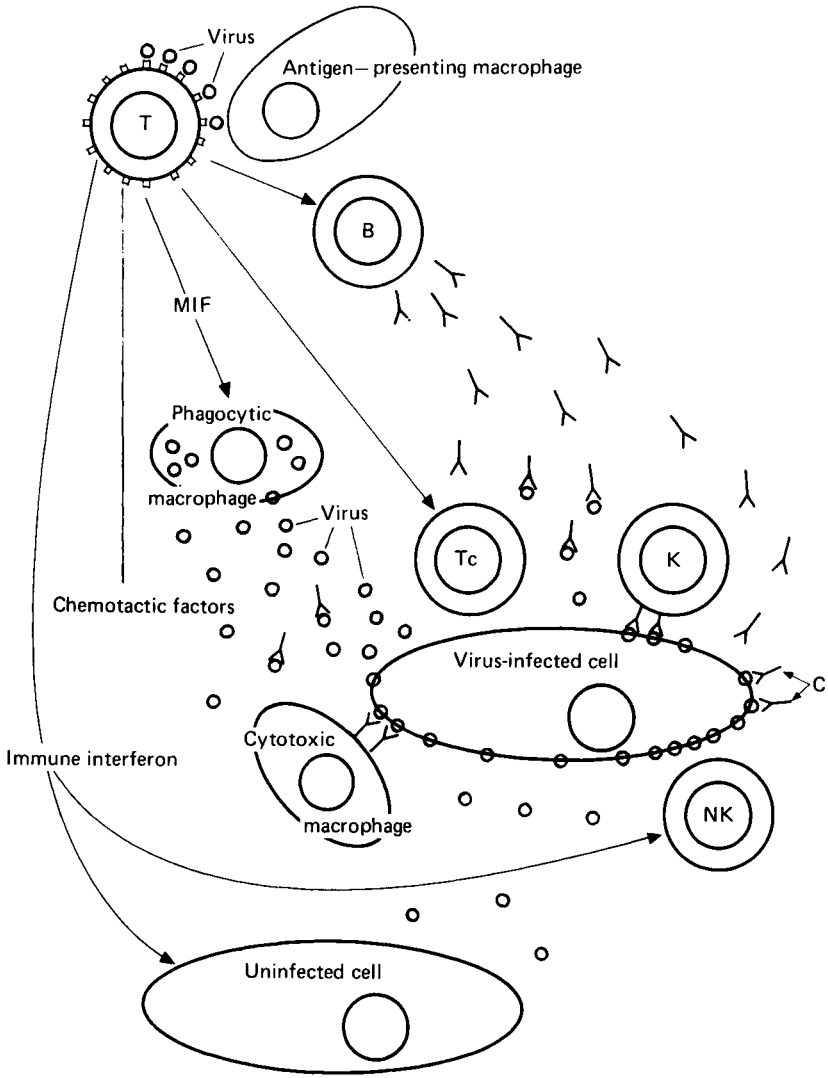

Figure 19.2. Mechanisms of action of cell-mediated immunity against virus infections. The induction of an immune response against viral antigens is complex and includes a cooperation between T cells and macrophages. As an end result of the reaction between receptors on T cells and virus, the T cells can synthesize soluble factors (lymphokines) which have a capacity to activate different effector cells. Examples of lymphokines which are important in the defence against virus infections are MIF (macrophage migration-inhibitory factor), immune interferon (IFN-γ) which directly may protect against a virus infection and also has a capacity to activate NK (natural killer) cells, and finally chemotactic factors which attract phagocytic cells.

The virus-infected cells may be killed by the influence of (1) complement (C) which is activated by membrane-bound virus–antibody complexes; (2) cytotoxic T cells, (Tc), which are activated by inducer T cells; (3) macrophages and K (killer) cells, which act via an antibody-dependent cellular cytotoxicity; and (4) NK (natural killer) cells, which are activated by IFN-γ or interferon which is produced by infected cells. The destruction of the infected cells may lead to a release of virus particles. These particles can become phagocytized by activated macrophages or neutralized by antibodies.

measles virus can replicate in T cells and that the infected cells lose their capacity to respond to stimulation with tuberculin. This observation is related to the finding that tuberculin-positive individuals temporarily become tuberculin-negative for several weeks after an infection with measles and that they have a decreased number of T cells in their circulation. In this context the old observation that a measles virus infection may cause activation of dormant tuberculosis should be mentioned. There are several other examples of virus infections that may lead to immune depression. This is particularly striking in leukaemia virus infections in animals which lead to a suppression of both cell-mediated and humoral immunity. In certain cases the reverse effect can be observed, i.e. a stimulation of the immune defence mechanisms. EB virus, which can cause mononucleosis, has been found to have a mitogenic effect on B lymphocytes. This can probably explain why an increased production of immunoglobulins, in particular IgE and IgM, is seen during EB virus infections.

It is obvious that in certain virus infections it is the immune response and not the virus replication which is the major cause of the pathologic changes. This has been most clearly demonstrated in infections of lymphocytic choriomeningitis (LCM) virus in mice. Adult mice which are injected intracerebrally with LCM virus contract a lethal infection, but they survive if they receive immunosuppressive treatment, for example with cyclophosphamide or antilymphocytic serum. Neonatally thymectomized or irradiated animals also survive, in spite of the fact that replication of the virus in the tissues can be demonstrated. If immune T cells from LCM virus-infected mice are transferred to immunosuppressed mice with a persistent LCM virus infection, pathological changes develop in the brain of these animals and cause a fatal outcome to the infection. The reason for this phenomenon is presumably that the immune T cells, at the same time as they may restrict the virus infection, can cause an extensive lysis of cells in the brain. Also, the humoral immunity developing during infection may cause immunopathological effects. Newborn animals which are infected with LCM virus develop a very poor cell-mediated immunity against the virus. Consequently they do not die of the infection but, instead, develop a persistent infection with an extensive replication of virus in the body. Connected herewith there is a continuous production of antibodies which can react with virus in the blood and form immune-complexes. These complexes are trapped in kidney glomeruli and the animals therefore develop glomerulonephritis.

There are several indications that a virus infection may lead to immunopathological diseases in man also. It is highly likely that cell-mediated immunity plays a major pathogenetic role in connection with postinfectious encephalitis which develops about one to two weeks after certain acute infections, for example measles and varicella, or after vaccination against smallpox. With all generalized virus infections there is a transient phase during which conditions exist that allow formation of immune-complexes. It is therefore likely that arthritis and arteritis, for example, which occasionally develop during acute virus infections, are caused by viral antigen–antibody complexes. However, persistent virus infections are associated with formation of immune-complexes much more frequently than acute infection. Examples of persistent infections which lead to a formation of immune-complexes are hepatitis B and subacute sclerosing panencephalitis.

Immune-complex formation appears to have some causal relationship with the development of bronchiolitis in infants in connection with RS virus infections. The explanation why infants frequently develop severe symptoms during this infection

is probably that they lack IgA antibodies, which protect against infection, but that they usually have maternally derived IgG antibodies, which can form immune-complexes with virus and activate complement. The same mechanism probably is responsible for the severe reactions which have been encountered in children who have been infected with RS or measles virus after a previous immunization with inactivated vaccines against these viruses. Immunopathological conditions also appear to play a role in the development of pulmonary changes in influenza and RS virus infections. Influenza virus infection has been shown to give less pronounced pulmonary changes in T cell-deficient mice than in normal animals in spite of the fact that T-cell defect is associated with increased concentration of virus in the lungs. This suggests that a part of the cell destruction which appears in the lungs during an influenza infection is caused by cytotoxic T cells.

Virus infections in connection with malignant diseases and immune suppression

Infections which have a particular propensity to develop in individuals with a defective immune defence are often referred to as 'opportunistic infections'. The most important virus infections within this category are diseases caused by viruses belonging to the herpesvirus group. This is due to the fact that herpesviruses which occur in a latent form in cells can be activated and, in individuals with a depressed immunological defence, replicate to a larger extent than normally. Accordingly, patients with Hodgkin's disease, who have a decreased cellular immunity, frequently develop generalized herpes zoster. Patients with haematological malignant diseases have a markedly increased frequency of infection with CMV and EB virus. Patients who have received kidney or heart transplants and in connection with this have received immunosuppressive treatment often develop CMV infections, usually as a consequence of reactivation of virus. Patients with leukaemia or lymphomas frequently develop an atypical form of measles characterized by an unrestricted virus replication, causing development of pneumonia and/or encephalitis that often runs a fatal course. Also vaccinia-virus infections are known to take a serious course in patients who have a depressed cell-mediated immunity.

The relative importance of humoral and cell-mediated immunity in the defence against virus infections is reflected by the fact that these infections run a similar course in patients with B-lymphocyte defects, i.e. hypogammaglobulinaemia as in normal individuals. However, picornavirus infections are an exception and can have serious consequences in hypogammaglobulinaemic patients. It is well known that, for example, polio virus infections carry an increased risk of development of serious disease with severe pareses in these patients. In patients with immunodeficiencies that concern the cell-mediated immunity, severe forms of vaccinia, measles, varicella-zoster, herpes simplex and cytomegalovirus infections are seen instead.

Immunological defence against congenital virus infections

An intrauterine infection affects an immunologically immature individual, and the active cell division which occurs during embryonic development provides a good milieu for virus replication. This is why virus infection in many cases leads to fetal death and abortion (*see also* Chapter 15). In cases where the fetus survives.

malformations of different kinds may develop. It has been suggested that interferon, that is produced during the viral infection, may be partially responsible for these malformations because of its antiproliferative effect on cells. Immunological disturbances of several kinds may develop as a consequence of intrauterine infection. A classic example of this is found in mice which have been infected *in utero* with LCM virus and have an immunological abnormality suggesting partial tolerance against this virus. In connection with congenital rubella, there frequently are signs of reduced cell-mediated immunity. In addition, congenital infection with rubella, CMV as well as varicella virus, are associated with signs of disturbed immunological functions in the form of abnormal relationships between the quantities of different immunoglobulin classes. The influence of intrauterine infections on immunological functions may probably explain why children with congenital rubella or CMV infection develop a persistent infection with these viruses for months or years after birth. The gradual disappearance of excreted virus in connection with a congenital infection presumably reflects an increasing maturity of the cell-mediated immune defence. A child infected *in utero* develops an antibody response against virus which includes the formation of IgG and IgM antibodies even during fetal life. Since IgG but not IgM antibodies can pass the placental barrier, identification of IgM antibodies against, for example, rubellavirus or CMV, either in cord serum or in serum taken immediately after birth, represents an important sign of an ongoing infection with the virus in the newborn child.

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20

Laboratory techniques for diagnosis of virus infections

Monica Grandien and Arne Svedmyr

An exact virological diagnosis is necessary for studies of the epidemiology of viral infections and for the control of epidemics and nosocomial infections. In some situations the clinical work is entirely dependent on the virological diagnosis, for example when rubella is suspected in early pregnancy, and for excluding hepatitis B carriers from becoming blood donors. The need for a rapid aetiological diagnosis will increase as more antiviral chemotherapeutics become available for clinical use, and the choice of drug and the development of drug resistance become problematic. Laboratory diagnosis of virus infections, including the specification of serotypes, has formed a necessary background to the development of viral vaccines and the continued control of the efficacy of vaccines.

Three different principles are used in the diagnosis of viral infections:

(1) Isolation of infectious virus by inoculation into cell cultures or experimental animals.
(2) Identification of virus or viral antigens directly in samples from patients. In the future the highly sensitive methods such as antigen detection by new serological tests or genome identification by nucleic acid hybridization techniques will probably be used routinely for direct identification of virus in samples from patients.
(3) Serological analysis to demonstrate a specific antibody response.

Isolation of viruses

Most known viruses causing disease in man can be demonstrated by use of cell cultures or experimental animals. The virus infection is indicated by the more or less characteristic changes that the virus causes in cell cultures (cytopathic changes and cell death, the formation of viral antigens, haemagglutinin, or interferon etc.; *see* Chapter 11) or by the appearance of disease in experimental animals. In some cases, for example in myxovirus infections, the production of haemagglutinin is readily demonstrated by the addition of erythrocytes, which adhere to the surface of the infected tissue-culture cells (haemadsorption, HAd). An isolate of virus can be identified, *typed*, by neutralization tests or other antigen-antibody reactions, for example by immunofluorescence staining of infected cells, haemagglutination inhibition etc. (*see* description of methods below).

TABLE 20.1. Scheme for collection of samples for virus diagnosis

	Virus isolation or direct identification						*Serology*		
Disease	*Nasopharynx*	*Throat*	*Faeces*	*Cerebrospinal fluid*	*Urine*	*Other materials*	*Acute*	*Conv. 10–20 days*	*Con. 4–6 weeks*[a]
Aseptic meningitis, encephalitis, myelitis		(+)	+	+	(+)	Vesicle fluid Brain biopsy	+	+	+
Conjunctivitis, keratitis						Conjunctival secretion Cornea scraping	+	+	
Mumps				+	(+)	Saliva	+	+	
Respiratory infection	+	+	(+)[b]				+	+	
Stomatogingivitis		+				Vesicle fluid	+	+	
Pleuritis			+			Pleuric fluid	+	+	
Myocarditis-pericarditis			+			Pericardium fluid	+	+	
Hepatitis					(+)		+[c]	(+)	(+)
Mononucleosis					(+)		+	+	(+)
Cytomegalic inclusion disease					+		+	+	+
Orchitis (without parotitis)			+		+	Saliva	+	+	
Diarrhoeal disease			+				+	+	
Maculopapular exanthema		(+)	+[d]				+	+	
Exanthematous disease with vesicles			+			Vesicle fluid, scrapings from bottom of vesicles, crustal material	+	+	
Rabies						Corneal imprint, mouth scraping or skin biopsy, saliva	+	(+)	

(+) Supplementary material in special cases.

[a] To be collected if an earlier convalescent serum has not provided aetiological diagnosis. The time for collection of samples is calculated from the day of appearance of disease.

[b] In connection with pharyngoconjunctivitis, herpangina or diarrhoea.

[c] If HBsAg is demonstrated, certain rules for the follow-up should be applied (*see* Chapter 30).

[d] With consideration taken of enterovirus and adenovirus infections.

Investigation of a suspected congenital or neonatal virus disease

The child: Blood sample (without additives).

Samples for virus isolation: Rubellavirus and CMV: pharynx swab, urine, cerebrospinal fluid. Herpes simplex virus or varicella virus: vesicle fluid, pharynx swab, cerebrospinal fluid. Enterovirus: faeces, cerebrospinal fluid, pharynx swab.

The mother: Blood sample (without additives). Indicate on the referral any diagnostic suggestions, symptoms of infections in the mother, and data about the child.

Samples for a direct diagnosis of viral antigen or virus particles in patient's material

Samples for immunofluorescence analysis (nasopharyngeal or tracheal secretions in respiratory infections, scrapings from conjunctiva and cornea in eye infections, and vesicular fluid from cases with vesiculated exanthema) should reach the laboratory within two hours, unless the cell smears are prepared at the hospital. Specimens for antigen detection by other methods or faecal samples for electron microscopy can withstand longer transport times.

Although virus isolation is still the technique most generally applicable for diagnosis of a virus infection, there are several important viruses which do not grow readily in ordinary cell culture systems (for example hepatitis virus A and B, rotavirus and certain strains of adenovirus), or which are not excreted or accessible at the time of disease (for example tick-borne encephalitis virus).

Samples for virus isolation must be collected by the correct method (*Table 20.1*) as early as possible during the disease since the concentration of virus is maximal at this stage and is often reduced rapidly as time passes. However, the infectious agent may in some cases be demonstrated for weeks, months or sometimes even years after the acute infection. Thus enteroviruses and adenoviruses can be excreted for a long time with faeces; CMV in urine and rubellavirus in pharynx and cataracts can be isolated for periods of months to years from congenitally infected children.

The time required for the laboratory to isolate a virus can vary between one to two days in the case of, for example, influenza, polio and herpes simplex viruses, to many weeks in the case of CMV.

Cell cultures

Virus isolation is most often performed in cultures of various kinds of cells (*see also* Chapter 5). By use of such techniques it has been possible to define the aetiology of many virus diseases, to isolate and characterize hundreds of previously unknown viruses, and to produce effective virus vaccines.

Cells of different origin and age may have a markedly varying susceptibility to different viruses. For diagnostic purposes a virus laboratory must therefore have access to a battery of cell lines from animals and man. As both diploid and heteroploid cell lines can be stored for an indefinite time at low temperature (liquid nitrogen − 196°C) the laboratory can easily keep appropriate cell lines in stock.

Some viruses grow poorly or not at all in cell cultures but can be isolated in organ cultures of highly differentiated tissue, for example some strains of coronaviruses in cultures of human nasal mucosa or tracheal epithelium. For isolation of virus from patients with persistent infection, cocultivation or even production of cell hybrids between susceptible cells and surviving infected cells from the patient may be needed. This technique has been used for the isolation of measles virus from explanted brain tissue in cases of subacute sclerosing panencephalitis (SSPE, *see* Chapter 16).

Experimental animals

In addition to cell cultures, laboratory mice and embryonated hen's eggs are still used for routine isolation of viruses. Poxviruses and herpes simplex viruses can be isolated by infection of the chorioallantoic membrane of the embryonated egg, giving rise to localized tissue changes. These changes are frequently characteristic enough to be used not only as an index of infection but also for differential diagnosis, for example between smallpox and vaccinia virus. The amnion and the allantoic sacs of the embryonated egg were previously used extensively for diagnosis of certain myxovirus infections, primarily with influenza and mumps viruses. Today this method has to a major extent been replaced by cell culture or by the direct identification of viral antigens. Laboratory mice are still being used for isolation of many coxsackie A viruses and arboviruses.

Direct identification of virus or viral antigens in samples from patients

A specific diagnosis can be obtained within a few hours by direct demonstration of virus or viral antigens in samples from patients. The aetiological diagnosis will reach the hospital while the patient is still ill and it can thus give important assistance in the choice of treatment and the epidemiological measures to be taken. Viruses which have a restricted capacity to grow in cell cultures may be demonstrated by this technique. It can be predicted that methods of this kind will become of increasing importance in future diagnostic work in virus laboratories.

Electron microscopy (EM)

EM analysis is used for estimation of the size, form and structure of virus particles. It is thus possible to identify to which family a virus belongs. For further characterization immune EM with specific antibodies is used.

For diagnostic EM the negative contrast technique is commonly used. The virus particles are surrounded by an electron-dense contrast which reveals the surface structure of the particles. If the outer part of the virion is damaged the contrast may penetrate and allow identification of internal components. The investigation is

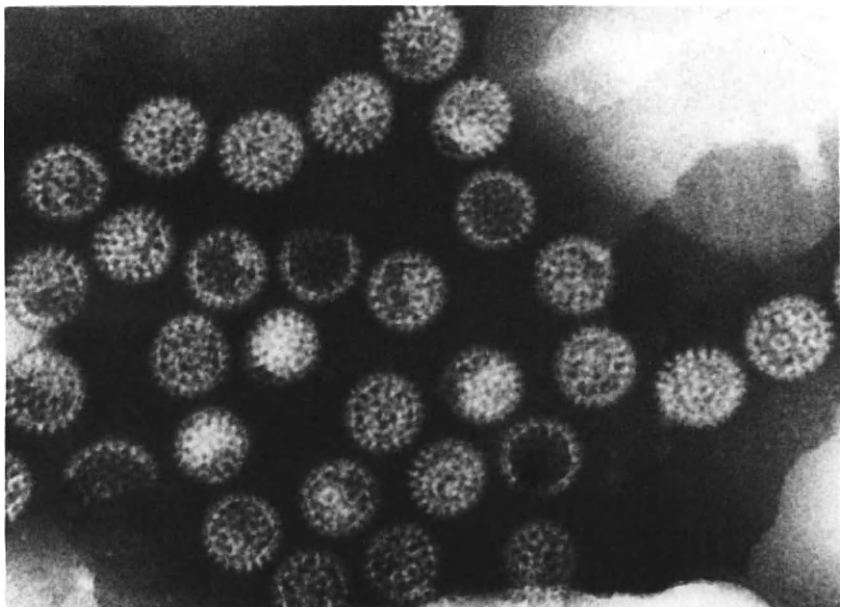

Figure 20.1. EM picture of rotavirus in a faecal sample from a child with diarrhoea. (Magnification: ×~160 000. Photo: L. Svensson)

readily performed by first allowing the test material and then the contrast to spread over a carbon and plastic coated copper grid for 15–30 seconds whereafter excess fluid is removed by a filter paper. The analysis can be performed in less than one hour. However, a relatively high concentration of virions is needed to give a positive result. Particles which occur in a low concentration in material from patients may be concentrated before examination, for example by ultracentrifugation of agglutination by specific antibodies.

EM analyses are often performed on faecal samples (e.g. rotavirus and adenovirus; *Figure 20.1*) but also on vesicle fluid (poxviruses and herpesviruses) and on brain biopsies (herpesvirus and rabies virus). Samples of serum and respiratory secretions are not suitable for routine EM analysis.

Immunological methods for identification of virus-specific antigens

For diagnostic purposes the presence of viral antigens may be demonstrated in cells as well as in suspension.

If collected at the proper time and from the appropriate locality most specimens will contain infected cells. The immunofluorescence (IF) technique has been shown to be a reliable method for specific identification of viral antigens in such cells. Immune peroxidase staining is a similar method, but since certain tissues contain endogenous peroxidases and artifacts may be encountered thereby it is less often used.

Immunofluorescence (IF) analyses

These are performed with sampled cells placed on a microscope slide. Fixation in acetone is used to bind viral antigens and cell components in cells and to make the cell membrane permeable to antibodies. The presence of viral antigens is demonstrated with specific antibodies which are either themselves fluorescein-conjugated (the direct method) or which are traced by fluorescein-conjugated antiglobulins (the indirect method). Fluorescein gives a yellow-green light in the fluorescence microscope. The more-or-less-typical topographical distribution of viral antigens in infected cells may give additional support to the diagnosis (*Figure 20.2*). A diagnosis can be given within three hours.

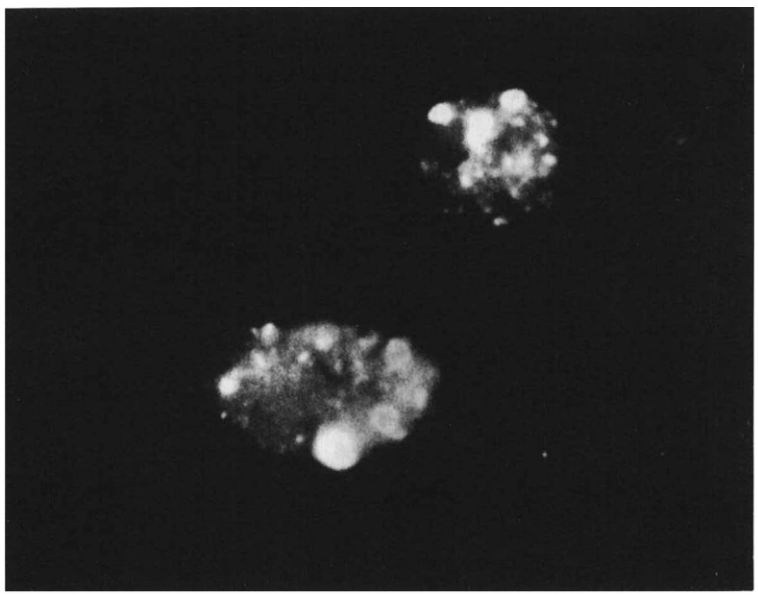

Figure 20.2. Cells containing RS virus antigen visualized by immunofluorescence, from a nasopharyngeal sample of a six-months-old child with obstructive bronchiolitis

The method is useful for the routine diagnosis of viral infections in the respiratory tract, skin, eyes and brain. In the case of very labile viruses, some of which are difficult to isolate, the method occasionally is the only practical means of obtaining a diagnosis. In experienced hands it is considered to be as reliable as virus isolation for the diagnosis of rabies infections.

Other immunological techniques

Viral antigens in suspension may be demonstrated by enzyme-linked immunosorbant assay (ELISA) or radioimmunoassay (RIA) as well as by the less sensitive techniques of immunodiffusion (ID) and immunoelectroosmophoresis (IEOP). In ELISA and RIA specific antibodies attached to a plastic surface bind viral antigens present in the patients' sample. The antigen is then demonstrated by specific antibodies coupled to a marker, i.e. an enzyme or a radioactive compound. Alternatively, the presence of antigen may be indicated through its competition with labelled antigen which is added instead of the labelled serum (*see Figure 20.5*).

ELISA and RIA methods are currently used for demonstration of hepatitis B antigens in serum, for rotavirus, adenovirus and hepatitis A virus antigen in faecal samples, but also for the demonstration of herpesvirus antigens and antigens of certain respiratory viruses. The sensitivity of the tests is high. For this reason, strict requirements have to be applied on the specificity and purity of the reagents used. Results of the tests may be obtained within a day.

Analysis of antibody response in patients

Serological methods are used for demonstration of specific antibody responses in patients with an infection.

Usually two sera are needed to perform a serological analysis. The first sample is taken as early as possible after appearance of symptoms, the other 1–2 weeks later. A methodologically significant increase of the antibodies against a certain viral antigen between the two samples, indicates that the patient at that time has been infected with the specific agent tested for or at least with an agent that is antigenically related. The serum titre (antibody concentration) is given as the highest dilution of serum which gives a positive reaction (for example 1:512), alternatively as the inverted value of this dilution (512).

Antibodies of IgM class appear earlier than, or at the same time as, IgG antibodies, but disappear more rapidly. With prolonged or chronic infections the IgM may remain detectable for a longer period of time. Virus-specific IgM antibodies frequently can be demonstrated in the first serum sample, which makes rapid diagnosis possible. If the first sample has been taken late the patient may already have developed a pronounced IgG antibody response. Identification of specific IgM antibodies may then provide the only possibility of serological diagnosis. As pointed out in Chapter 15, IgM analyses are of particular importance in the diagnosis of congenital infections since maternal IgM, contrary to maternal IgG, does not pass the placental barrier. Presence of specific IgM antibodies in the blood of a newborn child demonstrates, therefore, that the child must have been infected before birth.

Viruses induce the synthesis of several different antigenic proteins, and the antibody response in the infected individual is therefore complex. Antibodies

against antigens on the surface of the infectious particle are usually type-specific and antibodies of this kind may neutralize virus infectivity and consequently also provide immunity against infection. IgG antibody responses of this kind are usually durable. Antibody responses against other antigens, for example internal structural antigens and non-structural antigens, may be less durable and may cross-react with the corresponding antigens from related viruses. Different antigens may dominate in different serological techniques if one and the same antigen material has been used. It is therefore necessary to comment on the principles of some of the more important techniques and for the interpretation of results obtained.

The neutralization test (NT)

This measures antibodies which react with the surface of virions and neutralize their infectivity. The test is usually performed by incubating a series of dilutions of serum with a fixed dose of infectious virus, usually 100 ID_{50} (= 100 times the dose of virus which can infect 50 per cent of inoculated cell cultures), during a fixed time, for example one hour at +37°C or overnight at +4°C. The mixtures are then inoculated into susceptible cultures or experimental animals. The dilution of serum which can inhibit virus growth in 50 per cent of the infected cultures or animals is taken as the neutralization titre. In order to obtain reproducible results it is important that constant time and temperature for incubation of virus and antibodies are maintained, that the same cell type is used, and that the tests are read after a fixed time period. Preparations of certain virus strains contain aggregates of virions which are difficult to neutralize and therefore have to be removed, for example by ultrafiltration, before the neutralization test is carried out. The serum samples are usually inactivated at +56°C for 30 minutes before use, but for an effective neutralization of certain enveloped viruses, for example rubellavirus, there is need for the presence of a complement which may be added in the form of fresh serum.

Several technical variations of the neutralization test may be mentioned. The inoculated cultures can be covered by a layer of agar-containing medium and the antibody titre scored as the dilution which gives a 50 per cent reduction in the number of plaques. In order to allow automation and to save reagents, the cells may be cultivated and the neutralization test performed in the small wells of a microtitre plate made of disposable plastic. The end point of the serum titration can often be read on the bases of metabolic inhibition: cells which are actively metabolizing acidify the medium and the colour of the pH indicator is changed. This occurs in the wells where the virus has been neutralized but not in cultures where the virus infection has interfered with the cellular metabolism or destroyed the cells. If needed, the type-specificity of the neutralization tests can be increased to allow a comparison between closely related virus strains. This is performed either in the classic way by crosswise absorption of sera with the respective antigens or by kinetic neutralization. In the latter case the neutralization effect is measured by inoculation into cell cultures after varying times of incubation of the virus –serum mixture.

The haemagglutination-inhibition (HI) test

Many viruses can agglutinate red blood cells from one or more species at a suitable pH and this reaction can be blocked by antibodies directed against the antigen on the surface of virions which is attached to cellular receptors (*see also* Chapter 4).

Either whole virus particles or isolated haemagglutinin is used as antigen in the test. By use of the latter kind of antigen, somewhat higher antigen and antibody titres may be obtained. It is important as a first step to remove non-specific inhibitors of the haemagglutination which are present in most sera. These inhibitors are of a varying nature for the different viruses; glyoproteins and thermolabile inhibitors in the case of influenza virus; lipoproteins in the case of rubellavirus and other togaviruses etc. Consequently, the methods used to remove these inhibitors vary. Serial dilutions of sera which have been properly treated are incubated with an antigen amount corresponding to 4 times the minimum haemagglutinating dose. After these two ingredients have been allowed to interact, red blood cells are added and allowed to sediment. They form different bottom patterns depending upon whether they are agglutinated or agglutination has been blocked by antibodies. The serum titre is the highest 2-fold serial dilution of serum capable of inhibiting the haemagglutination (*see Figure 20.3*).

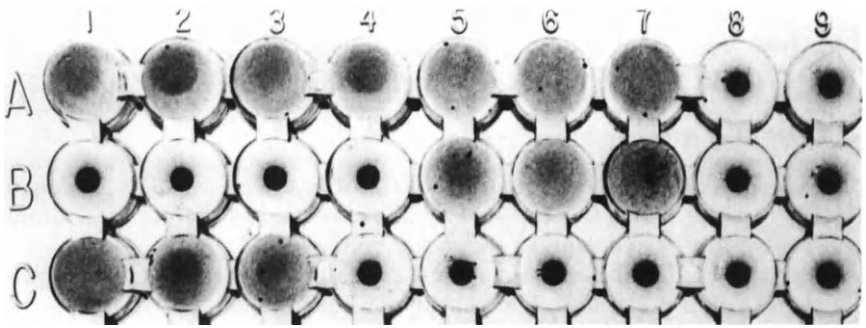

Figure 20.3. Demonstration of antibodies by a haemagglutination-inhibition (HI) test. The photograph shows the analysis of a pair of sera in serial 2-fold dilutions, starting from the left. The acute serum (row A) does not show any capacity to influence the haemagglutination by the virus. In contrast, the convalescent serum (row B) inhibits the agglutination in 4 dilution steps. Row C includes a control of the amount of haemagglutinating virus in the test; agglutination occurs in three 2-fold dilution steps. At the far right are controls showing that the sera do not by themselves agglutinate the erythrocytes

The HI test is relatively sensitive and only indicates antibodies against the haemagglutinin. In most cases this reaction is type-specific and correlates well with neutralizing antibodies and immunity. However, togaviruses represent an exception since different alphaviruses and flaviviruses cross-react in the test.

The complement fixation (CF) test

This test has a broad application and has therefore been one of the most extensively used serological techniques in virus-diagnostic laboratories.

The test is performed by incubating dilutions of heat-inactivated serum (56°C for 30 minutes) with 2–8 units of viral antigen and 2 units of complement (guinea pig serum) at +4°C overnight. An indicator system which contains sheep erythrocytes sensitized with amboceptor (antibodies against the erythrocytes) is then added and the mixture is incubated at +37°C. If the patient's serum contains antibodies against the viral antigen a complex is formed which 'fixes' the complement during the first antigen–antibody reaction. Consequently, no lysis of the sensitized red

blood cells added later will occur. Various purified antigens may be used in the CF test, but usually relatively crude and complex preparations are employed and the test therefore frequently measures antibodies against antigens other than the surface antigens of the virus. As already mentioned, these antigens are often group-specific rather than type-specific. Several controls have to be included in each CF test. In one control, no antigen is added to the serum dilution in order to exclude the possibility that the serum by itself fixes complement. This may occur if it has been contaminated by bacteria or contains circulating antigen–antibody complexes. Another control includes antigen but not serum, to exclude the possibility that the antigen by itself reacts with complement. Finally, a control serum and non-infectious antigen is included, to exclude the possibility that the antibody detected reacts with non-viral antigen from the cells or medium used for antigen preparation.

CF tests with viral antigens primarily measure IgG antibodies, rarely IgM antibodies. The CF antibody response therefore develops relatively slowly and an increase in antibody titres may be shown later than by the HI test, for example, which demonstrates both IgG and IgM antibodies. The titres obtained in CF tests are relatively low and the reaction is less sensitive than the HI test.

The immunodiffusion (ID) test

The ID test is relatively insensitive. In spite of this it has been used extensively in virological work, since it has the capacity to discriminate between several simultaneous reactions of different antigen–antibody systems.

When antigen and antibodies diffuse against each other from two wells punched in a thin agarose gel, a precipitate line can form where the two reactants meet in optimal proportions. Identical precipitate lines fuse to a continuous curve whereas lines representing different antigen–antibody complexes cross each other. Both the speed and the sensitivity of immunodiffusion tests can be increased by driving antigen and antibodies against each other in an electrical field, *immunoelectro-osmophoresis* (IEOP).

A quantitative determination of antibody concentration is most readily performed by inclusion of antigen in the gel. Under these conditions the precipitate occurs around the well in a circular zone, the diameter of which is proportional to the antibody titre. A variant of this technique which has been found very useful in serological diagnosis and immunity determination in rubella is the single radial diffusion haemolysis test (*Figure 20.4*). In this test red blood cells covered by rubella haemagglutinin are mixed into the agarose gel. After diffusion of antibodies into the gel it is overlayered with complement. This leads to development of zones of haemolysis since red blood cells carrying antigen–antibody complexes are destroyed.

The immunofluorescence (IF) test

The IF test has been used for determination of antibodies in routine diagnostic work and for research purposes it has been used extensively, for example in the characterization of different antigen–antibody systems in infections with EBV.

IF methods offer possibilities for discrimination between antibodies belonging to different immunoglobulin classes. Thus the test can be used for demonstration of IgM antibodies against, for example, EBV and CMV. Virus-infected cells fixed with acetone are used as antigen. They are incubated first with dilutions of the

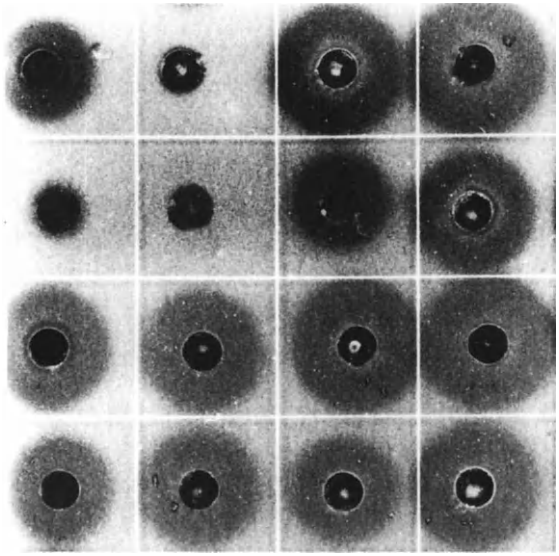

Figure 20.4. Single radial diffusion haemolysis test for determination of antibodies against rubellavirus. The uppermost row includes one negative specimen (only the well itself can be seen) and three positive control samples (clear zones around the wells). Rows 2 and 3 show test results with serum pairs from patients with rubella

Each convalescent serum in row 3 has a matching acute serum just above it in row 2. The bottom row includes sera from patients analysed for immunity against rubella; there are four positive samples

patient's serum and then, after washing, with fluorescein-conjugated antibodies against human IgM. If IgM is adsorbed to the cells it can then be visualized by fluorescence microscopy. A false-positive reaction may be encountered in this test if the patient's serum contains specific IgG and rheumatoid factor, i.e. IgM autoantibodies directed against IgG. False-negative results may be encountered in the presence of high concentrations of specific IgG since this may block the binding of IgM to the antigen.

RIA and ELISA

These techniques are as a rule more sensitive than other techniques for antibody determinations. The tests may be designed on different principles, employing either labelled antibodies or labelled antigen as an indicator of the possible occurrence of specific antibodies in the serum of a patient (*Figure 20.5*).

Because of the high sensitivity of the tests, it is necessary that the indicator reagent (antigen or antibodies) which is coupled to a radioactive isotope (usually iodine) or an enzyme (alkaline phosphatase or peroxidase) is available in a highly purified form in order to give the test a satisfactory specificity. Commercial RIA and ELISA tests are becoming available to an increasing extent, and many virus laboratories have developed their own RIA and ELISA techniques for various viruses. Once the specificity requirements are met with, these assays can readily be standardized and automized. The RIA technique involves the use of radioactive

isotopes, and therefore carries certain disadvantages for the working environment as well as for the stability of the reagents. Also, the reading of results of the RIA technique requires the availability of more expensive equipment (i.e. gamma counter) than does the ELISA test.

Both techniques can be used for determination of specific antibodies of different Ig classes. However, when these sensitive methods are used for indirect determination of IgM by use of antibodies against human IgM, it is particularly important to be aware of the risk of non-specific reactions because of the presence of rheumatoid

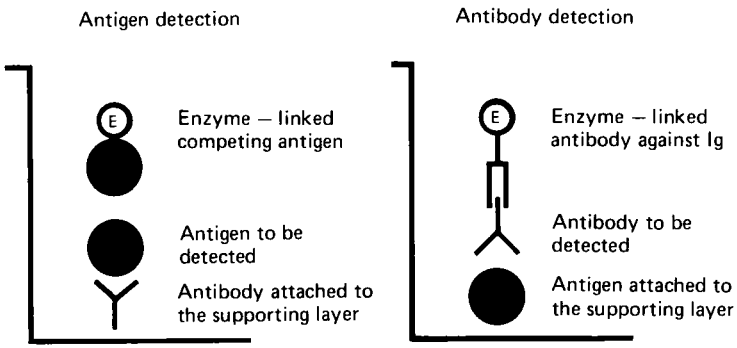

Figure 20.5. The principle behind an ELISA test used for antigen and for antibody determination. In this variant of the technique the determination of antigen is made by allowing an unlabelled antigen of a sample to compete with an enzyme-labelled known antigen. Antibodies are demonstrated by allowing them to bind to antigen on a solid phase and then indicating them by an enzyme-labelled anti-Ig. The presence or absence of an enzyme-labelled antigen or antibody is determined by addition of a substrate which changes colour if the enzyme is present

factors. In order to avoid this problem the factor or IgG has to be eliminated before performance of the test. Alternatively, antibodies against human IgM adsorbed onto the plastic surface may be employed to capture IgM from the test sample. Specific IgM is then tested for by incubation with viral antigen labelled with the indicator (radioactive isotope or enzyme). This variant also excludes the competition by high-titre specific IgG present in patient serum.

Evaluation of laboratory results

It should be pointed out that demonstration, by use of laboratory techniques, of a virus infection in a patient does not necessarily imply that this infection is the cause of the patient's disease. Many viruses often give inapparent infections or are excreted for a long time after the acute disease. Serological cross-reactions between related viruses should also be considered, for example between mumps and other paramyxoviruses, between herpes simplex and varicella-zoster virus and between different enteroviruses. These cross-reactions, which most often are encountered in CF tests, are common if the patient has been previously exposed to a related virus. On the other hand, cross-reactions are used routinely for group diagnosis of certain infections such as those caused by influenza and adenoviruses.

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21

The epidemiology of virus diseases

Margareta Böttiger and Erling Norrby

The epidemiological occurrence of different virus diseases shows marked variations. These concern seasonal and geographical distribution as well as the occurrence of diseases in different ages, sexes and races. The word *epidemiology* means, literally translated from the Greek language, 'the knowledge of what is over the people'. The traditional meaning of epidemiology is the science dealing with the occurrence of infections and disease. Many viruses may cause both clinical and subclinical infections. Both of these are important in the spread of infections.

The term *epidemic* is arbitrarily defined to mean the occurrence of a particular disease in a frequency exceeding that which should be expected under normal conditions. When a disease shows instead a habitual presence within a more restricted area the term *endemic disease* is used. The term *pandemic disease* is used to denote an outbreak of infections of exceptional proportions, including a spread over continents.

Surveillance of the epidemiology of infectious diseases is important for many different reasons. The awareness of the development of an epidemic may have a number of consequences. Since general therapeutic measures for the control of virus diseases are not yet available, various preventive interventions have to be made. These may include isolation of infectious individuals, mechanical protection (gloves, coats etc), use of disinfectants to prevent the spread of virus and appropriate passive and active immunizations, when applicable. The introduction of different schemes for limited or general vaccination against different virus diseases has often had a dramatic influence on the occurrence of these diseases. It has become increasingly important to monitor these changes in the epidemiological occurrence of certain diseases by different methods of surveillance.

Methods of surveillance of the epidemiological occurrence of infectious diseases

Notification of communicable diseases

Various reporting systems can be developed. These may register the *morbidity* and *mortality* in a certain disease. This information can be expressed as *prevalence* – the number of cases occurring at a fixed time point; *incidence* – the number of new cases during a predetermined period of time; or *frequency* – the total number of ongoing cases in a given group during a specified period of time. These systems may vary from the most simple one, i.e. the counting of new cases (numerical system) to

a somewhat more informative one in which name and age of patients are also noted (nominative system). In more extensive information-notification systems further data of epidemiological value may be included, such as the suspected source and place of infection, data on the onset of disease, occurrence of other similar cases etc. All reporting systems have their weaknesses and have to be evaluated with great caution and by persons who are aware of the limitations. A low incidence may be due to a poor reporting system; conversely, an improvement of a reporting system may give a false impression of an increase in the number of cases.

Almost all countries in the world try to follow the epidemiological situation by different reporting systems. At the World Health Organization in Geneva reports from all over the world are compiled by a special viral disease unit. A special group is surveying the epidemiology of poliomyelities. In addition, special centres and working groups deal with specific viral diseases. Thus there exist two influenza virus centres, one in London (UK), and one in Atlanta (USA).

Studies on the presence of immunity

The distribution of a disease in a population can also be determined by measurements of immunity to the infectious agent in different age groups, so called *seroepidemiology*. A prerequisite for such studies is that suitable serological methods are used and that samples are collected from a representative fraction of the population. It is important that the serological technique used has a high sensitivity and that it gives reliable results.

In contrast to notification systems registering disease, the seroepidemiological approach allows the identification also of subclinical infections. As an example it can be mentioned that the true nature of poliomyelitis infections was not discovered until it was possible to determine the presence of serum antibodies. It then became evident that apart from poliomyelitis causing paralysis there was also a large number of subclinical infections. The disease was found to be most prevalent in countries where the infectious agent spread less readily and where naturally-induced immunity deriving from infections early in life was relatively low.

Another means of studying immunity to a certain diseases is to use provocation tests. Attenuated virus strains are used as the agent of provocation. The response to or resistance against infection with such a virus can provide information on the absence or presence of immunity. Occasionally it is found that immunity is present even when no antibodies can be demonstrated. In these cases a state of sensitization exits and the body reacts on provocation with a booster reaction

Identification of virus

In the beginning of an acute epidemic it is particularly important to identify the causative agent. Isolation and a detailed characterization of virus have a particular epidemiological importance, for example in the case of genetically labile viruses such as influenza virus and foot-and-mouth-disease virus. The possibility of confirming or excluding long-term carriers of virus, for example hepatitis B carriers, is also of epidemiological interest. The diagnostic possibilities of identifying virus at an early stage of the disease are now rapidly improving. This will improve the possibilities of introducing preventive measures and, in the future, of using antiviral drugs effectively

The dissemination mechanisms of virus infections

In order to understand the mechanisms of the spread of infections three basic circumstances must be taken into consideration. These are the reservoir and source of infection, the transmission from this source, and the availability of susceptible hosts.

Reservoir and source of infection

Viruses may spread from many different sources and the contagiosity of the diseases they cause varies extensively. Most virus infections spread from man to man but animals or insects may also be a source of infection. The latter kind of infections are referred to as *zoonoses*. Concerning the spread of infections between humans is important to define whether it is dependent on an uninterrupted chain of contacts between acutely infected individuals or whether the virus may be continuously reintroduced into circulation from chronic infections or activated latent infections.

The contagiosity of an infection is dependent on (1) the amount of virus excreted, (2) the stability of the virus, (3) the duration of time of the excretion of virus and the relationship of this time to the possible occurrence of clinical symptoms, and (4) the route of exit of virus from infected individuals. It should be emphasized that healthy infected individuals are a relatively more important source of infection than diseased bed-ridden individuals and, further, that diseased individuals frequently spread large quantities of virus either before the appearance of overt symptoms or during the prodromal phase of disease. The incubation period for viral diseases varies from between a few days and many months. In these days of extensive international travelling it is a fact that within the incubation period of most viral diseases any part of our world may be reached. Consequently, a broader spectrum of viral diseases is now being encountered, in particular in industrialized countries. A broader assortment of available methods for the laboratory diagnosis of imported virus infections therefore is required in these countries.

Transmission of infection

Virus infections may be transmitted by a variety of different routes. The route of exit from the individual or animal which is the source of infection is of decisive importance. Respiratory infections may spread by aerosol. However, the importance of this route of transmission probably is overrated. Instead, spread of infected mucosal secretion from mouth-nose via hand (object) may play a dominating role. In special cases infected saliva may spread the infection in connection with kissing, for example, EBV in teenagers, or biting, for example rabies from a dog or other animal. Gastrointestinal infections spread from stool via hand or contaminated water. An example of the latter situation is hepatitis A; the virus may be excreted into sewage water for more than a week before the appearance of disease and this virus may accumulate in clams, for example, and spread via the ingestion of contaminated seafood. Swimming pools are another potential source of water-borne virus infections.

Virus may also exit from mucous membranes other than those in the respiratory and gastrointestinal tracts. Eye secretions may be infected and a spread of adenovirus infections from this source has been observed in connection with the use

of tonometers in ophthalmological clinics. That the urogenital tract may be an important source of virus infections has become inreasingly apparent only recently. Urine may be contaminated with viruses, for example large quantities of CMV is excreted by children with urogenital disease. However, the presence of viruses in secretions or blood in the genital tract probably plays a much larger role than virus-contaminated urine in the spread of infections. Sexual transmission can be a vehicle for herpes simplex virus type 2, hepatitis B, CMV and other infections. Transmission of virus via infected blood is seen in the case of hepatitis B and non-A and non-B hepatitis from transfusions or contaminated needles and syringes and, inevitably, in the wide spectrum of infections spread by vectors such as mosquitos, ticks, etc. Infected skin is the origin of the spread by contact for infections caused by poxviruses, some herpesviruses (HSV, varicella) and papillomaviruses. A final route of transmission to be mentioned is the vertical (transplacental) spread of virus from a pregnant woman to the fetus.

Availability of susceptible hosts

The third prerequisite for the continued spread of infections is the availability of susceptible hosts. Some virus infections induce an immunity of limited duration. This pertains to certain local infections in the respiratory tract and probably also in the gastroenteric tract. In this case repeated infections may occur. However, it is a characteristic feature of most generalized virus infections that they induce a highly efficient immunity which confers a lifelong protection against disease. Local reinfections without symptoms may occur in the immune individuals, but these limited infections probably do not represent a source of further spread of infection. With epidemics of generalized virus disease, for example measles, there is an accumulation of immune individuals. When a certain fraction of the population is immune the further spread of an infection, which is dependent on a chain of transmission between acutely infected individuals, is restrained. The percentage of the population which needs to be immune to establish this *herd immunity* varies for different viruses. In the case of a highly contagious virus like measles it has been shown to exceed 90 per cent. The size of a population and the frequency of contact between individuals also influence the possibilities for maintaining a continued, and most of the time, low-grade, endemic spread of infections. It has been found that under conditions prevailing in industrialized countries a population in excess of 300 000 is required to allow a continued circulation of measles virus. Thus in a country like Iceland measles epidemics will 'burn out'. New epidemics will occur provided that a sufficient number of non-immune individuals have accumulated and the virus is reintroduced into the society from without.

The eradication of smallpox

Smallpox is an example of a disease caused by one type of virus. Infections with this virus can be prevented by vaccination. There is no animal reservoir for smallpox virus and the infection can only be transmitted by direct contact between acutely diseased individuals. These were the conditions that set the stage for the global eradication of smallpox which was achieved in 1977. In order to reach this goal it was not found necessary to establish a herd immunity in populations in all countries. Instead it was experienced that the chain of transmission of disease could be effectively interrupted by vaccination of individuals in the surrounding areas of the infected population, a technique referred to as *containment measures.*

Epidemiological patterns of virus infections

As already discussed, the fraction of immune individuals determines the epidemic vs. endemic occurrence of virus infections. This ratio fluctuates in the yearly occurrence of different infections and in some cases patterns of epidemic occurrence can be discerned. In non-immunized populations in industrialized countries, measles epidemics occur at 2–5 year intervals and each epidemic lasts for 3–4 months. The intervals between epidemics of, for example, mumps and rubella, are some years longer owing partly to the relatively lower degree of contagiosity of these infections.

Many virus infections also show characteristic patterns of seasonal occurrence. In temperate climates enterovirus infections occur during the late summer and autumn whereas respiratory infections like influenza are winter diseases (cf. *Figure 21.3*). Obviously the patterns of social life determining the intensity and form of contacts between individuals play a role in this contagion, but there must also be other important but as yet undefined factors.

Examples of the epidemiology of some virus diseases

Measles, mumps, rubella and varicella

The reservoir and source of infection of all these common childhood diseases is man alone. Only one serotype of each virus has been identified throughout the world. Both clinical and subclinical infections occur although the latter are rare in the case of measles. The immunity following disease is long-lasting – for practical purposes a life-long immunity. In temperate climates these diseases appear in

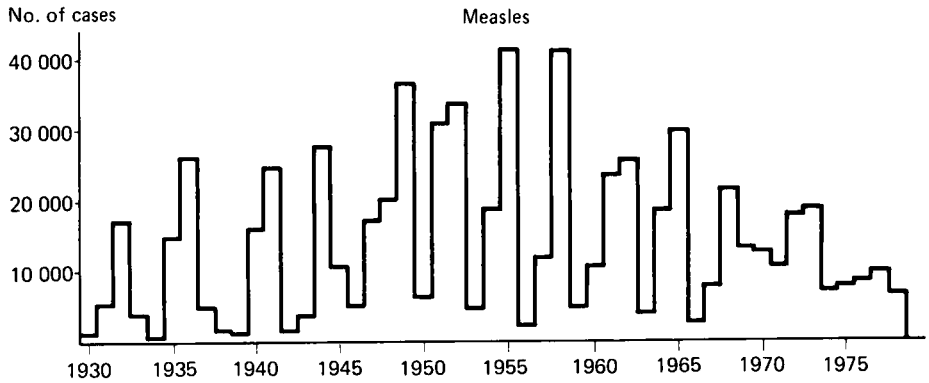

Figure 21.1. Yearly occurrence of measles in Sweden as reported by regional medical officers. Vaccination on a limited scale was introduced in 1972

waves at 5–10 year intervals. *Figure 21.1* gives the annual number of cases of measles reported from district officers in Sweden. In this relatively sparsely populated country with a temperate climate, measles returned in waves with 4–7 year intervals before vaccination was introduced on a limited scale in 1972. In tropical countries both the patterns of seasonal and yearly appearance are different. Serological epidemiological studies (*Figure 21.2*) show that the other childhood diseases occur at somewhat higher ages than measles, in part a reflection

of their lower contagiosity. Furthermore the intervals between epidemics of mumps, rubella and varicella are somewhat longer than for measles.

In northern Europe most children have been infected with measles by the age of 12. The percentage of individuals immune to mumps and varicella is 80–90 per cent and to rubella only 40–50 per cent at this age. This is of importance since men contracting mumps during or after adolescence may develop orchitis which occasionally leads to infertility, and women contracting rubella during early pregnancy may transmit the infection to the fetus, which may cause fetal damage.

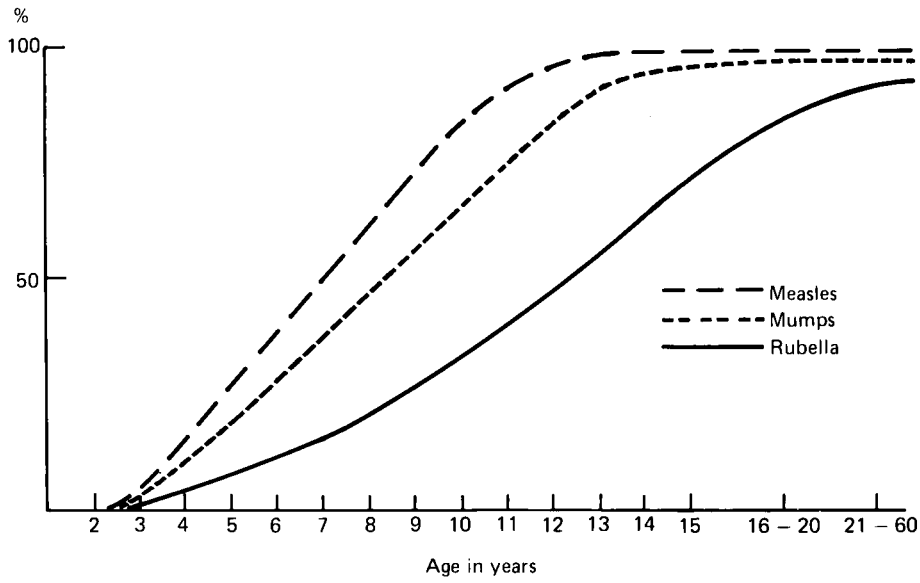

Figure 21.2. Percentage of children presenting demonstrable antibodies to measles, mumps and rubella in different age groups in Sweden before the introduction of vaccination

From the epidemiological point of view, varicella differs from the other three diseases in that it gives latent infections. When an endogenous infection with varicella virus is activated and causes a development of zoster, a spread of virus from skin vesicles may cause varicella in non-immune children in the surrounding area.

Influenza

Influenza virus is an example of a genetically labile virus. It can change its antigenic character abruptly and extensively – *antigenic shift*, at 10–20 year intervals, and also gradually from year to year – *antigenic drift*.

Influenza A appears in pandemics which occur after an antigenic shift. The antigenic composition of the virus has been so extensively altered that the immunity derived from earlier influenza virus infections no longer provides protection. In particular, the H (haemagglutinin) antigen is important for the immunity, but also the N (neuraminidase) antigen may play a role. The nomenclature used for classification of the different influenza virus variants include identification of the

serotype A, B or C, the place where the strain was first isolated and its H and N characters. The PR8 variant from the 1930s was named A/PR8/34 (HON1). In the years 1947 to 1956 a variant with the H and N characters H1N1 prevailed. In 1957 a new extensive pandemic wave started, the 'Asiatic flu' caused by influenza A/Asia/57 (H2N2), and in 1968 there was again an antigenic shift to influenza A/Hong Kong/68 (H3N2). The most likely explanation of the occurrence of antigenic shifts in influenza virus is a genetic reassortment in connection with a simultaneous infection of cells with a human and animal influenza A virus. For unknown reasons new strains appear primarily to originate from Asia. In 1978 the old strain from the 1940s, A(H1N1), unexpectedly returned. It remains to be explained how this strain had managed to survive in nature.

So far mainly people born after 1957 have become infected by this strain which illustrates that influenza virus infections induce a long-lasting immunity.

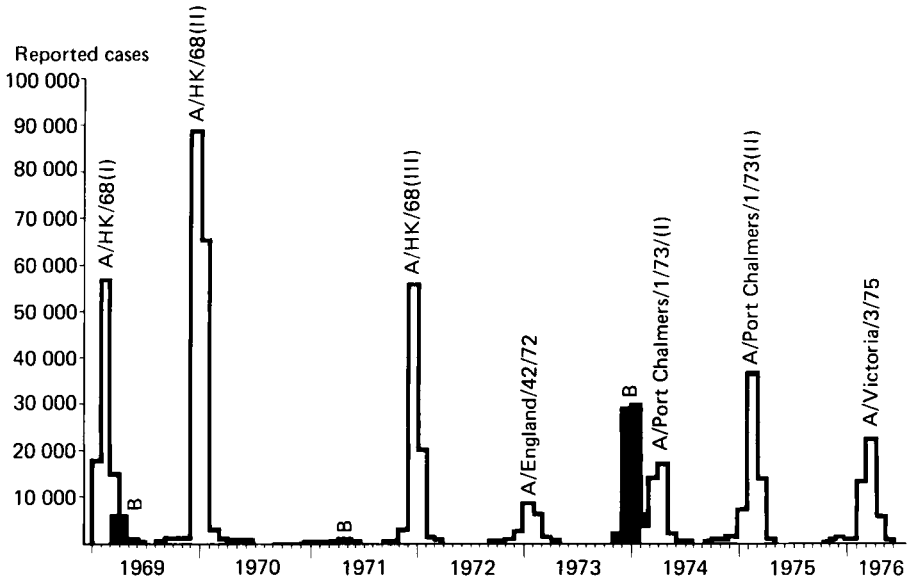

Figure 21.3. Monthly appearance of influenza A and B infections in Sweden during the period 1969–1976. The occurrence of antigenic drift is indicated by the varying designation of strains

Between the shifts of H and occasionally also N antigens, antigenic drift causing minor changes in these virus surface components can be registered. Example of variants generated by drift are influenza A/England/72 and A/Texas/77 both of which derived consecutively from the A/Hong Kong/68 virus (*Figure 21.3*). The mechanism behind antigenic drift in influenza is mutational changes in the virus haemagglutinin and possibly also neuraminidase.

Other respiratory infections

The common cold is the most common of all viral diseases. It is caused by numerous different rhinoviruses and coronaviruses and many still unidentified

viruses (*see* Chapter 34). Many of these viruses probably induce a fairly shortlived local immunity. However, the great number of agents involved in this disease has hampered detailed epidemiological evaluations.

Other respiratory infections may give a more severe disease than coryza because of involvement of lower parts of the respiratory tract. The importance of different viruses varies partly at different ages. In the first years of life RS virus is an important cause of capillary bronchitis. The epidemiology of RS virus infections is unique because of the apparent absence of any transfer of immunity during the prenatal period.

Enteric infections

It is characteristic of enteric viruses that they are relatively resistant to heat and to many disinfectants, that they are excreted in large quantities in the stools, and that the course of the infection in many cases is subclinical.

Polio virus infections, which are the ones most thoroughly studied, may serve as an example of enterovirus epidemiology. Under poor hygienic conditions the virus circulates more or less constantly in the community and most individuals are infected during the first years of life. At this age many of the children are protected against viraemia by maternal antibodies. Other unknown, age-related mechanisms also seem to play a role in preventing a central nervous system involvement of the infection at this age. There is an increasing risk of contracting paralytic disease when a person is infected later in life. Thus the frequency of paralytic cases among subclinical cases is about 1:1000 to 1:10 000 when the infection occurs in the first years of life but may be as high as 1:10 when adults are infected.

Polio in an epidemic form started to emerge in countries with improving hygiene. The first epidemics involved children and were encountered around 1860 in Sweden. With improving hygiene the disease in Scandinavia gradually came to attack more teenagers and adults. Because of this epidemiological pattern, poliomyelitis is referred to as a *disease of civilization*.

In recent years it has been demonstrated that a broad spectrum of different viruses can cause enteric infections. Rotaviruses in particular have been disclosed as an important aetiological agent in infantile diarrhoea.

Sexually transmitted virus diseases

Herpes simplex virus (HSV) type 2 is the most extensively studied sexually transmitted virus disease. Both acute and activated latent infections may be the source of spread of virus. Serological epidemiology has shown that whereas HSV type 1 infections accumulate during the preadolescent years, infections with type 2 occur in connection with the establishment of sexual relationships. It has been noted that the availability of modern contraceptive techniques has increased the frequency of sexually transmitted diseases, including infections with HSV type 2. Other viruses also may be spread by sexual contacts but the relative importance of this route of transmission is poorly defined. Hepatitis B is discussed in this context and the possibility of this virus spreading has a special relevance because of its capacity to establish chronic infections. Chronic hepatitis B represents a particular problem in homosexual men.

Virus diseases transmitted from a pregnant woman to the fetus – (vertical transmission)

The *vertical transmission* is used when a virus is transmitted either during pregnancy to the fetus or to a child in connection with delivery. The most important cases of fetal damage caused by such a transmission are congenital rubella and CMV infections (*see* Chapter 15). From the epidemiological point of view it is of importance that children with these congenital diseases are excreting virus for periods of months to years after birth. The paradoxical effect – that a pregnancy causes a physiological immunosuppression which leads to activation of some latent infections – should also be mentioned. One example is activated CMV infections. This virus can be isolated from 4 per cent of pregnant women at the time of delivery. However, in terms of fetal damage, primary infections with CMV appear to play a major role.

Slow virus infections with non-immunogenic infectious agents

Certain very unusual epidemiological circumstances were revealed concerning the non-inflammatory and lethal infectious disease, kuru, which mainly afflicted women and children in a special area of New Guinea (*see* Chapter 17). It was revealed that the causative agent was likely to have been transmitted in connection with funeral rituals. The practising of these rituals ceased in 1957 and no person born after that year has developed the disease. However, cases of kuru still appear. The routes of natural transmission of Jakob–Creutzfeld disease, which is the equivalent of kuru in countries other than New Guinea, are unknown. Clusters of cases at various times and locations have been encountered, but the source of infection has not been detected. Certain cases of Jakob–Creutzfeld disease may be due to intrafamiliar spread. In a few cases transmission in connection with medical interventions has been identified.

Diseases spread by artificial inoculation

Diseases hitherto unrecognized in industrialized countries became noticeable when they were found to be spread by the increasing use of injection and transfusion techniques. Among these diseases hepatitis B has been studied most extensively (*see* Chapter 30). Also other as yet not well defined viruses (non-A – non-B hepatitis virus) are transmitted in a similar way giving rise to what has become known as *nosocomial* or *iatrogenic infections* (diseases spread in connection with medical treatments). Rigid measures with the particular aim of preventing the spread of infected blood have been successful in reducing the circulation of hepatitis B within hospitals.

Hepatitis of both B and non-A, non-B types can be transmitted outside hospitals especially by addicts using intravenously injected drugs and within family milieus.

In connection with organ transplantation virus may be carried over from the donor to the recipient. CMV has been found to be transferred commonly in this way in connection with both kidney and heart transplantation. Corneal transplantation has caused a transmission of Jakob–Creutzfeld disease in one case and rabies in a few cases. Jakob–Creutzfeld disease has been also transmitted to two individuals in connection with stereotactic electroencephalography.

Zoonoses

Certain virus diseases in man emanate from animal reservoirs. Like humans, the animals may show symptoms or be healthy carriers. One of the most dreaded zoonoses, rabies, is transmitted by direct inoculation by a bite of, for example, a dog, wolf or bat. Examples of indirect transmission are the disease nephropatia epidemica in Scandinavia and the closely related Korean haemorrhagic fever, which are transmitted by contact with material contaminated by virus-infected rodents.

However, the most common route of spreading of a zoonosis is transmission by a vector. At least 50 different haemorrhagic fevers have been categorized, mainly from tropical areas (*see* Chapter 26). Yellow fever is the most wellknown disease. In Europe tick-borne encephalitis of eastern and Scandinavian types are prevalent. The reservoirs are wild and domestic animals. Characteristics of this kind of disease are the endemicity and, in temperate climates, seasonal appearance. The epidemiology of these diseases is determined by a complex interaction between factors such as infected animals, environment, climate, season and immunity. From the epidemiological point of view zoonotic infections in man in most cases represent a blind alley, i.e. no further spread of infection to other individuals can occur. The only exception is second- and occasionally third-generation spread of Ebola–Marburg virus infections to relatives and hospital personnel from close contact with patients under primitive conditions. The risk of transfer to laboratory personnel in connection with analysis of samples from patients with these kinds of rare diseases, including Lassa fever, should be appreciated.

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22

Inactivation of viral infectivity and disinfection

Erik Lycke

Inactivation of virus, i.e. destruction of its capacity to infect cells, can be achieved by means of several physical and chemical methods. Depending upon the desired end-result, different methods for virus inactivation have been elaborated. For disinfection purposes, coarse and destructive techniques may be used, while the inactivation of viral infectivity associated with production of vaccines requires a method eliminating the infectivity without significantly reducing the immunogenicity of the viral proteins.

Virus suspensions lose infectivity spontaneously upon storage. Rate-limiting factors for the inactivation are the temperature for storage and properties of the virus-suspending medium. Enveloped viruses are, as a rule, considerably more sensitive to environmental influences than non-enveloped viruses. Damage to the viral envelope will result in loss of infectivity. In the virus laboratory virus strains are stored at low temperatures. ($<-70°C$) and the suspending medium is designed to maintain the infectious virus (isotonic salt solution, neutral pH, etc). To protect the virions a stabilizing protein or other so-called protecting colloids are added or an admixture of DMSO (dimethylsulphoxide) is used. Lyophilization provides a most efficient method for storing virus strains.

In *Table 22.1* some methods used for *sterilization and disinfection* are listed. Effective techniques based on heating have had a long history. The standard programme for sterilization of microbially contaminated materials (autoclaving at 120°C for 30 min at $1\,kg/cm^2$) inactivates all viruses. Most viruses are rapidly inactivated at 50°C and within an hour more than 99 per cent of the infectivity is eliminated. In general, boiling for 20 to 30 minutes is sufficient to inactivate free virus particles but, as mentioned, the rate of inactivation varies with the amounts of other contaminating and protecting organic materials (blood, faeces etc.) present. Some viruses are more resistant to heat than others. Hepatitis B virus in particular has been referred to as a heat-resistant virus. However, this is probably due more to failures of sterilization, disinfection and the cleaning procedures used in the past than to the properties of the virus. Nosocomial infections were not uncommon and could often be traced to contaminating blood from insufficiently cleaned and non-sterile syringes and instruments. The introduction of better methods and the control of sterilization techniques and laboratory methods for the diagnosis of viral hepatitis have efficiently reduced hepatitis B virus infection in hospitals.

In recent years the remarkable stability of infectious agents causing the progressive encephalopathies has been emphasized (cf. kuru, Jakob–Creutzfeld disease, Chapter 17). Their resistance to inactivating irradiation has suggested that these

agents must have a unique nature, thus explaining their unusual stability. However, it should be noted that some chemical treatments (lipid solvents, detergents) inactivate the agents and may, like heat-sterilization, be used for prevention of the infections.

Dehydration Dehydration at room-temperature causes inactivation of many viruses. Again it should be recalled that viruses in organic material might maintain infectivity for a considerable time. Outbreaks of smallpox and varicella have been mediated by virus spread through ventilation systems of hospitals. The infectivity of the virus in crust material and dust seems to be preserved.

Irradiation Irradiation with ultraviolet light is a long established method of disinfecting surfaces and air in microbiological laboratories. Prerequisites for effective disinfection by irradiation are that the virus is exposed to the irradiation

TABLE 22.1. The most common methods for sterilization and disinfection and methods for production of inactivated virus vaccines

Method	*Purpose*	*Reaction*
Autoclaving	Sterilization	Denaturation of proteins
Heating	Disinfection	Denaturation of proteins, hydrolysis
Irradiation	Disinfection	Damage to the virus-genome
Photodynamic inactivation	Disinfection	Damage to the virus-genome
Oxidative inactivation	Disinfection	Hydrolysis, denaturation of proteins, damage to the virus-genome
Organic solvents	Disinfection	Destruction of the envelope
Formalin treatment	Vaccine production	Interaction between HCHO and the virus capsid and nucleic acid
β-propiolactone	Inactivation of whole virus in vaccines	Damage to the virus-genome
Detergents	Production of vaccines containing purified envelope components	Inactivation of enveloped virus by release of viral peplomers as a step in production of a vaccine

and not sheltered by other particulate materials present and that the intensity of the irradiation is adequate for the inactivation. The wavelength ranges most efficient for inactivation are between 2000 and 3000 Å, a range which covers absorption optima of nucleic acids. The inactivation capacity is reduced proportional to the square of the distance between the irradiation source and the target, and is dependent upon direct hits on the virus-genome.

Photodynamic reactions Photodynamic reactions by means of staining with acridine orange, neutral red, proflavin, methylene blue, etc. have been used to disinfect infected tissues locally. The photodynamic inactivation of viruses has been tested clinically for the treatment of cutaneous changes induced by herpes simplex virus. The stain is integrated in viral nucleic acid during the virus replication process. When the progeny virus is exposed to light, the dye emits energy and causes changes in the virus-genome resulting in loss of infectivity. Herpesvirus inactivated by photodynamic reactions has been shown to cause cell transformation and the method is therefore not accepted for therapeutic application.

Chemicals Chemicals for inactivation of viruses can, like irradition, act by reacting with the viral nucleic acid. However it is also possible to use chemicals reacting with virus proteins or the lipids of the virus envelope. Enveloped viruses are inactivated if the envelope is destroyed by, for example, extraction of the lipids. Different compounds with affinity for lipids (alcohol, ether, chloroform, etc.) can therefore be used for disinfection purposes against enveloped viruses. Exceptions are the poxviruses which have envelopes which are relatively resistant to organic solvents.

Non-enveloped viruses (for example, picornaviruses, adenoviruses) are less effectively inactivated by organic solvents. Instead, oxidation is the reaction most often utilized for disinfection. Halogens (Cl^-, J^- and F^-) react with S–S linkages of proteins and cause inactivation by oxidation. Other compounds affecting the stability of the protein capsid and thus destructive to infectious viruses are urea and phenols. It is important to remember that the presence of other organic materials (cell debris, serum, etc.) will influence the rate of inactivation and the result of the disinfection. The phenomenon of break-point chlorination for treatment of sewage is well known. Hypochlorite should be added in concentrations leading to a presence of free hypochlorite ions and not to chloramines formed by reactions with ammonia and amines. As a rule, effective disinfection is attained when the redox-potential of the virus-containing fluid exceeds 500 mV.

Formalin Formalin is the reagent conventionally used in vaccine production, i.e. for the inactivation of the virus preparation without destroying its immunogenic properties. Obtaining the correct balance in the formaldehyde treatment, i.e. an effective inactivation of virus infectivity together with maintained immunogenicity, has sometimes been difficult. Production of inactivated polio virus vaccines was for some time discredited by inadequately inactivated vaccine batches which caused a number of cases of paralytic polio in vaccinees. There have also been formaldehyde-treated vaccines which have been 'hyperinactivated' and thus have become poor immunogens. Formaldehyde reacts with the proteins as well as with the virus nucleic acid. Reactions between the virus capsid proteins and formaldehyde seem to cause fixation or a tanning of the capsid. This seems to render the capsid gradually less permeable to HCHO and delays the reactions with the viral nucleic acid. The kinetics of the inactivation of polio virus by formaldehyde deviate therefore from that of a first-order reaction. The second most common chemical for inactivation of virus vaccines has been β-propiolactone which has a greater affinity for nucleic acids than for proteins.

The inactivated virus vaccines are still much in use. Treatment with formaldehyde is used for production of influenza virus vaccines and in some countries for production of inactivated polio virus vaccines. The influenza virus vaccines have

been criticized for low immunogenicity. Since it is difficult to produce inactivated vaccines of enveloped viruses without lessening immunogenic qualities, attempts have been made to replace these vaccines with so-called *split virus vaccines* (*Figure 22.1*). Treatment of the virus with detergents releases the viral peplomers of the envelope. Nucleocapsids and glycoproteins are separated and the remaining nucleic

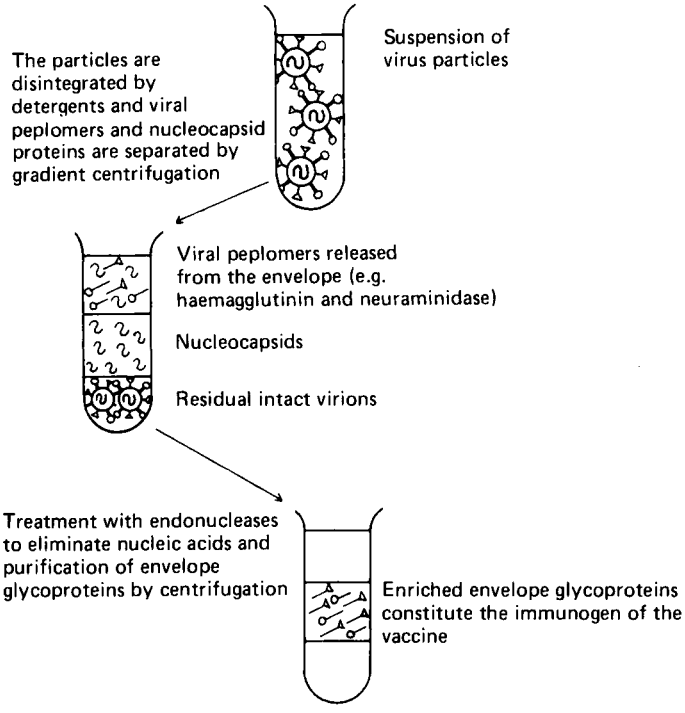

Figure 22.1. Production of split virus vaccines

acids of the glycoprotein fraction are digested with nucleases. The viral glycoproteins are used as the immunogens of the vaccine. The method has so far mainly been used for the production of vaccine against influenza but may, theoretically, be used for the production of split virus vaccines against any virus infection.

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23

Immune prophylaxis

Erling Norrby

The immunological defence reactions carry the important dual function of removing an infection from the diseased individual and of preventing a renewed attack by the same infectious agent. Many virus infections lead to the development of a lifelong immunity. This immunity may imply both a complete resistance against replication of virus in connection with a renewed exposure and a prevention of a new bout of disease. In the latter case, a local infection without symptoms may occur. This may lead to a boosting of the pre-existing immunity.

The immune defence mechanisms can be used in two different ways in order to provide protection against infections. One method is to provide individuals with specific antibodies by injection of immune globulin, *passive immunization*. The second method is *vaccination*, which is based on the stimulation of an individual to develop his own immune reaction, *active immunization*. All immune prophylaxis is specific and directed against a certain infectious agent.

The relative efficacy of different methods of immune prophylaxis is determined by a number of factors. Passive immunization aims at providing individuals with a shortlasting protection against a generalized virus infection. In contrast, an active immunization may provide protection for the vaccinee and in addition also for persons in his vicinity by a comprehensive usage of vaccines. After appropriate immunization the protection may be lifelong and pertain not only to generalized but also to local infections. The use of virus vaccines has provided some of the most spectacular advances in modern medicine. Examples are the elimination of poliomyelitis from industrialized countries and the global eradication of smallpox.

Passive immunization

Antibodies which are transferred from a pregnant woman to her fetus (maternal antibodies) represent a form of passive immunization which provides an important immunity against generalized infections in the newborn, immunologically-immature individual. In man, as in certain other species, immunoglobulins are actively transported through the placenta during the later part of the pregnancy. Maternal antibodies protect against virus infections such as measles and poliomyelitis. The duration of the protection correlates with the concentration of circulating specific antibodies in the mother. It has been shown that measles antibodies of maternal origin can in many cases negatively influence the take of a live measles vaccine even at 12–14 months after birth. Consequently, live measles vaccine and

also certain other live vaccines should not be administered until after the age of 14 months.

Breast milk is a source of passively transferred antibodies. In man antibodies in milk are not resorbed, but they provide a local immunity against infections in the gastrointestinal tract.

Antibodies can be passively transferred in the form of immunoglobulin. This type of preparation occasionally referred to as gammaglobulin, represents a concentrate (16.5 per cent solution) of purified human IgG proteins from a large number of different donors. The effect of passive immunization is dependent on (a) the concentration of specific antibodies in the immunoglobulin and the dose which is given, (b) the time interval between the injection of the preparation and exposure to the infectious agent, and (c) the pathogenesis of the virus infection. In order to increase the concentration of specific antibodies, a selection of donors with especially high titres of antibodies against a certain infectious agent is sometimes made. For example, blood collected from individuals who recently have had herpes zoster may be used to prevent severe forms of varicella. In connection with a herpes zoster patients boost their titres of antibodies against varicella virus. Preparations of this kind are referred to as immunoglobulin against a certain disease, in the given example varicella.

With passive immunization, the protective effect is established almost immediately, whereas it does not develop until several days after active immunization. Since only limited amounts of immunoglobulin can be given to an individual, the protective effect works exclusively against viruses which have a viraemic phase as an important step in their pathogenesis. Thus immunoglobulins cannot provide any protection against local infections. The protection after an injection of an immunoglobulin in optimal cases can have a duration of 2–3 months; administered IgG is eliminated with a half-time of 3–4 weeks. The proteins in an immunoglobulin preparation have a tendency to aggregate. The preparation therefore usually is given intramuscularly, even though preparations are available today which can also be injected intravenously. Treatment with immunoglobulins is specially important for individuals with a yielding immune defence caused, for example, by a congenital defect, by being a secondary adjunct to some other disease, or by certain medical treatments. In addition immunoglobulin is used in the following situations.

Measles Susceptible children under one year of age, who have been exposed to measles in industrialized countries, should be treated with immunoglobulin since a generalized infection may be a heavy burden at this age. The passive immunization should be given within five days after exposure. The amount of immunoglobulin given can be adjusted so that the infection either is completely prevented or is allowed to occur in a mild form. The latter form of infection may provide a durable immunity. However, results of this procedure are somewhat unpredictable. In order to obtain an immune protection of definite duration it is therefore recommended that a higher dose of immunoglobulin be given followed by a vaccination with live measles vaccine half a year later.

Hepatitis A The protective effect of immunoglobulin against the epidemic form of hepatitis is well documented. It should be used in persons who are in contact with individuals infected with hepatitis A and in people travelling to countries where the risk of contracting an infection is large. The relatively long incubation time of this disease provides good opportunities for a passive immunization also after exposure. However the treatment should be performed as early as possible.

Rubella In special cases it is necessary to consider the use of immunoglobulin as a treatment for a non-immune pregnant woman who has been exposed to rubella-virus. However the prevention effect of immunoglobulin on congenital rubella is limited. Preferably, therefore, a specific immunoglobulin against rubella should be given but even that does not provide good protection. Thus passive immune prophylaxis against rubella should be used very selectively and it must always be combined with a collection of blood samples before and 4–5 weeks after the treatment. The serological analysis of the first sample will show if the woman already has an immunity, whereas analysis of the later sample will indicate whether the treatment has been effective.

Specific immunoglobulins are used in the following situations.

Varicella This specific immunoglobulin is obtained by fractionation of plasma collected from patients who recently have had a herpes zoster infection. This preparation can be used to prevent serious infections during the neonatal period and in immunosuppressed patients.

Hepatitis B Blood donors are analyzed for the occurrence of antibodies against hepatitis B. Immunoglobulin is prepared from selected donors with high antibody titres and is used in the first place for treatment of persons who are suspected to have been infected with hepatitis B.

Active immunization

By the end of the eighteenth century Jenner had developed a prophylactic method against smallpox, one of the most severe infectious diseases occurring in those days. Thus Jenner's method was introduced more than fifty years before Pasteur could elucidate the nature of infectious agents and before the term, *immunity*, had obtained any conceptual meaning. Jenner noted that individuals, who had contracted the mild disease, cowpox, after contact with diseased cattle, showed a protection against the severe disease, smallpox. He found that a cowpox infection could be transmitted experimentally from cow to man and, further, from man to man. Thereby, possibilities of providing protection against smallpox were feasible. The substance used for inoculation came to be called vaccine (L. *vacca* = cow) because of its origin. This term is now being used to denote all kinds of preparations which are used for active immunization. By use of Jenner's vaccine it has now been made possible to eradicate smallpox from the world.

Two principally different kinds of vaccines – inactivated and live – can be distinguished. Inactivated vaccine either contains killed complete infectious agents or isolated antigens of a varying degree of purity. Live vaccines contain infectious material of selected strains of the agent which have a reduced pathogenicity. They act by inducing mild or symptomless infections leaving an immunity optimally similar to that which follows a regular disease caused by virulent virus.

Viral vaccines can be used to protect a selected group of individuals or for a general vaccination. In the latter case marked effects on the epidemiological occurrence of both the disease and virus can be obtained. Different viruses have different contagiosity. The fraction of susceptible persons in the population in many cases therefore has to be of a certain order of magnitude in order to maintain the chain of man-to-man spread of virus. With the introduction of a general

vaccination it is necessary to have careful follow-up so that the overall majority of non-immune children in low age groups are immunized annually. If circulation of a virus in the community is markedly reduced or eliminated it is important that a non-immunized fraction of the population does not accumulate and become infected with a reintroduction of virus.

Inactivated vaccines

The great advantage with this kind of vaccine is that the immunization occurs without any replication of the infectious agent in the body. The immune defence which develops primarily includes a formation of antibodies whereas cellular immunity normally does not develop. However, addition of an adjuvant may lead to an induction also of the latter kind of immunity. Inactivated vaccines are usually given parenterally but in certain situations a local application on mucosal surfaces has been tried. However, the efficacy of the latter procedure needs to be further evaluated, especially concerning the durability of the immunity. Parenterally administered vaccine induces the production of circulating antibodies. The content of these antibodies in the circulation depends on the amount of antigen in the vaccine and the immunization scheme used. In most cases, repeated injections are required at varying time intervals so that a high titre of antibodies in the circulation can be obtained as a consequence of booster effects. The primary purpose of using an inactivated vaccine is to induce a humoral immunity which prevents a spread of the infectious agent from the primary focus of replication. However, in special cases, the immunity can also include a protection against a local replication of virus at the entry point of the infection. As an example of such a protection can be mentioned the effect of immunization with inactivated polio vaccine in Sweden. The concentration of circulating antibodies reached by the adopted scheme of immunization exceeds that after natural infection. Since the concentration of circulating IgG antibodies is relatively high a certain fraction of these antibodies (about 1/200–1/300) will appear at the mucosal surface. Hereby, the occurrence of reinfections is prevented. The duration of the immune protection also is related to the content of circulating antibodies. In the case of inactivated polio vaccine it now appears that the immunity obtained after four separate injections may last for a lifetime. Thus it appears that in this case there is no need for a continued administration of antigen to provide a lasting immunity.

The ideal inactivated vaccine should contain only purified isolated antigens of the kinds which are needed to induce a protective immunity. Elimination of contaminating antigens offers the advantage that there will be less competition with the specific immunogens and that complications in the form of allergic reactions will be reduced. However, only a few currently available inactivated vaccines fulfil these requirements. Most inactivated vaccines contain inactivated complete virions and frequently also antigens remaining from the milieu of cultivation, for example cellular debris. Furthermore, the final vaccine product will have some kind of conservation substance added.

The choice of inactivating treatment is critical since it aims at a selective destruction of the infectious properties without any reduction of the immunizing capacity of the preparation (cf. Chapter 22). In the early phase of development of inactivated vaccines relatively crude methods were used. Treatment with formalin has been used extensively and it is only during later years that alternative

procedures with a more direct effect on the genetic material, such as treatment with β-propiolactone and hydroxylamine, have been tried.

In field trials with inactivated measles and mumps virus vaccines it was found to be important that the inactivation procedure does not destroy any of the essential surface antigens. Both these viruses have two dominating surface structures, the haemagglutinin and the haemolysin (fusion factor). When formalin was used for inactivation, parts of the immunizing capacity of the haemolysin were destroyed whereas the haemagglutinin remained essentially intact. Immunization with this kind of vaccine induced a production of high titres of circulating HI and neutralizing antibodies. The appearance of neutralizing antibodies generally is considered to be the best evidence of a situation of immunological protection. However, in the case of measles virus, there appears to be a difference between the humoral immune response which is required for virus neutralization *in vitro* and *in vivo*, respectively. Neutralization of virus under laboratory conditions only requires the presence of antibodies against the haemagglutinin, whereas *in vivo* antibodies against both the haemagglutinin and the haemolysin are needed to block virus replication. Immunoglobulin preparations which are used for passive immunization contain antibodies against both surface antigens. The formalin-inactivated measles vaccine did not only have the disadvantage that it gave an immunity of poor duration. In addition, cases of atypical measles appeared in individuals who had received this vaccine and had later been exposed to wild virus. This atypical form of measles (pneumonia, atypical vesicular rash, etc.) has an immunopathological background and is based on the occurrence of reactions of the Arthus type. Thus it is important that an inactivated vaccine contains all the antigens that are essential for the development of an effective immunity since a vaccinated individual in other situations could be placed in a worse situation than if he was not vaccinated at all.

The control of inactivated vaccine preparations involves both their harmlessness and their efficacy. The controls include analysis for the absence of any infectious virus, absence of contamination with other infectious or inactivated infectious agents, and analysis to determine the presence of acceptable amounts of potentially harmful additions, such as conservation substances, foreign proteins, etc. The strength of the preparations is determined by measuring the relevant antigen(s). The content of these antigens is compared with that of a standard preparation which has been documented to have a satisfactory immunizing capacity under field conditions. In general, inactivated vaccines are highly stable.

The effect of an inactivated vaccine can be studied by analysis of the appearance of specific circulating antibodies, for example, neutralizing antibodies after immunization. However, the decisive measure on the effect of immunization is the capacity of the vaccinated individual not to contract the disease after natural exposure.

Side-reactions of inactivated vaccines are primarily of two different kinds. When a vaccine contains an adsorbent such as aluminum phosphate or aluminum hydroxide local infiltrates or abscesses may develop. This complication is most easily avoided by deposition of the material intramuscularly. The most important kind of side-reaction is the hypersensitivity reaction. These may be induced by foreign proteins or remains of antibiotics which are present in the vaccine. It is important that instruments and drugs for treatment of an anaphylactic shock are available when vaccinations are performed.

A large number of different types of inactivated virus vaccines have been produced. Vaccines against rabies, influenza and polio viruses (*Table 23.1*) are of

TABLE 23.1. Viral vaccines

Disease	*Vaccine strain*	*Cell substrate*	*Character of vaccine*	*Route of injection*
Rabies	Pitman-Moore	WI-38*	Inactivated; β-propiolactone	Intramuscularly
Influenza	Relevant influenza A strains and influenza B virus	Chicken embryo	Inactivated; formalin alt. peplomers isolated after treatment with detergent	Subcutaneous
Poliomyelitis	Varying in different countries	Primary monkey kidney	Inactivated; formalin	Subcutaneous
	Sabin 1,2,3	WI-38	Live, attenuated	Oral
Smallpox	Vaccinia	Calf skin, chorioallantoic membrane of embryonated hen's eggs	Live, attenuated	Intradermal
Yellow fever	17D	Chicken embryo	Live, attenuated	Subcutaneous
Measles	Schwarz, Moraten	Chicken fibroblasts	Live, attenuated	Subcutaneous
Mumps	Jeryl Lynn	Chicken fibroblasts	Live, attenuated	Subcutaneous
Rubella	RA 27/3, Cendehill	WI-38	Live, attenuated	Subcutaneous
Adenovirus infections	Types 4 and 7	WI-38	Live virus in capsules which dissolve in the intestinal tract	Oral

*WI-38 is a human diploid fibroblast cell line.

major practical importance. As already mentioned, formalin-inactivated vaccines against measles and mumps have given problems since the inactivation procedures destroyed certain critical antigens. Corresponding negative experiences have occurred in connection with the testing of a formalin-inactivated vaccine against RS virus and parainfluenza virus.

Rabies vaccines

Pasteur prepared the first vaccine against rabies. This contained rabies virus with an attenuated characted in material from dried rabbit brain. Thereafter, inactivated

preparations were developed which contained virus propagated in duck embryo tissue. Today the inactivated vaccines are produced from virus propagated in human diploid cells. This kind of preparation has been found to have an efficient immunizing capacity. In spite of this, prophylactic use of the vaccine should be rather selective and considered in relation to whatever risk of exposure is present. Inactivated rabies vaccine is also used for the treatment of individuals suspected to have been infected with rabies virus. However, the problem in this case is that the formation of a protective concentration of antibodies in an unimmunized individual occurs slowly and that, therefore, the virus frequently does not become neutralized before it has reached an inaccessible intra-axonal localization from which it can spread relentlessly to the brain. The most important methods for dealing with rabies infections therefore are a local revision of the wound in which the virus has been deposited and the immediate administration of immunoglobulin against rabies in such cases where this can be made available.

Inactivated influenza vaccine

Vaccines of this kind have been used ever since the Second World War. Virus is propagated in the allantoic cavity in embryonated eggs. After purification and concentration the infectious properties of the virus are destroyed by formalin treatment. In later years vaccines containing purified peplomers have been introduced. The virions have been split by a detergent and the two surface components of the virus, the haemagglutinin and the neuraminidase, have been isolated by physiochemical methods (cf. Chapter 22).

The efficacy of the vaccine is determined by a number of factors. The major one is the capacity of the virus to gradually (drift) and stepwise (shift) change the character of its surface antigens. Because thereof the virus strains which should be included in a vaccine have to be determined separately for each year. The guideline in this selection is that the antigenic character of the strains used in a vaccine should be as similar as possible to the antigenic properties of the virus which is expected to circulate in the community. Influenza virus type A is of major importance from an epidemic point of view. However, the vaccine also contains a strain of influenza type B which has a more limited tendency to change its antigenic character and which also gives more limited epidemics. Usually the vaccine is given as a single parenteral injection. This immunization should not be expected to provide a satisfactory protection in the respiratory tract but, because of a previous sensibilization with related antigens via natural infections, a protective effect is achieved. The protective effect has been estimated to vary between 40 and 80 per cent. The large variations depend among other things on the degree of antigenic similarity between wild virus and vaccine virus. In order to obtain an effective immunization with new antigenic determinants two injections with an interval of one month may be considered. The influenza vaccine is primarily used in 'risk groups' which include persons with heart, lung and kidney diseases. In many countries doubt has been expressed as to whether it is appropriate to give yearly vaccinations to such groups. Some data indicate that the efficacy of protection is gradually reduced during the course of repeated, yearly infections. If an expected epidemic depends on a completely new variant of virus, which in addition may have a pronounced disease-inducing capacity, these vaccinations can be directed to a greater part of the population. In this situation, children may also be vaccinated but as a general rule younger individuals should not be immunized against influenza. The reason for this

is partly that the influenza disease normally is milder in children than in adults and partly that it appears to be of value if children can develop their baseline immunity against influenza via natural infection.

Inactivated polio vaccine

This vaccine is produced by formalin inactivation of virus propagated in primary monkey kidney cell cultures. In the future alternate cell substrates, including also heteroploid cells, will be used. The inactivating treatment originally was allowed to take place at 37°C for one week. However, a reduction of the temperature at which inactivation is performed and an extension of the time of inactivation has provided preparations of improved antigenicity.

Only a few countries in the world, for example, Sweden, have retained a vaccine programme which includes the use of inactivated polio vaccine only. In most countries a gradual change to the use of live vaccine has been made. Both vaccine programmes have been highly successful in terms of preventing disease. However, there are some differences in the effect on the circulation of the virus in the community. It was originally believed that the inactivated vaccine did not have a capacity to establish a local immunity in the intestinal tract and therefore was not capable of restricting the circulation of virus in the community. However, it has been found that four injections of antigen preparations with a good antigenicity lead to the production of circulating antibodies on such levels that some of these antibodies also appear on mucosal membranes and give a local immunity. Circulation of wild virus in a community like Sweden, therefore, has ceased. A consequence of this is that no boostering of immunity based on subclinical infections occur. In this situation it is obvious that a continued highly organized vaccination programme which reaches the overall majority of non-immunized individuals is necessary. Available data show that no booster injection is needed after the fourth dose of vaccine. This means that clones of antibody-producing cells, which are established during the course of immunization, can maintain an antibody production throughout life.

Inactivated vaccines for the future

It is to be expected that a number of additional inactivated vaccines will be produced during the forthcoming decade. A vaccine against hepatitis B virus is already subject to testing and results indicating an efficient immunogenicity have been obtained. Since hepatitis B virus cannot be propagated in cell cultures, antigen material is recovered from persons who are carriers of persistent hepatitis B virus infections. The vaccine contains parts of the hepatitis B virus structures (HB_sAg) but for the sake of safety the product is inactivated with formalin.

Different herpesviruses have a tendency similar to hepatitis B virus in that they give persistent infections in the organism. Preferably, therefore, a live vaccine should not be used against this type of virus, even though some field trials have been conducted with a live cytomegalovirus and varicella-zoster vaccine. One additional problem with herpesviruses is the fact that fractions of active viral genomes can give a transformation of cells in experimental systems. Attempts are therefore made to develop vaccines which only contain the envelope components of the virus.

In addition, future developments will involve the use of hybrid-DNA technology. Foot and mouth disease vaccine already has been produced in bacteria and is currently being tested in the field. Also other viral antigens have been produced in bacteria and will be subjected to corresponding trials. Another line of development concerns the production of synthetic vaccines. This problem has been approached firstly by characterizing the nucleotide sequence of the gene responsible for the formation of particular protein. From this sequence, the amino acid sequence can be deduced and by computer technology the tertiary structure of a protein formed by folding of the deduced polypeptide can be determined. It is also possible to decide the part of the polypeptide which carries the antigenic determinants which may be of importance for immunization. Synthetic small polypeptides which provide immunity against influenza virus in experimental animals have already been obtained by use of this approach.

Live vaccines

A live vaccine is used to establish a restricted infection in the organism. In connection with this infection relatively large amounts of antigens are produced in the body and the immunity which is obtained shows may qualitative similarities with the immunity that develops after infection with wild virus. The vaccine virus should be attenuated to the extent that it gives no symptoms or only very mild symptoms. However, it is important to compare the possible mild reaction given by a vaccine strain with the more severe symptom caused by wild virus. With the usage of live vaccines it is important to attempt to distinguish between vaccine reactions, i.e. to distinguish expected acceptable reactions from side-reactions. The border between these two forms of reaction obviously is not distinct, but the term *side-reaction* is used to indicate an abnormally strong or unexpected reaction to the vaccine. In this context it should be mentioned that a live vaccine administered to an individual who has a defective cell-bound immunity may give infections of a severe nature, occasionally even with a fatal outcome.

A special kind of live vaccine is used in the USA to prevent respiratory infections with adenoviruses. The vaccine contains ordinary wild virus strains enclosed in a capsule which after oral intake is dissolved in the intestinal tract. This infection does not spread to the respiratory tract but in spite of this the immune reactions which develop as a consequence of the infection in the enteric tract also provide protection against future infections in the respiratory tract.

The first live vaccine which was developed had a natural background. As was mentioned, Jenner observed that a poxvirus which occurred in cows could protect against smallpox in man. Yellow fever, measles, mumps and rubella vaccines were developed by repeated passages of virus in cultures of cells from a species which does not represent the normal host. This led to a modification in capacity of the virus to give infection in man. In later experimental work genetic methods have been used to allow a more systematic approach to the development of vaccine strains.

Many attempts have been made to produce temperature-sensitive mutants – in the first place, different respiratory viruses. These mutants, which replicate well at 32–33°C but not at 37°C, would be expected to give only a limited infection after local application in the respiratory tract. However, as yet no useful vaccine of this kind has been developed. The main problem encountered is the lack of genetic

stability. In connection with replication of vaccine virus for several consecutive generations in the body, there are possibilities for modifications leading to the regaining of virulence. The problem of genetic instability is particularly difficult in the case of vaccine viruses which can spread from a vaccine to non-immune individuals in the surroundings. This is the case with the infection established by live polio vaccine, but not with yellow fever, measles, mumps and rubella vaccines.

Live vaccines have the advantage of being relatively easy to administer and in many cases they give an immunity of long duration after a single injection. Certain live vaccines, however, have to be given repeatedly at regular time intervals. Concerning the live polio vaccine, three consecutive immunizations are given at monthly intervals. The reason for this is that the three types of live polio virus which are included in the vaccine have a tendency to compete with each other during the replication in the intestinal tract. This interference leads to one type dominating during the infection at the first immunization, another type at the second immunization, etc. It is important to consider the possible risk of interference phenomena, both in the case of immunizations with different live vaccines following each other closely in time and also when live vaccine virus is given in a milieu including naturally-circulating wild viruses replicating in the same organ. As a rule the interval between administration of two live vaccines should be at least four weeks. However, it has been experienced that a concomitantly-initiated infection with live measles, rubella and mumps vaccines gives an immunity which is comparable to that obtained when each vaccine is given separately. This type of combined live vaccine has been used extensively in the USA and is now gradually being introduced in other countries. An interference between vaccine virus and wild virus of the corresponding or a different kind has created great problems in the use of live polio vaccines in developing countries. In these milieus the take-rate frequently does not exceed 50 per cent.

Another problem in developing countries concerns the stability of live virus vaccines. The use of freeze-dried products properly stored at +4°C makes possible a conversion frequency which lies between 90 and 100 per cent. However, in developing countries, the possibilities of establishing an efficient 'cold chain' frequently are poor. The absence of a sufficient quantity of infectious virus in the vaccine is a common reason for vaccine failures. In some cases more heat-stable live vaccines are now being introduced.

The occurrence of circulating antibodies can prevent an efficient replication of a vaccine virus and lead to only a limited replication taking place. A live vaccine therefore should not be given to individuals who may be expected to have antibodies which are of maternal origin for example or are derived from recently administered immunoglobulin or transfused blood. Certain live vaccines should not be used on individuals under the age of 14 months.

Live vaccines are subject to a number of different controls. The presence of a satisfactory quantity of infectious virus is determined. The dose usually varies between 10^3 and 10^5 infectious units. The degree of attenuation of the virus strain used can in some cases be defined by characterization of certain genetic markers. However, in the case of most vaccines, useful markers are not available. Finally, it is also ensured that the vaccine does not contain any contaminating infectious agents. Regarding viral vaccines which are produced in eggs there are controls for the absence of leucosis virus.

Live vaccines against 7 different virus infections are currently available (*Table 23.1*).

Smallpox vaccine

During the latter decades, Jenner's vaccine has been used so effectively that probably there will be no need for future use. The origin of the virus strain which is used in currently available vaccines is unknown. However, it probably is of cowpox origin (cf. Chapter 32). The vaccine was originally produced by the relatively primitive procedure of propagating the virus in the skin of calves. Since the beginning of the 1960s, embryonated hen's eggs in many cases have been used as an alternative substrate.

The result of vaccination is dependent on the strength of the preparation, i.e. the content of infectious virus, the immune status of the vaccinated and the method for application of the vaccine. The vaccination aims at depositing vaccine virus in the epidermal epithelium to allow a local replication. A vaccine of normal strength is given to an individual who lacks immunity whereas an individual with a partial immunity should receive an extra strong vaccine. Occasionally the vaccination gives complications. If the normal barrier function of the skin is not functioning due to eczematous changes, for example, the local vaccine virus infection can spread and cause a severe generalized skin infection. Furthermore, a postvaccination encephalitis occurs with a certain frequency. This encephalitis most likely has an immunopathological background. It appears about a week after the normal vaccine reactions have started to disappear and is characterized by demyelinating changes in the brain and the absence of identifiable infectious virus.

The efficacy of the vaccine has been well documented during the last few decades. In 1967 the WHO initiated a programme for the global eradication of smallpox. Three circumstances were of importance to the success of this programme. The first was that only one serological type of virus circulated and that the vaccine gave efficient protection against this type. The second was the fact that the virus can give only acute infections. There is no evidence that smallpox virus can remain in the organism and reappear in some form a long time after the acute infection. The third prerequisite which was verified was that there did not appear to be any reservoir for the virus in animals. As a result of the vaccination programme organized by WHO smallpox has now been eliminated from the world. The last case of smallpox occurred in Somalia in 1977 and after a two-year interval the world was then declared smallpox free. Consequently most countries have now ceased to use the smallpox vaccine except for certain groups of individuals who might be the first to be exposed to virus if an epidemic should reappear.

Yellow fever vaccine

This vaccine is prepared from an attenuated virus strain with the designation 17D. The vaccine only rarely gives side-reactions and the duration of the immunity is good. Revaccinations are recommended at 10-year intervals.

Live polio vaccine

This vaccine has been used extensively in many parts of the world. The strains which are used today were developed by Sabin around the year 1960. The vaccine is given orally in different forms. The intestinal infection which is initiated does not give any symptoms, but an excretion of vaccine virus takes place. The vaccine which contains all three types of polio viruses is given three times at monthly

intervals. In an efficiently vaccinated individual, there should be no need to repeat the vaccination. However, one important prerequisite is that no concomitant infection with other intestinal viruses have prevented the replication of either of the three poliovirus types.

As mentioned above, the genetic instability of vaccine virus causes certain problems. A careful analysis of the situation in the USA has shown that polio immunization is associated with paralytic poliomyelitis in one case per 3.2 million doses of vaccine distributed. Paralytic disease occurs either in the vaccinated individual or in a close contact. From 1969 to 1980, 92 cases of paralytic poliomyelitis associated with vaccination were reported. Twenty-five of these cases occurred in otherwise healthy vaccine recipients, 55 cases occurred in healthy close contacts of recipients, and 12 cases in individuals (recipients or contacts) who had an immune deficiency condition. Although today in the USA there are more cases of poliomyelitis caused by vaccine virus than by wild virus, it should be noted that this is because of the marked restriction of wild virus circulation as a consequence of the overall efficiency of the vaccination programme. However, the vaccine-associated cases of paralytic poliomyelitis represent a problem and hopefully it will be possible in the future to prepare vaccine strains which show an improved genetic stability.

The protection against disease after vaccination with a live vaccine is good and it includes an efficient local immunity in the gut. However, the durability of this local immunity is limited and harmless reinfections may occur some years after vaccination.

Live measles vaccine

In parallel with the development of an inactivated measles vaccine in the beginning of the 1960s, Enders and collaborators developed a live vaccine by adapting measles virus to grow in chicken embryo cultures. Since the inactivated vaccine did not provide the expected effects as mentioned above, the live vaccine has become of dominating importance. The vaccine strain which was produced originally gave certain reactions in the form of fever and exanthema, but today some different further-attenuated virus strains are available and the clinical reactions after vaccination are negligible. Some slight fever and, occasionally, a mild rash may develop, but usually this does not interfere with the normal activity of children. The vaccine infection is not contagious.

Vaccination may be given within five days after exposure to wild measles virus. By this procedure the mild vaccine virus infection can substitute for the regular disease. Side-reactions in connection with vaccination are rare. A special evaluation has been made of the occurrence of complications from the central nervous system. The frequency of occurrence of acute encephalitis is of the order of one case per million vaccinated to be compared with one case per 1000 individuals after natural measles. The incidence of the more severe, but rare, late complication to natural measles, SSPE, is reduced at least 5–10 times after vaccination. The antibody response after vaccination is weaker than after infection with wild virus. However, it has been found to be of good durability also in the absence of reinfection and most evidence indicates that the protection against infection will be lifelong.

The protective efficacy of the vaccine has been shown to exceed 90 per cent under field conditions. The occurrence of vaccine failures may have several

different explanations. In some cases the replication of vaccine virus has been reduced because of the occurrence of low concentrations of antibodies, deriving either from the mother in the case of very young children or from previously administered immunoglobulin or vaccination with an inactivated vaccine. In other cases the vaccination has not been effective owing to improper storage of vaccine. This is a common cause of poor vaccination results in developing countries. Vaccines with an improved stability are now becoming available. A special problem in developing countries is the fact that measles gives severe infections even during the first year of life. If possible therefore the first vaccination should be given at the age of 6–9 months and should be renewed at the age of 1½ years. Today there is no evidence indicating that vaccination has to be repeated when used in industrialized countries.

Live mumps vaccine

Several problems were encountered in early attempts to obtain an attenuated strain of mumps virus. The major problem was that repeated passaging of virus in cell cultures readily led to an over pronounced attenuation. However, since about 1965, an efficient live vaccine containing the Jeryl Lynn strain of virus is available. This vaccine is produced in chick embryo cultures. It gives an infection which does not spread from the vaccinated individual. Complications are rare. The antibody response in connection with vaccination is markedly lower than after a regular mumps virus infection, but it appears to be of good durability. The protective efficacy of vaccination has been found to exceed 90 per cent. The use of mumps vaccine prevents the occurrence of mumps orchitis during and after puberty, but more importantly, it prevents the common complications from the central nervous system in connection with acute mumps.

Live rubella vaccine

The aim of using this vaccine is not primarily to protect the vaccinated individual against disease, but instead to prevent the transmission of an infection to the fetus in a pregnant woman. Thus, vaccination primarily should provide protection to fertile women. The vaccine has been applied somewhat differently in different countries. In the USA, vaccination programmes were originally aimed at reducing the circulation of rubellavirus by vaccination of all children at young age. Such an effect was achieved but the virus has continued to circulate in adult women and to cause congenital disease. In Europe, most countries have chosen to vaccinate one or more of the three following categories: (1) girls at the age of 12–13 years; (2) women who in connection with their first pregnancy have been found to lack immunity to rubella – these women are vaccinated in the postpartum period when the chance of a new pregnancy is negligible; and (3) women who run a high risk of being exposed to infection and who lack immunity. Vaccination of individuals in the last group is combined with an anticonceptional treatment for at least two months. The reason for this is that the vaccine virus possibly can be transmitted transplacentally to a fetus and cause damage. However, experiences gained so far indicate that even when such a transmission occurs the risk of fetal damage is very low.

Like the infection caused by wild virus, the vaccine reaction is mild, but occasional cases of arthralgia have been observed. The frequency of seroconversion

exceeds 90 per cent. The vaccine virus infection does not spread from the vaccinated individual. The duration of immunity after vaccintion is still being evaluated but all evidence indicates that it is longlasting. However, local infections may occur in the respiratory tract as early as 6–12 months after vaccination.

Future live vaccines

Live vaccines for the future will be developed primarily to provide protection against various infections in the respiratory tract, caused by, for example, influenza, parainfluenza or RS viruses, and in the enteric tract, for example, rotaviruses. Live influenza vaccines have been tested in field trials, but no vaccine strain with a suitable degree of attenuation and a satisfactory genetic stability has yet become available. The problem concerning RS virus infections is that the maternal antibodies do not provide the protection which might be anticipated. Thus severe infections occur even during the first year of life. A live vaccine against RS virus is presently under evaluation, but the appropriate time for administration of this product has to be considered further. Live vaccines against CMV and varicella are also being evaluated.

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24

Antiviral drugs

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In spite of relatively extensive efforts to develop antiviral drugs, only a limited number of compounds which can be used in man have become available. The reason for this is the difficulty in finding substances which selectively influence the replication of virus without having toxic effects on cells. Another problem concerns the fact that cell damage caused by a virus infection is often very extensive already at the stage when the first symptoms appear. During recent years our knowledge about the molecular events in virus replication has increased markedly and it is therefore now possible to search more systematically for drugs which block virus-specific processes without damaging normal cellular functions. Interferon, which was discovered in 1956, was for a long time the only antiviral drug which fulfilled the criteria of efficient inhibition of virus replication and low toxicity. However, it has been possible recently to produce several other relatively non-toxic substances with a verified or a potential therapeutic effect on virus infections.

The work with antiviral drugs has been concentrated on substances which may have an effect on herpes and influenza virus infections, even though hepatitis B virus and rhinovirus infections also have been considered as important targets for antiviral treatments. In this chapter the mechanisms of action of some antiviral drugs are described as well as some preclinical and clinical studies with these drugs. A separate part of the chapter is devoted to interferon.

Theoretically one might consider blocking virus replication at either of the following stages; adsorption, penetration, uncoating, the synthesis of viral nucleic acids and proteins and the assembly and release of virus particles. It is primarily in connection with the viral nucleic acid synthesis, that viral enzymes different from cellular enzymes play a major role. The differences have been exploited to obtain a selective blocking of virus replication.

Mechanisms of action of antiviral drugs

Antiviral drugs can roughly be divided into three classes depending upon their mechanisms of action:

I. Substances which selectively inhibit viral functions without any significant influence on the metabolism of uninfected cells. Example: phosphonoformic acid (Foscarnet).

II. Substances which in the infected cells are converted by a viral enzyme to compounds which inhibit virus replication. Examples: acycloguanosine (Acyclovir).

III. Substances which act by influencing the cell, thereby establishing an antiviral state. Examples: interferon, immunostimulators, ribavirin and possibly also amantadine.

In most virus infections, treatment has to be started immediately after the appearance of the first symptoms in order to have a therapeutic effect. The use of compounds in classes I and II requires a rapid and precise diagnosis since these compounds do not have a general antiviral effect, but only block a certain type or group of viruses. *Table 24.1* describes the mechanism of action of some different antiviral drugs and gives examples of viruses which can be inhibited by these drugs. The compounds belonging to classes I and II can induce the development of resistant strains but this appears less likely as regards substances in class III.

There are a number of virus-induced enzymes which are possible points of attack for antiviral drugs. *Table 24.2* describes some virus-induced enzymes which have

TABLE 24.1. Examples of some antiviral compounds

Substance	*Virus infection*	*Mechanism of action**
Clinical usage:		
Acyclovir	Herpes simplex (HS)	I+II
Adenine arabinoside, ara-A	HS	I
Amantadine	Influenza	I+III(?)
Idoxuridine, IDU	HS	II
Methisazone	Pox	I?
Trifluorothymidine, TFT	HS	II
Clinical trials:		
Acyclovir	HS, varicella-zoster	I+II
Adenine arabinoside, ara-A	Hepatitis B	I
Foscarnet	HS, cytomegalovirus	I
Bromvinyl deoxyuridine, BVDU	HS, varicella-zoster	II
Interferon	HS, hepatitis B, influenza	III
Isoprinosine	HS, hepatitis B, influenza	III
Levamisole	HS, hepatitis B, influenza	III
Ribavirin	HS, hepatitis B, influenza	III

* See the text for explanation of mechanisms of action

TABLE 24.2. Examples of virus-induced enzymes against which antiviral therapy may be directed

Virus	*Enzyme activity*
Rhinoviruses	RNA-dependent RNA polymerase
Influenza virus	Protein kinase, nucleoside diphosphate kinase, RNA-dependent RNA polymerase, neuraminidase, nucleoside-triphosphatase
Herpes simplex virus	Ribonucleotide reductase, protein kinase, thymidine kinase, DNA-dependent DNA polymerase, DNase, deoxycytidine deaminase
Hepatitis B virus	DNA-dependent DNA polymerase

been identified in cells infected with rhino-, influenza, herpes simplex and hepatitis B viruses. These enzymes represent the primary point of attack for antiviral compounds studied today. Also a number of other viruses have their own enzymes which might be blocked selectively by suitable antiviral compounds. The majority of all known viral enzymes participate in nucleic acid synthesis. Even in a situation when the viral enzyme forms a complex with cellular proteins it may be possible to block selectively the viral function.

Antiviral drugs used for clinical chemotherapy

At present there are only a limited number of antiviral drugs which have been registered for clinical use. These drugs, which have been shown in clinical trials to have significant effect against viral infections in man, are discussed below.

Acyclovir (Zovirax^R)

Acyclovir has recently been registered for topical use against herpes keratitis and primary genital herpes. The effect on herpes keratitis is similar to that of IDU and TFT but the toxicity of Acyclovir is lower. A marginal clinical effect is obtained on primary, but not on recurrent genital herpes. Acyclovir has also been approved for intravenous use in severe herpes infections of immuno-compromised patients

Adenine arabinoside

Adenine arabinoside (9-β-D-arabinofuranosyladenine, ara-A, Vidarabine) is similar to deoxyadenosine, but as can be seen in *Figure 24.1*, the deoxyribose unit is exchanged for arabinose. Ara-A is phosphorylated in both infected and in uninfected cells to ara-A-triphosphate. This metabolite blocks herpesvirus-DNA polymerase to a larger extent than cellular DNA polymerase. Ara-A also inhibits most other DNA viruses in cell cultures, but in addition it also has a toxic effect on cells. A certain incorporation of ara-A occurs both into cellular and viral DNA.

In later years, ara-A has been used for the treatment of herpes encephalitis. In one study the mortality decreased from 70 to 28 per cent after intravenous treatment for 10 days. In the case of patients who were comatose when the treatment was initiated, the survival was prolonged although the patient suffered from severe permanent brain damage as a consequence of the disease. Ara-A has also been shown to be effective as an intravenous treatment of herpes zoster. Because of the marked risk of side-reactions, systemic treatment with ara-A should be reserved only for very severe herpesvirus infections. When used for local treatment, ara-A gives good results on herpes keratitis but not on herpes labialis. Attempts to treat chronic hepatitis B infections with ara-A have not given uniform results.

Amantadine

The structure of amantadine (1-adamantanamine) is shown in *Figure 24.1*. Amantadine blocks the replication of influenza virus type A but not B. Different strains of influenza virus show a different sensitivity to the drug in cell culture experiments and it is possible to select strains of the virus which are resistant to amantadine. The mechanism for inhibition has not been clarified but amantadine possibly influences

the virus penetration step immediately after the adsorption. This step may involve an effect of cellular proteolytic enzymes on the virus and fusion with cellular membranes. In addition, amantadine may influence the immune response to influenza virus, since it has been shown to have an effect on T cells. There are several drugs (for example, spiroamantadine and rimantadine) which are structurally similar to amantadine and which appear to have a similar protective effect against influenza.

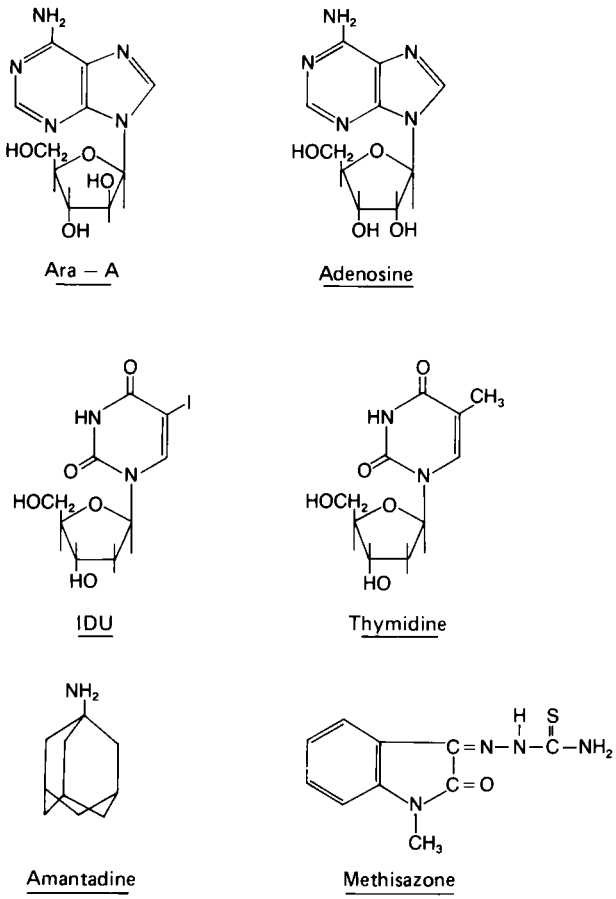

Figure 24.1. Antiviral compounds which are used for treatment of virus infections. The nucleoside analogues are shown to the left and the corresponding nucleosides to the right. In addition the figure shows amantadine and methisazone which are used for treatment of influenza and poxvirus infections, respectively

The treatment with amantadine must be started very early during the infection in order to have any effect. Therapeutic oral treatment of amantadine in influenza markedly reduces the number of hours with fever. It has further been shown that therapy with amantadine accelerates the normalization of lung functions after the infection and that the therapy therefore probably reduces the risk of lung

complications. When used prophylactically, amantadine has given significantly reduced periods of clinical symptoms.

Amantadine, which also is used in the treatment of Parkinson's disease, causes mild side-effects from the central nervous system. A teratogenic effect has been demonstrated when high doses were given to animals. It is a matter of dispute whether the prophylactic and therapeutic effects are sufficiently good in relation to the side-effects to encourage a more general use of amantadine, but during epidemics it seems justifiable to use the compound at least in certain risk groups.

Idoxuridine

5-Iodo-2′-deoxyuridine (idoxuridine, iduridine, IDU) is a structural analogue to thymidine. Instead of a methyl group in 5 position, IDU has an iodine atom. This similarity with thymidine (*Figure 24.1*) makes it possible for the herpesvirus-induced enzyme thymidine kinase, which normally phosphorylates thymidine, to phosphorylate the 5′-hydroxyl group in IDU and the 5′-monophosphate is then further phosphorylated to IDU-triphosphate. This metabolite is incorporated into both herpesvirus-DNA and cellular DNA in infected cells. The incorporation leads to an incorrect coding in connection with replication and transcription, resulting in a synthesis of non-functional proteins. Herpesvirus mutants which are resistant to IDU either lack viral thymidine kinase or have a modified enzyme which does not phosphorylate IDU. However, these mutants are sensitive to ara-A. Cellular thymidine kinase also can phosphorylate IDU and this leads to a high toxicity for cells. Also DNA viruses other than herpes simplex virus can be blocked by IDU.

IDU is used clinically for the treatment of herpes keratitis and it has been used both in the form of eye drops and eye ointment. As with other antiviral drugs, IDU treatment does not eliminate the latent virus infections which occur in sensory neurons but it is considered to have therapeutic effects on the ongoing infection in the cornea. IDU does not have any significant therapeutic effect on cutaneous and genital herpes simplex and herpes zoster infections. A local treatment with IDU dissolved in dimethylsulphoxide in order to increase the penetration of the skin has an effect but is not to be recommended because of the high toxicity.

Methisazone

This compound (Marboran), the chemical name of which is *N*-methylisatin-β-thiosemicarbazone (*Figure 24.1*), has been used for treatment of smallpox and vaccinia infections. When used for oral treatment within 24 hours after a smallpox infection, methisazone has been found to have a marginal effect on the infection. The mechanism for blocking has not been clarified but methisazone can chelate metal ions and this may be of importance.

Trifluorothymidine (TFT)

The structure of this compound is shown in *Figure 24.2*. The mechanism for blocking of herpesvirus replication is similar to that of IDU, but the clinical effect of TFT on herpes keratitis is better. Neither IDU nor TFT are effective on more penetrating infections and both of them are too toxic to be used for systemic treatment.

Antiviral drugs with a potential clinical usefulness

In recent years several antiviral compounds have been produced which are now being tested in clinical trials. It is likely that the use of combinations of compounds with different mechanisms of action may turn out to be necessary in order to reduce the risk of development of resistant strains. The following are compounds which are currently evaluated for possible clinical use in the future.

Substances which selectively block virus functions

The substance in this category which has given the best results in clinical trials is phosphonoformic acid (foscarnet sodium, PFA). Foscarnet has a structure which is similar to pyrophosphate (*Figure 24.2*) and binds at a pyrophosphate-binding site of some nucleic acid polymerases. Foscarnet blocks the DNA polymerase from herpes

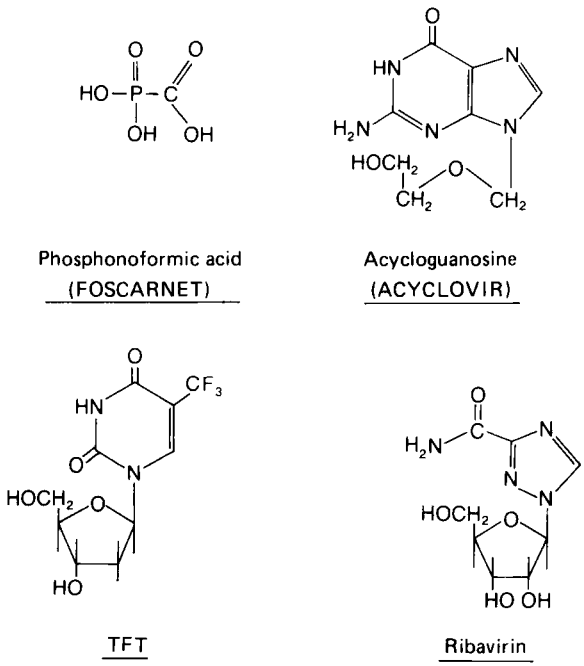

Figure 24.2. Some antiviral substances which are currently being subjected to clinical testing

simplex virus types 1 and 2, cytomegalovirus, varicella-zoster virus, Epstein–Barr virus and hepatitis B virus and also reverse transcriptase from retroviruses. This blocking occurs at concentrations which do not inhibit cellular nucleic acid polymerases. In animal models, Foscarnet has been shown to be effective in cutaneous herpes, genital herpes and herpes keratitis. Clinical trials in man have shown the therapeutic effect of 3% Foscarnet cream on recurrent cutaneous herpes infections and of 0.3% Foscarnet cream on recurrent genital herpes infections.

Substances which are activated selectively by viral enzymes

Some nucleoside analogues, which are selectively phosphorylated by herpesvirus thymidine kinase and then further phosphorylated, can be incorporated into DNA and/or block DNA polymerase. One such substance is Acyclovir (*Figure 24.2*). This substance has a clinical effect on herpes keratitis and is less toxic than IDU. Acyclovir does not block replication of cytomegalovirus since this virus does not induce any thymidine kinase. Acyclovir is now being tested clinically against several herpesvirus infections.

Other inhibitors of herpesvirus replication, for example bromovinyldeoxyuridine with a similar mechanism of action, are currently being developed in different laboratories.

Substances which influence cellular functions

Several immunostimulating compounds, e.g. isoprinosine and levamisole, have been tested clinically, but no therapeutic effects have been ascertained. These compounds are subject to further testing.

Ribavirin (Virazol: *Figure 24.2*) seems primarily to influence the concentrations of dGTP and GTP in cells and has a broad antiviral spectrum in cell cultures. Clinical tests on influenza virus infections have given varying results but a therapeutic effect has recently been shown using an aerosol. The substance might be too toxic to allow general clinical use. One possible area of usage for ribavirin could be life-threatening infections such as Lassa fever.

Interferon

Interferon was first described by Isaacs and Lindenmann in 1957 as an antiviral substance which is produced by virus-infected cells. Extensive later investigations have shown that there are several different types of interferon-like molecules and the name 'interferon' is therefore used for a group of proteins and glycoproteins which have the common property of inhibiting virus replication. The types of interferon which have been identified and purified in man have a molecular weight of about 20 000. There are at least three different categories.

1. IFN-α, Leucocyte interferon, which is produced by lymphocytes, is characterized by high stability at low pH and shows heat stability, i.e. it withstands incubation at +70°C for 30 minutes.
2. IFN-β, Fibroblast interferon, which primarily is produced by fibroblasts, shows the same acid stability but is more thermolabile.
3. IFN-γ, Immune interferon is produced by T cells after stimulation with antigens or mitogens. This interferon has also been called type 2 interferon in contrast to leucocyte and fibroblast interferon which frequently have been referred to as type 1 interferon. IFN-γ is acid labile and thermolabile.

The interferon activity is measured in international units and 1 unit represents the smallest concentration of interferon which can induce demonstrable antiviral activity in cell cultures under certain standardized conditions. The specific activity of interferon has been calculated to be about 5×10^8 units per milligram of protein.

This means that the specific activity is as high or even higher than that calculated for peptide hormones like ACTH or insulin and that only one or few interferon molecules are needed to make a cell resistant to a virus infection. Interferon is characterized by having a broad antiviral activity, i.e. it can inhibit the replication of most viruses.

With certain exceptions, interferon has a species-specific effect, i.e. human interferon has an effect in human cells but no effect or only a limited effect in cells from other species. Interferon appears at an early stage during a virus infection, before the development of cell-mediated and humoral immunity. Even during the acute phase of the infection serum concentrations are very low (less than 1 ng/ml), but because of the high specific activity of interferon a pronounced antiviral effect can be exerted.

Induction and mechanism of action

Formation of interferon can be induced not only by viruses but also by polynucleotides, bacteria, protozoa, endotoxin and also certain plastic polymers. Many of these inducers probably act indirectly, for example through the formation of metabolites. The most effective inducers are double-stranded RNA and virus. It is possible also that viruses act because of the formation of double-stranded RNA during some phase of replication. Synthetic double-stranded RNA, polyinosine-polycytidylic acid (poly-IC), is frequently used for experimental induction of interferon.

The main steps in the process of induction and the mechanism of action of interferon is described in *Figure 24.3*. The source of information for the production of interferon is probably located on chromosome 9 in man. Under normal conditions the interferon gene appears to be repressed. After a cell has come into contact with a virus or some other interferon-inducing agent, a derepression occurs and the interferon gene is transcribed into mRNA which then is translated into interferon. The interferon is secreted and reacts with receptors on other cells. These receptors have not been characterized, but are probably associated with gangliosides. It is possible that the synthesis of the interferon receptor is directed from chromosome 21 in human cells, since cells with a trisomy 21 are much more sensitive to interferon than cells with a normal number of chromosomes. After contact with the cell receptor, interferon induces the formation of proteins which have various effects on cells. Some of these proteins have a blocking effect on virus replication.

The mechanisms for the effects of the interferon-induced proteins on virus synthesis are partly known. Among these proteins are several enzymes. One of these is an enzyme, 2,5A polymerase, the product of which is 2′, 5′ oligo-isoadenylate. This product activates an endonuclease, which breaks down mRNA and thereby blocks the synthesis of virus proteins. Another enzyme, protein kinase, which also is induced by interferon, gives as an end-result in a chain of reactions, a blocking of viral protein synthesis. Blocking of virus replication thus primarily occurs on the level of translation. Certain results indicate that in the case of some viruses interferon blocks the transcription process and other cases indicate that interferon may cause a restriction in the release of virus from cells.

The basal mechanisms behind the non-antiviral activities of interferon have not been fully clarified. In general, it appears that the concentrations of interferon which are needed to give these effects on cells are often higher than the

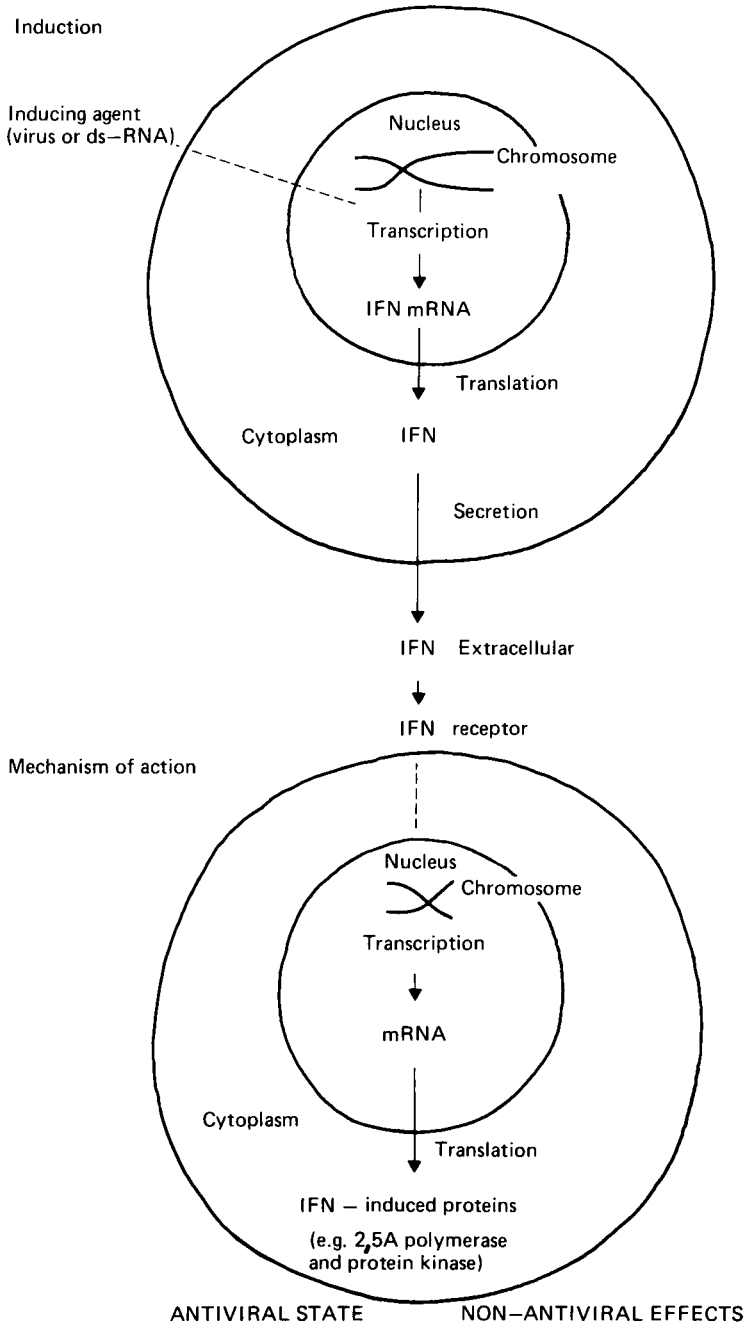

Figure 24.3. Mechanisms of induction and action of interferon. The inducing agent (usually a virus or double-stranded RNA) activates the gene for interferon (IFN) formation by derepression. IFN is bound to receptors on cells which leads to a chain of reactions causing the induction of certain proteins. These proteins modify different cellular functions and they interfere with translation of viral mRNA

concentrations which give antiviral effects. Thus low doses of interferon may have a relatively selective effect on virus replication.

Production of interferon

Because of the potential usefulness of interferon as an agent against virus infections and tumours, major efforts have been made to find systems to produce large quantities of interferon. For this purpose human cells have been used primarily. Various attempts have been made to increase the yield of interferon in cell cultures. This can be achieved by an addition of a low dose of interferon immediately before the addition of the interferon-inducing agent, an effect referred to as *priming*. Another method relies on attempts to block the synthesis of interferon repressor by use of RNA and protein-synthesis inhibitors (superinduction). In a third approach a selection is made of cells with multiple copies of chromosomes in which the interferon gene is located. By use of these different methods it has been possible to induce interferon in sufficient quantities to conduct preliminary clinical tests on its effect on virus infections and tumours.

Recently, the genes, and hence polypeptide structures of human IFN-α, IFN-β and IFN-γ, have been identified, thus paving the way for production of all these types of interferon by recombinant-DNA techniques. Using such techniques, it has been possible to produce human interferon in bacteria and yeast. In the future, large scale production of interferon will therefore probably rely mainly upon genetic engineering methods.

The effects of interferon on cells

In addition to its antiviral effect interferon has a number of regulatory effects on cells, and it appears reasonable to view interferon as a hormone with importance for the maintenance of homeostasis in the body. Examples of regulatory effects are:

1. Stimulation of synthesis of interferon. A cell which is treated with a low dose of interferon (priming) shows an increased capacity to synthesize interferon.
2. Inhibition of cell growth. *In vitro* experiments have shown that interferon in very low concentrations has a pronounced inhibitory effect on the proliferation of certain cell types, whereas other kinds of cells are completely resistant to this effect. Some types of tumour cells are unusually sensitive to the antiproliferative effect of interferon.
3. Increase in the amount of certain surface antigens on cells, for example histocompatibility antigens.
4. Increase in the intracellular concentration of certain enzymes, for example phosphodiesterase and protein kinase.
5. Modulating effects on the immune response by influences on immunocompetent cells. Cytotoxic reactions caused by T and K cells and, in particular, NK cells, are stimulated. Recently, considerable interest has been focused on the capacity of interferon to stimulate NK cells since these cells may represent an important part of the immunological defence against viruses and tumours (*see* Chapter 19). Also the function of macrophages is stimulated by interferon which thus has a phagocytosis-promoting effect. Because of the general inhibitory effect on cell growth, interferon may also have an inhibitory effect

on the immune response. Thus it has been found that antibody responses, graft rejections and delayed hypersensitivity reactions under certain circumstances can be inhibited by interferon.

The effect of interferon on virus infections

The importance of interferon in the defence against virus infections has been established by injection of anti-interferon serum. Animals treated in this way contract severe and progressive infections, frequently with a fatal outcome, when they are infected with a virus which under normal conditions only gives a mild disease. Against the background of these experiments it appears justifiable to attempt to treat virus infections in man with interferon. One possibility might be to stimulate endogenous interferon synthesis by treatment with interferon-inducers of different kinds. Several such attempts have been made but for two reasons the results have been discouraging. Firstly, the most effective inducers have been found to be toxic and potentially cancer-inducing and, secondly, a refractory period frequently develops after the treatment and, consequently, repeated administrations of an interferon-inducer result in the production of only small amounts of interferon. Thus it appears to be more feasible to treat virus infections with exogenous interferon.

One difficulty with intravenous administration of interferon is that the substance rapidly disappears from the circulation in spite of a rather limited excretion in the urine. The reason for this is probably that interferon either accumulates extravasally or is bound to cell receptors. Only a small fraction of administered interferon diffuses into mucosal membranes and cerebrospinal fluid. In the early attempts to treat virus infections, these circumstances were virtually unknown. Relatively low doses of interferon were used and the results were discouraging. In recent years, experiments have been performed with about 1000 times higher doses and the results have then been more favourable. Interferon's prophylactic and therapeutic effect against respiratory infections caused by rhinoviruses can be demonstrated provided that high doses are administered. Locally applied interferon has been found to have a therapeutic effect on herpes simplex and adenovirus infections in the eye. A similar effect has been shown on herpes zoster. Thus interferon reduces the frequency of meningoencephalitis and other neurological sequelae and the course of the zoster infection generally becomes mild. Interferon therapy has also been used in some very severe virus diseases such as infections with Marburg virus and rabies virus. The results obtained are difficult to evaluate but since an alternative therapy for these diseases is unavailable it is important to continue these studies. In recent years treatment of persistent hepatitis B infections with both IFN-α and IFN-β has been attempted. Varying results have been obtained and further experiments are needed in order to evaluate the possible effect of interferon on persistent virus infections.

Treatment of tumour diseases with interferon

It has been shown in animal experiments that interferon has an inhibitory effect on the development of tumours. With reference to the above described effects of interferon, the inhibition of tumour growth can be explained by either antiviral phenomena, cell growth inhibition, stimulation of cytotoxic cells, in particular NK cells, and an increased exposition of surface antigens on tumour cells, which make these cells more susceptible to immunological cytolysis.

Experiments in mice have shown that interferon treatment reduces mortality, prolongs survival time and influences the frequency of formation of metastases in connection with several tumour diseases. It is important that both virus-induced and chemically induced and spontaneously appearing tumours can be inhibited. This shows that not only the antiviral effect but also other effects of interferon can be utilized in the treatment of tumours. In man, interferon treatment has been attempted in several tumour diseases. The most comprehensive investigations have been made with IFN-α given to patients with osteogenic sarcoma. The results obtained indicate that interferon markedly reduces the mortality from this disease. Side-effects attributable to the treatment are loss of hair, fever reactions and granulocytopenia, but these reactions have not been severe enough to interrupt the treatment and generally the reactions are much weaker than those which are seen after treatment with cytostatic drugs. Further attempts to treat tumours with interferon have been made in different forms of lymphomas, myelomas, larynx papillomas and bladder papillomas. The results, particularly concerning myelomas and larynx papillomas, have been encouraging. Interferon has a marked suppressing effect on the development of these tumours but after interruption of the treatment the tumours have usually reappeared. Thus it is still unclear whether interferon can give a permanent cure for tumour diseases. Future investigations aim to define in which different situations interferon can be used for treatment, to compare the effects of different doses and types of interferon, and to evaluate the results of treatment with interferon in combination with other forms of therapy.

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25

Picornaviruses

Gerolf von Zeipel

A large number of small (*pico*) RNA viruses are classified in the picornavirus family. The four subgroups of the family are composed of viruses responsible for many of our most common infections (*Table 25.1*). The viruses most important for man are grouped as *rhinoviruses* (common cold viruses) and *enteroviruses*. Among the enteroviruses are also classified viruses pathogenic for monkey, swine, cattle and mouse. The foot-and-mouth disease virus belongs to the *aphtovirus group* while encephalomyocarditis virus of mice is representative of the cardiovirus group.

TABLE 25.1. Viruses of the picornavirus family

Group:	Enterovirus (Polio, coxsackie A and B, echo and other enteroviruses)	Rhinovirus (Human and animal rhinoviruses)	Aphtovirus (Foot and mouth disease virus)	Cardiovirus (Encephalo-myocarditis virus of mice)
No. of types pathogenic for man	> 70	> 113	–	–
No. of types pathogenic for other mammals	34	2	7	1

In contrast to the rhinoviruses, enteroviruses may induce a multitude of symptoms of disease including pareses, meningitis and disorders of muscle and heart. However, the number of cases demonstrating symptoms of disease represents only a small fraction of the infections as latent or clinically-mild and undifferentiated infections are in the majority. The picornaviruses which are pathogenic for man are ubiquitously spread but are most common in densely populated countries with low standards of hygiene and a warm climate.

The basis for the classification of the picornaviruses in enteroviruses and rhinoviruses is described in *Table 25.2*. The most important difference is the relative sensitivity of the rhinovirus capsid to acid hydrolysis, inactivating rhinoviruses but not affecting the infectivity of the enteroviruses. Earlier, before this

TABLE 25.2. Differentiation between entero- and rhinoviruses

Character	*Enterovirus*	*Rhinovirus*
Capsid at pH3	Stable	Non-stable
Density g/cm^3 (CsCl gradient)	1.33–1.35	1.38–1.41
Optimal temperature for replication	36–37°C	33–34°C

difference in stability was known, rhinoviruses were classified as enteroviruses. This is the reason why one of the first common cold viruses to be isolated was designated echovirus type 28.

Properties of picornaviruses

Picornaviruses (*see also* Chapter 8) are only 28 nm in diameter and have a molecular weight of 8–9 × 10^6. The icosahedral nucleocapsid is not enveloped and is resistant to ether. It is composed of 60 structural units, each formed by 4 proteins, VP 1–4. In addition a minor protein, Vg, linked to the viral RNA is demonstrable. The RNA is single-stranded and has a molecular weight of 2.6 × 10^6. The virion-RNA functions as mRNA and is translated from the 3′ end which carries a poly(A)-complex. Replication occurs in the cytoplasm of infected cells and replicative intermediate double-stranded RNA molecules are demonstrable. The structural proteins (VP 1–4 and Vg) are formed by cleavage of the one large polypeptide initially synthesized by translation of the viral RNA molecule.

Enteroviruses

At present the enterovirus group is composed of about 70 viruses pathogenic for man. The subgrouping (*Table 25.3*) of the enteroviruses reflects the progress of virological methodology over a long period. In 1908, polio virus was demonstrated to induce paralysis in chimpanzees after intracerebral inoculation of the virus. The three types of polio viruses were for a long time the only recognized types of the picornavirus family. The first strains of coxsackie viruses were isolated in 1948. Newborn mice were used for the isolation and inoculated with faecal specimens of patients with suspected poliomyelitis. The animals developed characteristic symptoms not observed after inoculation with polio virus. The virus isolated by inoculation of the infant mice was designated coxsackie virus after Coxsackie, the suburb outside New York where most of the patients lived. A few years later a large number of mouse-pathogenic coxsackie virus strains had been isolated and several different serotypes were recognized. The histopathological changes induced in infant mice suggested that two large subgroups, coxsackie A and coxsackie B viruses, were discernible. Viruses of the subgroup A cause a generalized lethal myositis in mice with flaccid pareses, while viruses of subgroup B, affecting several organs including the brain, induce spastic pareses in addition to focal myositis and necrotic degeneration of adipose tissues.

Isolation of the echoviruses began after 1949 as a result of the introduction of the tissue culture technique by Enders, Weller and Robbins. The designation 'echo' originated from the initial letters in *e*nteric *c*ytopathogenic *h*uman *o*rphans, 'orphans' referring to the isolation of these viruses before their importance as causes of disease was known.

TABLE 25.3. Enteroviruses pathogenic for man

Virus	*Type*	*Year of first isolation*
Polio virus	1–3	1908
Coxsackie A virus	1–22, 24	1948
Coxsackie B virus	1–6	1949
Echovirus	1–9, 11–27, 29–33	1951
Enterovirus	68–71	1969*

*Since 1969 all new enteroviruses are designated 'enterovirus' and given a consecutive number starting with 68.

In the last few years the subgrouping of enteroviruses has been superseded. Some echovirus strains have been found to be pathogenic for mice and on the other hand some coxsackie virus strains which, by definition, should be pathogenic for mice turned out to be virtually non-pathogenic. New types of enterovirus are therefore designated enterovirus and numbered from 68 onwards. Recently the hepatitis A virus has been classified as an enterovirus (cf. Chapter 30).

Antigenic properties

Antigens inducing formation of neutralizing antibodies responsible for immunity are virus type-specific. They are genetically stable and are rarely demonstrably modified. That antigenic changes do occur is apparent from the relatively large differences sometimes found between strains of the same type of virus.

Cross-reactions between two types which are in other respects different mean that the types have one or more antigens in common. Examples can be found between types of polio viruses as well as between types of coxsackie or echoviruses. These cross-reactions may sometimes complicate type determinations of newly isolated enterovirus strains.

Antigens inducing type-specific immunity are observed in the native virus particle and are therefore called N antigens. Treatment causing denaturation (for example heating to +56°C) will deprive the capsid of RNA and result in the loss of the VP 4. Moreover, the type-specific N antigen is destroyed while group-specific H antigen (H = heat treatment), common to several enteroviruses, is exposed. Such H antigen is used diagnostically and reveals group-reacting antibodies when used in complement-fixation tests.

Clinical features

Individuals susceptible to enteroviruses will demonstrate infection by formation of antibodies and development of immunity. However, only a few per cent of infected

individuals reveal symptoms of disease. The incubation time varies from 7 to 14 days but occasionally ranges from 2 to 35 days.

Polio-, coxsackie- and echoviruses, as well as the more recently detected enteroviruses, may induce similar clinical pictures. Such common syndromes may differ in character and take the form of a minor illness or a meningitis.

The uncomplicated infection proceeds with fever, upper-respiratory-tract infection, occasionally diarrhoea, headache, dizziness and nausea. The illness is usually over within a few days.

In cases of *meningitis* there may be a biphasic onset of disease in about half of the cases. The most important symptoms in addition to those already mentioned above are severe headache and stiffness of the neck. An additional sign of meningeal inflammation is an increased number of white blood cells in the cerebrospinal fluid. Symptoms of involvement of the brain may be present. As a rule, the disease will subside within 2–10 days but the duration of the convalescence may be some weeks. Earlier, when there was no possibility of typing enteroviruses, cases of viral meningitis were often called 'non-paralytic polio', especially when occurring during epidemics of paralytic poliomyelitis. In countries where vaccinations against polio have been performed many forms of meningitis nowadays associated with enterovirus infections are mainly caused by coxsackie, echo or enterovirus 71.

Polio virus infection Febrile illness associated with paralysis in a non-polio-vaccinated or incompletely immunized individual should always be suspected to result from a polio virus infection. The paresis may follow after an uncharacteristic clinical picture of infection but is often seen during the onset of the febrile illness. Fever, muscular spasm and pain sometimes precede the pareses, which are flaccid, asymetrically distributed and not associated with sensory disturbances.

Muscle functions of the lower extremities are usually the functions affected. Infections affecting the posterior cranial nerves will create the serious bulbar form of poliomyelitis. This form of the disease calls for treatment in a respirator, as do polio infections reaching the parts of the brain responsible for maintaining the respiratory functions. The frequency of paralysis is considerably higher after infection with type 1 virus than after infection with polio virus types 2 or 3.

Paresis resulting from polio virus infection will often be persistent, in contrast to the more uncommon, slight and transient pareses induced by the coxsackie and echovirus infections. The recently observed enterovirus 71, however, seems capable of causing disease as serious as the paralytic polio.

Coxsackie virus infections Meningitis may be caused by about 10 group A viruses, mainly those with low numbers (types 1, 2, 4, 5, 7, 9, 10, 14), and by all the group B viruses. From patients with paralysis the A types 2, 7 and 9 and the B types 3–5 have been isolated. Type A 7 has been associated with two larger epidemics of paralytic disease, one in the USSR and one in Scotland. Not infrequently a rash appears in association with infections with types A 2, 4, 9 and 16.

Some syndromes particularly caused by the coxsackie A viruses are herpangina, hand-foot-and-mouth disease, and conjunctivitis.

Herpangina Herpangina may result from infections with types A 2, 4, 5, 6, 8 and 10. It is an infection of the pharynx, mainly appearing in children, and characterized by fever, nausea, blisters or small gray ulcers in the faucial area or on the soft palate, the tonsils or the tongue. Herpangina will create pain in association with swallowing.

Hand-foot-and-mouth disease This is seen in infections with types A 5, 9 and 16. It is a mild infection with small vesicles on the palms of the hands, the soles of the feet and in the mouth. The disease can also be induced by enterovirus 71.

A widespread epidemic of conjunctivitis in Asia has been attributed to infections with type A 24 which, together with enterovirus 70, seems to be the most important enteroviral aetiology for conjunctivitis.

Coxsackie viruses of the B group are responsible for, among others, *epidemic pleurodynia* (syn. myalgia epidemica, Bornholm disease) and infections of the heart. The disease is characterized by fever, myalgia of the thorax and the abdomen, and pleurisy. The duration of the myalgia ranges from days to weeks.

Myocarditis and pericarditis These, not uncommon in children as well as in adults, are often overlooked infections of coxsackie B virus. The neonatal form of coxsackie B virus infections is commented on in the description of perinatal infections in Chapter 34.

Echovirus infection Almost all types of echoviruses can cause meningitis although this more frequently is seen in association with some of the types (4, 6, 9, 11, 14, 25 and 30). Paralytic disease has been observed in patients infected with types 2 and 4. Children infected with types 4 and 9, particularly, may demonstrate a rash.

Other enterovirus types Enterovirus 70, as mentioned above, has been responsible for large epidemics of a severe haemorrhagic conjunctivitis in Asia and Africa in the early 1970s and recently (1981) also elsewhere, for example, in South and Central America as well as in Florida, USA. The clinical picture in patients with enterovirus 71 infections has varied from hand-foot-and-mouth disease to meningitis and a severe poliomyelitis-like disease.

Pathogenesis

Enterovirus infections are mainly spread via the faecal-oral route. The conjunctivitis is dependent, however, on virus directly infecting the conjunctiva. Virus from secretions of nose and throat or from faecal contaminations may be transmitted with food and water or via fingers and toys. Children may spread viruses more efficiently than adults. Milk- and waterborne enterovirus infections were previously more common than they are today.

Virus is replicated in the lymph glands and tonsils and is demonstrable in the oropharynx for some days, sometimes for weeks. The infection is rapidly spread to the Peyer's patches of the intestines and to the mesenteric lymph glands. Virus is often as early demonstrable by faecal specimens as by throat swabs but the excretion from the intestines can persist for many months with up to a million infectives doses of virus per gram of faeces during the initial period.

The infection of the throat and intestines is followed by a viraemia carrying the virus to many organs including the meninges, CNS, heart and skin. In meningitis caused by some types of echo- and coxsackie viruses, infective virus is often demonstrated in the cerebrospinal fluid. This is very rarely seen in polio virus infections.

Pareses are secondary to destruction of nerve cells, for example pareses of the motor neurons of the spinal cord's anterior horn in poliomyelitis. Transient paretic

conditions may reflect the effects of an inflammatory oedema. It has been assumed that the spread of virus to the CNS may be effected also by an axonal transmission via peripheral nerves, for example, after tonsillectomy or injections.

Immunity

A few days after infection, specific antibodies, which neutralize the enterovirus responsible for the infection, are demonstrable in the blood of the patient. Often the neutralizing antibodies are detectable throughout the life of the patient and serum antibodies, together with secretory antibodies present on the mucosa of throat and intestines, are probably responsible for the patient's lifelong resistance to the enterovirus in question. Antibodies, which are transferred across the placenta, and the secretory IgA of the milk will provide the child with an enterovirus type-specific protection for at least 6 months. In the developing countries children receiving artificial feeding run a higher risk of contracting polio infection than do breast-fed babies.

Epidemiology

In densely populated countries with unsatisfactory sanitary and hygienic conditions most young children are infected with enteroviruses. The infection may occur soon after birth when the presence of maternal IgG antibodies and antibodies provided with the breast milk permit only a subclinical infection with a development of immunity. A rise in standards of living associated with improved hygiene reduces the dissemination of virus in the population and delays the debut of infection. The infection then might occur in older children and adults who, more than the very young children, are apt to become seriously ill, possibly with paralysis. With improved socioeconomic standards, the number of susceptible non-immune adults is growing, and without the introduction of prophylactic methods the risks of epidemic paralytic poliomyelitis are increasing (cf. Chapter 21). From the end of the nineteenth century until the introduction of polio vaccination in 1955–58, increasingly widespread epidemics occurred in Europe, North America and Australia. Epidemics including thousands of cases were encountered with attack rates of 5–10 per 100 000. Today, similar and even higher attack rates are noted in some Asiatic and African countries like Burma and Ghana where successful vaccination programmes have not as yet been carried through.

During the poliomyelitis-like epidemics occurring in Bulgaria in 1975 and in Hungary in 1978, about 1000 cases were registered in each of the countries. The epidemics were caused by enterovirus 71, a virus which until then had not been circulating in these countries. Enterovirus 71, in contrast to polio virus, has attacked siblings with high incidence rates of paralysis and death. Epidemics with the same virus had earlier been observed in several western countries (for example in Sweden in 1973) but then had been associated with a benign meningitis or the hand-foot-and-mouth syndrome.

In 1957, echovirus type 9 was observed in association with cases of epidemic meningitis occurring in pandemic proportions. Also other types of echovirus have been implicated in widespread epidemics but because of the low-grade incidence of severe illness these epidemics attracted little attention.

In countries with a tropical climate enteroviruses are constantly circulating in the population. Infections with two or more virus types simultaneously are not

uncommon. This is in contrast to the situation with countries of the temperate zones where enterovirus infections occur seasonally with a peak in the late summer or autumn.

Prophylaxis

Prevention of enterovirus infections should be based on improving the standards of hygiene, sanitary precautions and vaccination. The poliovaccines are the only vaccines available against enterovirus infections. An inactivated vaccine against enterovirus 71 has been elaborated in the USSR and used with some success in the USSR and Bulgaria.

The laboratory diagnosis

The demonstration of enterovirus infections is based on isolation of the virus and an analysis of the serological response. Virus is isolated from faeces, cerebrospinal fluid, nose- and throat secretions, conjunctival fluid and/or vesicles. Suspended specimens are inoculated into cell cultures of human or simian origin. However, many coxsackie viruses of group A are most readily isolated in suckling mice.

Enterovirus-infected cultures often show cytopathic changes which are characteristic enough to allow a preliminary diagnosis of an enterovirus infection. Typing of the isolated virus is performed by laborious neutralization tests using hyperimmune sera against the various types of virus.Typing can also be performed by means of other tests, for example complement-fixation tests or haemagglutination-inhibition. Combination pools of sera for typing of isolates have been made available internationally by WHO in order to facilitate and standardize the typing.

Neutralizing antibodies which are virus type-specific are determined quantitatively in two serum samples, one obtained as soon as possible after the onset of illness and the other obtained 2–3 weeks later. A 4-fold, or greater, rise in antibody titre from the acute phase to convalescence (the second sample) is suggestive of an acute infection (cf. Chapter 20). Assays of complement-fixing (CF) antibodies by means of the H antigens can also be of diagnostic use. In small children, the CF antibody response might be virus type-specific. In older children and adults, antibodies against the H antigen are often group-reactive. Demonstration of a 4-fold rise in CF antibodies suggests an enterovirus aetiology of the disease and may initiate a search for the type of virus involved by means of type-specific antibody tests, for example NT or possibly ELISA. It must be remembered, however, that a diagnosis based on serological data alone is uncertain.

In evaluating the laboratory results, it should be emphasized that the isolation of an enterovirus from the cerebrospinal fluid, conjunctiva or vesicle fluid is diagnostically significant. This is in contrast to the isolation of virus from a faecal specimen, which may reflect a transient virus-carrier state unrelated to the patient's illness. For a further discussion of the association between laboratory findings and clinical disease, see Chapter 20.

Rhinoviruses

Upper-respiratory-tract infections, common colds, are probably the most frequent virus infections in man with billions of cases occurring yearly. Between 25 to 50 per

cent of these infections are supposed to be caused by rhinoviruses. By 1978 a total of 113 types of rhinovirus had been isolated, the true number of rhinovirus types most probably being much greater.

Clinical features and pathogenesis

After an incubation time of 1–4 days, the well-known symptoms appear: nasal discharge and obstruction, sneezing, mild pharyngitis, cough and headache. Symptomatology peaks in 2–3 days but usually lasts only for a week. Sometimes 1–2 weeks of convalescence are required to reach complete restitution. Complications in terms of secondary bacterial infections are, primarily, otitis and sinusitis. Common colds may elicit attacks of asthma in allergic children and adversely affect the chronic bronchitis of many smokers.

In experiments with volunteers it has been clearly demonstrated that the relative susceptibility of man to rhinovirus infections is not influenced by the lowering of the body temperature. No causal relationship seems to exist between cold, rainy weather and epidemics of the common cold.

In the tropics, epidemics of the common cold nevertheless start during the rainy season, whereas in countries of temperate zones two waves are discernible, one appearing during the early spring, the other during the beginning of autumn. The latter as a rule coincides with the time when the schools start after vacation and many carriers with different rhinovirus types possibly might meet. The high incidence of common colds during the rainy and cold seasons might be influenced by people gathering indoors and in public transportation. Children are victims of common colds twice as often as adults, and parents are more susceptible than families without children or singles. In small isolated communities common colds gradually disappear. They return when visitors break the isolation. The transmission of infection by hand, from fingers to the nose and conjunctiva, seems to be more efficient than by exposure to aerosols from sneezing and coughing. The nasal mucosa is considerably more susceptible to the virus infection than the pharynx and reacts with hyperaemia, oedema and the increased activity of secretory glands. The ciliated epithelium may, as in organ cultures, be completely or partially destroyed. Virus-shedding in nasal mucus peaks on the second and third day after the onset of illness, and will reach concentrations 10–100 times higher than in the pharyngeal secretions. The necessity of a relatively low temperature for optimal virus replication might contribute to the limiting of the spread of virus to the lower respiratory tract. Instability at a low pH may explain the absence of rhinovirus in the intestines.

IgA antibodies in nasal discharges and tears, and IgG antibodies in serum are demonstrable one to two weeks after infection. The immunity is type-specific and has a duration extending into years. It is more attributable to the presence of nasal IgA than to serum IgG antibodies.

Patients are refractory to reinfections of common cold for 3–4 weeks after infection, although other rhinovirus types might be operating. It is not known if this resistance, as well as the healing of the infection, is associated with the production of interferon.

The laboratory diagnosis

It is difficult to propagate rhinoviruses in cultures. Often it is necessary to use actively multiplying cell cultures of human embryonic lung or kidney tissues. Some

rhinoviruses are, initially, most readily isolated by using ciliated cells in organ cultures of embryonic nasal mucosa. The cytopathogenicity of the virus is relatively weak and inhibition of the ciliary activity sometimes is the only criterion demonstrable of virus infection.

Isolated virus is typed by neutralization tests. The typing of rhinoviruses is difficult owing to the many cross-reactions between the strains. A serological technique applicable for routine diagnostic use is not available.

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26

Togaviruses

Marianne Forsgren and Gerolf von Zeipel

The togavirus family consists of more than 80 single-stranded RNA viruses. The icosahedral nucleocapsid has a tight-fitting envelope (L. *toga* = outer garment). Of the 4 subgroups (*Table 26.1*), *alphavirus* was designated arbovirus group A in earlier classifications while *flavivirus*, named after yellow fever (L. *flavus* = yellow) corresponds to arbovirus group B. All the alphaviruses and most of the flaviviruses are *ar*thropod *bo*rne, i.e. *arboviruses*, and transmitted by arthropod vectors, primarily mosquitoes and ticks.

TABLE 26.1. Relations between togaviruses and arboviruses

*Arbovirus**	*Togavirus*	*Vector*	*Disease*
Arbovirus group A	Alphavirus	Mosquitoes	Fevers, haemorrhagic fevers, encephalitides
Arbovirus group B	Flavivirus	Ticks and mosquitoes	Fevers (dengue), haemorrhagic fevers (yellow fever), encephalitides
–	Rubivirus	–	Rubella
–	Pestivirus	–	Infections of swine and cattle

*Arboviruses include also viruses classified among arena-, bunya-, reo- and rhabdoviruses (*see* Chapter 33)

This mode of transmission does not apply to *rubivirus*, the only member of the subgroup being rubellavirus, or to *pestivirus*, causing plague-like diseases with fever and diarrhoea in swine and cattle.

In addition to the arboviruses mentioned above, there are approximately 300 arboviruses belonging to the arena-, bunya-, reo- and rhabdoviruses. Of more than 350 types of arbovirus, about 50 are pathogenic for man. The mosquitoes, ticks, etc. transmitting the arboviruses become infected for the rest of their lives after feeding on a viraemic host, which includes birds as well as small and large mammals. Virus replicates for about two weeks in the vector, which then transmits the infection to new hosts and these transmit the infection to new vectors in a continuous circuit. Vectors and hosts together constitute the reservoir of the viruses (*Figure 26.1*).

Arboviruses are found in temperate as well as tropical climate zones, but particularly in the latter where large numbers of vectors and hosts are continuously present. Vectors, hosts and environmental conditions are the rate-limiting factors for the geographical distribution of the arboviruses. The various viruses are named after diseases or geographical places. Many of the haemorrhagic fevers and fatal encephalitides have been efficient obstacles to the colonization and exploitation of the tropics.

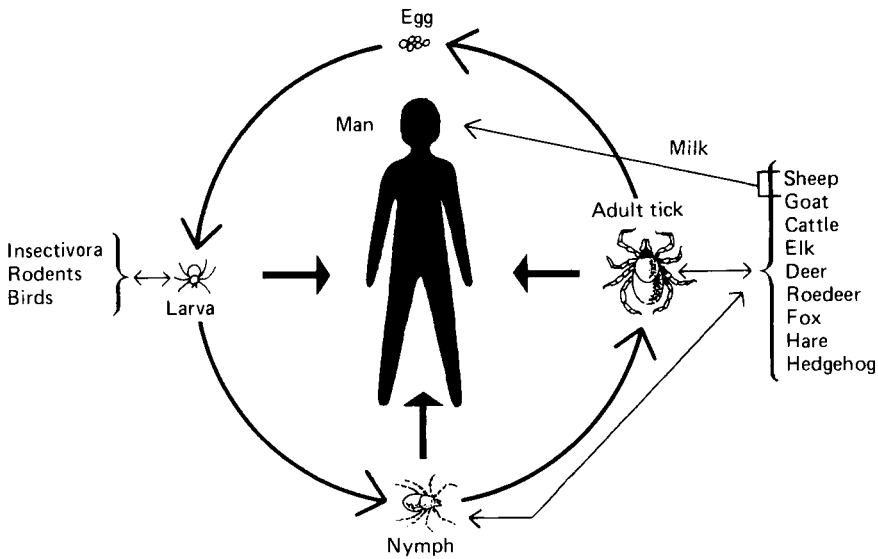

Figure 26.1. Circulation of tick-borne encephalitis virus. In addition, the immature forms of the tick (*Ixodes ricinus*) can transmit the virus from and to the host. The infection may be transmitted to man with milk from the sheep and goat also.

Of the togaviruses, alphaviruses are somewhat larger than flaviviruses, the diameter of the virion varying in size from 40 to 70 nm. Virus replicates in the cytoplasm of the host cell, and is released by budding from the plasma membrane, thus receiving its envelope. The 1–3 glycoproteins of the envelope react as haemagglutinin and complement-fixation antigen. There is no antigenic relationship between the alpha- and flaviviruses. However, within both groups cross-reactions are to some extent demonstrable in haemagglutination and complement-fixation tests. In contrast to what is known about other viruses, the haemagglutinin is less type-specific than the complement-fixation antigen. More type-specific are the antigens responsible for the induction of neutralizing antibodies. Most important as experimental animal models are newborn or young white mice which are susceptible to the majority of both alpha- and flaviviruses. They are not yet readily replaceable by tissue culture methods.

Each type of alpha- and flavivirus infects as a rule only one species of vector, but the vector may feed on many hosts, birds and/or mammals. Usually hosts within the endemic areas are latently ill but demonstrate a viraemia which is high enough to enable transmission of the virus to vectors. A majority of the infections are only exceptionally spread to humans. However, man is the only host recognized for dengue and the urban form of yellow fever.

Alphaviruses

The subgroup is composed of about 20 types of virus. The most important medically are three viruses responsible for the equine encephalitides.

Eastern equine encephalitis (EEE) The virus is endemic in horses of the eastern states of the USA but is responsible for up to a dozen human cases per year. Clinically manifested EEE has a mortality rate of 50–70 per cent in man. The number of latent cases is 4 to 10 times greater. EEE is present also in regions of South America.

Western equine encephalitis (WEE) In the western and southwest states of USA, this virus is registered as causing from a few to about 100 cases yearly. Clinically mild, WEE has a mortality rate in man of about 2 per cent. The number of latent cases is 50 to 1000 times larger, however. WEE is reported also from countries in South America.

Both these encephalitides are seen during summer and autumn in rural areas as well as in urban slums. Virus is transmitted by mosquitoes (Culex) and has birds as hosts. Vaccination of horses has efficiently reduced economic and veterinary damage. However, as a consequence of vaccination, it is no longer possible to observe the seasonal occurrence of the virus by the registration of sick horses.

Venezuelan equine encephalitis (VEE) From South and Central America and the West Indian islands, this virus is spread by mosquitoes and has small mammals and horses as hosts. In man the infection can cause an influenza-like disease or a clinically mild encephalitis.

O'nyong-nyong in Africa and *Chikungunya* virus in Africa and Asia are two other mosquito-borne alphaviruses. They induce a dengue-like disease (*see below*).

Flaviviruses

Just over 40 viruses transmitted by mosquitoes (Culex or Aedes) or in some cases, spread by ticks, are classified in the flavivirus group. *Table 26.2* summarizes the infections which are most important medically. Many of the infections may progress latently or with uncharacteristic clinical signs. Three syndromes will be discussed: fever with arthralgia and exanthema, haemorrhagic fever and encephalitis.

Fever with arthralgia Sometimes occurring with a rash, this can be induced by many arboviruses but is usually referred to as the *dengue triad* as it comprises the main symptomatology of the well-known dengue infections. Virus is transmitted by Aedes mosquitoes; 5–8 days after infection, the patient experiences fever-chills, headache, retro-ocular pains, erythema of the face and pains in the joints and muscles of the extremities. The pains are said to be as intense as those of a fractured leg. After 2–3 days of fever, the body temperature is normalized but rises again on the following day. Associated with the second peak of fever, a rash appears covering the body but sparing the face. Adults recovering from the disease require some weeks of convalescence. In South-East Asia children who have overcome a dengue infection but have later been infected by one of the other 3

types of dengue virus may develop a haemorrhagic fever, sometimes with a hypotensive shock. The mortality rate may exceed 10 per cent.

Haemorrhagic fever The feared yellow fever virus can induce an abortive infection clinically similar to the initial phase of the dengue fever. The progress of the disease is indicated by a second rise to a high temperature although the pulse rate still remains low.

Thrombocytopenia and a reduced number of leucocytes are some of the characteristic blood features. During the course of the fever, the clinical picture is dominated by hepatitis, nephritis and bleeding of the skin and mucous membranes,

TABLE 26.2. The most important flavivirus infections

Area	*Disease*	*Vector*	*Host*
Mediterranean, Europe, Middle-East, North Africa, East Asia, West Indies	Dengue	Mosquitoes	Man
Africa, Asia	West Nile fever	Mosquitoes	Birds
Africa, South America	Yellow fever	Mosquitoes	Monkeys, Man
North and South America	St Louis encephalitis	Mosquitoes	Birds
East Asia	Japanese B encephalitis	Mosquitoes	Birds, Swine
Australia	Murray Valley encephalitis	Mosquitoes	Birds
East Soviet Union	Russian Spring–Summer encephalitis (Eastern tick-borne encephalitis)	Ticks	Rodents and other mammals
West Soviet Union, Balkans, Central and Northern Europe	Western tick-borne encephalitis	Ticks	Rodents and other mammals
East Soviet Union	Omsk haemorrhagic fever	Ticks	Rodents and other mammals
India	Kyasanur forest fever	Ticks	Monkeys, rodents
North America and Canada	Powassan encephalitis	Ticks	Rodents

and black vomiting. The mortality rate is approximately 10 per cent. Survivors recover completely and surprisingly rapidly. Yellow fever virus is transmitted by mosquitoes (Aedes). The virus is circulated to non-immune humans in the urban form of yellow fever while virus of the so-called jungle fever is circulated to monkeys.

Omsk and *Kyasanur forest* are two haemorrhagic fevers with ticks as vectors and small mammals and monkeys as the main hosts. The viruses are closely related to the viruses of the tick-borne encephalitides.

Four viral infections, of which three are encephalitides, are transmitted by mosquitoes from birds. The most serious clinically of these infections is the

Japanese encephalitis. In addition to birds, swine play an important role as hosts. After an incubation time varying between 4 to 21 days, there is a sudden onset with severe headache, high fever, nausea and arthralgia. A day later, the patient demonstrates stiffness of the neck and confusion, and may die in convulsions and coma. A mortality rate of 80 per cent among old patients has been registered, but it can be high also in younger patients. The disease is widely disseminated in Asian countries. In the south the endemic area borders on the area of the *Murray Valley encephalitis* of New Guinea and Australia. The latter disease has caused several large epidemics predominantly in very rainy and mosquito-rich summers. Another related encephalitis is caused by St. Louis virus found all over the USA. During epidemic years thousands of cases of *St. Louis encephalitis* have been registered, the mortality rate amounting to 5–10 per cent in the older patients. West Nile virus in Africa and Asia is antigenically related to the three encephalitogenic viruses just mentioned. However, West Nile virus causes a clinically-milder infection with dengue-like symptomatology.

Around 1930 a disease appeared among timber workers of the Urals, Siberia, and the Far East. The disease was named *Russian Spring–Summer encephalitis (RSSE)* due to its seasonal appearance. It is also called *Eastern tick-borne encephalitis*. The clinical picture is characterized by a severe acute encephalomyelitis which has a monophasic course. Pareses are observed particularly in the muscles of the arms and shoulders. The death rate may reach 20–30 per cent. The incubation time is 1–2 weeks and the infection is caused by a virus transmitted by ticks (*Ixodes persulcatus, haemophysalis* and *dermacentor*). The virus infects rodents, deer and elks, but also domestic animals like sheep and goats. In the tick, transovarian passage of the virus has been demonstrated to occur although not regularly.

A milder form of the disease was reported in the 1940s from the western parts of the Soviet Union and subsequently found in most countries of Europe. This form is called the *Central-European* or *Western tick-borne encephalitis*. The vector is *Ixodes ricinus*. In endemic areas about 1 per million of the tick population carries the virus transmitting it during spring and autumn. Large endemics in Czechoslovakia have been associated with the consumption of unpasteurized goat milk. The disease is usually diphasic. It may start with an influenza-like onset. A second phase, after a period with normalized temperature, is characterized by a meningoencephalitis which occasionally is complicated by a usually-transient paralysis. A relatively long convalescence may be required. The mortality rate is low (1–2 per cent).

In the UK, *Ixodes ricinus* transmits a virus closely related to the tick-borne encephalitis virus. The virus causes *louping-ill* in sheep and is only rarely pathogenic for man.

Finally, the *Powassan encephalitis* of the USA and Canada should be mentioned. The disease is transmitted by ticks which, however, only rarely feed on humans.

The laboratory diagnosis and prophylaxis of alphaviruses and flaviviruses

Alpha- and flaviviruses replicate in cells of the reticuloendothelial system. A viraemia lasting for 2–5 days often accompanies the onset of disease. Isolation of the virus from blood specimens sampled early in the disease or from autopsy materials can be difficult and hazardous, because among other reasons the use of animals for virus isolation will increase the risk of infecting the laboratory staff. The diagnosis is therefore mainly based on serological tests. The HI antibodies of IgM

class appear very early, CF antibodies (IgG) may appear later. Preferably, three blood samples should be examined: one in the early phase of the disease and two collected 2 and 4–5 weeks later. A four-fold or greater increase of antibody titre is compulsory for a reliable diagnosis. In many cases an early diagnosis may be achieved by demonstration of IgM antibodies in blood specimens drawn during the first week of the disease. The serological tests most often used in this connection are the HI and ELISA tests. As for many other HI tests, the serum has to be treated with, for example, acetone to exclude the influence of non-specific inhibitors.

In areas where one or a few alpha- and flaviviruses are circulating, the antibody response will indicate specifically the virus type causing the current infection.

If many types of related viruses are present in the area, patients previously infected by one or more viruses may develop a broad antibody response at a new infection. An analysis in HI and CF tests with several antigens is then required. Even neutralization tests may be required to reveal the virus type presently involved.

An effective vaccine against yellow fever has been available for a long time (cf. Chapter 23). The vaccine contains live attenuated virus produced in embryonated hens' eggs and lyophilized. The virus strain (the 17D strain) has been attenuated by passaging in laboratory animals and tissue cultures. Immunization against yellow fever should be renewed every tenth year. Against Japanese encephalitis a formaldehyde-inactivated vaccine of mouse brain origin has been successfully employed. Recently a formaldehyde-treated tissue culture vaccine was introduced against tick-borne encephalitis. Dengue fever vaccination is hampered by the risk of hypersensitivity reactions which may lead to haemorrhagic disease and shock. Apparently there are immunopathological reactions involved which are directed against viral antigens of the plasma membranes of infected cells.

To reduce the dissemination of alpha- and flaviviruses, mosquitoes are controlled by insecticides, restriction of agricultural irrigation and draining of swamps. Larger domestic animals like cattle, horses, sheep and goats, are vaccinated, as are people within endemic areas. For personal protection, mosquito-nets, so-called repellents, and adequate clothes as used in malaria-endemic areas, are required.

Rubellavirus (Rubivirus)

It is only in recent years that rubellavirus has been included in the togavirus family. When the physical and chemical properties were thoroughly analysed and the morphology characterized by electron microscopy, it became obvious that rubellavirus should be classified as a togavirus. The diameter of the virions varies between 40 and 60 nm. All strains isolated are of the same serotype. No cross-reactions with other togaviruses have been demonstrated. One structural antigen is a haemagglutinin and two others are detectable by complement-fixation tests. In addition to man, the virus is infective to cattle, swine, sheep and goats. Virus transmission requires no vector.

Rubella belongs to the diseases of childhood. It is a gentle disease with a rash but few complications. If the infection is contracted by a pregnant woman, virus may be transferred to the fetus and cause fetal damage. This is the most important medical consequence of the rubella infection. In the following the clinical virology of the congenital rubella infection is therefore emphasized.

Postnatally acquired rubella

The symptomatology of rubella is as a rule discrete. After an incubation time of 2–3 weeks, on average 17 days, the patient may fall ill, at first with only fever and other undifferentiated symptoms of infection. Simultaneously, a rash is seen, first appearing on the cheek and then spreading to the trunk and extremities. Seldom does the rash last for more than 3 days. In adults the rash is demonstrable a few days later than in children. The rash is caused by immune reactions directed against rubella antigens which are exposed on surfaces of infected skin cells and capillary

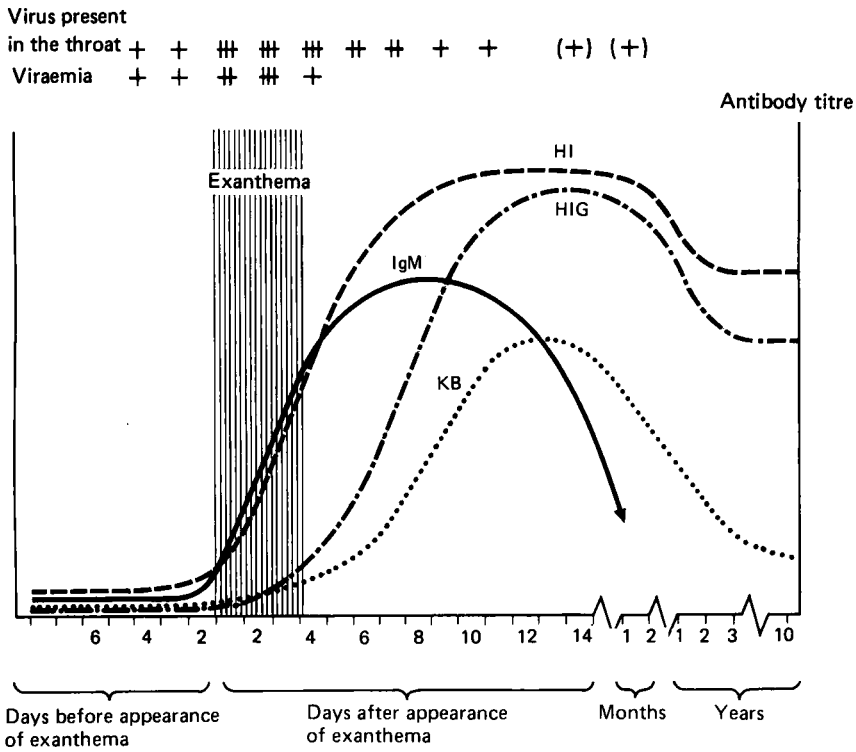

Figure 26.2. Laboratory findings in cases of postnatally acquired rubella. Antibodies assayed with haemagglutination-inhibition (HI), single radial diffusion or haemolysis-in-gel (HIG) and complement-fixation (CF). Presence of IgM antibodies is depicted separately

endothelial cells. Lymphadenopathy of the neck and retroauricular lymph glands, as well as a conjunctivitis, are often observed. However, the rubellavirus infection may be subclinical or demonstrate only a transient, itching exanthema. The frequency of subclinical infections in the studies reported varies but subclinical infections are considered to be almost as common as the infections which are clinically manifested. Frequent complications, particularly in women, are arthralgia and arthritis, while thrombocytopenia and encephalitis are rarely seen.

For transmission of the virus close contact is required. Virus is demonstrable in the throat 5–7 days before, and up to 2 weeks after, the onset of the disease. The contagiousness is highest at the onset and during the last days of the incubation

period. Virus probably replicates in respiratory epithelial cells and/or in the lymph nodes of the neck. Viraemia starts after about 1 week and continues until antibodies are present in the blood (*Figure 26.2*). At this time the rash develops. Subclinical infections may also be accompanied by viraemia. The arthritis is a result of an infection of the synovia of the joints.

Immunity after acquired infection is longlasting and in most individuals probably lifelong. Subclinical reinfections occur in about 5 per cent of the naturally immune while clinically overt reinfections seem to be exceptional.

Laboratory diagnosis

There is no characteristic clinical picture of rubella; the classic pattern of symptoms, including the arthritis, is seen also in other viral diseases. On the other hand, the rubella infection may be clinically uncharacteristic or subclinical. The diagnosis of rubella based on symptoms of an acute febrile illness with rash needs diagnostic help from a laboratory.

Rubellavirus is isolated in cell cultures. Presence of virus is demonstrable by viral cytopathogenic changes in certain cells while in other cell lines there is no detectable cellular change. Rubellavirus-infected cells then have to be traced by various methods: immunoperoxidase techniques or immunofluorescence, or indirectly by superinfecting the cells with echovirus type 11. If the cells are infected with rubellavirus, the echovirus will not be able to multiply (interference). On the other hand if the cells are not infected with rubellavirus, they will demonstrate the cytopathic changes of echovirus. Rubellavirus is unstable in room temperature and does not withstand drying or heat. Specimens for virus isolation should be refrigerated and virus is kept at −70°C in the laboratory. Isolation of rubellavirus is time-consuming and there is as yet no rapid method for the detection of available viral antigens.

The laboratory diagnosis is therefore based on serology. Demonstration of rubella-specific IgM and/or a rise in antibody titre from the acute phase to the convalescence offers the means for the diagnosis (*Figure 26.2*). The following methods are used for rubella serology: haemolysis-in-gel, also referred to as single radial diffusion-in-gel, haemagglutination inhibition (HI), enzyme-linked immunosorbent assays (ELISA), radioimmuno assays (RIA) and complement-fixation tests. It is necessary to determine the IgM antibody content if specimens are obtained late after exposure or late after the rash. In cases of uncomplicated rubella, serum-IgM titres persist for a limited period only (4–10 weeks, the time varying somewhat, depending upon the sensitivity of the method used for detection of the IgM antibodies).

Evaluation of the immune status Natural infection or vaccination will both induce immunity. Immunity is demonstrated by the antibody assays referred to above. These assays will indicate if a pregnant woman exposed to rubella infection or demonstrating a rash is susceptible to rubella. It is important to inform the laboratory not only about the clinical status of the patient but also about i.e. the date of the last menstrual bleeding, the date when she was exposed to rubella and when the symptoms of disease appeared. These data are important for the selection of the correct laboratory method of analysis and for the evaluation of laboratory data.

Epidemiology

Rubella is endemic in most countries and causes epidemic outbreaks about every fifth year in Europe and North America (*Figure 26.3; see also* Chapter 21). Seasonal variation is evident; cases accumulate at the end of December and peak in March/April. Closing of schools in June reduces the incidence of cases, but these may be seen sporadically all the year around. Transmission of infection is dependent upon person-to-person contacts indoors and the infection is therefore effectively spread between family members, in military camps and in institutions.

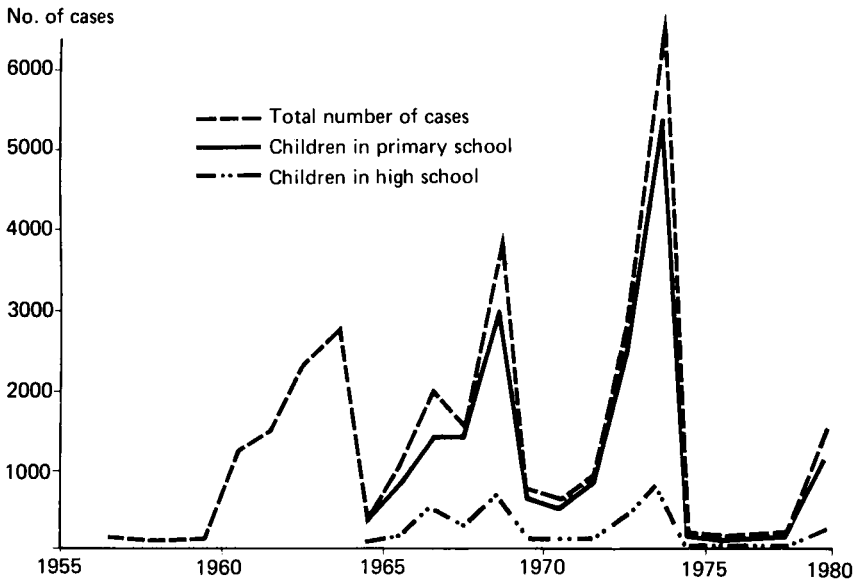

Figure 26.3. Cases of rubella reported in Swedish school children, 1956–1979. Vaccination of 12 to 13 year-old girls against rubella was introduced in Sweden in 1974

Before programmes for immunization were introduced, about half of the child population of the industrialized countries, in the age groups between 10 and 14 years, were non-immune and 10–12 per cent of non-vaccinated women of child-bearing age were still not naturally immunized (*see Figure 21.2*). The incidence of rubella infections in non-vaccinated pregnant women would amount to 1–2 per cent during epidemic years and an increased number of children were born with congenital rubella.

Congenital rubella

In the congenital form of rubella, virus is transferred across the placenta of the viraemic pregnant woman (cf. Chapter 15). It is particularly during the first trimester of the pregnancy, that the risk for fetal infection is high. After the eighteenth week the risk of infection of the fetus is reduced. Infection of the fetus during the early period of gestation may develop into a persistent infection and, at birth, the child demonstrates signs of infection (*see Figure 15.3*). The newborn child is highly contagious and may shed virus for months, sometimes for years. Infective virus may persist in infected tissues even after virus ceases to be shed and virus has been isolated from a cataractic lens.

Further evidence for a persistent infection are cases of progressive rubella panencephalitis observed in children of 11 to 12 years of age and who are congenitally infected with rubella (*see* Chapter 16).

The congenital infection may cause embryopathy with retardation of growth and failure in organogenesis. Consequently, damage of most organs may be induced: in the heart and the circulatory system (patent ductus arteriosus, pulmonary stenosis, ventricular and atrial septal defects), in the eyes (cataract, retinopathy, glaucoma), in the brain (encephalitis of varying degrees of severity), in the ears (often a double-sided impairment of hearing), etc. A generalized intrauterine growth retardation is a common feature of congenital rubella. The prognosis for survival and recovery is bad concerning the most damaged children, and the mortality rate is high, particularly during the first 6 months of life.

The risk of infection of the fetus is reported to be almost 100 per cent during the first trimester, but the risk for embryopathy is less. Fifteen to 20 per cent of the children demonstrate sequelae when they are examined during the neonatal period. At re-examination several years later, further signs of disease are usually demonstrable and the hearing in particular is affected. The risk of damage in the children will thus increase to 30–35 per cent. As a result of infections occurring during the second trimester, losses of hearing are predominant. The risk of damage to the fetus after the sixteenth week of gestation is small.

The fetus produces IgM antibodies and, later, also IgG antibodies against rubella (*cf. Figure 16.5*). IgM antibodies are detected in the majority of the children demonstrating the congenital rubella syndrome at birth and these antibodies persist for months to years concurrently with shedding of virus. There is still an IgG antibody activity detectable in the blood of the child when the maternal antibodies have disappeared.

Laboratory diagnosis

Clinical virological examinations of children, who are suspected to be congenitally infected with rubella, should include (from the neonatal period until the child is about 2 months of age): (1) attempts to isolate virus from throat, urine, possibly heparinized blood (buffy coat) and cerebrospinal fluid; (2) demonstration of anti-rubella-specified IgM activity in the blood of the child. When positive, these are indicative of a congenitally acquired infection. Blood samples of the mother must be examined concurrently. The tests should be followed up by determination of rubella antibodies when the child is 6–9 months old, to ascertain the presence of antibodies when the maternal antibodies will no longer complicate the evaluation of serological results. A retrospective diagnosis is often possible until the child is 3–4 years old, since the incidence of postnatally acquired rubella is relatively low during the first 4 years. However, the possibility of a postnatal infection must always be considered when the results are being evaluated.

Prophylaxis

If rubella is verified during the first 14–16 weeks of pregnancy, termination of pregnancy should be considered.

Immune prophylaxis with immunoglobulin of a pregnant woman after rubella exposure has not effectively prevented the infection. Even the effect of hyperimmune globulin with higher anti-rubella activity is uncertain but this may be used

in special cases. A serological follow-up of the rubella-susceptible woman, 6–8 weeks after the prophylaxis, is necessary to trace a subclinical infection.

Live rubella vaccines are available for use alone or for use combined with measles and mumps virus vaccines (*see* Chapter 23). Following vaccine administration, antibody develops in > 95 per cent of susceptibles. The antibody levels are somewhat lower than after natural infection but antibody activity has persisted in the majority of individuals during a 12-year observation period. A higher frequency of reinfection is observed in vaccinees than in individuals who are immune after natural rubella. Longterm observations are needed to clarify whether there is a risk of viraemia – and fetal infection – in a reinfected vaccinee. Available data suggest that this risk is small.

Vaccine reactions in children and teenagers are few but symptoms of mild rubella may develop (mainly transient arthritis and arthralgia) particularly in women over 20 years of age. Contact spread of vaccine virus to susceptible pregnant women is negligible. Blood transfusion, but not anti-D prophylaxis, may prevent vaccine infection.

The most important group to be protected against rubella is women of childbearing age. However, vaccine virus can infect the fetus and our knowledge of the teratogenic potential of the vaccine strains is still incomplete. Studies of more than 100 children born by mothers inadvertently vaccinated during pregnancy have not shown any case of fetal damage. Still, vaccination is contraindicated during pregnancy and should be undertaken in fertile age only if the woman is known not to be pregnant and birth control measures are undertaken for at least two months following vaccination (recommendable for susceptible women working in schools, day nurseries and hospitals, for example). While vaccination is contraindicated during pregnancy, large-scale immunization of women of childbearing age is possible a few days postpartum. Postpartum vaccination programmes are in use in many countries and have reduced susceptibility among pregnant women (for example, in the Stockholm area from 12 to 5 percent in 7 years).

Different strategies have been adopted for child immunization. Children between 1 year to the age of puberty are vaccinated in order to induce herd immunity and to block the circulation of rubellavirus in the population, for example, in the USA. Another approach focuses on girls close to puberty, leaving the wild virus circulating among boys and younger girls, for example in the UK. The effect of such programmes has been slow. To achieve a more rapid result, immunization of boys and girls at 18 months of age followed by revaccination at 6 or 12 years of age is now being introduced, for example in Finland and Sweden. Follow-up programmes with registration of congenital rubella and rubella during pregnancy are used to determine the effect of vaccination.

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27

Orthomyxoviruses (influenza viruses)

Svante Hermodsson

Since the fourteenth century, influenza has been known as a disease with a remarkable capacity for spreading and the ability to cause widespread epidemics. The name 'influenza' was established in Italy since it was believed that the appearance of the disease was 'influenced' by the positions of the stars. There are still features of the epidemiology of the disease which have not been satisfactorily explained. A detailed analysis of viral structure and replication, however, has elucidated some of the mechanisms essential for the antigenic changes of the virus, one of the important factors contributing to the epidemic character of the disease.

Three types (A, B, C) of influenza virus are recognized, all of which cause respiratory tract infections. The so-called Spanish grippe was a severe epidemic in 1918 and 1919. It has been estimated that approximately 20 million people died as a result of the epidemic. Probably the bacterial infections following the influenza were the direct causes of death in most cases. Also, in recent years, influenza epidemics have been associated with marked increases in death rates and, compared with other virus infections, influenza is associated with the greatest number of deaths.

Properties of the virus

Electron microscopy shows that the influenza virus exhibits particles of varying size and morphology. This is because many virus particles are incomplete and the envelope of the virions is labile. The envelope originates from the plasma membrane of the host cell. The virion is spherical or filamentous (diameter 100 nm) and contains a helical nucleocapsid with single-stranded RNA. The nucleic acid has a total molecular weight of about 5×10^6 and is divided into fragments. Many fragments code for one protein only, and the genome thus consists of monocistronic RNA strands. Both influenza A and B have 8 RNA fragments and 7 of these code for structural proteins of the virion. Glycoproteins are demonstrable in the envelope as projections (*Figure 27.1*). Inside the bipolar lipid layer are the membrane protein (M) and the 4 proteins making up the nucleocapsid. The dominating nucleocapsid protein is designated nucleoprotein (NP); the others which have polymerase activities are called P1, P2 and P3. The 5 proteins of the interior of the virus are antigenically similar in all virus strains of the same type. The outer proteins demonstrate a greater antigenic variation. This is particularly pronounced among influenza virus type A strains which are grouped into several

subtypes containing several virus strains within each subtype. Influenza virus types B and C are divided into virus strains only. There are two kinds of glycoproteins on the surface of the virus, the haemagglutinin (H) and the neuraminidase (N). The haemagglutinin is the predominant outer protein. It can attach virus to specific cellular receptors and harbours the binding sites for neutralizing antibodies. Neuraminidase is an enzyme capable of releasing sialic acid from the receptor by hydrolysis. The enzyme plays a role in the liberation of influenza virus from infected cells and in the spread of virus in the tissues and secretions. Influenza type C virus lacks neuraminidase but exhibits another receptor-destroying activity. This virus has not been as thoroughly studied as influenza A and B. The virions of influenza type C seem to have at least 4–5 fragments of RNA and a corresponding number of proteins.

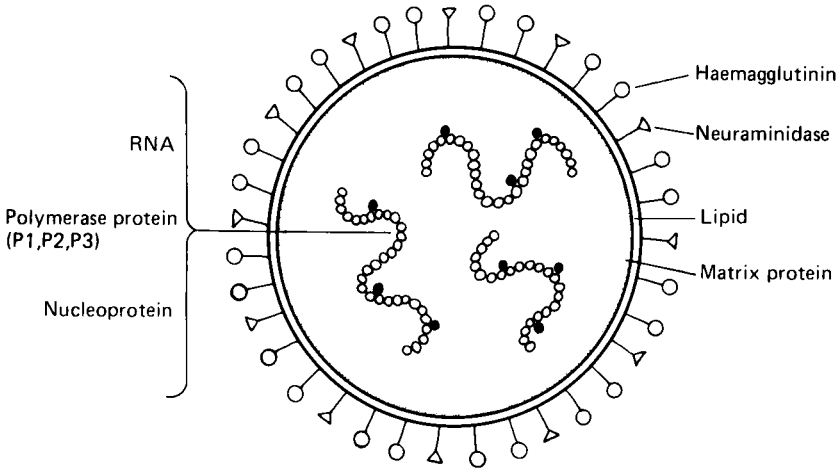


Figure 27.1. Schematic diagram showing the morphology of the influenza virion

The synthesis of influenza virus in permissive cells displays several interesting features. The RNA fragments of the virion are transported to the cell nucleus and are transcribed to mRNA by means of a capsid-associated enzyme. This primary transcription produces incomplete copies of the viral RNA. A secondary transcription is needed for replication. This is dependent upon protein synthesis as well as simultaneous transcription of cell DNA, probably for the synthesis of a primer. Aggregation of RNA and proteins into nucleocapsid structures is demonstrable in the nucleus and the nucleocapsid, as well as the M protein, migrate to the plasma membrane which has been modified by insertion of the viral glycoproteins. The contact between M protein and nucleocapsid leads to an evagination, and by this budding process virus is released from the cell. The synthesis of glycoproteins is located to the endoplasmatic reticulum. Hydrophobic domains of the newly formed polypeptide span the membrane. After glycosylation, processed by cellular glycosyl transferases, the haemagglutinin and neuraminidase migrate to the plasma membrane. During this transportation the haemagglutinin is exposed to cellular proteases splitting the protein into two parts. The cleavage does not affect the haemagglutinating properties but is essential for infectivity.

The majority of the virus particles formed are non-infectious. Experiments mimicking the natural infection have shown that only one out of 10 virus particles is

infectious. If the cells are infected with large quantities of virus, a much larger proportion of the virus is incomplete. The so-called defective, interfering virus which is produced contains a complete set of proteins but a modified RNA content. The larger RNA fragments are replaced by molecules of lower molecular weight.

It is a characteristic of influenza virus that recombinents appear in high frequency when cells are simultaneously infected with two viruses. Due to the fragmentation of the viral genome, virus with a varying combination of genes may be produced during maturation. The reassortment of genes may lead to formation of strains with new antigenic properties, changed virulence, a different host-cell dependence, etc. This form of genetic interaction is seen between viruses of the same type. It is utilized not only in laboratories for production of vaccine viruses but probably also in nature for the creation of strains capable of causing new epidemics of influenza.

The classification of influenza viruses is at present based on the antigenic properties of the viral NP, H and N proteins. The study of the immunological properties of these antigens can determine the degree of relatedness between different viruses. For the designation of influenza virus strains a complicated system is used which categorizes (1) the type of virus, (2) the host (provided the virus is not isolated from man), (3) the geographic area where the virus was isolated, (4) the number of the isolated strain, (5) the year of isolation and (6) the subtype, by marking the antigenic specificities of H and N. Examples of influenza virus strains are influenza A/Swine/Taiwan/1/70 (H3N2) and influenza B/Hong Kong/5/72. The subtyping of influenza A virus has previously been somewhat confusing since antigenically related proteins have been designated in different ways (e.g. Hsw1, H0 and H1). At present 12 subtypes of haemagglutinin (H1–H12) and 9 subtypes of neuraminidase (N1–N9) are known.

Clinical features

Influenza A and B display similar clinical pictures which markedly differ from the one observed in infections with influenza C virus. This last virus is only seen in subclinical infections or mild upper-respiratory-tract infections with a common cold picture without fever. Infections with influenza A and B viruses may be mild as well and inapparent, but in most cases they are characterized by a sudden onset of shivering, high fever, painful headache and myalgia. Initially, these symptoms obscured symptoms from the respiratory tract. As a rule there are signs of rhinitis, pharyngitis and, particularly, dry coughing and other symptoms of respiratory illness. The blood picture may display changes and often there is leucopenia with an accompanying lymphocytosis. Initially there might be a transient leucocytosis with a relative lymphopenia. The incubation time is short, usually 1–2 days. An abrupt onset of disease is characteristic and in general the fever reaches a maximum within 12 hours. The fever often lasts for 3 days but may persist for a few more days in cases without signs of complications. The convalescence may be relatively long and coughing and fatigue may be present for some weeks when the other symptoms have vanished. Influenza is, particularly, a disease of childhood but in comparison with adults, children get a milder disease with less myalgia and fewer lung complications. Gastrointestinal symptoms are uncommon in adults but exist in children, especially up to the age of 6 months. The increased mortality during epidemics of influenza amounts to 0.1–0.5 per thousand. Most deaths occur among old people.

Complications of influenza are pneumonia, heart insufficiency, encephalomyelitis, polyneuritis, Guillain-Barré syndrome, Reye's syndrome, myositis and perimyocarditis. Cases of pneumonia are responsible for about half of the increased morbidity. This is mainly because of bacterial infection complications, but in some cases the virus infection itself causes the pneumonia. The secondary bacterial pneumonias affect, in particular, the elderly and patients with pulmonary diseases. The primary viral pneumonia is associated with a high mortality. It may occur in previously healthy individuals but it is also seen in patients with heart failure, pregnant women, and patients treated with corticosteroids. In patients dying of a primary influenza pneumonia, influenza virus can regularly be isolated from the brain. There are also other observations suggesting that influenza virus may spread to the CNS. Reye's syndrome is an unusual disease, characterized by encephalopathy and fatty degeneration of the liver. The onset of the disease occurs as a rule a week after recovery from an infection and it has been claimed that there exists an association between this syndrome and certain infections, in particular influenza B.

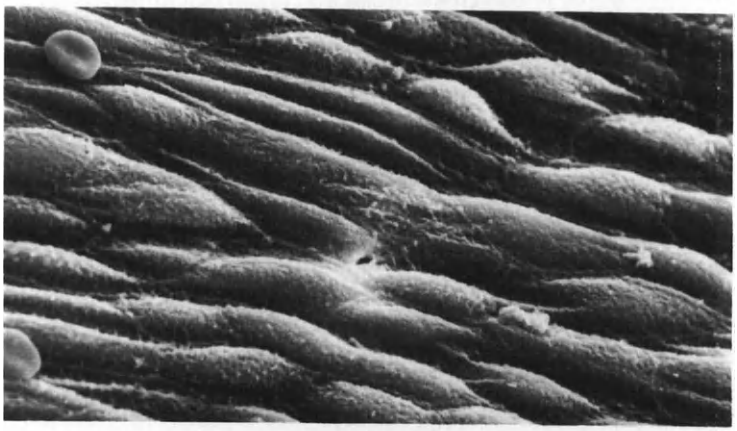
Pathogenesis

Virus enters the respiratory tract by inhalation of virus-containing microdroplets. The respiratory epithelium is protected by mucus containing mucoproteins with an affinity for influenza virus. The neuraminidase activity of the virus can digest the mucus and avoid the virus-blocking effect of the mucoproteins. If neutralizing antibodies are absent or present only in low concentrations in the respiratory tract, virus may establish an infection of the ciliated epithelium. This will degenerate in patches and may be destroyed down to the basal membrane. The nasopharynx, trachea, bronchi and bronchioli are variably affected. The production of mucus is inhibited in infected areas, possibly because of infections of mucus-producing cells. Regeneration of the epithelium is seen after about one week (*Figure 27.2*) but the respiratory tract and the lung functions are often not restored for several weeks. In the primary virus pneumonia necrosis of alveolar cells, capillary thrombosis, capillary bleedings and an interstitial oedema are seen. The alveoli contain a haemorrhagic exudate with hyaline membranes. In patients dying from secondary bacterial infections, *Staphylococcus aureus* is the most common bacterial finding. In most cases the influenza infection seems to be restricted to the respiratory tract and the lungs. Viraemia has been demonstrated in a few cases but, as mentioned, spread of virus to the CNS is a common finding in fatal virus pneumonias.

Similar to other infections, the course of the influenza virus infection is influenced by virulence factors pertaining to the virus, and to immunity and other factors of resistance of the host. It is difficult to evaluate if differences in the mortality rates of the epidemics may be attributable to virulence properties of the virus. Some data indicate that the virus of the Spanish grippe was particularly virulent. The average mortality rate was high, about 1 per cent. The high death rate was not dependent only on bacterial pneumonias, but also on a high frequency of non-bacterial lung infections. Also, young people were affected to a remarkable extent.

Changes in the virulence of influenza A virus may be a result of an exchange of one or more genes of the virus. Reassortment of genomic RNA of two virulent viruses or between two avirulent strains may create both virulent and non-virulent recombinant. Thus, there is not a single gene which controls the virulence but an optimal combination of several genes is required for high virulence. It is easily

(a)



(b)

(c)

Figure 27.2. Changes in tracheal mucosa after influenza infection. The pictures are obtained by scanning electron microscopy and are reproduced from an article by Ramphal *et al.* (1978) *Inf. Immun.* **25**, 995. Normal ciliated cells are shown in (*a*).(*b*) demonstrates the tracheal surface 3 days after infection. The basal layer of cells is exposed due to degeneration of the ciliated cells. (*c*) shows regeneration of ciliated epithelium 7 days after infection. (Reproduced by permission of the American Society of Microbiology, Washington D.C., USA)

understood that virulence reflects the capacity of viruses to replicate and spread. It is known that enzymatic cleavage of the haemagglutinin is required to make virus infectious. Other factors with an influence on virulence are the temperature-sensitive steps of virus replication. These may restrict the replication of virus to the upper respiratory tract. Attempts have been made to use temperature-sensitive mutants for the production of live vaccines.

The course of the infection is influenced by the relative resistance of the infected individual. It has been shown in experiments with mice that there is a genetically controlled resistance against influenza mediated by the degree of permissiveness of macrophages. Probably there is an inherited resistance also in man. An observation indicating such a resistance may be that influenza seems to occur more frequently in individuals of blood group 0 than in those of blood group A. A reverse situation seems to apply in the clinical reactions to influenza.

A previous influenza infection normally induces an immunity of long duration against homologous virus. The immunity not only results in fewer infections but also milder symptoms and a lower contagiousness. Usually the HI test is used to determine immunity and a titre of ≥ 40 is considered to indicate immunity.

Studies on experimental influenza in mice clearly show that passively transferred IgG antibodies give a protection against lethal pneumonia and inhibit virus replication in the lungs. On the other hand virus replication in other parts of the respiratory tract is not markedly inhibited, indicating that in these regions the immunity is dependent on locally produced IgA antibodies. In what way antibodies exert their protection is unknown but they might neutralize the virus, inhibit the release of virus from infected cells, interfere with the spread, and opsonize the virus. In addition, antibodies co-working with complement or K-cells may destroy virus and virus-infected cells. Antibodies appear in serum 4–7 days after infection. Maximal titres are reached after 14–21 days. The antibodies locally produced in the respiratory tract are seen at approximately the same time as the serum antibodies but disappear faster. Antibodies are formed against all viral proteins but only the antibodies against the viral glycoproteins have protective effects. Antibodies against neuraminidase inhibit the spread of virus but are of less protective importance than antibodies against haemagglutinin. Immunization with haemagglutinin leads to formation of antibodies against other viruses of the same subtype in addition to antibodies against the homologous virus. This has been explained as indicating that the haemagglutinin possesses both strain- and subtype-specific antigenic determinants. Another explanation, based on experiments with monoclonal antibodies, is that the antibodies have varying avidity but are directed against the same antigenic region. The immunity against influenza is primarily associated with strain-specific antibodies but repeated exposure to the virus increases the importance of cross-reacting antibodies.

Virus-specific cell-mediated immune reactions are demonstrable in influenza but the importance of these reactions is unclear. It is known that functioning T cells are needed for synthesis of HI antibodies. Also it has been demonstrated that absence of T cells may facilitate the spread of virus; in turn this may lead to development of encephalitis. At the same time absence of T cells is associated with less frequent lung complications suggesting an immunopathological role of the cell-mediated immunity. In addition, influenza virus infections may modify the cellular immune reactions in several ways. Suppression of T-cell functions and inhibition of phagocytic and bactericidal actions of macrophages may occur. It is possible that these effects may contribute to the secondary invasion with bacterial infections.

Epidemiology

Sporadic cases of influenza are observed all the year around but larger epidemic outbreaks occur in temperate climate zones, particularly during the winter; in the northern parts of Europe they occur usually during the first three months of the year. This seasonal appearance has been tentatively explained by assuming that spread of virus is favoured by low temperature, low relative humidity and indoor accumulation of people. Children are important transmitters as the incidence of infection is highest in the 5–19-year age group

The intervals between epidemics usually is 1–3 years for influenza A and 3–7 years for influenza B. Influenza C is endemic and only a few epidemics have been described. The susceptibility to influenza of a population depends on the relative immunity. Since this mainly depends upon immune reactions directed against the envelope proteins, the epidemic potential of a virus is influenced by the degree of antigenic change of the haemagglutinin and the neuraminidase. The antigenic variations of these proteins (cf. Chapter 12) are usually divided into two kinds. The first is called *antigenic drift* which is dependent upon mutations which gradually lead to changes of antigenic properties. The second kind is designated *antigenic shift* since it causes a shift from one subtype to another. The new subtype has completely new antigenic properties in one or both of the viral glycoproteins. Antigenic shift has been demonstrated in influenza A virus only and is probably due to the reassortment of RNA fragments between viruses of two subtypes. *Table 27.1* shows the different subtypes of influenza A virus which during the last century

TABLE 27.1. Subtypes of influenza A virus during different pandemics

Year	1889	1918	1929	1957	1968	1977
Haemagglutinin	H2 or H3?	H1?	H1	H2	H3	H1
Neuraminidase	N2?	N1?	N1	N2	N2	N1

have been associated with pandemics. This hypothesis is supported by studies of the virus of so-called Hong Kong influenza. This virus with the subtype designation H3N2 was first observed in 1968 when it replaced the previous H2N2 subtype. Both viruses were shown to be identical with the exception that the virus of Hong Kong influenza carried another haemagglutinin (H3) and another H gene. It has not been established from which virus the H3 gene originated but on the basis of antigenic relationship it is believed that the gene derived from a duck or a horse influenza virus.

Influenza A virus causes a natural infection in horses, pigs, and in many different wild and domestic birds. Virus adapts to its particular host but the swine influenza virus, for example, may be transmitted to man and induce clinical disease. Ducks may be infected simultaneously with several different influenza viruses, and all subtypes of influenza virus causing pandemics in man have been isolated from ducks. The birds shed large amounts of virus via the cloaca. This might be of importance in the dissemination of virus to other species. Since the three last pandemics have started in China it might be suspected that in China there exist the favourable ecological conditions for recombination between human and other influenza viruses. Until these problems have been more closely considered, the Peking duck must be regarded more as a gastronomic than a virological concept.

Influenza virus is unique in its ability to display major antigenic changes and by this to acquire a capacity for dissemination in a non-immune population. This leads to pandemics, the average morbidity rate of which is often about 25 per cent. With a few years' interval after the introduction of a new subtype, new epidemics appear which usually are less intense. In this way the immunity of the population increases but the accumulated immunity is counteracted by the antigenic drift, which creates improved conditions for influenza virus to establish new epidemics (*Figure 27.3*). In May 1977, a new epidemic started in the northern parts of China. It proceeded to

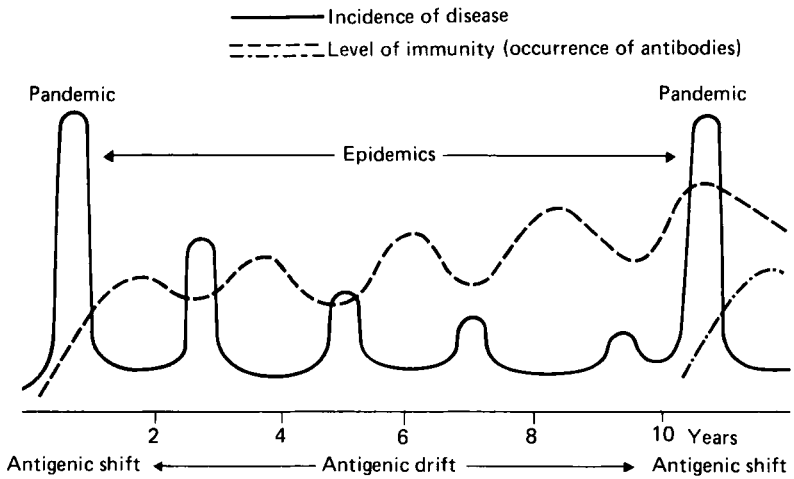


Figure 27.3. Schematic diagram showing the epidemic pattern of influenza A. The appearance of influenza epidemics is dependent on the immunity of the population. Each epidemic increases the immunity but this is counteracted by the antigenic drift of virus. An antigenic shift introduces a new subtype and since the immunity to this virus is low the result will be a new pandemic of influenza. The pandemic interval seems to vary between 10 and 40 years

South-East Asia and the Soviet Union and then continued to spread over the rest of the world. This pandemic affected children and young people in particular. A thorough antigenic and biochemical analysis has demonstrated that this influenza virus belongs to subtype H1N1 and is indistinguishable from the influenza virus strains which were observed in Europe and North America in 1950. It is remarkable that the virus has not been submitted to antigenic drift during a period of some decades and these findings therefore suggest that influenza virus might persist in nature for a long time without changing its antigenic properties.

Laboratory diagnosis

The laboratory analysis is important not only for providing a diagnosis when clinical signs are diffuse but also for the maintenance of epidemiological control of one of the most important epidemic diseases. Specimens which primarily should be collected are (1) nasopharyngeal secretions for isolation of virus and direct determination of viral antigens, and (2) serum samples for demonstration of an antibody response indicating past infection. Virus is present in large amounts in the

nasopharynx at the onset of disease but titres fall rapidly and at 7 days after infection virus is seldom isolated. For cultivation of virus, embryonated hen's eggs and cell cultures are used. In cell culture signs of infection can be observed a few days after inoculation if immunofluorescence and haemadsorption tests are included. Sometimes several passages are required to adapt the virus to embryonated eggs, and therefore, it may take days to weeks before results of a virus isolation attempt are available. For cultivation in chick embryo, the virus should be inoculated into the amniotic cavity to establish contact with the respiratory epithelium of the embryo. After adaptation (*see* Chapter 5), multiplication of virus may be achieved also in the cells of the chorioallantoic cavity.

For a more rapid diagnosis, cells of nasopharyngeal secretions are prepared on glass slides and the presence of viral antigens in infected cells demonstrated by an immunofluorescence test. If type-, subtype-, and strain-specific antisera are used it is possible to obtain a direct identification of the virus. For a more thorough characterization of the virus, cultivation is necessary. Many laboratories use radio- or enzyme-immunoassays as a rapid test for detection of viral antigens in secretions.

Serum antibodies may be demonstrated by several different methods and to date those most frequently used are the CF and HI tests. In the CF test, mainly type-specific nucleocapsid antigen is used, enabling detection of infections with all viruses of the same type. The HI test reacts with strain- and subtype-specific antibodies. It is used to determine immunity and reveal a rise in antibody titres after an infection. One difficulty with the HI test is its sensitivity to non-specific virus inhibitors in serum. Therefore, treatment of serum is required before testing. To overcome this problem HI can be replaced by the single radial immune test or haemolysis-in-gel neither of which require removal of serum inhibitors.

The antibody response after influenza is complex since cross-reactions may occur between the viruses. Repeated infections with closely related viruses may give a secondary response against cross-reacting antigens but a primary response against strain-specific antigens. If the antibody determination is performed using nucleocapsid antigens in CF tests a weak response is often encountered after the first influnza A virus infection. The antibodies demonstrated disappear relatively soon. Subsequent infections with influenza A viruses produce a better antibody response; antibody titres rise more rapidly, attain higher levels and remain for a longer period of time. In this context it is of interest to mention 'the doctrine of the original antigenic sin'. According to this doctrine, repeated infections with different but related influenza viruses always induce most antibodies against the antigenic determinants which are present in the virus causing the first infection.

Prophylaxis

For prophylaxis against influenza it is important to diagnose the early cases of an epidemic, to isolate and characterize the virus and to register epidemic outbreaks. There exists a worldwide organization built up for the collection of data, for distributing information about the epidemic situation and providing help in characterization of new isolates and the production of adequate vaccine virus strains. This organization is managed by WHO in Geneva and consists of about one hundred national laboratories and two international reference laboratories, one in London, UK, and one in Atlanta, USA.

Currently inactivated influenza virus vaccines are used most extensively. Virus is cultured in embryonated eggs and therefore the vaccine contains, in spite of purification attempts, small amounts of avian proteins potentially capable of inducing hypersensitivity reactions in individuals allergic to chickens. A few per cent of the vaccinated individuals react with fever, pain and local inflammation at the site of injection. These reactions are considered to be the results of toxic viral effects and are more common in children and old individuals previously unexposed to the virus in question. Less frequent side-reactions are noted with the 'split virus vaccine' (cf. Chapter 22) in which the virus is disintegrated by means of detergents. Purified viral glycoproteins are to be preferred since they induce fewer side-reactions but they require adjuvants to be efficient immunogens. An uncommon complication of influenza and vaccination against influenza is the Guillain–Barré syndrome. During the extensive vaccination campaign which was started in USA in 1976 after a few cases of swine influenza in a military camp, the syndrome was registered in one per 100 000 vaccinated.

Inactivated influenza virus vaccine has an average protection rate of 50–90 per cent and the protection remains for ½ to 1 year. Vaccination is most relevant at times of pandemics but for some risk groups such as the old and those with chronic heart and lung diseases a yearly vaccination is recommended in some countries. This recommendation seems somewhat dubious, since some investigations show that repeated yearly inoculations of inactivated influenza vaccine give a deficient protection.

Several live influenza virus vaccines have been tested. After intranasal instillation they induce a clinically mild infection and a satisfactory immunity. The possibilities of achieving an effective and longlasting immunity are greater with live than with inactivated influenza virus vaccines. Live vaccines have not come into practical use due to the difficulties of ensuring that the vaccines are innocuous. Reliable markers of avirulence are not available and therefore the vaccines have to be tested on a relatively large number of volunteers. One problem is that an attenuated virus may revert to a virulent strain when replicating in the respiratory tract. Attenuated strains are produced by adaptation to certain cells, adaptation to replication at +25°C ('cold mutants') and by selection of ts-mutants. Recombination of an attenuated virus strain and a wild-type strain may produce recombinants with H and N antigens of the virulent strain and the other genes of the avirulent strain. It is probable that this principle will be used in the production of future influenza virus vaccines.

An alternative to vaccination is chemoprophylaxis using amantadine hydrochloride. This compound has prophylactic and therapeutic effects against influenza A and is administered orally to adults in doses of 100 mg per day. Tests have indicated that this drug should be prescribed during an epidemic of influenza to non-vaccinated individuals belonging to the medical risk groups.

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28

Paramyxoviruses

Svante Hermodsson

In the family of Paramyxoviridae 7 viruses are pathogenic for man. They are divided into 3 genera; paramyxovirus (parainfluenza virus types 1–4 and mumps virus), morbillivirus (measles virus) and pneumovirus (RS-virus).

In many respects, these viruses are similar to the orthomyxoviruses. The virus particles are pleomorphic, i.e. they vary in size and morphology. Filamentous forms of virus exist but the typical virion is spherical and has an envelope containing two different peplomers formed by glycoproteins. Inside the lipid membrane there are the M protein and a helical nucleocapsid containing enzymes for transcription of single-stranded virion-RNA and mRNA.

In certain aspects paramyxoviruses differ from orthomyxoviruses. The virus particles are larger (diameter 100–300 nm) and the nucleocapsid is broader (13–18 nm) than the nucleocapsid of influenza virus (9 nm). The nucleic acid has a molecular weight of about 6×10^6 and the nucleocapsid is one single structure with a length of about 1000 nm. Since the RNA is not divided into fragments, genetic recombinations between paramyxoviruses are unusual. Instead these viruses show a high stability. Changes in the antigenic properties are rare and do not seem to play any role in the epidemiological occurrence of infections.

In contrast to orthomyxoviruses, the paramyxoviruses are not dependent upon cell-DNA for their replication. Synthesis and maturation occur in the cytoplasm. In measles-infected cells, however, an accumulation of nucleocapsids in the nucleus, demonstrable as intranuclear inclusions, may occur late during infection. A prominent property of paramyxoviruses is their capacity to cause fusion between the envelope and the plasma membrane of the cell. This capacity is dependent upon a viral glycoprotein (F) in the envelope. By the fusion mechanism the virus can cause haemolysis and formation of syncytia. The latter are formed by fusion of the plasma membranes of adjacent cells leading to giant cells with several nuclei.

An interesting property of the paramyxoviruses is the capacity to induce persistent infections in cell cultures. A more or less complete replication of virus may occur without obvious cell degeneration or inhibition of the synthesis of cellular products. The overall clinical importance of persistent paramyxovirus infections is relatively unknown. Measles may persist in the CNS and cause subacute sclerosing panencephalitis.

Most paramyxoviruses are adsorbed to structures on host cells and erythrocytes containing sialic acid. The binding of the virus to the cells is achieved by one of the two glycoproteins on the surface of the virus. In parainfluenza and mumps viruses this glycoprotein has haemagglutinating as well as neuraminidase activities and is

therefore designated HN. Measles virus has a haemagglutinating activity but no neuraminidase activity, while RS virus lacks both of these activities. A morphological difference between RS virus and other paramyxoviruses is the more narrow nucleocapsid of RS virus (12–13 nm). Regarding antigenic properties there is a certain similarity between viruses of the same genus but not between viruses of different genera of the paramyxovirus family. It is possible that RS virus in the future will be classified as a family of its own.

Parainfluenza virus

Parainfluenza virus is ranked next to RS virus as the most important cause of lower-respiratory-tract infections in small children. Reinfections are common both in children and adults but usually appear as mild upper-respiratory-tract infections or asymptomatic infections. Infections with parainfluenza viruses occur among several animal species but there is no definite evidence for a spread of virus between animals and man.

Properties of the virus

The chemical composition of the parainfluenza virus in per cent of the dry weight is about 1 per cent RNA, 73 per cent protein, 20 per cent lipids and 6 per cent carbohydrates. The lipid content is mainly determined by the host cells since the lipids originate from the plasma membrane of the infected cell. The composition of

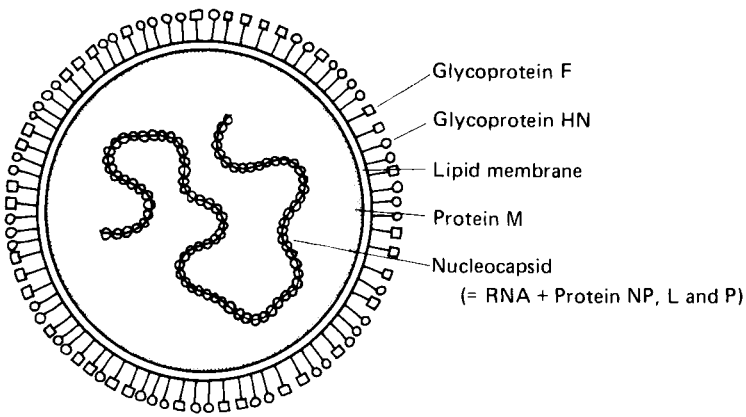

Figure 28.1. Schematic diagram showing the structure of a parainfluenza virion

lipids in the envelope differs from that of the membrane, however, since viral neuraminidase activity eliminates glycolipids containing neuraminic acid. Carbohydrates are present in glycolipids and glycoproteins.As glycosylation is achieved through cellular transferases, the content of carbohydrates is influenced by the type of cells in which virus is replicating. In the virion there are six virus-coded proteins (*Figure 28.1*). The M protein is linked to the lipid membrane as are the glycoproteins HN and F. The M protein is in contact with the helical nucleocapsid, formed by RNA, the nucleoprotein (NP) and two other proteins usually designated L (large) and P (polymerase).

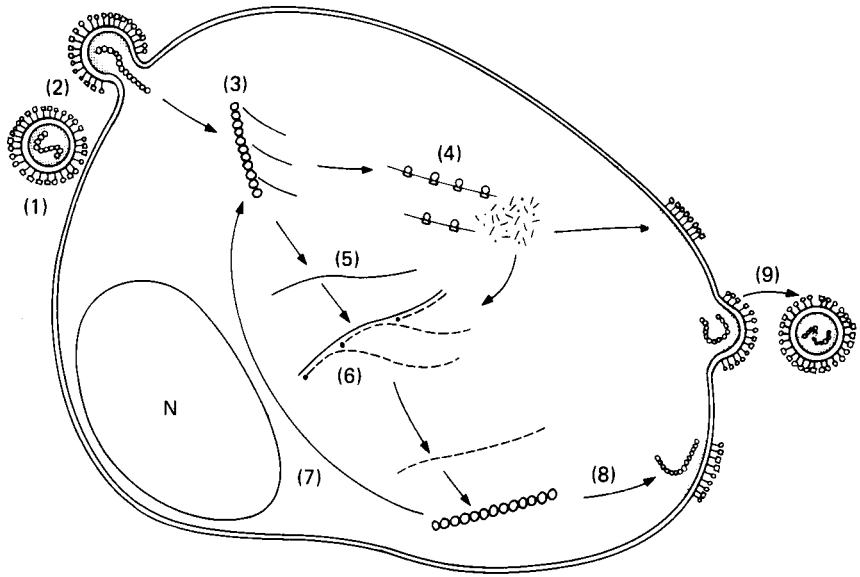

Figure 28.2. Schematic description of the replication of a paramyxovirus. Adsorption of virus (1) occurs by the binding of glycoprotein HN to specific receptors on the surface of the cell. The attachment may be inhibited if the cells have been pretreated by neuraminidase or if the virus has reacted with inhibitors or specific antibodies. The glycoprotein F causes fusion (2) between the envelope and the plasma membrane. This glycoprotein occurs in an inactive form and may require a proteolytic cleavage to be active and to make the virus infectious. A primary transcription (3) is established by the enzyme incorporated in the nucleocapsid and the product is complementary RNA strands of varying length. Translation of mRNA (4) leads to synthesis of viral proteins, some of which are glycosylated and incorporated in the plasma membrane. Complete RNA strands (5) are used for the replication of virion-RNA (6), which is utilized both as template for a secondary transcription (7) and for formation of nucleocapsid structures (8). Virions are produced by budding (9) of the modified membrane. M protein seems to be involved in this process. It has affinity to the membrane and the nucleocapsid and, by changing the fluidity of the membrane M protein, probably causes the patchy accumulation of viral glycoproteins on the surface of the cell

Parainfluenza virus is divided into five serotypes (1–5). These viruses demonstrate some antigenic similarities and immunological cross-reactions are also seen between parainfluenza and mumps viruses. Among the human parainfluenza viruses, types 1–3 are antigenically stable and homogeneous, while type 4 has been subdivided into two subtypes (4A and 4B) due to antigenic differences of the viral glycoproteins. An outline of the virus replication is shown schematically in *Figure 28.2*.

Epidemiology and clinical features

Infections with parainfluenza viruses are common and a majority of children demonstrate antibodies before school age. In one study on children of 5 years of age, 95 per cent had antibodies against parainfluenza 3, 74 per cent against parainfluenza 1, and 59 per cent against parainfluenza virus type 2. Virus is transmitted as an airborne infection or by direct contact. Parainfluenza virus type 3, in particular, is very effectively spread from one individual to another.

Infections with parainfluenza viruses are present all the year around but there is often a seasonal distribution of cases. In England, parainfluenza virus type 3 has been reported to cause annual epidemics during the summer, while the parainfluenza viruses types 1 and 2 cause epidemics every other year during the autumn. This indicates that epidemiological differences exist not only between different parainfluenza viruses but also between these viruses and typical winter-season viruses such as the RS and influenza viruses.

The incubation period is short, lasting only for a few days. Experimental infections of adults have revealed an incubation time of 3–6 days for parainfluenza virus 1, 2–4 days for type 2, and 3–4 days for type 3.

All four types of parainfluenza viruses induce acute respiratory tract infections. Although there is some evidence of viraemia, symptoms from other organs have not been clearly documented. Parainfluenza viruses are important pathogens particularly in children. The severity of the infections ranges from mild upper-respiratory-tract infections to life-threatening laryngotracheobronchitis and pneumonias. Severe symptoms are found in primary infections during the first years of life. It is uncommon to find parainfluenza infections of the lower respiratory tract after the age of 6.

Parainfluenza virus type 1 is the most common cause of acute laryngotracheobronchitis (croup) in children. Those affected are, in particular, boys at the age of ½ to 3 years. The disease often starts as a common cold after which cough, stridor and asphyxia appear. In connection with primary infection with parainfluenza virus type 1, subclinical infections are found in about half of the individuals and pneumonia and bronchitis are diagnosed in about a quarter of the infected individuals. Parainfluenza virus type 2 may induce similar clinical mainfestations but croup dominates the clinical picture. Parainfluenza virus type 3 is considered to be the most virulent type and is, second to RS virus, the most common cause of pneumonia and bronchiolitis in children less than 6 months of age. Parainfluenza type 4 is also a common infection but symptoms are mild and rare. Reinfections with parainfluenza virus occur both in children and adults but these infections as a rule are subclinical or are associated with mild symptoms from the upper respiratory tract (*see* Chapter 34).

Pathogenesis

The parainfluenza virus infections seem to be restricted to the respiratory tract. It is the mucosa of the nasopharynx that is primarily involved. The infection leads to epithelial degeneration and inflammatory reactions. When virus is spread further down in the respiratory tract, types 1 and 2 often cause laryngotracheobronchitis while type 3 more often leads to bronchitis and pneumonia. The parainfluenza viruses differ in virulence. Type 3 is the most virulent, followed by 1, 2 and 4.

Relatively little is known about the influence of non-specific factors and specific immune reactions on the course of the infection. Experiments with animals might indicate some similarities in the reactions against parainfluenza and influenza viruses. Antibodies seem to be more important than T cells and interferon for the defence. Maternally transferred antibodies may to some degree provide protection of the newborn, but passively transferred antibodies may also inhibit the immune defence and in this way contribute to a more severe infection upon reinfection.

A past infection induces immunity which often is incomplete and of a relatively short duration. After repeated exposure to the viruses, the symptoms will be milder

and the contagiousness reduced. Immunity does not prevent the establishment of infection in the respiratory tract. Reinfections with parainfluenza viruses are also common. In one study in which children were followed for a long period, 17 per cent contracted a second infection within 9 months of the primary infection with parainfluenza virus type 3. Reinfections are less common with the other parainfluenza viruses. There is a clear connection between immunity and occurrence of neutralizing antibodies in serum although immunity is correlated better with the occurrence of antibodies in nasal secretions. Therefore it has been assumed that secretory IgA is a factor of primary importance for immunity.

Laboratory diagnosis

It is difficult to diagnose parainfluenza clinically but with knowledge of the epidemiological situation and recognizable clinical features of these infections it is possible to make an intelligent guess. An accurate diagnosis is obtained only by means of virological examinations. The laboratory diagnosis is based primarily on the detection of virus in the nasopharynx. This is made by cultivation of virus in cell cultures or by a direct identification of virus antigen in nasopharyngeal secretions.

Specimens for isolation of virus may be obtained by rubbing cotton swabs against the posterior pharyngeal wall or by collecting nasopharyngeal secretions. As paramyxoviruses are labile, specimens should be stored at +4°C and examined as soon as possible in the laboratory. Most parainfluenza viruses cause only slight cytopathic changes and the presence of virus is therefore often demonstrated by HAd or IF tests. Isolation of parainfluenza viruses in cell culture as a rule takes from one to several weeks. Therefore it is advisable to use techniques which permit a direct demonstration of viral antigens in exudate or in separated cells of the nasopharyngeal specimen. The cells are normally fixed on glass slides and examined by IF.

The serological diagnosis is usually based on the demonstration of a significant rise of antibody titres in paired serum samples. One problem is the occurrence of heterotypic antibody responses, due to the antigenic relatedness of the different parainfluenza viruses and mumps virus. This explains why children previously infected with parainfluenza virus type 3 may exhibit an antibody response also against this virus after an infection with parainfluenza virus type 1 or mumps virus. The evaluation of the serology is further complicated by the fact that these viruses sometimes give rise to a heterotypic but not to a homotypic antibody response.

Prophylaxis

It is difficult to avoid infection with parainfluenza viruses. The infections are common and easily transmitted. Also, individuals lacking clinical symptoms may disseminate virus. There is no effective vaccine available. Inactivated vaccines have been tested but no protection was obtained although most vaccinees responded to seroconversion. Experiences with vaccination of cattle suggest, however, that immunity may be obtained by intranasal instillation of live virus vaccine.

Mumps virus

Mumps virus usually causes infection of the parotid glands, but virus may spread to other organs as well. The virus is an important aetiological factor in meningoencephalitis and orchitis. It is seldom that serious sequelae are observed and an

efficient protection can be achieved by vaccination with a live vaccine. Mumps virus is related to parainfluenza viruses and the reader is referred to the previous description for information about structure and replication.

Epidemiology and clinical features

Mumps is seen all the year around with cases accumulating during the winter. Major epidemics are observed at intervals of 2–7 years. Children in the 5–9 year age group are most commonly affected and the majority of 10-year-old children in Europe and North America have experienced infection with mumps virus. In countries of the tropical and subtropical zone, mumps is an infection of early childhood. Virus is present in saliva from 6 days before to 9 days after the debut of the disease, but the risk of transmission is high only a few days before and after the onset of illness. Virus is disseminated by droplets or contact.

The incubation time is, on average, 18 days but may vary between 14 and 24 days. Subclinical infections are produced in 30–40 per cent of the infected. Mumps usually begin with a moderate fever and, 1–2 days later, enlargement of one or more of the salivary glands is seen. It is primarily the parotid glands which are affected and clinically a diffuse tender swelling is found in front of and below the ear. The fever usually persists for a few days but may return if other organs are affected. The symptoms then appearing are seen 2–10 days after the onset of the parotitis. In some cases these symptoms may precede the parotitis or may appear without clinical signs of salivary gland infection. Common complications are orchitis (in about 20 per cent) and meningoencephalitis (in about 1–3 per cent). Severe infections often are seen in adults, and orchitis is an unusual complication before adolescence. Orchitis may cause atrophy of the testis but since the disease generally is unilateral the average risk for sterility does not exceed 2 per cent.

Symptoms from the nervous system also seem to be a 'male' affliction, at least in 3 out of 4 cases. Mumps virus has been associated with meningitis, encephalitis, acute cerebellar ataxia, myelitis and neuritis. Less commonly, these clinical manifestations lead to a permanent neurological defect, but there are cases of a persistent one-sided deafness. A prominent feature of mumps virus is the affection of different glands, not only salivary glands and testes but also the lacrimal glands, mammary glands, pancreas, ovaries, thyroid and kidneys. The virus seems also able to cause conjunctivitis and polyarthritis and to induce diabetes. In spite of the apparent ability of mumps virus to infect many different tissues there is no evidence of congenital infections with malformations. An interesting detail in this context is that mumps may produce hydrocephalus in hamsters. Ependymocytes are destroyed by the virus replication and when the lesions heal the aqueductus cerebri is obliterated. It is not known if congenital mumps virus infection may cause hydrocephalus in man.

Pathogenesis

The primary focus of infection is probably the upper respiratory tract and by viraemia virus is transmitted to various organs. Subclinical infections are common and degenerative changes are few, even in infections with clinical symptoms. In the infected salivary glands some degenerated epithelial cells are deposited in the excretory ducts but the histopathological picture is dominated by inflammatory cells and an interstitial oedema. Oedema is also found in surrounding tissues, which explains the common finding of a diffuse palpatory swelling. The tunica albuginea

of the testis is rigid and oedema may therefore cause necrosis due to the increased pressure. In addition, a hormonal influence seems to be of importance since orchitis is uncommon before adolescence.

As a rule a mumps virus infection induces lifelong immunity. This is valid not only for the double-sided parotitis but also when the infection is one-sided or subclinical. Reinfections occur but are rarely followed by clinical manifestations. Clinical observations on reinfections which are not confirmed by laboratory examinations may be unreliable, since it has been suggested that other viruses such as parainfluenza and coxsackie viruses also have a capacity to cause parotitis.

It is difficult to assess the role of various immune reactions in the pathogenesis. Interferon is present in saliva and serum soon after the onset of infection and has also been detected in the cerebrospinal fluid. Some findings might speak for a protective effect of antibodies. The symptoms are preceded by viraemia and parotitis is an uncommon disease before 9 months of age, suggesting a protective effect of passively transferred maternal antibodies. Against this background it is remarkable that mumps immune globulin is not effective when administered to individuals exposed to virus.

Laboratory diagnosis

Specimens for virus isolation should be collected as soon as possible after the onset of the disease. This precaution should be followed although mumps virus may be recovered for several days after infection in saliva, urine and cerebrospinal fluid. At a late stage of the infection, urine should always be examined for isolation of virus. Mumps virus is readily cultivated in cell cultures and produces a cytopathic effect with syncytium formation. The identification of isolated virus is performed by means of IF- and HAd-inhibition tests.

Serology is supplementary to the isolation of the virus. Paired sera should be collected with an interval of 2–3 weeks. It is important to remember the existence of heterotypic antibody responses when the results are evaluated. This means that an infection with parainfluenza virus may induce an antibody response directed against mumps virus. Evidence of a recent infection may be obtained by determination of IgM-antibodies. It is also possible to utilize the fact that antibodies against nucleocapsid antigens appear and disappear earlier than antibodies against the viral glycoproteins. Immunity is established by NT but many laboratories use other tests which are easier and more rapidly performed such as the single radial diffusion in gel and ELISA.

Prophylaxis

An effective protection against mumps is achieved by subcutaneous injection of live attenuated vaccine.

Antibodies develop in 95 per cent of the vaccinees and presence of the antibodies is correlated to protection. Virus has been attenuated by repeated passages in embryonated hens' eggs, also used for the production of vaccine. There are no side-reactions. The vaccine has been used since 1967 and seems to induce a longlasting immunity. Immunoglobulin has been used prophylactically in individuals exposed to mumps virus but the protection rate is unsatisfactory and, at present, there are no indications for its use.

Measles virus

Measles virus (morbillivirus) causes a most contagious, acute, respiratory-tract infection accompanied by a characteristic rash of skin and mucous membranes. Virus replicates in macrophages and lymphocytes and this may be of importance for the spread of the virus to other organs. Virus may persist in tissues for several years after an acute infection and can be responsible for a chronic lethal disease of the nervous system, subacute sclerosing panencephalitis (SSPE). Other severe complications are giant-cell pneumonia and encephalomyelitis, but most common are the complications caused by secondary bacterial infections of the respiratory tract and the middle ear. At the start of the twentieth century, measles was associated with a high mortality rate and it is still a problem in developing countries where the mortality rate may be 5 per cent or more. Man is the only natural host but antigenically related viruses cause distemper in dogs and rinderpest in cattle. The structure and the replication of measles virus is similar to that of other paramyxoviruses. A short description is offered in the beginning of the present chapter.

Epidemiology and clinical features

Measles is endemic all over the world and is responsible for epidemics with intervals of 2–5 years. Virus is spread as an airborne infection and its high contagiousness depends to a large extent on the intense dry cough which efficiently produces aerosols. The majority of children in developing countries are infected before the age of 5 years, while in industrialized countries children contract measles between 5 and 10 years of age. The use of vaccines has in some countries delayed the debut to higher ages. Virus is demonstrable in the respiratory tract two days, at the earliest, before the onset of fever, and, at the latest, two days after the appearance of the exanthema. The incubation time is about 10 days but may be longer in older individuals and in those who have received gamma globulin injections prophylactically.

Measles starts as a rule with fever, coryza, cough and conjunctivitis. A very early sign of the disease is leucopenia with a relative lymphopenia. About four days after the onset of illness, a rash is seen, first on the face and the neck and then on the trunk and extremities. Before the exanthema is visible the so-called Koplik's spots are detectable on the buccal mucosa close to the orifice of the parotid duct. The fever usually disappears within a week and is often biphasic with a second peak when the rash appears. Mild forms of measles occur, but latent infections are uncommon in contrast to what is the case in many other virus infections. Individuals who have received inactivated measles vaccine may develop an atypical measles with high fever, pneumonia and an uncharacteristic rash.

Severe but uncommon complications are bronchiolitis, giant-cell pneumonia, encephalitis and some other neurological diseases. In individuals with immune defects, for example leukaemic patients treated with cytotoxic agents, measles may lead to a fatal interstitial pneumonia. Remarkably enough, patients with this disease often have no rash of the skin and mucous membranes. Patients with immune defects may acquire a lethal encephalitis with a massive replication of virus in the brain. Another type of encephalitis has been designated *postinfectious* since it appears after measles infections, usually on the sixth day after the debut of the exanthema. Postinfectious encephalitis is seen in about one out of 2000 cases of

measles. The disease is more frequent in adults than in children. It has been assumed that the encephalitis is due to an autoimmune reaction, since the virus only exceptionally has been isolated from the brain and cerebrospinal fluid of the patient. The histopathological picture is similar to that observed in experimentally induced allergic encephalitis, i.e. demyelination is a prominent feature. In a few cases, however, multinucleated cells have been found in brain preparations, suggesting a viral infection with cytopathic changes. The majority of the patients with postinfectious encephalitis recover completely but deaths and sequelae due to brain damage have been reported.

Another type of measles-induced encephalitis is the subacute, sclerosing panencephalitis (SSPE). This unusual disease appears on average 6 years after a past measles infection (*see also* Chapter 16). The incidence is 1 case out of 1 000 000 children. The disease affects, in particular, boys growing up in rural areas and suffering from measles during the first two years of life. SSPE starts insidiously with personality changes and memory defects and gradually causes serious disturbances of mental as well as motor and sensory functions. The disease will inevitably lead to death which may occur after a few months or after several years. Other neurological complications of measles are the opticus neuritis and the Guillain–Barré syndrome. Common complications are the secondary bacterial infections, manifested as otitis, bronchitis or bronchopneumonia. Secondary bacterial infections should be suspected if the fever persists for more than a week or increases after the rash has vanished.

Pathogenesis

The primary focus of infection is probably localized to the epithelium of the respiratory tract. Virus is spread with lymph to the lymphoid tissues and the blood. By viraemia the infection is transmitted to various organs such as the spleen, liver, kidneys, intestines, skin and brain. The dissemination is effected by infected leucocytes due to virus replication in T and B lymphocytes as well as in other mononuclear blood cells. The replication of virus in these cells might explain the leucopenia seen in the early stages of infection. In immune defects, particularly those involving cellular immunity, the measles infection readily causes fatal pneumonia and encephalitis. There seems to be a spread of virus to the brain also in uncomplicated measles. This assumption is supported by the observation that as many as 90 per cent of the patients demonstrate electroencephalographical changes and many of the patients also show an increased number of cells in the cerebrospinal fluid.

Like several other generalized virus infections, measles leads to a lymphoid hyperplasia and the infected tissues show an inflammatory exudate with mononuclear cells. The multinuclear cells formed by fusion of adjacent infected cells are a characteristic histopathological finding. Cell fusion has been observed also in infections with other paramyxoviruses but it is a common finding in measles-infected tissues. The giant cells may be utilized diagnostically and are regularly present not only in infected tissues but also in urine and secretions from the respiratory tract. Measles virus infection will induce a lifelong immunity and occurrence of reinfection with clinical symptoms has to be considered as an extraordinary rare event. Immunity is based on the presence of both antibodies and immunocompetent cells. Most newborns receive a relatively efficient protection during their first year of life from maternal antibodies. It is also known that persons

with agammaglobulinaemia develop immunity to measles after infection. Thus the immunity seems to depend on the activity of both B- and T-lymphocytes.

Some of the symptoms of measles are probably the result of immunopathological reactions. Exanthema and enanthema occur normally in individuals with hypogammaglobulinaemia but, on the other hand, are absent in those with combined immune defects. Therefore it seems probable that the rash is caused by cell-mediated immune reactions directed against viral antigens on infected cells. The pathogenesis of the postinfectious encephalitis is unknown but an autoimmune reaction has been assumed. A contributory cause might be the incorporation of host-cell antigen in the envelope of measles virus.

A temporary depression of various cell-mediated immune reactions is observed during acute measles infection. The delayed hypersensitivity reaction against tuberculin may disappear. This immunosuppression may lead to an activation of tuberculosis and is probably unfavourable for those who already have an immune deficiency, for example undernourished children. In these children measles may cause severe enteric infections, which is a serious complication since it further deteriorates their nutritional state.

The disease SSPE is an indication that measles may persist in an individual for many years without causing any symptoms. It is not known if a persistent infection is established in the brain during the acute infection, but it is known that patients with SSPE may have virus also in other organs, for example lymph nodes. It is possible even *in vitro* to establish a persistent infection in lymphocytes with measles virus. Presence of antibodies favours the development of persistency and causes a release of viral antigens from infected cells by capping phenomena. The cells will then be less susceptible to the cytotoxic reactions which are important for the elimination of infected cells. Persistent virus infections may be induced relatively easily *in vitro* by ts mutants. It is therefore of interest that some virus strains isolated from patients with SSPE have ts properties. Antigenically, SSPE strains differ somewhat from the common measles virus strains, but there is an antigenic variation also between different SSPE strains. It has been assumed that SSPE strains originate from common measles virus by a selection of virus with SSPE properties. The infected nerve cells and oligodendroglia of SSPE patients contain large amounts of nucleocapsids in intranuclear inclusions, whereas mature virus is absent. One explanation for the defective replication might be that SSPE virus codes for a non-functional M protein which inhibits the normal maturation of virus.

Laboratory diagnosis

Virus may be isolated from the throat, urine, blood and conjunctivae during the early stage of measles but usually not later than 1–2 days after the appearance of rash. The isolation procedures are complicated and require special tissue cultures, which are not always available. Therefore, it is often preferable to utilize other methods for the detection of virus. The IF test has been used for the direct identification of measles virus antigen in infected cells from nasopharyngeal secretions or urine. This method may also be utilized for detection of virus in biopsy materials.

A laboratory diagnosis can often be made by serology alone. Recent infection is diagnosed by a rise in antibody titres or the occurrence of IgM antibodies. Measles antibodies appear at about the same time as the rash, and maximal IgM and IgG titres are achieved about 10 and 30 days after the onset of illness, respectively. An

antibody response may be absent in immunocompromised patients and to obtain the diagnosis it may be necessary to perform virus isolation and a direct demonstration of virus by IF test. In CNS infections antibody assays should be performed on both serum and cerebrospinal fluid. A decreased serum/CSF ratio indicates local production of antibodies within the CNS. Immunity is best revealed by neutralization tests, often replaced however by more simple tests such as the HI test.

Prophylaxis

Prophylaxis against measles can be attained by the injection of immunoglobulin or by vaccination with a live vaccine.

Immunoglobulin affords a protection lasting for three or more weeks depending upon the dose administered. It may be effective if given even after the patient has been exposed to the virus, but no protection is obtained if 6 or more days have elapsed since the exposure. Usually immunoglobulin is given in doses of 0.2 ml/kg bodyweight. Smaller dosages have been used to modify the disease. It is doubtful if immunoglobulin should be utilized in such a way since vaccines with insignificant side-reactions are available.

The attenuated virus of the live vaccine has been produced by repeated passages of virus in cell cultures and embryonated eggs. In comparison with wild measles virus strains, the vaccine virus induces a mild infection with few side-reactions. One third of the vaccinees will develop fever and in about half of these an exanthema is observed. Properly used, the vaccine affords protection in 95 per cent of the vaccinees. It should not be administered to children under 15 months of age as residual maternal antibodies may inhibit development of immunity. In these children there is a risk that vaccination may not induce a complete immunization. It has as yet not been definitely proved that immunization with live measles virus vaccine induces a lifelong immunity, although the information available indicates that this is the case. For further details *see* Chapter 23.

Respiratory syncytial (RS) virus

RS virus induces the formation of syncytia both in cell cultures and *in vivo*. This virus is the most important aetiological agent of lower-respiratory-tract infections in infants. Reinfections are common but are usually only associated with symptoms of the upper respiratory tracts. Epidemics are reported yearly during the winter season. RS virus infections may be a life-threatening condition in infants and a nosocomial transmission of virus should be considered in paediatric wards. Regarding the structure and replication, RS virus shows similarities with other paramyxoviruses (see the first part of the present chapter).

Epidemiology and clinical features

A prominent feature of RS virus infections is the annual epidemic. In the northern temperate climate zone the peaks of epidemics usually are observed from January to March; in the tropics, during the rain season. Urban epidemics often last 4–5 months, but the duration is shorter in smaller population groups.

RS epidemics are signalled by an increased number of cases of bronchiolitis and pneumonia registered among infants, i.e. children at the age of 1–12 months. Most

of the children are affected at about 2 months of age. As illustrated in *Figure 28.3* RS virus is a common finding in cases with bronchiolitis, a disease mainly occurring in children during their first year of life. Pneumonias are also common and RS virus infections have been detected in about a quarter of the cases. The incubation time is on average 4–5 days. The bronchiolitis usually starts as an upper-respiratory-tract infection, but suddenly dyspnoea sets in with expiratory wheezing and cyanosis. Death has been reported in children with bronchiolitis and some cases of sudden death in children have been associated with RS virus infections.

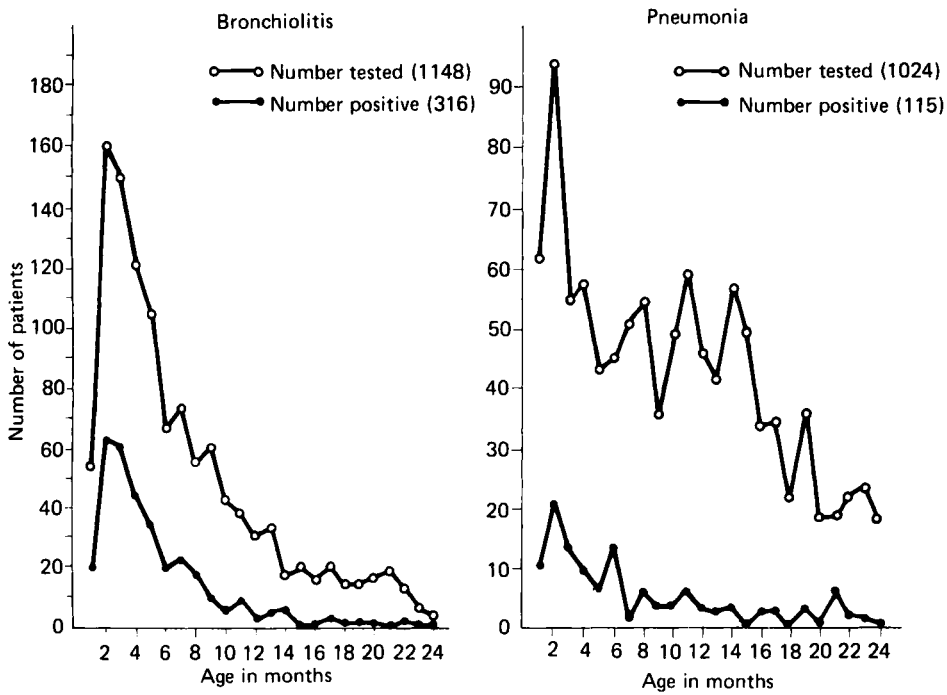

Figure 28.3. The occurrence of bronchiolitis and pneumonia in children of different age according to an investigation reported by R. H. Parrott et al. (1973) *American Journal of Epidemiology* **98,** 291. The figure shows both the total number of diseased children and the number of cases with virological evidence of RS virus infection. (Reproduced by permission)

Since most children are infected with RS virus before 3 years of age the infections in older children are reinfections. The risk of RS virus infections in children of school age may be 20–40 per cent during an epidemic. In reinfected children the symptoms are generally mild and restricted to the upper respiratory tract. RS virus has been demonstrated in association with infections of the middle ear and in attacks of asthmatic and chronic bronchitis. In old people reinfections may cause troublesome lower-respiratory tract infections.

Pathogenesis

RS virus is transmitted as an airborne infection and virus replicates in the respiratory epithelium of the nose, throat and bronchi. Virus is present also in high titres in nasal secretions in cases with lower-respiratory-tract infection. Virus is

often recovered within 5 to 7 days after the onset of illness. In some cases virus may be detected even later but it is not known whether chronic virus carriers may occur. The infection results in a patchy degeneration of the respiratory epithelium which then is desquamated. The infected tissue shows an infiltration of inflammatory cells and there is an accumulation of exudate in the bronchi and bronchioli. This may lead to respiratory obstruction. As the lumen of bronchioli is narrower during expiration than it is during inhalation a relative obstruction will impair particularly the expiration and cause emphysema and expiratory wheezing. The result of a complete obstruction is a collapse of the pulmonary parenchyma. An inflammatory reaction in the walls of the alveoli contributes to the picture of an interstitial pneumonia. All these changes may be found simultaneously and therefore it may be difficult to distinguish clinically between bronchiolitis and pneumonia.

A past infection induces immunity of relatively short duration but the immunity may be maintained and amplified by reinfections. The immunity prevents only to some extent new infections but reduces the severity of clinical symptoms and the spread of virus to the lower respiratory tract. Interferon frequently is not detectable in RS virus infections and probably has no influence on the course of the infection. The presence of specific antibodies and immunocompetent cells in blood is not always equivalent to immunity to the virus. Multiplication of virus seems to be affected mainly by the local concentration of IgA antibodies in the respiratory tract and not by the content of antibodies in serum.

Immunization with killed RS virus vaccines has shown that immunized persons often develop more severe infections of the lower respiratory tract when exposed to live virus. These findings might suggest that bronchiolitis is a consequence of immunopathological reactions. It has also been assumed that maternal antibodies are responsible for bronchiolitis almost exclusively occurring in infants. Antibodies unable to block the replication and dissemination of virus might produce immune-complexes and interfere with the normal immune response. There is no definitive evidence for this hypothesis. Bronchiolitis may occur in children lacking demonstrable maternal antibodies. Furthermore, the disease is less common in newborn than in somewhat older children, although the former have a higher concentration of maternal antibodies. On the contrary, there seems to exist a connection between bronchiolitis and allergy. Infants who develop bronchiolitis after an RS virus infection are more prone than other infants to acquire asthmatic bronchitis later in life when exposed to various respiratory virus infections.

Laboratory diagnosis

The isolation of virus in cell cultures or the direct demonstration of virus-antigen in infected cells, requires specimens of secretions from the nasopharynx. The procedure employed has been described in previous chapters. RS virus is a labile virus and specimens should be stored at +4°C and transferred to the laboratory within a few hours of sampling. Freezing of specimens at −70°C may cause inactivation of the virus if the virus is not suspended in a proper medium.

Nasopharyngeal secretions may also be used for demonstration of antibodies. At an early stage of the infection the antibodies are bound to virus and virus-infected cells but a few days later, when less virus is produced, free antibodies may be detected. The predominant class of immunoglobulins is IgA but both IgM and IgG are present in nasopharyngeal secretions.

The laboratory diagnosis of RS virus infections is based primarily on the direct demonstration of virus. However, the outcome of the virological examinations is improved if, in addition, the antibody response is studied. In children under 7 months of age the serological examination is less useful since maternal antibodies may mask, suppress and delay the antibody response.

Prophylaxis

There is a need for a vaccine to prevent severe RS virus infections and suitable target groups for vaccination are children less than 1 year of age and old people.

Several different vaccines have been tested but none has been accepted yet for general use. No clear protective effect has been obtained by killed virus vaccine and indeed pneumonias are more frequently encountered in vaccinated persons than in the non-vaccinated. The live vaccines have been prepared from ts mutants and virus strains adapted to growth at low temperature. In order to attain a local immunity in the respiratory tract, live virus vaccines have been administered intranasally. Serious complications have not been observed after vaccination but the live vaccines have nevertheless not been accepted for use since the viruses have not been avirulent and genetically stable. Live virus vaccines administered subcutaneously induce no clinical reactions but the antibody response has been inferior in children with maternal antibodies.

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29

Adenoviruses

Göran Wadell

The first isolates of adenoviruses (Gk. *aden* = gland) were made from lymph glands. Members of the adenovirus family have been found among amphibians, birds and marsupials and each species of animal which has been studied. Usually adenoviruses show a restricted host-specificity. In 1953 the first type of adenovirus was isolated from an explant of tonsillar tissue. Hitherto 40 serotypes of human adenoviruses have been identified. These can be divided into seven subgroups (*Table 29.1*).

TABLE 29.1. Properties of the seven subgroups of human adenoviruses

Sub-group	*Type*	*Pathogenicity*	*Tumour-inducing capacity in newborn hamsters*
A	12, 18, 31	Not defined	Tumours within 4 months in most animals
B	3, 7, 11, 14, 16, 21, 34, 35	Acute respiratory infections with fever, conjunctivitis, pharyngitis, pneumonia, gastroenteritis, haemorrhagic cystitis (Ad 11)	Tumours within 4–18 months in a limited number of animals
C	1, 2, 5, 6	Respiratory infection in small children, latent infection in lymphatic tissue	No tumours
D	8, 9, 10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36, 37, 39	Ad 8 and Ad 19 cause epidemic- and Ad 37 sporadic-keratoconjunctivitis	No tumours
E	4	Acute respiratory infections with fever, epidemic keratoconjunctivitis	No tumours
F	38	Diarrhoea in children	No tumours
G	40	Diarrhoea in children	Not determined

Properties of the virus

The adenovirus particle is composed of at least ten different structural polypeptides (*Figure 29.1*). It has a diameter of 80 nm and lacks an envelope. The virus capsid is an icosahedron which is composed of 252 capsomers. Among these, 240 capsomers are symmetrically arranged so that each capsomer (hexon) is surrounded by six other capsomers. Each hexon is formed from three copies of polypeptide II. The 12 vertices of the virion contain a capsomer from which an antenna-like protection (fibre) extends. The vertex capsomer together with its projection is called 'penton', since it shows a five-fold symmetry, i.e. it is surrounded by five hexons. Each penton capsomer is formed by five copies of polypeptide III. The fibre is a glycoprotein, which has varying length in adenoviruses belonging to different

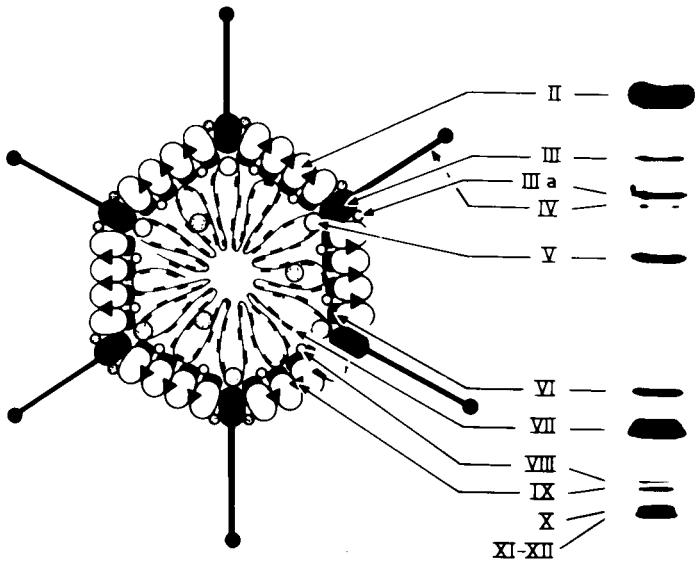

Figure 29.1. Location of different structural proteins in a virion of adenovirus type 2. The structure of the virion is illustrated schematically and to the right is shown different polypeptides identified by electrophoretic separation in polyacrylamide gel in the presence of a strong detergent (SDS). Large polypeptides, for example II, migrate slowly whereas the small polypeptides X–XII migrate a longer distance. The exact location of the latter proteins in the virion has not been defined

subgroups. Each fibre is formed from three copies of polypeptide IV. The polypeptides IIIa, VI, VIII and IX connect hexons and penton capsomers into a dense capsid. This capsid encloses a nucleoprotein complex which is composed of the polypeptides V and VII and the linear double-stranded DNA molecule containing 33 000–45 000 base pairs. The ends of the DNA molecule are covalently bound to a protein with a molecular weight of 55 000. The adenovirus particle is stable against the low pH in the ventricle, the emulgating effect of the bile products and the proteolytic enzymes of pancreatic origin. For this reason adenoviruses can replicate to high titres in the intestinal tract.

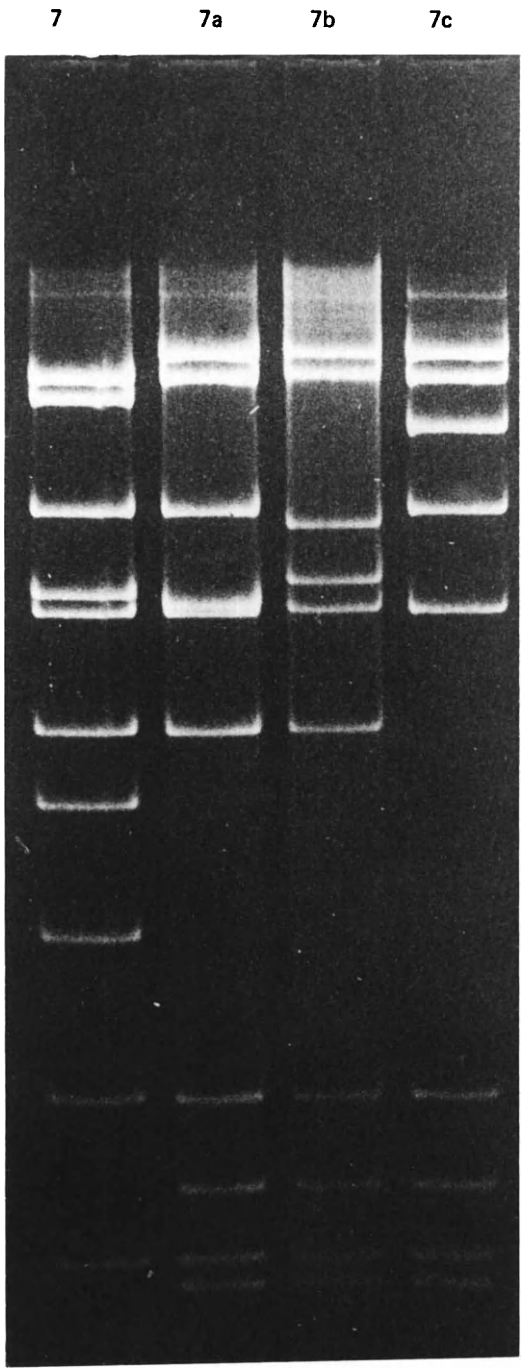

Figure 29.2. Identification of different subtypes of adenovirus type 7 by means of properties of virion-DNA. Virion-DNA has been purified and hereafter cleaved by a selected DNA restriction enzyme. The number and the size of the DNA fragments obtained have been determined by electrophoresis. Different patterns are obtained with different strains, which depends on there being a variation in the occurrence of the specific base sequence recognized by the endonuclease. This implies that the strains, in spite of their close serological similarity, are genetically dissimilar. They are therefore referred to as subtypes

The genetic variability among human adenoviruses has become expressed in the emergence of 40 different serological adenovirus types. Each serotype of adenoviruses carries two different type-specific antigens located in the outermost parts of hexons and fibres. Recombinants between two known serotypes, intermediate types, which carry hexon antigen and fibre antigen from two different serotypes, have been demonstrated. By use of restriction enzyme analysis of the adenovirus-genome, distinct differences can be distinguished between strains belonging to the same serotype(*Figure 29.2*). The internal polypeptides carry antigenic determinants with a broad immunological specificity. Similarly, determinants on the inside of hexons are shared between all animal adenoviruses.

Molecular characterization of virus-DNA from the 40 human adenovirus serotypes shows that they can be divided into seven subgroups (A–G). The degree of homology between viral genomes belonging to the same subgroup is at least 90 per cent with the exception of subgenus A which shows a larger heterogeneity. Virus-genomes belonging to different subgroups show a homology of DNA which is lower than 25 per cent. Ad 4, which cross-reacts with members of several subgroups, has been suggested to be most closely related to the archetypes of human adenoviruses.

Replication of DNA, transcription and maturation of adenoviruses, take place in the cell nucleus, whereas the spliced mRNA is translated in the cytoplasm. The infection is initiated by an attachment of virions via their fibre structure to receptors on the surface of susceptible cells. The penetration of virus particles occurs through an unknown mechanism. The vertex capsomers are removed in the cytoplasm and the remaining virus capsid is lost in the deposition of the virus core structure in the cell nucleus. The transcription and DNA synthesis of the virus occurs in this compartment. The virus-specific cell surface antigen and the tumour antigen (T antigen) and about ten other early virus-specific proteins are formed before viral DNA synthesis can be initiated. This occurs 7 hours after infection. Five hours later the synthesis of viral structural proteins is initiated, whereafter the synthesis of cellular proteins gradually ceases. The newly formed viral structural polypeptides are transported within a few minutes to the cell nucleus, where the virus particles assemble, starting 7 hours after infection. In the nucleus of each cell about 10^5 virus particles are assembled, within 30 hours after infection, with the most rapidly growing types of adenoviruses. The proportion of infectious particles among these varies between different serotypes from 1:10 to 1:10 000. The translation of viral mRNA which codes for capsid proteins continues uninterrupted for 40 hours. This leads to accumulation of a 10-fold excess of viral building stones. No virus-specific function has been shown to be responsible for the release of infectious virus from the cell nucleus. Virus is released in connection with disruption of the cells.

Clinical features

The different serotypes of adenoviruses can induce a spectrum of different symptoms but they still have common properties. They can infect via the conjunctiva, the pharynx or the small intestine and the infection may spread to regional lymph nodes but rarely beyond these. The incubation time is dose-dependent, but is usually 5–8 days.

Pharyngitis, conjunctivitis, symptoms from the upper and lower respiratory tracts with engagement of cervical lymph nodes, frequently with fever up to one week, characterize adenovirus infections. Pneumonia may develop in small children. This form of pneumonia cannot be distinguished from other viral pneumonias. In connection with the uncommon generalized adenovirus infections which may have a fatal outcome, virus can be isolated from lungs, liver, kidneys and brain. Histopathological analysis shows characteristic basophilic inclusions in the nuclei in the alveolar epithelium.

Adenovirus type 8 and enteric adenoviruses cause keratitis and diarrhoea, respectively. Adenoviruses have an affinity for lymphatic tissue in which types 1, 2 and 5 can give persistent infections. In contrast to most other respiratory infections, the adenovirus infections give relatively durable immunity and repeated infections with the same serotype are rare.

Table 29.1 presents a summary of the properties of the seven subgroups of human adenoviruses.

Subgroup A This subgroup includes adenovirus types (Ad) 12, 18 and 31. They replicate in the intestinal tract but this infection rarely leads to demonstrable symptoms. These adenovirus types induce sarcomas some months after being injected into newborn hamsters. However, gastrointestinal tumours in man do not contain adenovirus-DNA and thus there is no evidence for any aetiological relationship between adenovirus infections and tumours in man.

Subgroup B Ad 3, 7, 11, 14, 16, 21, 34 and 35. The members of subgroup B also can induce sarcomas in hamsters although the incubation time is about one year. In particular Ad 7, but also Ad 3, Ad 14 and Ad 21, cause epidemic outbreaks of respiratory infections both among children and military recruits. In small children Ad 7 can give a severe pneumonia which can become complicated by meningitis and gastroenteritis. Ad 11, Ad 34 and Ad 35 give latent infection in the kidneys and can be activated during pregnancy and during transplantations. Ad 11 also can cause haemorrhagic cystitis and haemorrhagic keratoconjunctivitis.

Subgroup C This subgroup includes Ad 1, 2, 5 and 6, which can cause respiratory infections with fever in children under 5 years of age. In older children more than half of the infections are subclinical. The primary infection of lymphatic tissue frequently develops into a persistent infection. The mechanism behind this infection is unknown. Ad 1, 2 and 5 can be excreted from the intestinal tract for more than three years after the primary infection. This is the main reason why these virus types are isolated more frequently from faecal specimens than are other adenovirus types. The isolations are made in the absence of any demonstrable seroconversion. Ad 1, 2 and 5 frequently have been isolated both from children with whooping-cough-like disease and children with mesenteric adenitis coupled with invagination, but the possible aetiological role of the viruses in these contexts is still debated.

Subgroup D Twenty-two types of adenoviruses belong to the subgroup. Among these are Ad 8 and Ad 19 which cause epidemic outbreaks of keratoconjunctivitis occasionally transmitted in eye clinics and clinics for industrial health care. The infections are spread by direct contact and tonometers are a potential source of infection. The sexually-transmitted Ad 37, which may cause cervicitis, belongs to

this subgroup. Other members of subgroup D are isolated only rarely in industrialized countries.

Subgroup E Ad 4 is a single member of subgroup E. This virus causes epidemic outbreaks of both conjunctivitis and acute respiratory infections, primarily among military recruits.

Subgroup F The subgroup includes an enteric adenovirus, candidate Ad 38. It causes diarrhoea, primarily in children. Enteric adenoviruses are fastidious and cannot be propagated in conventional cell cultures and thus are distinguishable from other adenovirus types in this regard.

Subgroup G Candidate Ad 40 also causes diarrhoea in children. This serotype is fastidious but less so than candidate Ad 38, since it can be grown in adenovirus-transformed human embryo kidney cells.

Laboratory diagnosis

Adenoviruses are most readily isolated from conjunctiva, pharynx, faeces and urine specimens in human epithelial cell cultures such as primary embryonal kidney or established cell lines like A549 or HeLa cells. Adenoviruses cause a characteristic swelling of the infected cell and a pronounced intranuclear accumulation of virus-specific material occurs.

Virus types which belong to subgroups B, C and E cause an aggregation of cells into grapelike clusters, whereas the other serotypes give a rounding off of individual cells. The cytoplasmic changes caused by virus types in subgenera A and D can develop relatively slowly and therefore a transfer of the virus-infected cell culture, a blind passage, may be needed for virus identification. This may take as long as three weeks. Diagnosis of adenoviruses in isolates can be verified by complement-fixation or immunofluorescence. Hereafter, the isolates can be typed by the HI technique or by neutralization tests. Adenoviruses can also be identified directly in lacrimal fluid, faeces or urine, by electron microscopy or ELISA tests. The enteric adenoviruses which cannot be propagated in conventional cell cultures by necessity have to be identified by electron microscopy or by ELISA, using type-specific reagents. Complement-fixation is most commonly used for serological diagnosis. However, this technique is not suitable for analysis of samples from small children. In this case, the neutralization test gives more reliable results.

Treatment with DNA restriction endonucleases can be used to identify human adenoviruses since DNA restriction patterns of the 40 adenovirus prototype strains have been defined.

Epidemiology

Adenoviruses are species-specific and the virus therefore spreads from man to man via droplet infections to the respiratory tract or via contact infection to the gastrointestinal tract. As a consequence of the stability of adenoviruses, they can also be transmitted by water in swimming pools, waste water and via ophthalmological instruments. Spread of virus may cause conjunctivitis, gastroenteritis and

epidemic keratoconjunctivitis. In industrialized countries adenoviruses are responsible for 2–5 per cent of all respiratory tract infections (8 per cent of all pneumonias in children) and 5–10 per cent of all cases of diarrhoea among children. As many as 80 per cent of all recruits who contract adenovirus infections in military camps develop upper-respiratory-tract symptoms or pneumonia.

Enteric adenoviruses and adenovirus types 1, 2 and 5 infect most children before the age of 3 years. Ad 1, Ad 2 and Ad 5 can be secreted for years after this primary infection. Ad 3 and Ad 7 cause epidemic outbreaks of respiratory-tract infections with fever in pre-school children and in school children. These adenovirus serotypes represent about 85 per cent of all adenoviruses isolated in industrialized countries.

A highly effective spread of adenoviruses may occur in countries where the limited supply of water leads to the same sources being used both for drinking water and for personal hygiene. In densely populated countries and in developing countries antibodies against adenovirus types 4, 8 and 19 can be demonstrated in most individuals. Adenovirus type Ad 8 gives a seasonally occurring outbreak of keratoconjunctivitis in children in Japan, whereas the same serotype in other industrialized countries only gives sporadic outbreaks. Children in Europe and USA lack immunity against Ad 4 and Ad 19, which therefore may cause epidemic outbreaks of acute respiratory disease (Ad 4) and eye infections (Ad 4 and Ad 19).

Prophylaxis

Live vaccines against Ad 4 and Ad 7, propagated in human embryonal fibroblasts, are used in the USA for immunization of military recruits (cf. Chapter 23). The vaccine strains have a low virulence but carry the same antigenic determinants as disease-inducing strains. The vaccine virus is swallowed in the form of gelatin-coated capsules. This method of inoculation gives a subclinical intestinal infection, which induces a local protection also in the respiratory tract, by which epidemic outbreaks can be prevented.

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30

Hepatitis viruses

Erik Nordenfelt

Hepatitis can be caused by several different viruses. The most important of the viruses involved are discussed together in this chapter although they are totally unrelated and classified in different virus families.

Hepatitis as a disease was described first by Hippocrates. The great wars have regularly provided a milieu for epidemics of hepatitis ('Soldatengelbsucht' and 'jaunisse des camps'). During the Second World War, more than 5 million people suffered from hepatitis in Germany. This form of hepatitis was formerly called infectious or epidemic hepatitis but is nowadays referred to as hepatitis A.

In 1885 an outbreak of hepatitis was reported among ship-yard workers vaccinated 2 months previously against smallpox. This is the first documented description of hepatitis caused by a therapeutic measure. It was caused by contamination with blood containing hepatitis virus. The latter form of hepatitis, previously denominated *serum hepatitis*, is nowadays called hepatitis B.

Virus was suggested as the aetiological agent of hepatitis in the beginning of the twentieth century. Convincing evidence for a viral aetiology was not obtained until 1940 when batches of yellow fever vaccine were incriminated as a source of infection, and experiments were performed with volunteers. In 1965 Blumberg discovered the Australia antigen (hepatitis B antigen) which was associated with hepatitis B infections. This marked the beginning of an era of intense research resulting in identification of both the hepatitis A and B viruses. The hepatitis-inducing viruses are spread over all parts of the world. In developing countries, hepatitis A virus is endemic and most individuals are infected at an early age; in the industrialized countries hepatitis A is epidemic. Hepatitis B virus causes both acute and chronic infections. It has been estimated that about 175 million people are chronic carriers of hepatitis B virus infections.

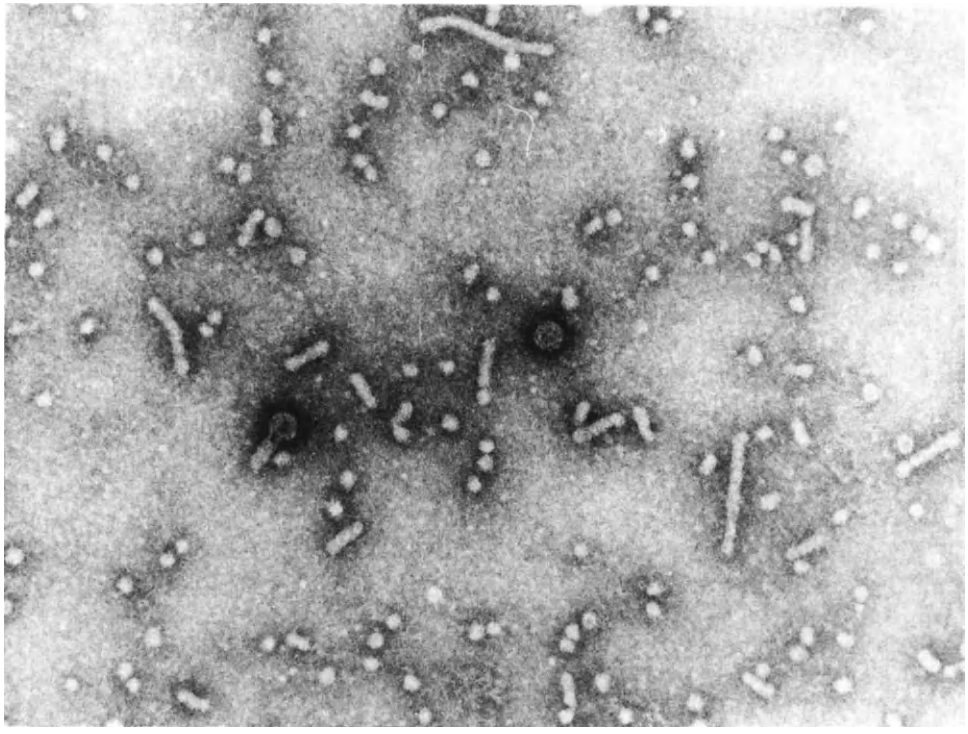
Properties of the virus

Hepatitis A

In 1973 hepatitis A virus (HAV) was identified by electron microscopy of faecal specimens from patients and of preparations of liver from experimentally infected marmoset monkeys. The virus has a diamter of 27 nm, an icosahedral structure and a density corresponding to 1.34 g/cm^3. The nucleic acid is single-stranded and the virions are relatively resistant to acids, ether and heat (maintaining infectivity at +60°C for one hour). The polypeptides of the capsid have molecular weights corresponding to proteins of enteroviruses. Consequently, the hepatitis A virus has been classified as a picornavirus. Recently it has been cultured *in vitro*.

Hepatitis B

Hepatitis B virus (HBV) has not been cultivated *in vitro* yet. It has been identified in serum samples of patients with acute hepatitis B as well as in chronic carriers. Three different kinds of particles are found in serum: spherical (diameter 22 nm), tubular (diameter 22 nm; length 40–400 nm), and the Dane particles (diameter



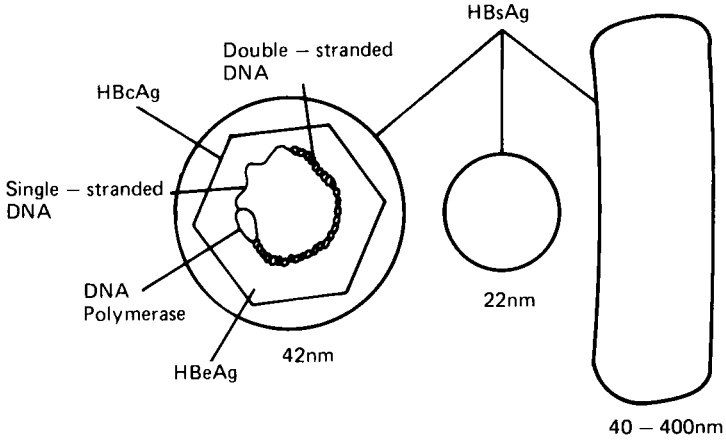


Figure 30.1(a). Electron micrograph demonstrating the different kinds of particles seen in serum of patients with hepatitis B (Magnification: ×120 000)
Figure 30.1(b). Diagrammatic description of HBV and viral antigens

42 nm). The Dane particles are the virions of HBV (*Figure 30.1a,b*). The particles are surrounded by a lipoprotein coat about 7 nm thick. (Since the surface of HBV differs in its chemical composition from the membrane of enveloped viruses, the term 'shell' is used in the following description). The shell protein on the surface of the particles is referred to as HBsAg (s = surface). This antigen carries antigenic specificities differentiating HBsAg into various subtypes. All carry a mutually dominating determinant denominated *a*. The other antigenic determinants, *d* and *y* and *w* and *r*, are present in alternating fashion so that *d* excludes *y*, *w* excludes *r* and vice versa. Thus four subtypes of HBV can be recognized and defined by the antigenic composition, *adw*, *adr*, *ayw* and *ayr*. Furthermore, some antigenic variants have been described. Purified HBsAg of subtype *ay* consists of 2 major polypeptides (P1 and P2) and the *s* gene coding for these polypeptides has been localized on the HBV genome.

HBV (the Dane particle) has an inner core with a diameter of about 27 nm. The core has a specific antigenic structure, HBcAg (c = core), and one further antigen, the e antigen or HBe. The core contains a circular double-stranded DNA, which is partly single-stranded, and a specific DNA polymerase. The size of DNA corresponds to about 3250 bases.

HBV has not been classified into any of the hitherto recognized virus families. Recently, a new virus in marmots (*Marmota monax*) has been reported. This virus is closely related to HBV and is designated WHV (Woodchuck hepatitis virus). Serum samples of infected animals contain particles which are morphologically and antigenically similar to HBV. Related viruses have been found also in ground squirrels and Peking ducks. The core of WHV has a DNA polymerase. Thus HBV appears to be the first identified member of a new group of viruses. A new antigen, delta antigen (δ Ag) has recently been described. This antigen is associated with a transmissible hepatitis agent (δ agent) which requires helper functions from HBV for its replication. δ Ag has been found isolated in the serum and encapsulated by HBsAg in 35–37 nm particles. These particles contain the putative genome of the δ agent, a low-molecular-weight RNA.

Non A- non B hepatitis

When diagnostic methods for demonstration of hepatitis A and B virus infections were introduced it became obvious that there remained a group of hepatitis viruses causing disease, for example, after blood transfusions. The group was called *non A-non B hepatitis*. Samples of infectious transfusion blood were inoculated into chimpanzees and they developed hepatitis. Additional studies have demonstrated that probably there exists at least two different non A-non B viruses. Results of preliminary experiments indicate that one of these agents may be closely related to HBV.

Clinical features

Hepatitis B is the most common form of hepatitis and represents about 60 per cent of the hepatitis cases in northern Europe and the USA. About 25 per cent is caused by hepatitis A and 15 per cent by non A-non B virus. The relative importance of

non A-non B virus infections seems to be increasing. The clinical course is principally the same for all three categories of hepatitis. Although certain clinical features of the infections may differ, when well defined groups of patients are considered, aetiological diagnosis cannot be based on clinical findings. The incubation time of hepatitis A varies from 15 to 50 days, usually 28–30 days. Hepatitis B has a longer period of incubation, 45–180 days, usually 60–90 days. The incubation time is inversely related to the dose of the infecting virus. Concerning non A- non B hepatitis, available data are uncertain but the incubation time seems to range from 15 to 150 days with a mean time of 40–50 days.

Cases with and without jaundice occur and they are referred to as icteric and anicteric cases of hepatitis. The anicteric hepatitis is common in children, about 10 times more common than the icteric form. The anicteric hepatitis is generally milder and may be almost inapparent, detectable only by biochemical tests.

Most patients experience prodromal symptoms from 1 to 14 days, usually 2–7 days, before appearance of the icterus. Predominant symptoms are signs of gastroenteritis with nausea and vomiting. An aversion to smoking is an often recognized symptom and myalgia and headache are also frequently observed. In cases of hepatitis A, symptoms of an upper-respiratory-tract infection are encountered initially. Arthritis, urticaria and/or a maculopapular rash are seen in 10 per cent of the hepatitis B patients. Most patients with hepatitis A notice a sudden onset of illness and can refer back to a day when symptoms began. In hepatitis B and non A- non B hepatitis the onset is insidious. Fever is an uncommon symptom of hepatitis B but is noted in 50–70 per cent of the patients with hepatitis A. Dark-brown stained urine due to excretion of bilirubin is often observed before the icterus, as are pale stools.

In connection with the appearance of the icterus, the nausea and vomiting become less frequent although the patients themselves may feel even more tired and weak than before. Enlargement and tenderness of the liver are seen in most patients during the icteric stage. The duration of the disease seems to be related to the degree of icterus. Symptoms, including the icterus, vanish gradually during 2–6 weeks but most patients are not completely restored until after 3–4 months.

Progress to chronic hepatitis is not observed in hepatitis A infections, while 5–10 per cent of the hepatitis B cases become chronic infections. Men, in particular, develop chronic infections. The greatest risk of chronic hepatitis is present after non A- non B infections, 25–45 per cent of cases in a study of a limited number of post-transfusion hepatitis. There are two types of chronic hepatitis, one called *chronic-persistent* the other *chronic-aggressive* hepatitis. The differential diagnosis can only be made by examination of liver biopsies. The chronic-persistent type is the more common and has a good prognosis even though healing may take many years. The chronic-aggressive type of hepatitis is more serious and associated with marked histological liver changes. The course may be varying. In many patients the disease remains stationary for many years, in some a gradual healing occurs, and in a few per cent of the patients a development of liver cirrhosis is encountered.

An episode of acute hepatitis in a chronic HBsAg carrier can be caused by a δ agent infection. This is commonly seen among addicts.

A rare but serious disease is fulminant hepatitis (acute yellow liver atrophy). It begins in connection with an acute hepatitis and has a high mortality rate. Liver coma with lethal outcome occurs within 1–2 weeks after the onset of disease and is dependent upon a massive necrosis of the liver. Hepatitis B virus is the most

common cause. Chronic carriership of HBsAg is associated with development of cancer of the liver. No aetiological relationship has been established, however.

Pathogenesis

In hepatitis virus infections the liver is the target organ, and the main locality for virus replication. Damage to hepatocytes is the basic result of the infectious process. Although HAV and HBV are completely unrelated viruses, the histopathological changes are very similar. The mechanisms behind the degenerative changes of the hepatocytes and the cell death are not known. Virus adsorption, penetration and replication processes have as yet not been defined. Hepatitis A antigens are demonstrated in the cytoplasm of hepatocytes and Kupffer cells only. In HBV infection, HBcAg is detectable in the cell nucleus and HBsAg in the cytoplasm. The HAV replication thus is similar to other infections with picornaviruses and HBV appears to replicate, as most other DNA viruses, in the nucleus of infected cells.

Studies of the pathogenesis of hepatitis A are as yet very limited. Available data would support the hypothesis that the liver damage is a direct result of viral cytocidal effects in the liver cells.

HBV seems to replicate without direct cytopathic effects in infected hepatocytes and the cell damage has been supposed to be mediated by immunological factors, cellular or humoral. It has been postulated that immune-complexes formed by viral antigens and antibodies would play a direct role in the liver cell damage. There is, however, no clearcut evidence. Immune-complexes are involved in a number of early extrahepatic symptoms due to hepatitis B or sequelae of hepatitis B infections (*see below*). The specific humoral antibody response directed against HBV antigens does not seem to play an important role in the development of cell damage. In patients with humoral immune defects, such as agammaglobulinaemia, a serious form of acute hepatitis may develop in spite of the absence of a normal antibody response. Since neither immune-complexes nor antibody-induced cytotoxic effects on the liver cells have been demonstrated, the cellular immunity remains as a plausible pathogenetic factor. The experimental possibilities of investigating the importance of cellular immunity are not readily available however and the results of reported studies are also contradictory. The acute liver damage is probably cell-mediated but how, and with what effector cells, is not known as yet. Specific cell-mediated immunity directed against HBsAg is demonstrable during convalescence, suggesting that it may be important for eliminating virus and healing after the infection. Moreover, a defective cellular immunity against HBsAg is considered to be involved in the development of a chronic carrier state. It is also plausible that impairment of some, as yet undefined, control mechanism of immunity is important for the attack on the HBV-infected liver cells.

Immune-complexes consist of HBsAg and anti-HBs with antigen in surplus. The complexes may induce several extrahepatic manifestations and cause a serum-sickness-like syndrome. In some patients urticaria, angioneurotic oedema, petechia and rash, often combined with arthritis, are observed in the prodromal stage. In chronic hepatitis B a number of cases with glomerulonephritis have been described with immune-complexes detected in the epithelium of the glomeruli. In 30–40 per cent of the patients with polyarthritis nodosa, there is an ongoing HBV infection. Immune-complexes of HBsAg and anti-HBs have been identified in arterial lesions

of these patients. Another syndrome associated with HBV infections is cryoglobulinaemia characterized by vasculitis, purpura, arthralgia and a progressing disease of the kidney. HBsAg and anti-HBs have been demonstrated in the cryoprecipitates.

Immunity

Only one type of HAV is known. After infection a lifelong immunity against hepatitis A is developed. Several subtypes of HBV are recognized. The predominant antigenic determinant *a* is type-common, and therefore infection with one type of HBV generally induces immunity also against other types of HBV. In rare cases, antibodies against only one type-specific antigen are developed, thereby creating possibilities for reinfection with another HBV subtype. In general, also, the HBV infections induce lifelong immunity.

The agents inducing non A-non B hepatitis have not been defined, but some studies on chimpanzees as well as clinical findings indicate that one infection will induce homologous but not heterologous immunity.

Laboratory diagnosis

Biochemical tests

Several enzymes are found in serum in pathological concentrations indicating liver damage in patients with viral hepatitis. Most important diagnostically are the alanine-aminotransferase (S-ALAT), the asparagate-aminotransferase (S-ASAT) and alkaline phosphatase (S-ALP). The transferase level will increase 5–10 days before, or simultaneously with, the debut of symptoms. The S-ALAT level is higher than the S-ASAT level. The maximum level may vary considerably but is usually within a range of 8–80 μkat/litre. There is no correlation between maximal levels and a clinical prognosis. S-ALP is normal or slightly increased.

The liver damage will cause a hyperbilirubinaemia. The serum level generally peaks within two weeks of onset of symptoms. Usually, values less than 170 μmol/litre are achieved but concentrations up to 350 μmol/litre may be seen in patients with uncomplicated acute hepatitis. The duration of the disease seems correlated to the level of the serum bilirubinaemia.

A series of other biochemical tests may also demonstrate liver cell damage and registration of plasma proteins may be of diagnostic significance but is not specific for viral hepatitis. A rise of the IgM concentration is encountered in hepatitis A but not in B or non A-non B hepatitis.

Clinical virology

As yet there are no methods for the isolation of HAV and HBV. HAV is demonstrable in faecal specimens by electron microscopy and can be grown in cell culture. Virus is excreted with faeces, reaching the maximum concentration before the onset of clinical symptoms. Diagnosis of hepatitis A can be performed by means of RIA and ELISA tests, demonstrating antibodies against HAV. The peak of the antibody response is rapidly attained and a rise in titres may therefore be difficult to detect. But since the presence of IgM antibodies against HAV only is seen in connection with a recent infection, the diagnosis can be made nevertheless. IgM

antibodies generally disappear about 3–6 months after the onset of disease. The course of infection is depicted in *Figure 30.2*.

Hepatitis B is diagnosed by the combined information of several tests demonstrating antigens and antibodies against the antigens in serum. The course of the HBV infection and appearance of the various antigens and antibodies are illustrated in *Figure 30.3*.

HBsAg is demonstrable up to about 6 weeks before the onset of clinical symptoms and usually disappears 6–12 weeks thereafter. Anti-HBs can be detected in most cases, but not until several weeks after the HBsAg have disappeared. Anti-HBc is detectable early during the disease and has often reached maximal titres when the patient is falling ill.

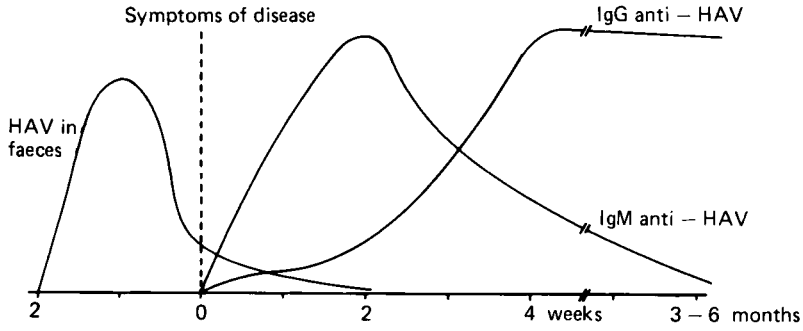


Figure 30.2. Virus shedding and antibody response in hepatitis A

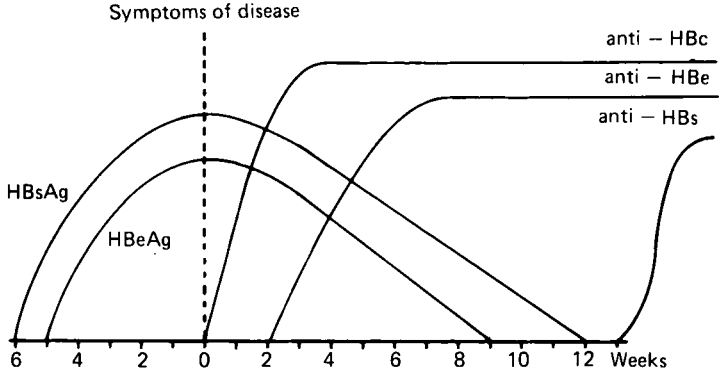


Figure 30.3. Presence of antigens and antibodies in the blood of patients with hepatitis B

HBeAg is detected later than HBsAg and vanishes earlier. Anti-HBe is demonstrable when HBeAg is disappearing. The markers which have to be followed routinely in patients with acute hepatitis B are HBsAg and HBeAg. Both antigens are assayed with sensitive RIA and ELISA tests. HBeAg can be used as a prognostic marker. If HBeAg persists longer than 8 weeks, the recovery will be delayed and there is a great risk of progression to chronic hepatitis.

HBsAg-positive individuals are often discovered otherwise than in association with acute hepatitis, for example by screening of blood donors and in healthy

controls. Repeated testing must be performed to evaluate if an acute hepatitis is being developed or if it is a chronic carrier state. Two kinds of chronic carriers may be diagnosed by HBeAg/anti-HBe (the prevalence and type of carriership differ between various parts of the world, *see below*), which are of practical importance for the estimation of contagiousness.

Patients with HBeAg have HBV in the blood and are contagious. The same patients also have biochemical signs of chronic hepatitis with raised transferase levels. Liver biopsies will indicate risks for chronic persistent and aggressive hepatitis. Continued sampling and testing are motivated as healing is associated with seroconversion of anti-HBe. This type of chronic carriership is observed after a clinically-recognized acute hepatitis.

Individuals with anti-HBe may be regarded as innocent from the point of view of contagiousness, and are generally healthy and without biochemical and histological signs of liver damage. No special follow-up is needed. These kinds of carriership are often detected accidentally and the carriers have no anamnestic experience of hepatitis. Probably they have been infected during the neonatal period or early childhood. In addition δ Ag and anti- δ can be detected by RIA tests.

Epidemiology

Hepatitis A

HAV is excreted with faeces. Virus can be demonstrated for about 14 days with maximal excretion at about 7 days before the icterus. In association with the debut of clinical symptoms, virus cannot be regularly detected by means of methods presently available but in single cases shedding of virus occurs up to 14 days after onset of the icterus. The presence of chronic excreters of virus has not been established.

During infection there is a short viraemic phase appearing shortly before and in connection with the onset of disease.

The predominant route of transmission of HAV is the faecal-oral route. Infected individuals spread the infection for a relatively short period. This means that the spread of hepatitis A is dependent upon continuous passage of virus from clinically or subclinically infected individuals to those susceptible to hepatitis A. The epidemiological pattern of hepatitis A differs in various countries and is directly associated with the level of hygienic and socioeconomic standards.

Different stages of epidemiological evolution can therefore be traced. In developing countries hepatitis A is endemic. In Africa, Asia and Latin America, more than 90 per cent of the population is immune by the age of 10 years. In some Mediterranean countries, the majority of those about 20 years old have attained immunity. In industrialized countries of the northern hemisphere, only 10–20 per cent of the population is immune at 40 years of age.

Epidemic outbreaks of hepatitis A are therefore encountered in the socioeconomically developed countries. Sporadic cases of hepatitis A may be seen, for example in non-immune individuals who become infected as tourists in endemic areas. A common route of infection is via virus-contaminated food, owing to unsatisfactory hygienic conditions or subclinically infected virus carriers handling the food.

Water-borne virus causing outbreaks of hepatitis A are well recognized. Most of these have been transmitted by sewage-contaminated fresh water supplies. HAV-infected shellfish, in particular oysters, have mediated large epidemics. Oysters

filter large amounts of water through their gills when feeding on plankton. Virus may be trapped and enriched in the gills and the alimentary canal of the oysters. A parenteral route of infection is possible by virus-contaminated blood. This route is of little importance, however. Epidemiological studies have demonstrated that multitransfused individuals do not have an increased prevalance of antibodies against HAV.

Hepatitis B

In hepatitis B infections, HBV and large amounts of the outer proteins (HBsAg) are demonstrable in the blood. The pathognomonic marker is HBsAg. In acute transient hepatitis B, the HBsAg may, as mentioned, be demonstrated for a long time, up to about 18 weeks. In addition, there is an extended carriership which may last for many years or for a whole lifetime. A prolonged carriership is seen in association with chronic hepatitis in patients with lowered immunity, and in patients infected during the neonatal period or early childhood. The risks of development of chronic carriership are greatest in men. Moreover, genetic and socioeconomic conditions influence the occurrence of chronic carriership of HBsAg. Children born by mothers who are chronic carriers of HBsAg will, in turn, be chronic carriers. It was demonstrated in an English study that while no children of white women were infected, 30 per cent of the coloured children and 65 per cent of Chinese children became infected. The contagiousness is correlated to the presence of HBeAg.

The prevalence of chronic carriers is different in different parts of the world. Carrier rates are 0.1–0.5 per cent in USA and western Europe, 1–2 per cent in South America and southern parts of Europe, 3–5 per cent in northern Africa and large parts of the Soviet Union, 6–10 per cent and even higher (up to 20 per cent in Oceania) in southern Africa and South-East Asia. As mentioned above, different subtypes of HBV dominate in different regions. Antigens *ayw* are predominant in USA and northern Europe. Drug addicts are spreading mainly *ayw* and today this subtype causes increasing numbers of acute hepatitis. The antigen complex *adr* predominates in HBV of the Far East and has been introduced to the US by soldiers returning home from Vietnam. Another source are the adopted children immigrating to western countries from Asia and Africa. Clinical differences between infections of different subtypes or a relative association of chronic carriership with infections of a particular subtype of HBV have not been revealed.

The pattern for a chronic carriership of HBsAg, i.e. if patients with HBsAg also demonstrate HBe or anti-HBe, varies between different regions. Studies of Asiatic countries, mainly Japan, have indicated that to a large extent asymptomatic carriers in Asiatic countries are HBeAg-positive. This is in contrast to findings in Europe and other western countries. Carriers of HBeAg are less numerous in older age groups. In individuals less than 10 years of age, HBeAg is detected in 75 per cent of the carriers; in carriers 20–30 years old, 25 per cent are positive; while only 10 per cent of those more than 50 years old carry HBsAg as well as HBeAg. In Asiatic countries most of the chronic carriers have been infected neonatally.

HBsAg is demonstrable also in other body fluids, such as saliva, urine and semen, and in faeces. Often this is due to a concealed bleeding but in some studies it has been possible to exclude contamination with blood. The predominant route of transmission of HBV is a direct parenteral transfer of infected blood. A microscopically small amount of infected blood, 10^{-7} ml, has been proved to be

sufficient. Transmission orally may occur but considerably larger infectious doses are needed than with parenteral inocula. The oral method of spreading the infection is without epidemiological significance. It should be emphasized however that not all carriers of HBsAg harbour HBV in their blood and thus these should not be considered as risks of infection. While a carrier of both HBsAg and HBeAg is contagious, this is not the case with carriers of HBsAg who demonstrate anti-HBeAg.

Parental infection with HBV can be achieved in many different ways. A vertical route of transmission from an HBsAg-HBeAg-positive mother to her child may occur congenitally or more likely during delivery. This transfer of the infection is a very rare event if the mother has anti-HBeAg. In hospitals hepatitis B infections represent a risk for both patients and personnel. Patients may be infected when, for example, receiving transfusions and hospital personnel in association with operative work, sampling of blood from infectious patients or testing the blood samples in the laboratory.

Hepatitis B is very common among drug addicts. Most of them will be infected after a few years of abuse due to sharing syringes, for example. About 5–10 per cent will become chronic carriers and about 60 per cent will demonstrate anti-HBs as a sign of a past infection.

Hepatitis B might also be sexually transmitted. Among homosexual males of USA and England there is an incidence rate as high as that among drug addicts. Outbreaks have been reported in association with tatooing when inadequately cleaned needles have been employed.

Prophylaxis

Hepatitis A

Spread of infection can be effectively prevented by means of good hygienic measures. Immunoglobulin injections to non-immune people travelling to endemic areas provide an effective prophylaxis against the disease. There are ample possibilities for production of a vaccine as it is now possible to propagate HAV in cell cultures.

Hepatitis B

The routes of transmission of HBV are well known. Simple precautions such as the use of gloves when blood samples are collected, use of disposable needles and syringes etc. reduce the risk of blood from carriers of HBV spreading the disease.

Carriers of HBV can be detected and the routine testing of transfusion blood for absence of HBsAg has further reduced risks of transfusion-induced hepatitis. The epidemiological search for cases of hepatitis will trace sources of infection and contribute to prevention of transmission.

An effective prophylaxis against hepatitis B is now being elaborated. Immunoglobulin offers no satisfactory protection. Hyperimmunoglobulin preparations from individuals with high titres of anti-HBs are available and are used prophylactically. They are usually administered to hospital personnel and others directly exposed to infection and should be injected shortly after the exposure. The protection offered is still debatable. A vaccine against HBV has recently been licensed for use. The vaccine gives an effective protection and is offered to some

groups which are at particular risk. It is produced from serum of chronic HBsAg carriers by the purification of the 20 nm particle which is used as the immunogenic vaccine component. These particles constitute parts of the HBV shell protein. Repeated injections will induce formation of anti-HBs and immunity. Recently the HBV genome has been cloned in *E. coli*. Thus the possibilities of vaccine production against HBV have been considerably amplified.

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31

Herpesviruses

Erik Lycke

The herpesviruses constitute a family with about 70 large DNA viruses. Of these 5 are pathogenic to man (*Table 31.1*). Herpesviruses are found in animals of all classes of the animal kingdom, vertebrate animals as well as invertebrate. Each class has its particular virus(es) and there are many apparent similarities in both the pathogenesis and the symptomatology of the different herpesvirus infections. The infections are mostly apathogenic and only exceptionally do they become life-threatening.

TABLE 31.1. Herpesviruses pathogenic for man

Herpes simplex virus type 1 (HSV-1)
Herpes simplex virus type 2 (HSV-2)
Varicella zoster virus (VZV)
Cytomegalovirus (CMV)
Epstein–Barr virus (EBV)

Herpes (Gk.) alludes to the slowly progressing ('creeping') vesicular rash of the skin.
Varicella (L.) means vesicle.
Zoster (Gk.) means girdle and alludes to the segmented area of the skin affected.
'Cytomegalo' refers to histologically demonstrable changes of infected cells.
EBV was first described in 1964 by M. A. Epstein, Y. M. Barr and B. G. Achon.
An additional virus to be mentioned is herpesvirus B which is a simian herpesvirus capable of causing a lethal encephalitis in man if it is transmitted to humans by bites or skin lesions caused by monkeys carrying the virus.

Herpesviruses are ubiquitous. Studies on prevalence of antibodies indicate that at least 50 per cent of the European and North American women of child-bearing ages have been immunized against CMV, and that most individuals have developed antibodies against VZV and one of the two types of HSV before the age of 30. In countries with a warm climate and large populations almost all become infected with herpesviruses during childhood. The number of clinically manifested infections is markedly less, thus mild and subclinical infections predominate.

Properties of the virus

The virion (*see Figure 2.6b*) has an icosahedral nucleocapsid composed of 162 capsomers. These have the shape of a pentagonal prism with a central depression. The nucleocapsid measures 100 nm in diameter and is surrounded by an envelope giving the virion a total diameter of 150–200 nm.

The herpesvirus-genome is a linear double-stranded DNA molecule with a molecular weight of 100–150 million. The principal structure of the genome is depicted in *Figure 31.1* which compares the genome anatomy of some herpesviruses. In HSV the unique nucleotide sequences, the long U_L and the short U_S, are surrounded by two pairs of repeated and inverted sequences (a,b,c,). In the CMV-genome U_L and U_S are enclosed by one pair only of repeated sequences. The EBV-genome on the other hand has 10 repeated redundant sequences.

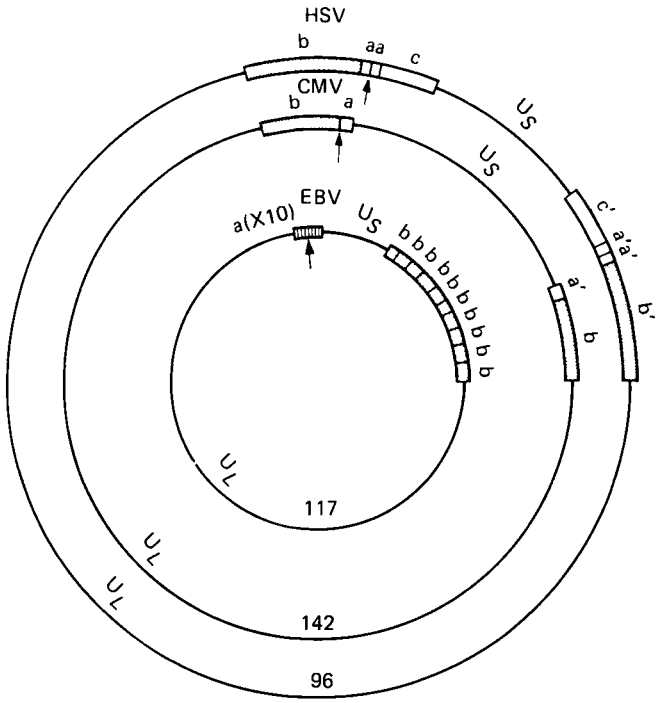

Figure 31.1. Comparison of DNA anatomy of three herpesviruses. Arrows indicate the beginning and end of the genome. Figures give molecular weights $\times 10^6$. For specification of the different sequences see text

The DNA molecule is wound on a toroid protein structure formed of basic histones and located in the centre of the nucleocapsid. Theoretically the HSV genome might code for at least 70–80 different polypeptides. Of these, some 48 proteins have been identified and 24 are referred to as *structural proteins*. The genes for the viral structural proteins are mainly located to the unique sequences U_L and U_S.

The area outside the nucleocapsid is usually referred to as the *tegument* and connects the capsid with the envelope. The virion acquires its envelope by budding from the inner nuclear membrane of the infected cell. The lipid skeleton of the envelope originates from the membrane, but the 7 glycoproteins (gA–gF and gY) of the envelope are coded for by the virus-genome. One or more of the proteins of the envelope function as VAP attaching the virion to the receptors on susceptible

cells. Only virus with an intact envelope is naturally infective. The functions of the oligosaccharides of the glycoproteins are as yet incompletely defined but they seem to be of importance in stabilizing the polypeptide, perhaps protecting it from digestion. They might also be important for the transportation of the viral glycoproteins to the plasma membrane in the infected cells. Glycoproteins gA and gB are antigenically closely related and glycoprotein gB is of importance for the penetration of HSV. In the antigenic mosaic of the herpesviruses, type-common as well as type-specific determinants are recognized. The type-common antigens may be shared between two or more herpesviruses. Glycoprotein gC of HSV-1 is a type-specific antigen. There are some observations indicating that the oligosaccharide of glycoprotein gC might have a more sophisticated biological function, perhaps capable of modulating the immune response against HSV.

Cells susceptible to herpesvirus infections carry receptors with a different affinity for different types of herpesviruses including the two types of HSV. The envelope of the virion fuses after attachment to the cellular plasma membrane, the nucleocapsid is transported to the cell nucleus and virus-DNA can then be demonstrated intranuclearly. Herpesvirus-DNA replicates according to a rolling circle model. Circularization of the genome is possible when the terminal complementary repeated and inverted sequences of the genome are uncovered. Transcription of the DNA involves splicing. At 4 hours after infection, practically all metabolic activities of the infected host cell are shut off, and during the following 12 hours virus-specified proteins and replication of DNA are demonstrable.

The virus-specified proteins are divided into 3 classes (α, β, γ). Viral immediate-early and early proteins (α and β) represent different virus enzymes (for example thymidine kinase and DNA polymerase); virus structural proteins belong to the γ class.

One of the HSV glycoproteins (gE) of the envelope which is also detected as an antigen inserted into the plasma membrane of infected cells can bind Fc fragments of immunoglobulins. These Fc receptors are transferred to the infected cell both when the virus envelope and the plasma membrane are fusing and later when *de novo* synthesized viral glycoproteins appear in the plasma membrane. The infected cell can therefore bind immunoglobulins in two ways – by the specific reaction between antibody and antigen and by the Fc receptor activity. The biological significance of the Fc receptors during the herpesvirus infection is unknown.

Latency and transformation

The ability to induce latent infections is a characteristic feature of the herpesviruses. HSV and VZV establish latent infections in nerve cells, while the lymphocyte is the main target cell for latency of EBV. Development of latent lymphocyte infections with EBV is connected with transformation and immortalization of the lymphocyte (*see also* Chapters 11 and 18). Latent CMV infections have been observed in lymphocytes but CMV latency in nerve tissues has not been completely ruled out. Neither has latency of HSV in lymphoid cells been excluded.

Latent HSV-1 infections have been demonstrated in neurons of peripheral nerve ganglia (cranial and spinal, as well as autonomic nerves) and in the central nervous system (CNS). Ganglia of sensory sacral nerves have been found harbouring latent HSV-2 infections. VZV has been detected in ganglia of several sensory nerves. In more than 50 per cent of examined cadavers, latent reactivated HSV infections

have been traced although in many of the cases an anamnesis for a clinical HSV infection was not registered. Latent infections with HSV and VZV in nervous tissues are obviously common.

Several findings indicate that the viral genomes of latently infected neurons are in a non-replicative state. Thus latent virus infections are not eliminated by antiviral drugs inhibitory to replication of viral DNA. The form of association between virus-DNA and the genome of the nerve cell and the control mechanism by which latent infection is maintained is unknown. It is reasonable to assume that there might exist virus strains more apt to induce latency than others and that nerve cells may be capable of restricting virus replication. An immunological control is probably important for the surveying of reactivated infections but may not significantly interfere with the reactivation process as such. The infections following reactivation of VZV, herpes zoster and of EBV, for example in association with immune defects and immunodepression, are apparently dependent upon immunological control functions, however.

TABLE 31.2. Some different factors possibly responsible for reactivation of latent herpes simplex virus infections

Effects acting via skin and peripheral nerves	
Irradiation	Sunshine Artificial u.v. light
Chemical agents	Acids (e.g. acetic acid, experimentally) Lipid solvents (e.g. xylene, experimentally)
Mechanical treatment	Skin injuries Nerve injuries Neurectomy
Effects acting via centrally elicited stimuli	
Hormones and emotions	Premenstrual reactivation Coitus Depression Neuro- and psycholeptic drugs, experimentally
Effects induced immunologically or by infections	Immunosuppression Intervening infections Fever

Stimuli leading to reactivation are well recognized from clinical observations of mucocutaneous HSV infections. In *Table 31.2* are listed some different reactivation-inducing factors. Three principally different sets of reactions seem capable of inducing reactivation of latent HSV infection in a trigeminal ganglia: (1) peripheral injuries by violence against skin and/or nerve, surgical operations or damage produced by irradiation or chemicals etc.; (2) central neuronal stimuli of emotional character (distress, anxiety, depression, etc.); and (3) changes in the hormonal status (menstruation, treatment with corticosteroids, etc.). Peripherally as well as centrally, triggered stimuli of latently infected neurons are capable of causing reactivation. Probably reactivation is a frequently occurring phenomenon and infectious virus is transported with the nerve from the ganglion to the innervated dermatome, but intervening immune reactions prevent the appearance of a

clinically overt infection of mucocutaneous tissues. Clinically demonstrable infection develops only when the immune defence for some reasons is handicapped and cannot prevent virus released from nerve endings from infecting epithelial cells. Factors of importance may be immunosuppression by treatment with corticosteroids or inhibition of interferon production by release of prostaglandin E_2 from skin irradiated by sunbathing or u.v. light.

Human B lymphocytes can be transformed and immortalized by EBV infection. The EBV-transformed cells can thus be established as cell lines. These cells contain multiple copies of the EBV-genome but do not produce infective virus. EBV-transformed cells may be isolated from the blood of EBV-infected patients, and from biopsies of Burkitt's lymphoma and some forms of nasopharyngeal carcinoma (*see below*). In the latently infected cells the EBV-genome is represented by a circular covalently-linked extrachromosomal DNA. This plasmid- or episome-like structure is localized to nucleosomes. In addition, latently infected lymphocytes contain EBV-DNA sequences which are integrated with cellular DNA. It has been observed that about 5 per cent of the EBV-genome is transcribed in latently infected cells and that all latently infected cells demonstrate EBNA (EB nuclear antigen) and LYDMA (lymphocyte-detected membrane antigen). Bromo- and iododesoxyuridine and other inhibitors, certain lymphocyte mitogens, arginine starvation, etc. may induce reactivation leading to virus production. Obviously this induction of synthesis of virus is associated with the loss of a cellular control mechanism.

The latent CMV infection has been incompletely explored. Human fibroblast cultures are most successfully used for the cultivation of CMV *in vitro* but infections *in vivo* reveal that different endothelial and epithelial cells and white blood cells may be infected with CMV. Two kinds of clinical findings suggest that lymphocytes might be carriers of latent CMV infection: (1) patients treated with immunosuppressive or cytostatic drugs may show signs of a CMV infection in association with or after the treatment (this has been interpreted as a result of a reactivation of latent infection); (2) patients receiving large blood transfusions may subsequently fall ill with a CMV-induced fever, which occasionally may result from the presence of infective virus in the transfusion blood, but which also can follow transfusions of blood not containing demonstrable CMV. In the latter case, it has been assumed that immunization with allogeneic antigens, transfused blood cells, has triggered a reactivation of CMV in the recipient. It cannot be excluded that a number of CMV infections in pregnant women may be reactivated infections. Influences on the immunological reactivity by fetal antigens, or other conditions pertaining to the pregnancy, have to be considered.

Herpes simplex virus (HSV) infections

Clinical features, pathogenesis and epidemiology

Primary HSV infection is an infection of mucocutaneous membranes and traumatized skin. Tissues which are the site of the primary infection are the same as those which reveal symptoms of recurrent infections. The feared herpes encephalitis is probably most often a consequence of a reactivated infection which has spread to the CNS.

Less than 1 per cent of all primary HSV infections are clinically overt. About 25 per cent of all individuals with latent HSV infections will at some time exhibit

symptoms of recurrent infections. Relationships between clinical, subclinical, primary and recurrent infections are outlined in *Figure 31.2*.

Of the two types of HSV, the type 1 infections mainly affect the oropharynx (*herpes labialis*) or the eyes. The type 2 infections are mainly localized to mucous membranes and skin of the genitals (*herpes genitalis*) and the adjacent areas of the skin. Neonatally acquired infections are predominantly of type 2 origin since the infection is transmitted when the child passes through the infected birth canal.

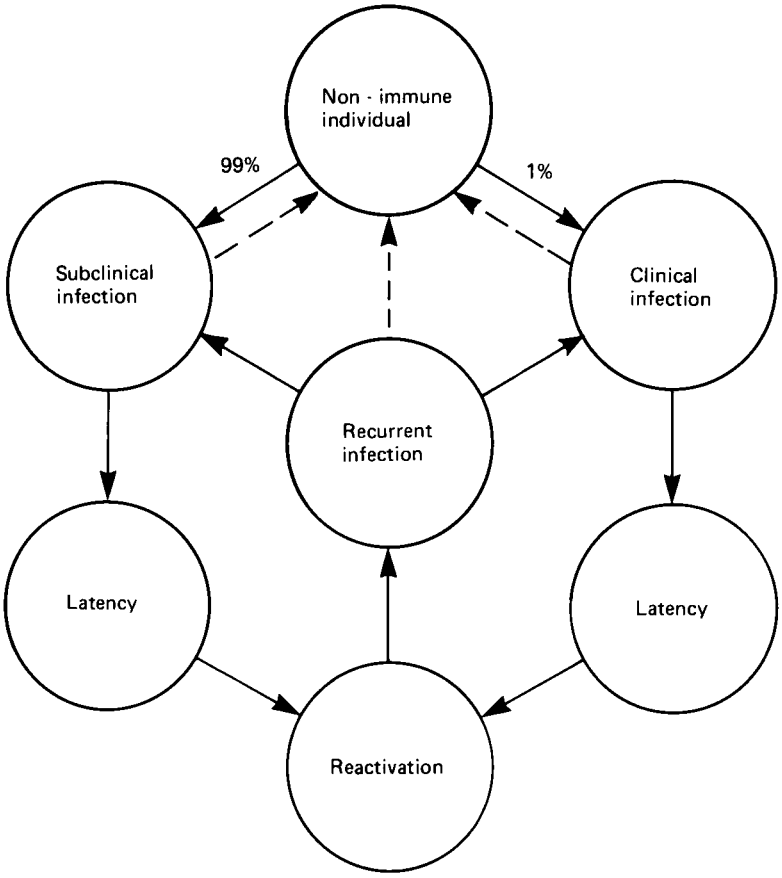

Figure 31.2. Infections with HSV. The interdependence between primary and recurrent infections: wholly drawn arrows. Spread of infective virus: dashed arrows

The epidemiology of the HSV infections is influenced – as many epidemic and endemic viral infections – mainly by socioeconomic factors. Limited standards of living are grounds for a high incidence of infection in the early ages with development of immunity and resistance to infection as a result. Recurrent infections become less frequent. The opposite, with more than twice as high a rate of recurrent infections, has been observed in socioeconomically favoured populations, for example, in USA and Europe. Low rates of recurrence are seen in Asians and American Indians. HSV infections are maintained endemically by the shedding of virus from recurrent infections. The clinical picture, including the severity of the

infections is, in addition, markedly influenced by age-dependent factors. There is a high mortality associated with neonatal infections, while infections in older children and adults, as a rule, are mild.

Table 31.3 gives a survey of primary and recurrent HSV infections. Primary infections of the mouth and throat (*gingivostomatitis and pharyngitis*) belong to the infections of 1–4-year-old children. The mucosa is red, prone to bleeding and covered with many large and small vesicles (*Figure 31.3*); occasionally there will be necrotic ulcerations and loosening of the teeth. *Tonsillitis* is more common in somewhat older children.

Primary infections with HSV may occur in children with *atopic eczema* and become a serious complication. Infections of an acute eczema or a skin previously

TABLE 31.3. HSV-1 and HSV-2 infections

Virus	*Pathogenesis*	*Symptoms*	*Target group*
HSV-1	Primary infection	Pharyngitis Tonsillitis Gingivostomatitis	Children and young adults
		Eczema herpeticum	Atopics
		Keratoconjunctivitis	Young adults
HSV-2	Primary infection	Vulvovaginitis Herpes progenitalis	Children and young adults
		Neonatal infections	Newborns
HSV-1	Recurrent infection	Herpes labialis Keratoconjunctivitis Encephalitis	Children and adults
HSV-2	Recurrent infection	Herpes genitalis Meningoencephalitis	Children and adults

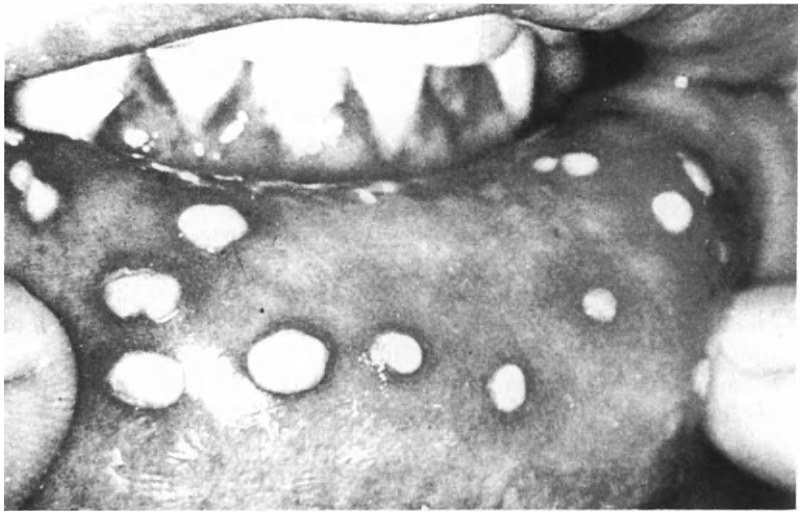

Figure 31.3. Gingivostomatitis in association with a primary HSV infection

affected by atopic changes may lead to prolonged infections, conditions refractory to therapy due to the usually less efficient immune defence of atopic patients.

Lacerations of the skin may provide portals of entry for HSV infections. In laboratory personnel and dentists, infections of cuticles on fingers (*herpetic whitlow*) and, in wrestlers, infected abrasions on the skin of the lower part of the back (*herpes gladiatorum*) are recognized.

Primary infections of eyelids, conjunctivae and cornea in children and adults may sometimes be serious, especially infections leading to *keratitis*, as ulcers of the cornea cause impairment of vision. Spread of virus to the inner parts of the eye, an *iridocyclitis*, occasionally causes blindness. Different clinical forms of herpesvirus-induced keratitis have been described, one of which is the characteristic branched *dendritic ulcerations* (*Figure 31.4*).

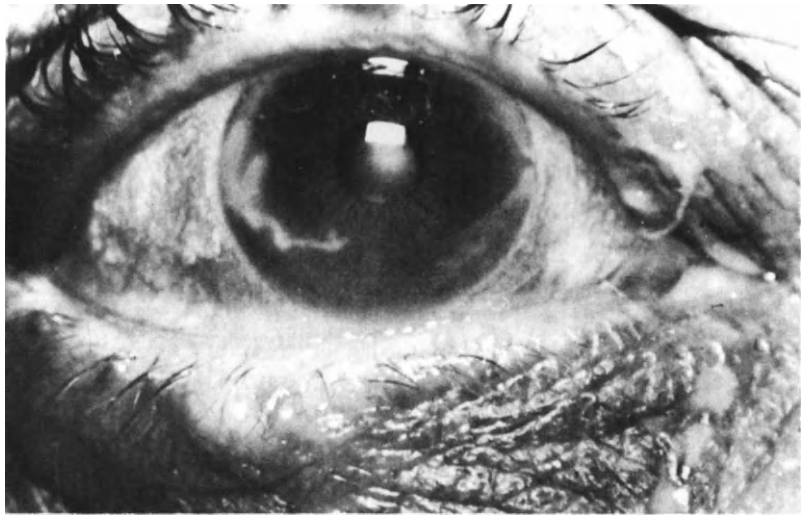

Figure 31.4. HSV keratoconjunctivitis, dendritic ulceration

Genital herpes simplex virus infection is in most cases a disease caused by type 2, but recently an increasing number of genital type 1 infections have been reported. Usually the infection is sexually transmitted and the occurrence of the genital HSV infections consequently depends upon the time of sexual debut, sexual activity and other associated social conditions. In small children, mostly girls with a primary gingivostomatitis, a self-inflicted transfer of the infection to the genitals of type 1 virus will produce a *vulvovaginitis*. The genital herpes infections appear in women as a vulvovaginitis with swollen inguinal lymph glands, and in men as an infection of the mucosa of the glans penis and the prepuce (*herpes progenitalis*). Genital herpes infections may be very painful, particularly in women.

Women, who at the end of pregnancy have an acute clinical or subclinical HSV infection, may spread the infection to the child during labour. Most serious are the consequences for the child if the mother has a primary infection and the child thus lacks the defence of transferred maternal antibodies. Also, when the mother has a recurrent genital infection there may be a risk of *neonatal infection* of the child. Clinically demonstrable genital infection in pregnant women close to term might

therefore be an indication for caesarean section. In some countries pools of human hyperimmunoglobulins with high antibody titres have been collected to be used for prevention of neonatal HSV. The preparations are administered to the exposed child directly after birth. In the infected newborn the infection becomes generalized in about one-third of the cases. A necrotizing infection with bleedings of several organs develops and the mortality rate is high.

The most common recurrent type 1 infections are herpes labialis and keratitis. Recurrent infections in the cornea may cause repeated ulcerations and healing leading to scarring with visual impairment. Sometimes recurrence may damage therapeutically grafted corneal transplants.

Primary infections, but probably much more often reactivated infections, may lead to an *encephalitis* usually of type 1 origin. The incidence of encephalitides (1–2 cases per million inhabitants) is roughly the same in all age groups suggesting that a reactivated infection for unknown reasons has spread from a sensory ganglion to the CNS. However, one cannot as yet exlude the possibility that a latent infection of the CNS has been reactivated. Herpes encephalitis is almost always localized to the temporal lobes. Without therapeutic intervention mortality is high, more than 70 per cent in some reports.

Meningitis, sometimes recurrent, is seen in association with cutaneous and genital type 2 infections. The symptoms of meningeal infection frequently occur a few days after the appearance of the peripheral infection. Sacrolumbar *neuralgia* has been reported in association with genital type 2 infection.

Laboratory diagnosis

Infections with HSV are diagnosed in the laboratory by isolation of infective virus or by direct demonstration of virus, viral DNA or viral antigens of infected cells. Attempts at virus isolation should be supplemented by serology.

Virus infectivity is lost if the envelope gets damaged. Specimens should therefore be stored in the cold, and then only for a few hours, before being transported to the laboratory. Very high isolation rates, >90 per cent, are obtained if the specimens originate from 'early viral changes' and are inoculated into susceptible cell cultures directly after sampling. Virus can be identified within 1–3 days of inoculation.

Often the collection and analysis of results for diagnosis of encephalitis cases encounter problems. Patients with herpes encephalitis as a rule lack infective virus in the cerebrospinal fluid (CSF) while virus is readily isolated from CSF in cases of meningitis. Brain biopsy material is required for the isolation of virus from cases of encephalitis. Attempts are made in many laboratories to detect minute amounts of HSV antigens in CSF by sensitive RIA and ELISA methods. The techniques used are often based on the finding that in a well balanced antigen-antibody system the addition of even very small amounts of antigen from, for example, a sample of CSF will disturb the balance. These methods require access to type-specific HSV antigens and antibodies (the latter may be monoclonal). However no generally accepted technique is as yet available.

Also, latent infections may be diagnosed in the laboratory. Peripheral ganglia have been shown to harbour virus which can be isolated when the ganglionic tissue is cultured alone or in the presence of HSV-susceptible laboratory cells (cocultivation). The observation that no virus can be isolated if the tissues are homogenized before mixing with laboratory cells has been interpreted as indicating that latent virus of the tissue specimens requires reactivation before the appearance of

demonstrable viral cpe. Presence of virus-DNA in ganglion tissues has also been revealed by DNA hybridization methods. In some reports more than half the number of cadaver tissues investigated have shown a presence of latent type 1 infection in trigeminal and spinal ganglia, while type 2 has been detected in sacral spinal ganglia.

The antibody response after a primary infection is usually clearcut. Also, conventional serological techniques such as complement fixation and neutralization tests will provide results adequate for a diagnosis. Although antibody levels tend to decline with age, a recurrent infection may not boost the levels of antibodies enough to make them demonstrable with traditional serology. However IF, and sensitive RIA and ELISA methods introduced in recent years have been found to be useful for diagnosis of infections of the nervous system. By comparing the ratio of CSF/serum antibody levels it is possible to decide whether or not an infection of the CNS is involved.

Therapy

Several antiviral drugs have been developed for the treatment of herpesvirus infections (*see also* Chapter 24). *Trifluorothymidine* is the antiviral drug generally used for treatment of HSV-induced keratitis. Two other drugs are expected to be licensed soon for treatment of HSV infections in general: Acyclovir (*acycloguanosine*) and Foscarnet (*phosphonoformic acid*). Both these drugs inhibit virus-induced enzymes relatively selectively. Acyclovir acts against the thymidine kinase and Foscarnet against the DNA polymerase of the virus. No drugs active against latent infections have been produced so far.

In the treatment of encephalitis, antiviral drugs (for example ara-A; *see* Chapter 24) have reduced the mortality markedly. Many of the surviving patients are left with lifelong invalidism however due to the brain damage caused by the encephalitis. The experiences available emphasize the importance of an early diagnosis of the encephalitis and a prompt administration of therapy.

HSV-2 infection and cervical cancer

Cancer of the cervix of the uterus is one of the most common tumours of the female reproductive organs. The first reports of a suspected association between genital HSV infections and cervical cancer were published in 1966. Serological findings suggested that prevalence and concentration of serum antibodies against HSV-2 but not against type 1 were significantly higher in women with cervical cancer than in healthy controls or women with cancer of the corpus uteri. Increased knowledge about the transforming capacity of herpesviruses, and the discovery of HSV-2 antigens and viral DNA in epithelial cells in some cervical cancers as well as in HSV-2-transformed tissue culture cells and in experimentally induced tumours in animals, have supported the assumption of a causal relationship between genital HSV-2 infection and cervix cancer. However HSV-2 infection of epithelial cells in the cervix uteri are probably common and predominantly subclinical.

In animal experiments tumours are induced in about 1 per cent of HSV-2-infected mice and hamsters. If infected animals are treated simultaneously with chemical carcinogens the percentage of cancer in the animals increases markedly. Apparently the virus infection may act as a cocarcinogen as well.

In an American study more than 600 000 women were examined histologically for the presence of cervical cancer. Signs of genital HSV-2 infection were noted in 0.3 per cent and 18 per cent of the HSV-2 infected women had a histologically verified cancer. In a follow-up study of women with HSV-2 infection cervical cancer was 4 times more common than in a control group without demonstrable experience of HSV-2. In women with primary genital herpes the risk of cervical neoplasia was 12 times higher than that for uninfected controls. It is important to remember that socioeconomic factors (sexual activity, hygiene, etc.) are of great relevance to the acquisition of HSV-2 infection as well as cervical cancer. Although it cannot be excluded that the association may be accidental, there remains a remarkable serological correlation between genital HSV-2 infection and cancer of the cervix uteri.

Varicella-zoster virus (VZV) infections

Clinical features, pathogenesis and epidemiology

As pointed out previously, the two syndromes *varicella* (chickenpox) and *zoster* (shingles), are caused by the same virus. Analysis with restriction enzymes has proved that the DNA of virus strains isolated from cases of varicella and zoster are identical.

VZV is spread as an airborne infection. Varicella is epidemic in pre-school children and children of the first two school years. Since transplacentally transferred antibodies have disappeared by about 6 months of age in children, outbreaks of varicella in small children in institutions and paediatric wards are not uncommon.

After an incubation period of 2–3 weeks, the exanthema appears with pocks mainly on the trunk. Enanthema with vesicles in the mucosa of the mouth and throat is not uncommon and probably represents only the visible part of the virus-induced changes in mucous membranes of inner organs. Clinically, more severe varicella is seen in adults and in patients with a tenuous immunological defence, for example leukaemic or immunodefective children, patients with Hodgkin's disease or patients treated with immunosuppressive drugs. Pneumonia is particularly feared. Encephalitis and hepatitis are two rare complications of varicella.

Varicella during the first 4 months of pregnancy may be transmitted to the fetus. It is possible that the physiological immunosuppression of the pregnancy may negatively influence the immune defence. Varicella in the ninth to twelfth weeks of the pregnancy may result in microphthalmia and anomalies of the extremities. Scars of the skin are often the first detectable symptoms of a congenitally acquired varicella.

A high proportion of babies born to mothers having chickenpox during one of the last 5 days preceding partus, will contract *neonatal infection*. If not mitigated by prophylactic immunoglobulin treatment, these infections may become serious. The longer the interval between the onset of the mother's infection and the time of the delivery, the greater are the possibilities for protection against, and mitigation of, the neonatal infection.

Also, congenitally and neonatally acquired infections may be reactivated and later sometimes appear as herpes zoster, even in a newborn child. The distribution of the shingles corresponds to a dermatoma and is usually unilateral. The cutaneous zoster infection will reach the middle line on the chest, abdomen or back. Often the

thoracic dorsal ganglia are involved but also other sensory nerves including the cranial nerves may harbour the latent infection. An intense neuralgia is a part of the clinical picture. Patients with *zoster ophthalmicus* feel the pains of the forehead and the eye regions particularly embarrassing; the zoster ophthalmicus may engage more than one branch of the ophthalmic nerve. Zoster spread to the eye may cause visual impairment and occasionally lead to blindness. *Table 31.4* gives a survey of the symptoms associated with herpes zoster.

TABLE 31.4. Symptomatology of herpes zoster

A chickenpox-like rash distributed over a dermatome
Zoster of one of the cranial nerves, usually the trigeminal nerve
Zoster sine herpete, unilateral pain as of a zoster but without exanthema
Paraesthesia
Lymphadenopathy
Encephalitis*
Hepatitis*
Pneumonia*

*Encephalitis, hepatitis and pneumonia are seen in 1–2 per cent of the zoster patients

Zoster may be seen in patients of all ages. The fact that zoster so often is seen in the old may indicate a deterioration of immunity allowing a clinical expression of the reactivated infection. A reduced immune defence may also be the reason for the high incidence of zoster in patients with leukaemia, Hodgkin's disease or lymphosarcoma as in patients receiving immunosuppressive drugs. The control of VZV infections is maintained primarily by the cell mediated-immunity.

VZV can be isolated only from cutaneous changes of the early phase of the infection. Later, the detection of infectious virus is difficult and on the fifth day after the appearance of the exanthema, patients may be regarded as non-infectious. Although varicella is disseminated as an airborne infection and is considered to be highly contagious, VZV has not been demonstrated by virological examination of throat swabs. Possibly the susceptibility of human lung fibroblast cultures considered to be the most sensitive for VZV isolation is nevertheless insufficient for the demonstration of small amounts of infectious virus. Therefore the direct detection of viral antigens is often of decisive importance (*see* Chapter 20).

It is usually possible to isolate VZV from about a quarter of the zoster patients with 'young' cutaneous efflorescences. More frequently IF-specific VZV antigens are demonstrable in cell preparations from vesicles of the skin. The immunological response (immunofluorescence, complement-fixation tests) as a rule is pronounced and in herpes zoster the antibodies are boosted to very high levels.

Therapy

Besides being used diagnostically, the boostered antibody response in herpes zoster patients is utilized in the production of immunoglobulins to be used specifically in prophylaxis against VZV. Children with leukaemia or lymphomas, or children whose mothers demonstrate clinically overt varicella, may be given human IgG prophylactically against varicella. Treatment with specific anti-varicella IgG prevents varicella in about two-thirds of the children and will make the symptoms less severe in the other cases.

Treatment of elderly patients with shingles by available antiviral drugs (IUDR, ara-A, Acyclovir etc.) has been found to enhance the healing. Favourable influences on neurological symptoms are less often encountered, suggesting that these symptoms derive from the infectious process in the nervous tissue. Treatment with corticosteroids is therefore often used in patients with shingles and then primarily to mitigate the pain.

Cytomegalovirus (CMV) infections

Clinical findings and pathogenesis

CID (*cytomegalic inclusion disease*) has been recognized since the beginning of the century and is the classic syndrome caused by a congenital CMV infection. Since the introduction of vaccination against rubella, CMV infection is the most important congenital virus infection medically (CID, intrauterine death, prematurity, malformations, somatic and mental retardation, cf. Chapter 15).

TABLE 31.5. Symptomatology of postnatally acquired and of reactivated CMV infection

Primary infection
Neonatal infection
CMV-induced mononucleosis-like syndrome
Hepatitis
Reactivated infection
Transfusion fever
Postoperative fever
Interstitial pneumonia
Chorioretinitis
Complication of a malignant disease

The symptomatology of CMV infections is presented in *Table 31.5*. A CMV infection acquired postnatally may display three different pathogenetic patterns.

1. CMV causes clinically detectable infection in patients who are immunologically stimulated by medical treatment or disease. In association with an allogeneic immunization it seems possible to reactivate latent CMV infection. Support for the occurrence of such a phenomenon has been obtained from experiments with animals as well as from clinical experiences. The *post-transfusion fever* which may afflict patients who have received blood transfusions, as well as the interstitial pneumonia occurring in patients receiving transplants, are both considered to be induced by reactivation of a latent CMV infection.
2. Immunosuppressive treatment of patients receiving kidney transplants, for example, and treatment of patients with malignant diseases with cytostatic drugs may cause complications in the form of a reactivated CMV infection. In these cases the reactivation would be the result of a deterioration in the patients' immunological control of the latent infection.
3. Exogenous CMV induces acute infections, the majority of which are subclinical. In adults and young non-immune patients the CMV infection may be sexually transmitted. This is indicated by the fact that the primary infection may occur at the time of sexual debut. It has been estimated that in women

2–3 per cent have a latent venereal CMV infection. However, in comparison with the oral route of infection, sexual transmission seems to be of secondary importance. As with HSV, the oral and genital tracts are the two main entry points for infection.

The clinically demonstrable infections of the neonatal period are not very characteristic (fever, enlargement of liver and/or spleen, sometimes a transient icterus, shedding of virus with the urine). In contrast to the congenital CMV infections which later may cause disease, mostly hearing defects, even when the children disclose no signs of illness at birth, no sequelae are recognized after the neonatal infections. The virus may be transmitted to the children with the breast milk. Reactivation of latent CMV infection seems more common in a milieu of low socioeconomic standards. Young women excrete CMV more often than older women. However, it has been documented that the CMV infection itself is capable of exerting immunosuppressive effects. Whereas American women of lower social class excreted virus in 1–2 per cent during the first trimester, 10–12 per cent of the same women excreted virus at the time of delivery.

In older children and adults, fever with lymphadenopathy, atypic lymphocytes and lymphopenia are the predominating symptoms. Thus the clinical picture may mimic that of an infectious mononucleosis. In single cases hepatitis is the main symptom of disease. It should be mentioned that a *'CMV-induced mononucleosis'* may be encountered also in individuals with antibodies from a previous CMV infection. Thus a reactivated CMV infection may display a symptomatology with clinical signs of mononucleosis.

In heart-transplanted patients, CMV infection has been considered to be of aetiological importance for the *chorioretinitis* observed in these patients 1–2 years after the transplantation. The chorioretinitis may result from an immunopathological reaction triggered by the CMV infection. About 20 per cent of CMV infections reveal the presence of circulating immune-complexes. Also, the rare haemolytic anaemias and the cases of the *Guillain–Barré syndrome* seen as sequelae of CMV infections have been interpreted as immunopathological reactions. In the Guillain–Barré cases, sometimes more than one of the cranial nerves are involved. Presence of a positive Coombs test, cold agglutinins and antinuclear antibodies in association with CMV infections, all speak for the capacity of the CMV infection to influence the immunological reactions of the body.

Recently the increased occurrence of *Kaposi's sarcomas* in young homosexual men in the USA and Northern Europe has attracted much attention. In these patients the disease is characterized by infiltrative tumour growth of skin and mucous membranes, lymphadenopathy, loss of weight, fever and pneumonia. Usually, the patients die within 2 years. Many homosexuals have signs of CMV infections in addition to compromised immune reactions. The CMV infection of these patients seems to be a part of an acquired immune deficiency syndrome (AIDS) (*see* Chapter 34).

Laboratory diagnosis

CMV-infected patients often excrete virus for a long time in saliva, urine, sperm, cervical secretions and faeces. The virus excretion can easily be detected by means of human fibroblast cultures. The cellular changes induced by the virus are usually characteristic enough to exclude serological typing routinely. If infected cultures are histologically stained the easily recognized intranuclear inclusions are revealed.

The laboratory diagnosis of a congenital CMV infection requires among other things that virus be isolated from the newborn within 2 weeks of birth. Since neonatal infections are common, many children become infected during the first months of life, shedding virus for weeks, sometimes months.

Isolation of virus may be achieved also from infected lymphocytes added to fibroblast cultures. The lymphocytes should be preincubated for 2 days in cell culture medium to allow a reactivation of a latent infection.

Immunofluorescence (IF) and immunoperoxidase techniques are used to demonstrate CMV antigens directly in cells, in urine or saliva and in post mortem specimens. Often there is an advantage in combining virus isolation and IF. The IF examination of inoculated cultures will markedly decrease the usual 3–6 weeks required for demonstration of virus.

The serological methods (complement-fixation, IF, RIA and ELISA techniques) for diagnosis of congenital infections must be focused on the demonstration of CMV-specific IgM antibodies in cord blood. Also, for the diagnosis of postnatally acquired infection, tests for IgM antibody responses are essential. Neutralization tests for antibody detection are less reliable partly because there are several serological types of CMV (tests using more than one strain of CMV are thus sometimes required), partly because preparations of CMV employed must be free of infected cells, which is difficult because standard virus suspensions contain mainly cell-associated virus.

Prophylaxis

Congenital CMV infection is discussed as a possible aetiology for mental retardation. One to 2 out of 10 000 newborn children have been reported to be at risk of being mentally retarded as a consequence of intrauterine CMV infection. Against this background, attempts have been made to develop avirulent CMV strains for vaccination. One of these strains, the so-called Towne strain, has demonstrated good immunogenicity after subcutaneous injections without severe side-reactions. However, the experimentally demonstrated association between herpesviruses and tumours has called for a cautious and restrained attitude to the introduction of live vaccines against CMV infections.

Epstein–Barr virus (EBV) infections

Clinical findings, pathogenesis and epidemiology

Like other herpesviruses pathogenic to man, EBV is disseminated ubiquitously and most individuals of all populations examined exhibit signs of having been immunized during childhood. *Table 31.6* presents a survey of various symptoms of disease either directly induced by EBV infection or resulting from an obvious influence of EBV infection on the course and development.

Infectious mononucleosis is a disease of the lymphatic system displaying lymphadenopathy with enlarged lymph glands of the neck and the axillae, *pharyngitis, tonsillitis* and enlargement of spleen, sometimes also of the liver. The patients complain of fatigue and fever. The CNS may be occasionally affected as well as heart and lung functions. Deaths associated with mononucleosis are often caused by rupture of the intensely swollen parenchyma of the spleen. The blood picture is

characterized by increased numbers of mononuclear cells, among which large atypical lymphoblastoid cells indicate that there is a proliferation of lymphoid cells.

Mononucleosis is the symptomatology of the infected adult non-immune individual. It is most commonly seen during adolescence but may sometimes also be seen in older ages. The oldest patient with a diagnosed infectious mononucleosis was 79 years of age. In developing countries, the majority of young children become subclinically infected and usually school children and older children are immune. Both clinically manifested and subclinical infections may initiate a longlasting carriership with virus excreted in the saliva. Virus replicates in epithelial

TABLE 31.6. Symptomatology of EBV infection

Mononucleosis
Pharyngitis
Pneumonia
Meningoencephalitis
Thrombocytopenic purpura
Myocarditis
Nephritis
Guillain–Barré syndrome

cells of the mouth and transmission may therefore be effected with virus-containing saliva. The EBV epidemiology is influenced by socioeconomic conditions. A genetic predisposition for the infection may also be discernible. The infection may be more or less severe depending upon the ability of the patient to react immunologically. Patients with immune defects, under influence of immunosuppressive drugs or suffering from diseases such as Hodgkin's disease, lymphomas or sarcoidosis, may demonstrate aggravated, sometimes life-threatening, mononucleosis. In rare cases with X-chromosome-linked, recessive B- and T-cell deficiency, the EBV infection develops into a lethal lymphoproliferative disease.

TABLE 31.7. EBV-antigens

Antigen	*Structure*	*Occurrence*
VCA	Virus-capsid antigen	In virus-replicating cells
EA-R EA-D	Early antigen. Restricted (R) or diffuse (D) distribution in the cytoplasm of the cell	In both virus-replicating and latently infected cells
MA	Viral peplomers, *m*embrane-*a*ssociated antigens	In virus-replicating cells
EBNA	EB-nuclear antigen, corresponding to T antigens	In EBV-transformed cells, the nuclear antigen
LYDMA	Plasma membrane EBV-antigen detectable by lymphocytes; corresponding to TST-antigen	In EBV-transformed cells, the membrane antigen

The B lymphocytes carry the receptors for EBV adsorption which are closely related to the receptors for the complement factor C'3. *In vitro* there is a proliferation of clones of immunoglobulin-synthesizing cells with production of heterophile IgM antibodies. Detection of these antibodies still has a certain diagnostic relevance (Paul–Bunnell, oxerythrocyte haemolysis, monospot tests, and others). In modern laboratory diagnostics, the serological methods determining specific antibodies against EBV-induced antigens have an increasingly larger diagnostic significance.

Patients with infectious mononucleosis due to EBV infection produce antibodies against virus structural antigens (VCA, virus-capsid antigens), virus-specified enzymes (EA, early antigens) and antigens of the plasma membrane of infected cells (MA, membrane antigen). During the acute phase of the disease no antibodies against EBNA (EB-nuclear antigen) can be demonstrated. These antibodies appear when the acute phase is over and the patient is in convalescence or in restored health.

VCA, MA and EA are antigens present in EBV-infected susceptible cells, while EBNA is an antigen characteristic of the transformed, non-virus producing cell. EBNA and an antigen detectable in plasma membranes of infected cells, LYDMA, recognized by cytotoxic cells, correspond to the T and TST antigens of other systems of virus-transformed cells (cf. Chapters 11 and 18). A description of the different EBV antigens is presented in *Table 31.7*.

Laboratory diagnosis

For detection of EBV *in vitro*, the immortalization of lymphocytes is a possible but technically complicated method. Nasopharyngeal secretions of EBV-seropositive patients are inoculated into a suspension of lymphocytes of a seronegative donor. Repeated passaging of the lymphocytes will reveal if immortalization, i.e. transformation, has been attained. Non-transformed lymphocytes cannot be continuously cultured.

Diagnostic methods based on serology or detection of antigens of infected cells are less laborious and time-consuming. By means of chronically infected cell lines, the virus laboratory can detect specific antibodies against EBV-induced antigens. Rise in antibody titres against VCA and EA is detectable serologically with IF not only in patients with acute mononucleosis but also in patients with a reactivated latent EBV infection. Thirty to 50 per cent of patients subjected to organ transplantation or treated with immunosuppressive drugs experience a reactivation of latent EBV infection with increasing antibody titres and excretion of virus with saliva.

Heterophile antibodies are demonstrable from the second week of the disease and usually disappear after a few weeks. Sheep and horse erythrocyte-reactive antibodies are demonstrable for a longer period after an EBV mononucleosis. The higher the antibody levels observed during the early phase of the disease, the longer the persistence of the symptoms.

EBV infection and Burkitt's lymphoma

EBV was isolated by Epstein and Barr in 1964 from certain tumours of lymphoblastoid cells, the Burkitt's lymphomas. Four years previously a British surgeon, Denis Burkitt, had described clinical and epidemiological features of a lymphoma geographically restricted to a belt across central tropical Africa. Tumour cells of

patients with Burkitt's lymphomas carry EBV-genomes and display EBNA and LYDMA antigens. Consequently, the EBV infection has been suspected to be an important aetiological component for the development of lymphoma. The tumour has its origins in the upper or the lower jaw and may sometimes reach as far as the orbit. Metastasizing to abdominal organs and to the cranium has been reported. The patients as a rule are boys 3–5 years of age. The Burkitt's lymphomas are sensitive to treatment with cytostatic drugs and in many cases regression and good healing are achieved by treatment.

Even if the EBV infection is not the sole factor responsible for development of Burkitt's lymphoma, it appears to be one important factor acting together with one or more other aetiological factors. Prospective studies have shown that individuals with antibodies against VCA run a 30 times greater risk than VCA-antibody negative controls of contracting Burkitt's lymphoma. For comparison, it should be mentioned that cigarette smoking is considered to increase the risk of lung cancer about 16 times. EBV-DNA is recovered from all cells of Burkitt's lymphomas with a few exceptions. The tumour cells demonstrate a translocation between chromosomes 8 and 14, which, however, is not found in *in vitro* immortalized cells.

The particular geographic distribution characterizing the Burkitt's lymphomas contributed to an early interest in the possible cocarcinogenic roles of different environmental factors. It has been suggested that the high incidence of malaria infections in the area may be important. Repeated exposures to malaria affects the immunological reactivity.

A limited number of Burkitt's lymphomas have been discovered also in the temperate climate zones of America and Europe. Examinations have indicated that European and American tumours are devoid of EBNA, similar to about 2 per cent of the African lymphomas, findings which have made the aetiological role of EBV dubious. Perhaps the lymphoma cells are susceptible to EBV infection and able to develop into latently infected carrier cells without EBV having anything to do with the development of the tumours.

However, the many similarities between Burkitt's lymphoma cells and cells transformed *in vitro* by EBV infection, as well as observations of oncogenic properties of EBV in experimental animal systems, are still to be considered as strong arguments for an aetiological relationship.

EBV infection and nasopharyngeal cancer

One further type of tumour, carcinoma of the nasopharynx, is virologically and immunologically linked to EBV infection. The tumours, undifferentiated epithelial tumours and lymphoepitheliomas, originate from the walls of the nasopharynx and metastasize to the lymph glands of the neck. In South-East Asian people nasopharyngeal cancers are more common than in North African people, who, in turn, more often get these cancers than European and American people. Among the Cantonese of China, the nasopharyngeal tumours are demonstrable in 30 out of 100 000 men, which is an incidence 30 times higher than the average prevalence in any other population.

A high concentration of dimethylnitrosamine has been observed in salted fish consumed in Japan. Salted fish also is the basis for many dishes consumed among the people of Canton and other ethnic groups in China. Possibly there are several cocarcinogenic agents responsible for the nasopharyngeal cancers in South-East Asian people.

The occurrence of antibodies against EBV-antigens in patients with nasopharyngeal cancer is correlated to the stage of development of the tumour. This connection is particularly pronounced when the tumour progresses and increasing titres of EBNA and EA are encountered. DNA hybridization experiments have demonstrated that EBV-genomes are present in epithelial tumour cells, but not in infiltrating lymphoid cells, and by means of immunofluorescence biopsies of tumour tissue and cultured tumour cells have been recognized as carriers of EBNA. Thus there are both virological and immunological observations linking the nasopharyngeal cancer with EBV infection. In addition, it is obvious that genetic factors particularly, but also social and environmental factors, have an influence on the development of the tumours.

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Poxviruses

Åke Espmark

Poxviruses are large DNA viruses of which there are numerous types and subtypes. Poxviruses have been found in vertebrates and non-vertebrates, even in insects.

Compared with other DNA viruses the poxviruses are exceptional with their replication localized to inclusions in the cytoplasm of the infected cell. The most well known type of the poxvirus family is variola or smallpox virus. The virus is considered eradicated as a wild virus but was formerly the cause of terrible epidemics and was endemic in some geographical areas. For a long time handling of poxvirus infections was unique in that there existed a prophylaxis by vaccination with a closely related and less pathogenic virus, vaccinia. Almost a hundred years would pass after this immunological discovery at the end of the eighteenth century before similar preventative methods were tried for other infectious diseases. The next application was vaccination against rabies introduced by Pasteur in 1885.

Classification

Based on antigenic relatedness, morphology, biological properties and to some extent host-cell specificity, the poxviruses are classified in the following 7 subgroups.

1. *Orthopox viruses* i.e. variola virus (smallpox virus), vaccinia virus (vaccine virus against smallpox), and several poxviruses of rabbit, cattle, buffalo, mouse (ectromelia virus), etc. Of differential diagnostic importance are those viruses which produce monkey pox and the so-called white pox which also belongs to this subgroup.
2. *Avipox viruses* (poxviruses of birds) cause pocks in hens, canaries, pigeons, sparrows, quails, turkeys and other birds.
3. *Capripox viruses* causing sheep pox, goat pox, lumpy skin disease in cattle.
4. *Leporipox viruses* (myxoma-fibroma group) causing myxoma in rabbits, fibroma in the Californian rabbit, squirrel and hare.
5. *Parapox viruses* which cause among other diseases orf (contagious pustulous skin infection) in sheep, and milkers' nodes.
6. *Suipox viruses* causing swine pox.
7. *Entomopox viruses* which occur in several insects.

In addition there is a group of some unclassified poxviruses causing pocks in horses, guinea pigs, marsupials, sea lions, rhinoceroses, etc. Yaba virus causes benign tumours in monkeys and molluscum contagiosum virus causes a skin disease in humans (*see below*).

Properties of the virus

Members of the different subgroups of poxvirus vary somewhat in size but mostly they fall within the range 220 × 330 nm, a size which is at the limit of the resolution of the light microscope. Orthopox viruses, viruses of the myxoma group and the avian poxviruses have the same surface appearance when extracted from the cytoplasm of infected cells. In the electron microscope the irregular surface pattern gives them a mulberry-like appearance. Parapox viruses on the other hand have a regular surface structure of cross-windings similar to that of a ball of yarn.

The morphology of poxvirus is described in Chapter 10. Vaccinia virus, which is the most studied member, may be referred to as the model for all orthopoxviruses and to some extent also for the other subgroups. In the centre of the virion there is a biconcave disc-formed core containing among others the virus-DNA-genome. The genome is surrounded by two lateral bodies composed of several proteins of mainly unknown nature. Outside of these proteins follow several membrane structures. One of the membranes is synthesized in the cytoplasm while the outer envelope is acquired when virus is released. Both enveloped virus and intracellular particles without envelope are infective. The virions are well provided with enzymes and practically all of the synthesis is carried out in the cytoplasm of infected cells. Probably only the assembly of the virus requires assistance from cellular functions. Their highly differentiated properties have caused speculation as to whether poxviruses may not in fact be degenerate forms of bacteria and thus the final entities in a series of evolution: bacterium – Rickettsia – Chlamydia – poxvirus.

Intracellular virus is covered by a membrane but lacks an envelope. For a short phase during the maturation, i.e. before its liberation from the cell, the naked virus is surrounded by a double envelope deriving from the Golgi apparatus. When virus is passing out from the cell the outer envelope fuses with the cell membrane and extracellular particles thus have one envelope. The antigenicity of enveloped extracellular particles differs from that of non-enveloped intracellular virus particles. These antigenic differences had relevance when the possibilities for making a killed vaccine against poxviruses were considered. Since under natural conditions the variola infection is disseminated in the body by enveloped virus, protection can only be anticipated after immunization with inactivated enveloped vaccinia virus. A killed virus vaccine prepared from naked virus particles extracted from cells would not be expected to induce protection against variola.

The genome of poxviruses is a double-stranded DNA molecule with a molecular weight of 130–160 million which would account for the coding of some hundred polypeptides with different functions. In older studies when detergents and enzymes were used to release proteins from suspensions of purified poxvirus, 10–12 different polypeptides were recovered. In more recently published reports using crossed immunoelectrophoresis, more than a hundred polypeptides of poxviruses have been identified.

Poxviruses are resistant to treatment with ether and are also otherwise relatively stable. They are inactivated by heating to +60°C for 30 minutes and by, for

example, cationic detergents such as a 2 per cent solution of quaternary ammonium compounds or strong chlorine solutions.

The commonly used serological tests demonstrate only a small number of the polypeptide antigens. All poxviruses have a common antigen (NP = nucleoprotein) but the subgroups are otherwise different serologically.

Within the subgroups, cross-reactions are observed serologically. This is most pronounced in the orthopoxvirus group (variola virus and others). In practice, serological tests are unsuitable for distinguishing between different types.

In the following variola and vaccinia virus infections will be described; also some of the viruses of subgroups other than the orthopoxviruses will be dealt with since some of these viruses tend to cause zoonosis.

Variola (smallpox)

Without doubt, the most important type, medically, of the poxviruses has for long been the variola or smallpox virus. The disease, variola, can be traced back in time for at least 5000 years in Asia and has perhaps been associated with the most devastating epidemics of history.

Variola was present in Asia Minor and the Orient around the beginning of modern historical time and for the last four to five centuries variola has been endemic all over the world. In the 1920s, 1930s and 1940s, variola was reduced markedly in countries of the temperate climate areas owing to strict vaccination rules among other causes. Since 1950 the disease has only been imported to these countries from tropical endemic areas, Africa, South America and Asia, particularly from India and the adjacent countries.

In the whole of the tropical belt variola was still endemic even during the beginning of the 1970s. It has been estimated that during the 1960s the number of smallpox cases was 10 million per year. Of these only a small fraction were reported and registered.

In the beginning of the 1960s a worldwide campaign for eradication of smallpox was planned. There were three basic features pertaining to this disease which contributed to a successful eradication: variola virus is strictly pathogenic only to man (there are no animal reservoirs); there are no carriers with latent infections; and an effective vaccine against the monotypic virus was available.

Smallpox vaccine (vaccinia virus)

Vaccinia virus is generally accepted as originating from cowpox virus but after several passages it now differs from cowpox by demonstrating changed requirements for culturing. A hypothesis that vaccinia virus would represent a recombinant of the cowpox and variola viruses has been postulated. The smallpox vaccine is produced by inoculation of the skin of calves or sheep or by inoculation of embryonated hen's eggs. Vaccinia virus which is inoculated into the skin by primary vaccination will cause only a skin reaction locally at the site of the inoculation, enlargement of the regional lymph glands, and fever on days 5–10. The immunity will be solid for several years. Vaccination carries risks of complications (generalized vaccinia, postvaccinial encephalitis, eczema vaccinatum, etc.). Vaccination against smallpox today is virtually unnecessary (*see below*).

The WHO smallpox eradication programme

In 1967 the World Health Organization started a large global campaign for eradication of smallpox. The goal has been achieved and no more cases have been reported since the last one found in Somalia in 1977. Very large efforts personally and economically were concentrated on the campaign and besides the attaining of the ultimate goal, experience was gained about methods and strategy for international cooperation in preventative programmes against infectious diseases in general. The results of the WHO campaign must be attributed partly to the large amounts of lyophilized vaccine available. The lyophilized vaccine was resistant to the high temperatures prevailing in the tropical areas with endemic smallpox. The successful eradication of smallpox has lead to the abandonment of vaccination against the disease in most countries.

In spite of the eradication of the disease there might also be reasons in the future to maintain means for prophylactic control and laboratory diagnosis. Some laboratories still keep smallpox virus stored in freezers and are allowed to perform experiments with the virus. WHO intends that 4 reference laboratories only should be allowed to keep variola virus in the future. It seems necessary at present to have variola strains as reference strains for continued studies on some types of animal pox, monkeypox and white pox, which both show antigenic and biological similarities with variola virus. Actually such studies have raised suspicions that white pox, claimed to be of monkey and/or rodent origin, might represent a laboratory contamination by variola virus. Although these latter viruses have so far demonstrated little or no tendency to spread among humans under present conditions, it is hard to predict how the viruses will behave in 20–30 years from now when most of the earth's population will be devoid of immunity against the orthopox viruses. Also, the risks of the deliberate spread of virus for terrorist purposes should be considered. WHO has decided to store more than 200 million doses of vaccine and member countries are keeping national vaccine reserves as well. These last mentioned apprehensions justify a short description of the disease.

Clinical features of variola

Smallpox begins in the upper respiratory tract by the inhalation of droplets of saliva carrying the virus from an acutely diseased patient. In the last few decades it has been found that the contagiousness of smallpox virus is less pronounced than was previously believed. During the incubation time of 12–13 days (extremes 8–17 days) when the patient is not contagious there is a primary virus replication probably in mucous membranes. Virus is spread to regional lymph nodes and, by a primary viraemia, also to more distant reticuloendothelial sites. After virus replication, the main viraemic phase – as a rule of 2 days duration – will transfer the virus to skin and to the mucous membranes of the upper respiratory tract. The acute onset of disease with fever, headache, myalgia and fatigue, coincides in time with the main viraemic phase. One or a few days later the exanthema appears. This is at first maculopapular and, later, vesicular with a clear fluid in the vesicles. Some days later the vesicles turn to pustules when white blood cells invade. Crusts from healing skin lesions in survivors will fall off as a rule after 3 weeks leaving characteristic scars (*Figure 32.1*). In smallpox the pocks are all at the same stage of development while pocks of varicella – the most common problem in differential diagnosis – may at the examination demonstrate different stages of development.

The mortality rate of smallpox has been found to vary geographically and the question has been raised whether or not there are different subtypes of variola virus. The Asian form of smallpox demonstrated a mortality rate of 15–40 per cent, while South American smallpox (alastrim) only had a mortality rate of 1 per cent. For this reason the Asiatic form has been referred to as variola major and the South American as variola minor. In Africa an intermediate form seems to have been predominant with a mortality rate in some areas of 1–10 per cent. The different

Figure 32.1. Child suffering from moderately severe variola. Note that the pocks seem to be synchronously developed (Photo: S. Hausson)

subtypes can be distinguished in the laboratory by the differences in optimum temperature for growth. Immunity after smallpox is usually lifelong; however, reinfections have been observed in environments with very intense exposure. Immunity after vaccination with vaccinia virus is good for 5 years and partial for 10 years. An old immunity induced by vaccination may, with exposure to smallpox, induce a modified type of infection, a condition which even the experienced clinician may have difficulty in recognizing.

Laboratory diagnosis

Samples are collected by means of a syringe from vesicles or pustles. The samples are sent immediately to a virus-diagnostic laboratory which should be informed in advance. Crusts and tissue specimens should be transported, as should the vesicle fluid, in well-sealed tubes and if possible with someone being personally responsible for the delivery of the specimens. Blood samples for serological diagnosis

should also be examined. Rapid testing is performed with electron microscopy, immunodiffusion and, possibly, immunofluorescence. The appearance of the pocks on the chorioallantoic membranes of inoculated embryonated eggs is decisive for the differential diagnosis. Serologically, HI and CF tests are equally useful. A very high antibody titre in a serum may be indicative of smallpox, as the titres after vaccination seldom reach high levels.

After the preliminary examination in national laboratories, specimens from suspected cases might be referred to some of the WHO reference laboratories for further examination.

Other medically important poxviruses

Molluscum contagiosum This is a benign disease of the skin and mucous membranes of man. It is caused by a poxvirus which has been disseminated all over the world. Cases of the disease have been seen also among primates other than man but not among domestic animals. Virus is transmitted by contact. Many patients are

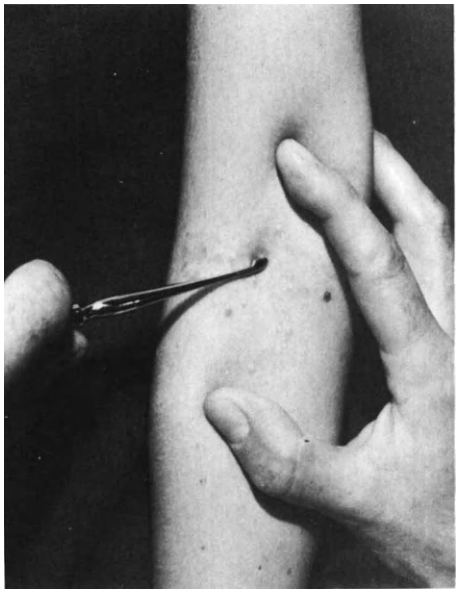

Figure 32.2. Molluscum contagiosum on the elbow. The molluscs are removed with a curette (Photo: L. Molin)

children, but the affection appears in older individuals as well and is then often sexually transmitted. The incubation time is 2–7 weeks. The lesions (*Figure 32.2*) are small umbilicated papules (1–5 mm) and are usually multiple. They have a bright lightly red to pearl-grey colour. During the first weeks, the lesions are relatively proliferative giving the impression of neoplastic growth. The lesions grow in 2–3 months to a diameter of 5–10 mm. As a rule, the molluscum contagiosum efflorescences heal without scarring in 6–9 months but they might occasionally persist for 3–4 years. If they are relatively few, the healing may be enhanced by the removal of the white central matrix with a curette, the skin area of the patient being locally anaesthesized. The diagnosis is mainly clinical and is based on the

appearance of the lesions. The white matrix is composed of, among other constituents, the molluscum virus particles which have not yet been serially propagated in cell culture or laboratory animals.

Cowpox The cowpox virus is considered to be the source of the smallpox vaccine virus (vaccinia) and is enzootic in Western Europe. The pocks are localized to the udders of the cow and consequently farmers, milkers, and others working with cattle may become infected. Usually there is one solitary pock identical in shape with the pock after vaccination against smallpox. The diagnosis is simple: cultivation on embryonated eggs or cell cultures and the conventional serology used for the orthopox virology.

Orf (contagious pustular dermatitis in sheep) This, like some other poxvirus infections, is a zoonosis. The source of the infection is sheep. Farmers, butchers and others handling sheep may be infected by contact with animals having orf lesions around the mouth and nose. In man the lesions (*Figure 32.3*) are most often

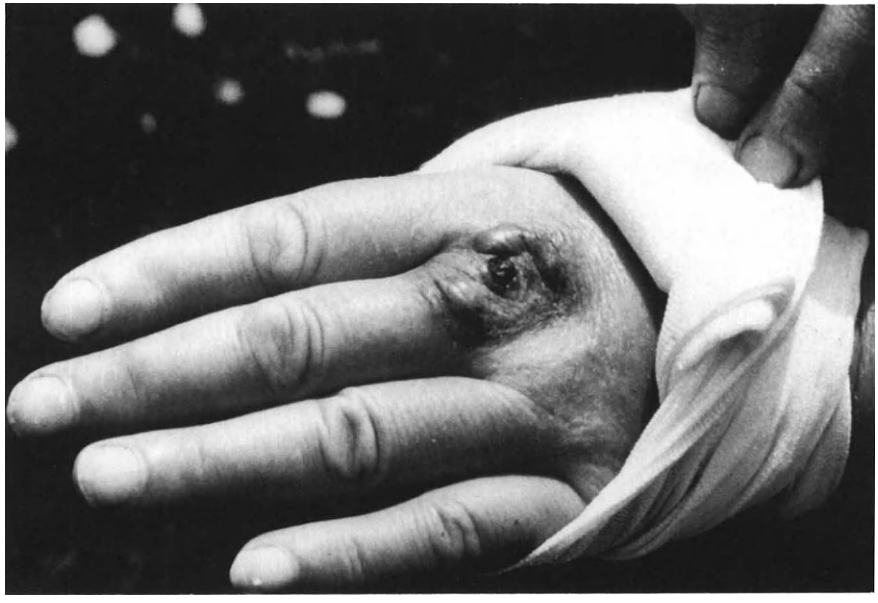

Figure 32.3. Orf infection on a finger (Photo: S. Lindgren and M. Skogh)

seen as a solitary ulcer or a pock, usually on the hands or on the face. The lesions will generally be 1.5–2 cm in diameter and demonstrate a hyperplasia which occasionally is referred to as neoplasm-like. The orf lesions heal in about 35 days.

The diagnosis is based on the electron-microscopic finding of 'ball of yarn'-like particles and on the isolation of virus by means of calf or sheep embryonic cultures. The cellular changes are similar to those seen in vaccinia – and herpes-simplex-virus-infected cultures, but the cellular degeneration propagates more slowly. The diagnosis may also be made serologically by means of indirect immunofluorescence or neutralization tests.

Orf is most common in countries where there is extensive breeding of sheep. It is reported from, for example, Australia, Continental Europe, Scotland, Scandinavia, etc. but it is probably as common in some Asian and African sheep-breeding countries.

Milkers' nodes (paravaccinia) This is a benign pock-producing disease in cows with the pocks localized to the udder. It is a zoonosis and has been transmitted to man in association with the milking. A small papule, usually on a finger, appears about 5 days after the exposure. It is later enlarged to a tender nodule with a crater in the centre and a formation of a crust. Sometimes there is a slight lymphangitis. The general condition is not affected and the lesion heals without scarring in 4–6 weeks. Milkers' nodes are difficult to distinguish from orf lesions. The anamnesis giving information on whether the contact has been mainly with sheep or cows may give some guiding information. Vaccination against smallpox protects against true cowpox infection but not against the virus causing milkers' nodes.

Virus isolation is difficult to attain in the laboratory and requires cultures of bovine embryonic cells. The virus grows more slowly in cell culture than does the orf virus. Orf virus and milkers' node virus are distinguished from each other by neutralization tests which, however, are relatively insensitive. Typing with immunofluorescence is probably feasible but it has not been reported.

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33

Other viruses

Åke Espmark and Monica Grandien

A number of viruses pathogenic to man do not naturally fit into the previous chapters. The most important of these viruses will be described in this chapter. Some of them occur in tropical climate zones, others present particular risks for hospital and laboratory personnel, whilst the medical importance of others is still under discussion.

Reoviruses

The reovirus family is composed of three groups – reovirus, orbivirus and rotavirus – and can cause infections in both man and animals. Other reoviruses are pathogenic to insects and plants.

The group of reoviruses (*r*espiratory *e*nteric) was identified at the end of the 1950s. It is questionable if any of the three types of this group is causing the induction of disease in humans. Antibodies against all three types are demonstrable in most individuals, however, and subclinical infections thus must be common. In contrast reovirus infections of mice are accompanied by symptoms.

The orbivirus group consists of a large number of members some of which are transmitted by vectors and therefore belong to the heterogenic arboviruses. Of the orbiviruses, the Colorado tick fever virus is the only one known to cause disease in man. As indicated by its name, the vector is a tick and the virus produces a febrile illness with myalgia.

The most important types, medically, are in the rotavirus group. They represent the most common aetiology of gastroenteritis in children (*see* Chapter 34).

Reoviruses have attracted much interest due to the molecular properties of the virus. The genome contains 10–12 fragments of double-stranded RNA. The virion is devoid of envelope but has two capsid layers. Each RNA fragment codes for synthesis of one defined protein. The structure and replication of reoviruses are described in Chapters 2, 3 and 8.

Retroviruses

This family includes the subgroups oncovirus (leucosis virus), lentivirus (visna-maedi virus) and a large group of spumaviruses ('foamy agents'). Oncoviruses and lentiviruses have been described in some detail in Chapters 18 and 16, respectively, in the context of tumourviruses and persistent virus infections.

The 'foamy' viruses have been given their name because of the picture of cytopathic changes induced by these viruses in cell cultures. This is characterized by the formation of syncytia and vacuolization as if the cells were foaming. Spumaviruses have hitherto been identified in different monkeys (at least 9 different serotypes), cattle, cats and hamsters, and probably also in man. These viruses have demonstrated a tendency to establish persistent infections but as yet no infections have been associated with signs of disease. Endogenous 'foamy' viruses often occur in cultures of primary monkey cells and may cause a considerable problem in diagnostic laboratory work. They also have a disturbing influence on the production of vaccines and controls of vaccines produced on monkey-derived cell cultures.

Bunyaviruses

Arboviruses, i.e. viruses borne by arthropod vectors and multiplying both in the arthropod and in the animal on which the arthropod is parasitic, show markedly variable morphological and biochemical characteristics. The arboviruses include at present more than 350 serologically different types. Among those are the togaviruses of about 80 types (*see* Chapter 26) and viruses among the orbiviruses and

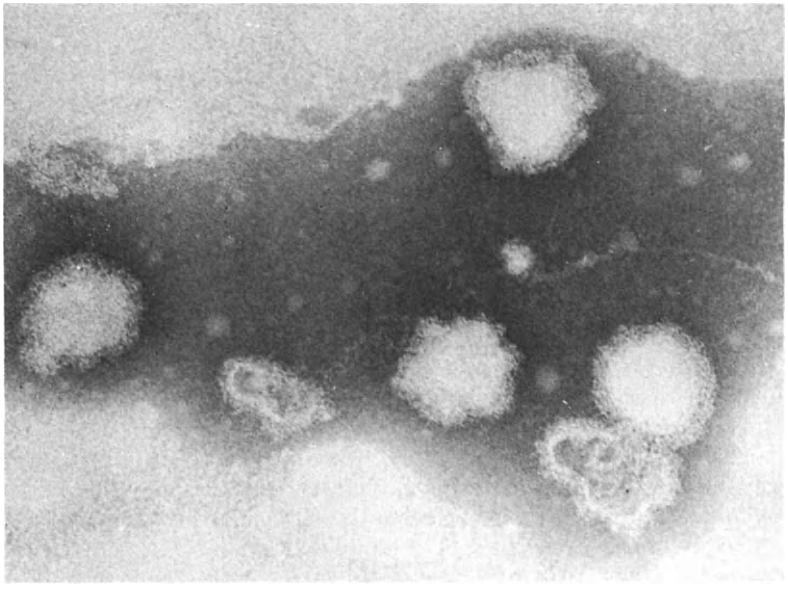

Figure 33.1. Electron micrograph of a bunyavirus. The thread-like helical nucleocapsids are discernible in two of the virus particles. (Magnification: ×200 000. Photo reproduced by permission of Dr C-H. von Bornsdorff, Department of Virology, University of Helsinki, Finland)

rhabdoviruses. However, the largest number of arboviruses belong to the bunyavirus family. Bunyaviruses have a single-stranded RNA genome, divided into 3 segments. The molecular weight of the RNA is 7×10^6 daltons, thus it is larger than the togavirus RNA (4×10^6). The virions are spherical, 90–100 nm in diameter, with a helical nucleocapsid, according to recent findings (*Figure 33.1*).

The bunyavirus family comprises at least 150 types divided into 4 genera (Bunya-, Nairo-, Phlebo-, and Uukuviruses). The *Bunyamwera supergroup* is composed of 87 serologically related types falling into 11 subgroups. Clinically important and much studied is the California subgroup including the viruses of *California encephalitis* and *La Crosse encephalitis*. Their pathogenicity to man is still discussed however and obviously they only rarely induce severe disease.

The other viruses of the bunyavirus family which are serologically distinguishable from the Bunyamwera supergroup amount to more than 60 members distributed in several subgroups. Viruses within each subgroup are often antigenically related but as a rule there is no antigenic relationship between the subgroups. Viruses causing the *Rift Valley fever* and *Crimean haemorrhagic fever* are members of the bunyaviruses. Both viruses cause serious epidemic outbreaks with considerable mortality. The Crimean haemorrhagic fever virus is closely related to the less severe *Congo fever* virus.

Rift Valley fever is mainly an epizootic infection of cattle and sheep. The infection is transmitted to man, farmers, veterinary personnel, butchers, etc. The symptoms are a fever, which reduces the general health condition, and headache; sometimes there is a haemorrhagic reaction. Symptoms of retinitis with reduced vision, which may be transient but is sometimes persistent, occur occasionally.

It has been demonstrated that diseased sheep have concentrations in the blood of 10^{10} infective units per ml. The mortality rate is high and the pathology shows, among other effects, necroses of the liver. The disease was first described in Kenya. It is known however from several large outbreaks in South Africa, Zimbabwe (Rhodesia) and East Africa. In recent years the disease seems to have migrated towards the north and a large epizootic occurred in Egypt in 1977 when also a large number of people fell ill. The statistics, which are of doubtful accuracy however, have reported between 20 000 and 200 000 cases with between 100 and 600 deaths. A vaccine against the disease has been produced in USA.

Crimean haemorrhagic fever, as the name indicates, was reported from the Crimea in 1954. The disease is mainly encountered in the rural population who work in the fields. It is spread with ticks and is manifested by general fatigue, headache, myalgia, hepatitis, a haemorrhagic rash and bleedings of inner organs. The mortality rate is, as a rule, 3–8 per cent but has in some outbreaks been as high as 30–50 per cent. The virus is antigenically related to the virus causing the Congo fever in Africa. The symptoms associated with the Congo fever are less severe however. There have been some reports of Crimean haemorrhagic fever also in other parts of the Soviet Union although no precise information is available. Some Siberian haemorrhagic fevers are induced by togaviruses related to the RSSE virus or TBE virus (*see* Chapter 26).

Phlebotomus fever (also called sandfly fever or Papataci fever) is a disease with a relatively slight fever and insignificant mortality rate. It is named after the mosquito, *Phlebotomus papatasii*, which is the most common vector of several related viruses. The disease is observed in Mediterranean countries, particularly around the Adriatic Sea.

Nephropathia epidemica is an acute epidemic nephrosis or nephritis first reported from Sweden. It has recently been demonstrated that its aetiological agent probably is a bunyavirus which is antigenically related to haemorrhagic fever virus, and is endemic in the Soviet Union and large parts of Asia (*Korean haemorrhagic fever, see also* Chapter 34).

The virus is transmitted by rodents. Preparations of lungs of infected mice have been used for determination of antibodies by means of immunofluorescence. Preliminary data indicate that cultivation of the virus is feasible and this also may provide a more detailed classification of the virus.

Arenaviruses

The family of arenaviruses consists of, among others, *LCM (lymphocytic choriomeningitis), Lassa, Junin- and Machupovirus.* In nature small rodents – mice and rats – are carriers of arenaviruses. The animals are infected perinatally and develop tolerance against the infecting virus which results in a chronic excretion of virus. Arenaviruses are RNA viruses with a single-stranded genome. The capsid symmetry is not known and the virus has an envelope which is irregular and varies in size (diameter 60–350 nm). The genome is divided into at least two fragments and the virion contains ribosomes of the host cell. The molecular weight of the genome is 3.2×10^6. All arenaviruses except LCM replicate with cytopathogenic effects on Vero cell cultures or some other cell lines. The Machupo- and Juninviruses are antigenically related, while there is only a distant relationship between the LCM and Lassa viruses.

The *Lassa fever* of Western Africa is reported to have a very high mortality rate (35–70 per cent) and a high contagiousness of infected individuals which may imply that there is a great risk of the infection spreading outside of Africa. There is a less pronounced contagiousness and a more restricted person-to-person transmission of virus in *Bolivian haemorrhagic fever* caused by Machupo virus and very little risk of secondary spread of the Junin virus which induces the *Argentine haemorrhagic fever.* Both the latter diseases demonstrate considerable mortality rates among those first infected, however, i.e. 5–30 and 10–20 per cent, respectively.

Cases of Lassa fever have been observed in the northern provinces of Nigeria since 1969. Several outbreaks were observed in Nigeria but in other West African states also, Liberia, Sierra Leone, Guinea and the Central African Republic.

As reported, secondary cases among hospital personnel are common and associated with a high mortality rate, while tertiary cases are unusual. Infections have been observed also in laboratory personnel handling specimens of patients.

Lassa fever should be suspected in patients with a fever lasting for more than 4 days and starting within 20 days of the patient's return from a Lassa fever endemic area. The clinical picture is similar to the one noted for the other haemorrhagic fevers, i.e. fatigue, fever, pharyngitis with blisters and ulcers and sometimes a maculopapular rash; in the more severe cases, bleeding of skin, mucous membranes and inner organs are seen. It is possible that the Lassa virus infections, at least in West Africans, are more common and often less fulminant than among the hospitalized patients. More than half of the cases in Sierra Leone demonstrated a slight fever only.

In non-endemic areas units for the hospital care of imported cases of Lassa fever are needed as are the high-risk units of the national virological laboratories permitting work with highly contagious viruses like that of the Lassa fever. The Microbiological Research Establishment at Porton in England and the Center for Disease Control (CDC) in Atlanta, USA, are geared for diagnostic work with Lassa fever virus. Several national laboratories of other countries have agreements

with these two mentioned laboratories in UK and USA for cooperation with laboratory work for diagnosis of Lassa fever virus. International rules for the packing and transportation of these specimens have to be followed.

Coronaviruses

In the first years of the 1960s viruses which later became designated as coronaviruses were isolated almost simultaneously in England and USA from cases of upper-respiratory-tract infections.

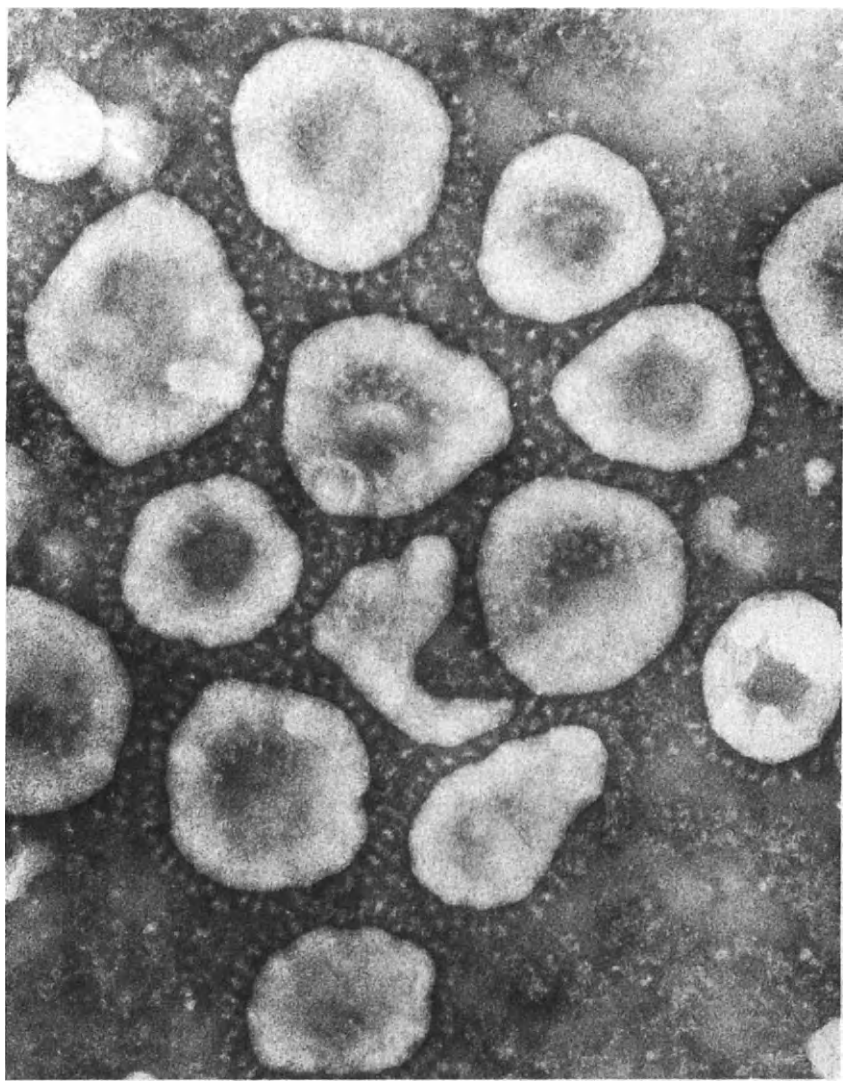

Figure 33.2. Electron micrograph of coronavirus. Note the club-like projections of the envelope (Magnification: ×~95 700. Photo reproduced by permission of Dr J. Almeida, The Wellcome Research Laboratories, Beckenham, Kent, UK)

Coronaviruses have a single-stranded RNA genome, unknown capsid symmetry and an envelope which possesses 'knob'-like projections. In the electron microscope the virus appears to have a corona-like structure (*Figure 33.2*). The virus has a diameter of 80–160 nm. It is released from infected cells by budding in cytoplasmic vesicles.

Organ cultures of mucous membranes of the respiratory tract are as a rule required to permit growth *in vitro* of human coronaviruses. This is the case with the types B814 of England and OC38 and OC43 of the USA. OC43 has later been adapted to suckling mice by intracerebral inoculation. Brain tissue homogenates of mice may be used as antigen in complement-fixation tests and as haemagglutinin in HI tests. The type 229E, on the other hand, grows well on primary cultures of human kidney cells and on human diploid cell strains (WI-38). Probably there are several types which have as yet not been propagated *in vitro* and characterized. More extended epidemiological studies have only been performed with the types 229E and OC43 which are antigenically distinguishable.

The respiratory coronavirus infections are transmitted by droplets (saliva, etc.) and have an incubation time of 2–4 days. The duration of the infection is usually 6–7 days. Reinfections are common although antibodies are present in the exposed individual.

Coronavirus infections (profuse rhinitis, pharyngitis and cough) appear with a certain periodicity every second to third year, often in epidemics infecting up to 15 per cent of the exposed population in the age range 15–29 years. About half of the cases infected with strain 229E are subclinical. Outbreaks are most common during January to April.

Coronavirus-like particles have been observed in faecal specimens of patients with gastroenteritis symptoms in India, Australia, Gambia, West Germany and other countries. As yet the evaluation of the role of coronaviruses in gastroenteritis seems incomplete. Since there are probably several as yet uncultured human coronaviruses, it is not impossible that one or more of these might be important in the aetiology of gastroenteritis in man.

Among the animal coronaviruses, mouse hepatitis virus (MHV) and avian infectious bronchitis virus (IBV) should be mentioned. It is not uncommon to find antibodies against MHV in humans. Antibodies against IBV are found in breeders of poultry but not in the population in general. In some animals (swine, cattle, horses, dogs, etc.) coronaviruses appear to be capable of causing gastroenteritis.

The methods of laboratory diagnosis of coronavirus infections are as yet difficult and insensitive. For the relatively common types 229E and OC43, serological methods (CF) are available. The direct demonstration of coronavirus antigens in nasopharyngeal specimens should be possible but this has not yet been tested routinely.

Rhabdoviruses (rabies virus)

In man and animals the rhabdoviruses are represented by rabies- and vesicular stomatitis virus (VSV). The latter virus is responsible for disease in cattle which induces vesicles of skin and mucous membranes. Rabies virus is of great medical importance. It is endemic in wild carnivorous animals, foxes, wolves etc., and is spreading in the highly endemic areas to dogs and other domestic animals and from them occasionally to man in association with bites.

Rabies is practically 100 per cent lethal in the infected higher animals, man included. The infection is mostly disseminated in countries of the warm tropical belt. Only areas with natural borders, such as Australia, the British Isles, Scandinavia, Iceland and some other islands have been kept free of rabies. In Europe an epizootic outbreak in the fox population has been slowly progressing since the 1940s. In the Americas, skunks, racoons and bats, in addition to foxes, are the most important reservoirs of rabies. Wolves, wild dogs and several small carnivora are other carriers of rabies in the eastern parts of the Soviet Union, and in Asia and Africa.

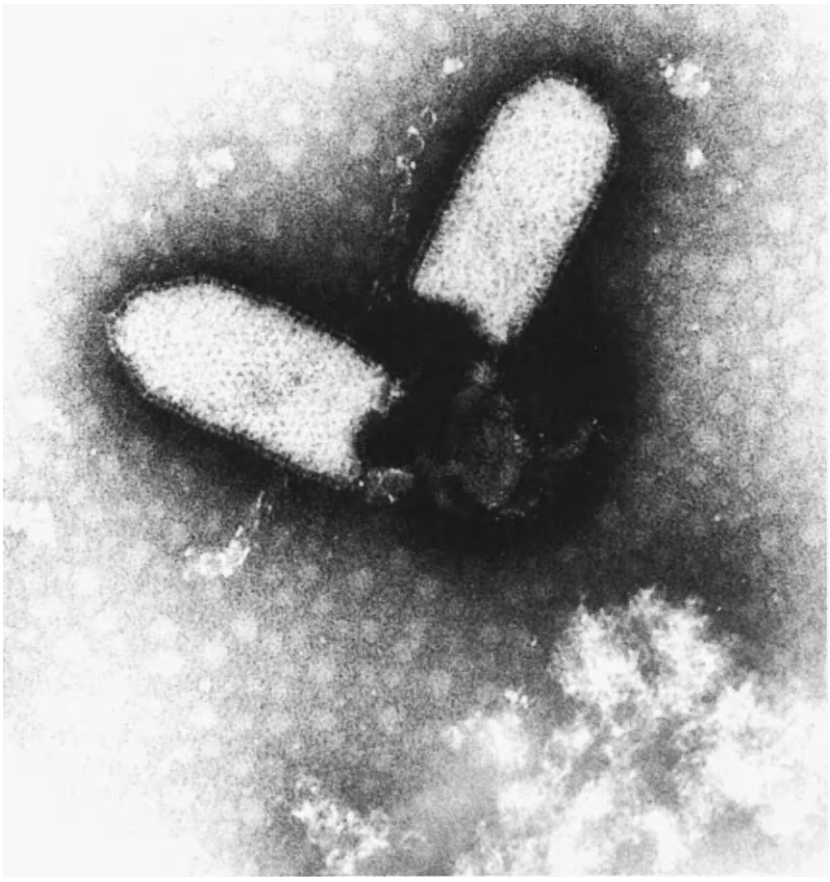

Figure 33.3. Electron micrograph of rabies virus. (Magnification ×~210 700. Photo: L. Svensson)

Rhabdoviruses are negative-strand RNA viruses shaped like bullets and having a size of about 175 × 70 nm (*Figure 33.3*). The envelope of the virion is covered with projections about 10 nm in length. Inside the envelope is a helical nucleocapsid. Antigenic determinants on the projections are responsible for virus type specificity while the nucleocapsid antigens are responsible for group and subgroup specificities. The infectious nucleocapsid carries an RNA-dependent RNA polymerase. Replicating RNA is transcribed to several mRNA segments. Rabies virus of

infected nerve cells buds from the endoplasmatic reticulum; in other infected cells the virus buds from the plasma membrane.

Rabies virus causes an acute CNS disease which is lethal for man. Virus is transmitted with saliva from a biting infected animal or by the animal licking abrased skin or mucous membranes. An airborne infection route has been observed occasionally in a few laboratory accidents and with scientists visiting caves in Texas with large populations of bats. The incubation time as a rule is 2–8 weeks but may be longer. As prodromal symptoms, malaise, fever, headache and pains in and around the wound, precede the dramatic onset of the disease. This is characterized by symptoms of the CNS infection, hyperexcitability, muscle spasms and convulsions. Convulsions may be elicited even at the thought of water. The spasms of the glottal and deglutition muscles lead to the hydrophobia. Respiratory and vasomotor paralysis and functional changes in the autonomic nervous system directly contribute to the patient's death.

Virus probably replicates in muscle cells after the introduction of the virus by, for example, a bite from an animal. It is then transported axonally in nerves to the CNS. After replication in the CNS, virus is again transported axonally from the CNS. Salivary glands, eyes and the skin of the neck and face are infected at an early stage of the disease.

Three different methods are used for the laboratory diagnosis of rabies: (1) the isolation of virus by newborn mice, a time-consuming and laborious method; (2) the demonstration of viral antigens with immunofluorescence directly in specimens from the diseased patient (corneal cells or skin biopsies may be used); (3) histological staining to detect special inclusions, the Negri bodies (this method is not reliable and yields a certain number of false-positive as well as false-negative results).

Rabies is a zoonosis and the transfer of virus from animals to man has been almost completely interrupted in the USA, for example, by compulsory vaccination of dogs and cats. Rabies can also be prevented by vaccination of non-immune but infected individuals. Inactivated virus vaccines are used (*see* Chapter 23).

Marburg/Ebola virus

In 1967 a number of cases of a haemorrhagic disease with a considerable mortality rate was reported from laboratories at Marburg in the Federal Republic of Germany. Some cases of a similar disease occurred at laboratories in Yugoslavia. Altogether there were 24 primary cases, seven with a lethal outcome. All of the seven secondary infected cases survived. The common finding in all the primary cases was the handling of blood or cells of a shipment of African green monkeys which had been imported via London from the places of capture in Uganda.

A virus was isolated in Vero cells, an established green monkey cell line. Electron microscopy revealed numerous filamentary forms of virus particles, about 100 nm in diameter and of varying length 300–1500 nm. The filaments were often club-like with an enlargement at one end and branched parts enclosing both nucleocapsid and envelope. The findings indicated helical symmetry. Morphologically, Marburg virus is not like any of the previously recognized viruses. The genome of the Marburg virus is probably a single-stranded RNA.

The clinical picture of the Marburg virus infection has features of a fulminant haemorrhagic fever. Death is usually due to shock as a consequence of extensive blood loss.

The Ebola virus infection occurring in 1976 in Sudan and Zaire caused a large outbreak of disease resembling the Marburg infection. Among more than 600 cases the mortality rate was 67 per cent. The Ebola virus demonstrated the same morphology and other properties of the Marburg virus. A serological comparison showed, however, that the Marburg and Ebola viruses are antigenically different.

The contagiousness for person-to-person transmission is low in Ebola virus infections compared to that of Lassa fever. Risks of secondary cases are present in longlasting exposure to the virus and particularly in laboratory environments when there are possibilities of exposure to infected blood, when, for example, laboratory analyses of blood samples are performed. No natural source of infection for Ebola or Marburg diseases has been established. Previous hypotheses suggesting that the African green monkey may be the host animal have not been wholly supported by extensive epidemiological and serological studies carried out by WHO in Africa during and after the Ebola virus disease outbreak.

Parvoviruses

The family of parvoviruses is composed of single-stranded non-enveloped DNA viruses displaying a cubical symmetry and with 32 capsomers. The diameter is about 22 nm and the parvoviruses are thus the smallest known animal viruses. The DNA has a molecular weight of $1.5–2.2 \times 10^6$ daltons. Parvoviruses are distributed in three genera: the true parvoviruses, the adenovirus-associated viruses (AAV) and the densoviruses (insect viruses).

Aetiologies of severe true parvovirus infections are recognized in several species: rat, goose, mink, cat, dog, swine, cattle and hamster. The Norwalk virus inducing gastroenteritis in older children and adults may possibly belong to this genus (*see* Chapter 34).

Adeno-associated viruses are present in man in 4 different types, and among animals (monkey, dog, mouse and chicken) there exist further types. AAV are dependent for their synthesis upon a simultaneously replicating adenovirus in the infected cell. Herpesviruses may also enhance AAV synthesis of DNA and some antigens. AAV has not been associated with symptoms of disease in man or animals.

Papovaviruses

The papovaviruses are DNA viruses which have been described in the chapters on persistent virus infections and tumour viruses. Some of these viruses are observed in patients with idiopathic immune defects or in patients with drug-induced immunopathology. Encephalopathies are diagnosed in these patients (progressive multifocal leucoencephalopathy, PML, Chapter 16). The human wart viruses

responsible for laryngeal papilloma and epidermodysplasia verruciformis in addition to warts and condyloma acuminatum, also belong to the papovaviruses (Chapter 18).

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34

Viral syndromes

Erik Lycke

Only occasionally do virus infections display clinical features distinctive enough to provide a diagnosis of the aetiology of the infection. More often the generalized virus infections make the clinical picture less characteristic and therefore many different viruses induce infections with a similar symptomatology. A listing of viruses which are aetiologically important in association with a particular pattern of clinical symptoms, a *syndrome*, often leads to a reiteration of the same groups of viruses under different syndrome titles. In the case of some viral infections, the relative importance is insufficiently known and often detailed knowledge about prevailing local conditions is required before any evaluation can take place. The prevalence of an infection is influenced not only by climate and environment but also by many socioeconomic factors. Thus it is far from certain whether data from the international literature should be adopted without question to illustrate the specific national situation. The following account of viral syndromes and the medical importance of various virus infections has deliberately been kept to an outline. It is presented mainly in tables and so-called 'cakes of etiology' using the information on the virus family level. There are, however, observations demonstrating that a particular type of virus is more frequently isolated than others in association with a clinical syndrome. Only the most important of these observations will be described. The pathogenetic background has been commented on in Chapters 13 and 14.

Respiratory tract infections

The majority of all *upper respiratory infections* are caused by viruses (*Figure 34.1*). The impression that viral infections dominate is strengthened if, in addition, the abundance of subclinical virus infections is considered, in particular those in children.

The possibilities of estimating the relative medical importance of various respiratory viruses are complicated by the epidemic occurrence of the infections, like influenza, parainfluenza, RS- and adenovirus infections. Epidemiological factors, seasonal variation, etc. markedly influence the overall number of cases. A comparison like the one in *Figure 34.1* between the medical importance of different virus infections will therefore, by necessity, be a rough approximation. Probably there are still a number of unknown viruses which play important roles in upper-respiratory-tract infections. The coronaviruses, a yet little recognized virus group, may turn out to be important inducers of respiratory tract infections in adults.

Acute *laryngotracheitis or croup* is associated primarily with infections of parainfluenza type 1. This type of virus is about 4 times more often isolated from children with croup as parainfluenza type 2 even though the latter in the earlier literature is designated as CA (croup-associated). Less common are RS-virus infections in cases of croup.

Common colds are probably most frequently caused by rhinoviruses but both enterovirus and coronavirus infections may cause a similar pattern of symptoms. By no means all of the common cold-associated viruses have been isolated. To use a well worn phrase we are only seeing the tip of the iceberg.

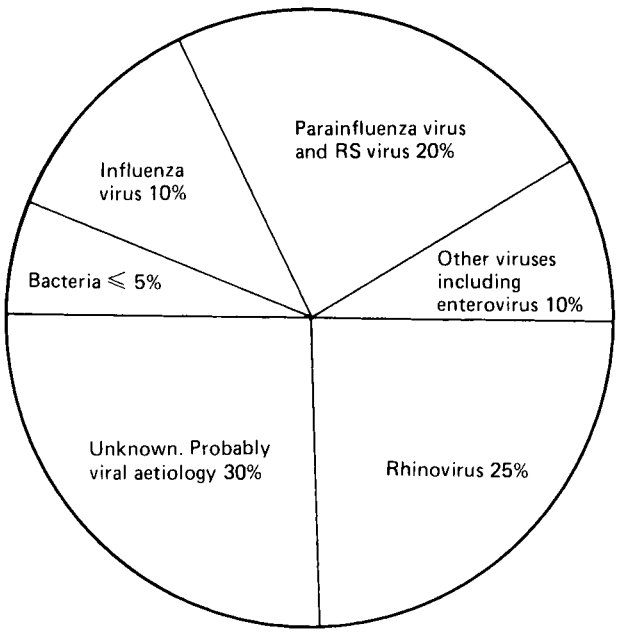

Figure 34.1. The relative importance of viruses in upper-respiratory-tract infections

A somewhat different distribution is discernible between bacteria and viruses as to their relative importance for upper and *lower-respiratory-tract infections* (*Figure 34.2*). Bacterial infections (infections with pneumococci and mycoplasmas) are responsible for many of the adult patients hospitalized with *pneumonia* while viruses (influenza-, parainfluenza-, RS- and adenoviruses) in adults are less often causes of the lower-respiratory-tract infections. However, pneumonia might commonly be a predominant symptom of a generalized virus infection or occur in infections complicated by immunosuppressive treatments. These cases are occasionally observed with entero-, EBV-, CMV- and HSV infections. It should also be kept in mind that measles, rubella and chickenpox all start as respiratory tract infections.

The most serious medical consequences of viral pneumonia are encountered in the old, the newborn or the patient with a circulatory or respiratory handicap. Influenza is a cause of increased mortality among the old whilst RS virus is the most important respiratory virus infection in small children. Particularly in individuals

with generally low nutritional and socioeconomic conditions, the RS-virus-induced bronchiolitis and pneumonitis are responsible for an increased mortality rate in infants.

Patients receiving treatment with immunosuppressive and cytostatic drugs, or who are otherwise immunologically handicapped, are, as mentioned, more susceptible to lower-respiratory-tract infections when infected with measles and adenoviruses, but pneumonia could also result from a reactivated CMV and HSV infection. The latter kind of infections, gradually becoming more common in

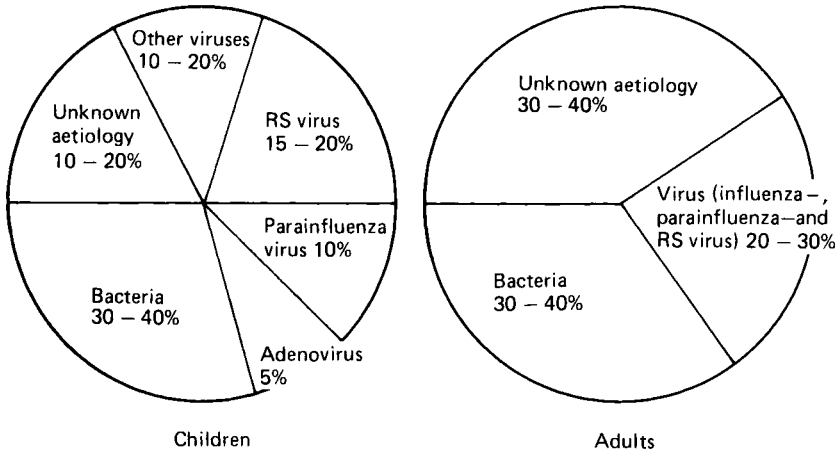

Figure 34.2. The relative importance of viruses and bacteria for lower-respiratory-tract infections in children and adults

medical clinics in industrialized countries, particularly in association with organ transplant surgery, are often referred to as *opportunistic infections*. These opportunistic infections, some of which are bacterial, viral or fungal, others of parasitic origin, are generally subclinical and latent but may become serious and life-threatening in the immunocompromised host.

The respiratory virus infections are diagnosed in the laboratory by the combined use of virus isolation methods, detection of viral antigens in cells of nasal and/or throat washings, and by serology.

Infections of the pharynx

Fever and a reddened sore throat are among the most common symptoms of infection and are seen in association with many virus infections. The predominant number of cases of *pharyngitis* in children and young adults are due to virus infections. *Table 34.1* presents some virus infections which may produce more characteristic pictures of *gingivostomatitis* and *tonsillitis*. Enanthemas with eruptions on the mucous membrane of the pharynx may be caused by the herpesviruses. Vesicular gingivostomatitis may result from both primary and recurrent HSV infections. The primary HSV stomatitis may lead to bleeding and ulcers of necrotic tissue. Patients with zoster sometimes present a distinctive picture with involvement of the mucosa of half the tongue to the middle line.

Herpangina with a few small blisters on the soft palate, the posterior wall of the throat or the tongue, is seen mainly in children or young adults. The cases of herpangina are a part of the symptomatology in epidemics of coxsackie A virus infections. Tonsillitis with a badly-smelling greyish exudate covering the tonsils is observed in about 50 per cent of cases of EBV-induced *mononucleosis*. On the other hand, pharyngitis is not a feature of the mononucleosis-like syndrome associated with CMV infections. Tonsillitis is not an uncommon finding also in the HSV-induced gingivostomatitis. In general, tender swollen tonsils may be a sign of the lymphoid reaction many virus infections can initiate. It is a common finding in adenovirus infections, usually in combination with enlargement of other lymph glands of the neck.

TABLE 34.1. Enanthematous virus infections of the oropharynx

Virus	*Clinical symptoms*
HSV-1	Gingivostomatitis, pharyngitis, tonsillitis
VZV	Enanthema of varicella and zoster
EBV	Mononucleosis tonsillitis
Measles virus	Pharyngitis, Koplik's spots
Rubellavirus	Pharyngitis
Coxsackie A virus	Herpangina, hand-foot-and-mouth disease
Other enteroviruses	Occasionally herpangina or undifferentiated pharyngitis

In this context it may be appropriate to recall the varying engagements of the salivary glands in mumps virus infections. Most commonly, the parotid glands are affected on both sides but sometimes the submandibular and sublingual salivary glands are infected. Infection of the glands on one side only is less frequent than the infection of the salivary glands of both sides.

Infections with exanthemas

The appearance, texture and consistency of the *pocks* and their distribution over the body were the four important signs for distinguishing clinically between smallpox and other vesicular and pock-producing infections. In smallpox the efflorescences develop synchronously while in chickenpox all the stages from the early maculopapular changes to the crusts are demonstrable. The pocks are hard as buttons in smallpox but soft and vulnerable in vaccinia and varicella virus infections. In chickenpox the exanthema is mainly located on the trunk while in smallpox most of the pocks are found on the head and limbs.

Maculopapular and vesicular rashes are common features also in several enterovirus infections in addition to the pox- and herpesvirus infections.

Coxsackie A viruses, mainly types 5, 9 and 16, have been associated with vesicular efflorescences on the palms, soles and in the mouth, so-called *hand-foot-and-mouth disease*. As with the pox- and herpesviruses, infective coxsackie virus can be isolated from vesicular fluid during the acute phase of the infection.

The maculopapular skin eruptions of measles and rubella cannot be distinguished from rashes seen in association with many enterovirus infections. In fact much confusion, anxiety, and even legal abortions, have resulted from epidemic outbreaks of enteroviruses in the past when the rash is similar to that of rubella and appears in pregnant women. Confusion has resulted also from the characterization of the appearance of a rash as morbilliform or rubelliform, terms which have been misinterpreted as indicating the aetiology. While in measles and rubella the laboratory diagnosis almost exclusively is obtained by serology studies, the aetiological characterization of enterovirus infections with rash is mainly based on the isolation of the virus. Maculopapular exanthemas may be encountered also in other virus infections, for example the adenovirus infections, and mainly in small children with fever and pharyngitis.

Distinctly different are the changes in the skin caused by the bleeding associated with the *haemorrhagic fevers*. The extensive diapedesis observed in these diseases is not restricted to capillaries of the skin but is present also in inner organs.

Hepatitis

Hepatitis A, B and so-called 'non A- non B' virus infections have been described in Chapter 30. Other virus infections may also affect the liver however and under some conditions lead to severe infections. Hepatitis may be part of a generalized infection affecting several organs; the infections may be congenital or neonatal infections but also affect adults. Newborn children with symptoms of a rubella-, cytomegalo- or herpes simplex virus infection may demonstrate extensive inflammatory and necrotizing engagement of the liver. In yellow fever, hepatitis and nephritis are parts of the haemorrhagic fever syndrome. A hepatitis may also be seen in mononucleosis, and signs suggestive of a pathological liver function in patients with enterovirus infections may sometimes be discovered in the laboratory. In immunosuppressed patients hospitalized for organ transplantation surgery, opportunistic herpesvirus infection may include infection of the liver.

Gastrointestinal infections

In adults the majority of epidemic diarrhoeal infections are probably bacterial but in children it is undoubtedly the virus infections which are the most important. *Table 34.2* presents the most important infections associated with symptoms of gastroenteritis. Common to these infections is the lack of cell culture systems for cultivation of the viruses. It has been claimed that only enterocytes are permissive of infections with gastroenteritis viruses. Even though other cell systems might be susceptible, there are at present no cell lines available for isolation and propagation of the different gastroenteritis viruses. They are routinely demonstrated by electron microscopy, demonstration of viral antigens, or serology.

Rotavirus is recognized by its characteristic double-layered capsid with cubical symmetry and by its size, 70 nm in diameter. There are rotaviruses of many different animal species and some have antigenic and biological properties in common with the three human serotypes.

Rotavirus is transmitted by the faecal-oral route; it replicates in the intestine villi which are destroyed by the infections. The first infection appears as early as the

neonatal period. In the absence of resources for rehydration and restoration of electrolyte balance, the diarrhoea may be life-threatening. Rotavirus infections therefore create medical problems particularly in developing countries. Presence of IgA and IgG antibodies in the intestines and the transfer of secretory IgA with the milk from mother to child have been considered essential for recovery and immunity against the rotavirus infections. The importance of passive local immunization via breast-feeding may be an essential argument against artificial feeding in developing countries. Rotavirus is, as mentioned, diagnosed by electron microscopy of faecal specimens or by detection of viral antigens. In serology the antigenic relatedness between animal and human rotaviruses are sometimes utilized. In contrast to human rotavirus, the calf rotaviruses may be grown in cell cultures. Also hybrids between human and calf rotaviruses have been used for, among other reasons, the production of antigens to be used in diagnostic work for detecting rotaviruses.

TABLE 34.2. Gastroenteritis viruses

Virus	*Family*
Rotavirus	Reovirus
Norwalk virus	Parvovirus-like*
Astrovirus	Picornavirus-like
Calicivirus	Picornavirus-like
Hawaii virus	Picornavirus-like
Adenovirus	Adenovirus**

* Although not classified, Norwalk virus displays several features in common with the parvoviruses.
** Adenoviruses associated with symptoms of gastroenteritis are genetically distinguishable from adenoviruses causing respiratory infections. They cannot as yet be cultivated *in vitro*

Also, other gastroenteritis viruses have been demonstrated by electron microscopy (Norwalk agent, Hawaii agent, astrovirus, calicivirus, etc; *Table 34.2*). Knowledge about these viruses and their association with acute gastroenteritis is as yet incomplete. Norwalk agent, as yet unclassified but perhaps a parvovirus, has been observed in patients suffering from so-called *winter vomiting disease* and for some of the 'non-cultivatable' or enteric adenoviruses there are strong associations between infection and epidemic diarrhoeal disease. Myxovirus infections (influenza in small children and measles in undernourished children) may induce symptoms of gastroenteritis. Sometimes diarrhoeal disease has been associated with enterovirus infections. However, the causal relationship between enteroviruses found in faecal specimens and gastroenteritis is doubtful.

Urogenital infections

In many virus infections virus is excreted with the urine but this finding does not signal an impaired kidney function. Histopathological findings in some infections with CMV and HSV suggest that the kidneys sometimes may be involved. Several arboviruses responsible for haemorrhagic fevers are associated with nephrosis or

nephritis. A *nephropathia epidemica* has been reported from northern parts of Scandinavia, Finland and the Soviet Union. The virus, which seems to be a bunyavirus, is immunologically related to the Korean haemorrhagic fever virus. A similar virus is responsible for a syndrome of nephritis described in Japan. Virus reservoirs have been traced to wild small rodents.

Glomerulonephritis as a symptom of the hepatitis B virus syndrome has been attributed to deposits of antigen-antibody complexes in glomeruli and arterioli.

An acute *haemorrhagic cystitis* with blood in the urine and dysuria has been observed in associtions with the adenovirus types 2, 11 and 21 infections. Symptoms of cystitis are often recorded in cases of genital HSV infections particularly in women.

Among the genital virus infections, the HSV type 2 and the genital wart virus infections are the most common clinically of the *sexually transmitted virus infections*. In patients attending outpatient clinics for venereal diseases, HSV-2 infections with symptoms of disease are recorded in about 3 per cent. However, there is a trend for an increasing medical implication of genital HSV-2 infections throughout the world. Also *condylomatosis*, the anogenital papillomas due to a wart virus infection (cf. Chapter 18), is a disease which has spread considerably among prostitutes and promiscuous individuals. CMV as well as the hepatitis B virus can both be transmitted sexually although clinical signs of genital infection are lacking. Moreover the immunosuppressive action of CMV infections may be important in the development of *Kaposi's sarcoma* among male homosexuals. Kaposi's sarcoma is usually associated with the *acquired immune deficiency syndrome (AIDS)*. Although the aetiology of the underlying immune deficiencies is unknown, several different infections are involved; among those are many virus infections (CMV, HSV, hepatitis B etc.) but also pneumocystis carinii, syphilis and other bacterial and protozoal infections. Most of these are well recognized opportunistic infections. In addition to in male homosexuals AIDS occasionally may be encountered in heterosexual women. On the other hand, promiscuous sexual life seems to be a common denominator. By 1983 more than 600 cases of AIDS had been reported from US and 40 to 50 cases from Europe.

Finally the spread of mumps virus to the testes should be recalled. The *orchitis* which complicates about every fifth case of mumps may, if it appears in boys after adolescence, cause a reduced fertility.

Infections of the nervous system

Chapters 13 and 14 described how virus infections occasionally might reach the nervous system by haematogenous or neuronal pathways. The different modes by which the infection is carried to and propagated in the CNS undoubtedly influences the course of an CNS infection. Haematogenously spread virus may initially cause a *meningitis* and induce a *meningoencephalitis* when further transmitted to the brain.

Axonal spread of virus may induce *encephalitis* without the virus passing the blood-brain barrier and symptoms of meningitis may be sparse or absent.

Most of the epidemic enterovirus infections may result in meningitis. Usually, in outbreaks of poliomyelitis, a number of cases are registered as meningitis; non-paralytic polio was a diagnosis previously much used. As a rule there are more cases of meningitis than of paresis. Meningitis as a part of a syndrome consisting alternately also of herpangina, hand-foot-and-mouth disease, myalgia or an undifferentiated febrile condition with or without rash, is seen in coxsackie- and

echovirus infections. In outbreaks of coxsackie B virus infections, cases of meningitis coupled with myalgia, pleurisy and/or pericarditis are the characteristic symptoms. Meningitis is a common symptom also in other virus infections. As a complication of mumps, a meningitis may appear before, simultaneously with or, as a rule, after symptoms from the parotid glands. Mumps is often accompanied by meningeal irritation, in most cases subclinically.

Polio- and some other enteroviruses (coxsackie A 7, coxsackie B, echo 2 and 4, entero 71) may affect motor neurons and cause *paralytic disease* (*Table 34.3*). Paralysis may in addition be a part of the syndrome of encephalitis. Many of the

TABLE 34.3. Virus infections of the nervous system

Syndrome	*Virus*
Symptoms of the central nervous system	
Poliomyelitis	Poliovirus 1–3
Poliomyelitis-like	Coxsackie virus A7, enterovirus 71, occasionally also coxsackie B- and some echoviruses
Encephalitis	Arboviruses, HSV, rabies virus, enterovirus 71, mumps- and measles virus
Postinfectious encephalitis	Measles-, mumps-, influenza-, rubella-, vaccinia- and varicella- zoster virus
Encephalopathy, Reye's syndrome	Influenza- and varicella-zoster virus
Subacute sclerosing panencephalitis (SSPE)	Measles virus
Progressive rubella panencephalitis (PRP)	Rubellavirus
Progressive multifocal leucoencephalopathy (PML)	Papovavirus
Jakob–Creutzfeld disease, Kuru	Unclassified agents (*see* Chapter 17)
Symptoms of the peripheral nervous system	
Guillain–Barré syndrome	All the herpes-, measles- and influenza viruses
Transverse myelitis	Varicella-zoster virus, Epstein–Barr virus
Bell's palsy	Herpes simplex virus, varicella-zoster virus, Epstein–Barr virus

arbovirus infections are accompanied by symptoms of meningoencephalitis and are, together with rabies, the most common encephalitogenic virus infections of the tropical and subtropical climate zones. In Europe, USA and Canada, herpes simplex virus type 1 is the virus most frequently isolated from cases of encephalitis. An incidence in these countries of one case yearly per million inhabitants is probably an underestimation. While the HSV type 2 infections are responsible for most of the meningoencephalitides observed in neonatally infected children and measles encephalitis is the feared complication of measles in children of school age, HSV type 1 is the most important cause of encephalitis in the adult population.

Occasionally the so-called *postinfectious encephalomyelitides* may be associated with symptoms of paralysis. Part of the pathogenesis of these diseases are myelin damages, probably immunologically elicited. Infective virus is not isolated. The

postinfectious encephalomyelitides develop after the acute phase of measles, mumps, rubella, influenza, varicella or vaccinia infection. Clinically it may be difficult to differentiate between postinfectious encephalomyelitis and forms of acute encephalitis.

Reye's syndrome is one of the sometimes fatal *encephalopathies* demonstrating degenerative changes in several parenchymatous organs and considered to be immunologically elicited. Reye's syndrome has been observed in association with varicella and influenza virus infections. Chronic degenerative encephalitis may follow as a sequela to measles (SSPE) and rubella (PRP). Belonging to the group of encephalopathies, but with a different pathogenesis, is the *progressive multifocal leucoencephalopathy* from which papovaviruses have been isolated. Although the infections are seen in the immunocompromised patients and the viruses may be referred to as opportunistic agents, they are believed to be aetiologically important for the disease. The *progressive spongiform encephalopathies* (Jakob–Creutzfeld disease and kuru) have been described in Chapter 17.

The *Guillain–Barré syndrome* is an acute febrile polyneuritis, and has been observed after infections with herpesviruses, measles, influenza and after vaccination against influenza. In rare cases, a *myelitis* with the symptomatology of a transverse lesion is observed in patients with varicella and EBV infections. Herpesvirus infections (HSV, EBV, and particularly VZV) have been discussed as a possible aetiology to *Bell's palsy*, a paresis of the facial nerve.

Apparently, virus infections may be responsible, both directly and indirectly, for acute as well as chronic diseases of the nervous system. Both the central and peripheral nervous systems may be affected. Even if the diseases are uncommon, they are of considerable medical importance due to their invalidating and life-threatening consequences.

Infections of the sensory organs

Virus infections of the CNS may occasionally result in symptoms from the optic nerve. Acute *optic neuritis* has been described in context with epidemic viral meningitis and acute mumps infection. A *retinitis* may be part of the syndrome of measles and SSPE. Haemorrhages and exudate over the retina may be demonstrable in the necrotizing neonatal HSV infection.

Impairment of vision as a consequence of infections which have reached the eye from without, however, is more frequent. *Conjunctivitis* may be encountered in adeno-, entero- and herpesvirus infections. If the infection affects the cornea as well the result may be a serious complication, a *keratoconjunctivitis*. Outbreaks of epidemic keratoconjunctivitis caused by adenovirus type 8 have only rarely caused persistent impairment of vision. A worldwide epidemic with severe acute *haemorrhagic conjunctivitis* was associated with enterovirus 70 but coxsackie A 24 has also been incriminated with haemorrhagic conjunctivitis. Necrotic changes of the cornea with scarring or ulcers may follow eye infections with herpes simplex virus. If untreated this infection may lead to perforation and an *iridocyclitis* with blindness. A similarly serious course may result from a *zoster ophthalmicus* affecting sclerae and cornea. Furthermore, one of the herpesviruses, CMV, may induce congenital eye infections as well as an acquired *haemorrhagic retinits* later in life. CMV and rubella virus constitute the medically most important aetiology to

virus-induced *eye malformations*. The symptomatology of congenital rubella eye infection includes in addition *cataract, retinitis* and *strabismus*.
Hearing defects following congenital rubella and CMV infections may be due to central as well as inner ear defects. Sensory-neural hearing damage is the most common symptom of the rubella syndrome and is demonstrable in 50 per cent of the children. Neurological defects combined with impairment of hearing have been observed also after mumps infections. Common in both children and adults are the infections which reach the middle ear from the nasopharynx via the auditory tube and cause an otitis. The aetiological importance of virus infections of the upper respiratory tract for induction of infections of the middle year has still not been evaluated satisfactorily. It is possible however that many cases of bacterial otitis develop secondarily to upper-respiratory-virus infections, the latter having reduced the defence barrier of the ciliated epithelium.

Patients with viral infections of the nasopharynx may find that their smell and taste sensations have disappeared. Usually the *anosmia* is present during or shortly after the infection. If the effects of the local inflammatory reaction are responsible normal sensations will return when the infection is over. Infections destroying the structure of the mucous membrane, including the basilar membrane, olfactory glands and nerve terminals, may however result in a longlasting functional impairment with absence of the sense of smell and taste.

Neonatal infections

Infections in a child acquired after the rupture of the fetal membranes when it is passing the birth canal or infections occurring during the earliest period of life are usually referred to as neonatal infections. The clinical picture is characterized by the greater susceptibility of the newborn to infections but is otherwise not principally different from the course of infections met later in life. Since the organogenesis is completed none of the malformations or organic defects which constitute the congenital infections are encountered (*see* Chapter 15). It is possible that a neonatal infection of the still immature CNS may have a particular negative influence, however.

The neonatally infected child first excretes virus several days after birth. Children infected with CMV shed virus 3–4 weeks postinfection and children becoming hepatitis B virus carriers first shed virus at 3–6 months after birth (*see* Chapter 30).

Table 34.4 gives an account of the most important and frequent neonatal infections. The majority of the infections are subclinical. When clinical symptoms are manifested they may range from short periods of nutrition difficulty to life-threatening infections. The generalized infections particularly are dangerous. Often these children are handicapped in other ways too, being born prematurely or being immunologically defective, etc.

Herpes simplex type 2 infections are in most cases transmitted to the child in association with birth and they become most serious in children born to mothers with a primary genital HSV infection. If the mother has a recurrence of her infection the disease of the child usually becomes restricted, perhaps because of passively transferred maternal antibodies. A caesarean section before rupture of the fetal membranes reduces the risk of neonatal infection as may also a prophylactic transfusion with anti-HSV immune globulin, although very limited experience is yet available. Subclinical infections are common also among neonatal

CMV infections. Only occasionally does neonatal CMV infection lead to clinical symptoms and sequelae seem rare although they might not be excluded. *Failure to thrive, pneumonitis and hepatosplenomegaly* are the most regularly observed symptoms of the clinically overt infection.

RS virus often produces serious symptoms of *bronchiolitis* with asphyxia in newborns. These symptoms also affect children of mothers immune to the infection. Reactions between maternally transferred antibodies and RS virus antigens of cells of the infected child have been considered as pathogenetically essential although this seems doubtful since it has been shown that children born to sero-negative mothers may be affected.

TABLE 34.4. The most common neonatal infections

Virus	*Clinical symptoms*
HSV	Restricted or generalized necrotizing infection
CMV	Thriving problems, pneumonitis, hepatitis
VZV	Neonatal varicella, pneumonitis
Measles virus	Pneumonia, measles
RS virus	Bronchiolitis, pneumonitis, undifferentiated fever
Poliovirus	Meningoencephalitis, paralytic poliomyelitis
Enterovirus 71	Meningoencephalitis
Hepatitis B virus	Generalized infection
Rotavirus	Gastroenteritis

Poliovirus infections occasionally appear as *neonatal paralytic infections*. The disease is present in children of non-immune mothers. Of the neonatal enterovirus infections, the coxsackie B virus is responsible for the most important infections. *Nosocomial coxsackie B virus infections* in nurseries and paediatric wards are particularly feared as the infections which affect heart, brain and liver are associated with high mortality rates. Often the disease is introduced to the wards by a subclinically infected patient, staff member or visitor.

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Appendix

Chlamydia

Erik Lycke and Per-Anders Mårdh

Although chlamydiae are bacteria and not viruses there are several reasons why a chapter on *Chlamydia* should be included in a textbook of medical virology. Chlamydiae were earlier believed to be large viruses and it was thus natural that methods for studying and diagnosing these infectious agents were considered to be a virological concern. Chlamydiae cannot be propagated in cell-free systems and the need for cell culture facilities to culture the organisms explains why many virological laboratories are still researching and diagnosing chlamydial infections.

Microbiology of chlamydiae

Chlamydiae are prokaryotic microorganisms which are classified in the genus of *Chlamydia* consisting of two species, viz. *Chlamydia trachomatis* and *Chlamydia psittaci* (*Table A.1*).

Chlamydiae, like other prokaryotic cells, multiply by binary fission. For multiplication chlamydiae are dependent on precursor substances and energy from the host cell, for example amino acids of which isoleucine is essential for chlamydial

TABLE A.1. Taxonomy of chlamydiae

Order	Chlamydiales	
Family	Chlamydiaceae	
Genus	*Chlamydia*	
Species	*C. psittaci*	*C. trachomatis*
Immunotypes	Not yet established	A, B, Ba, C, D, E, F, G, H, I, J, K, L1, L2, L3

growth, and adenosine triphosphate (ATP). Chlamydiae are incapable of producing ATP but synthesize their own nucleic acids, proteins and lipids.

Chlamydiae are susceptible to antibiotics. The relative difference in susceptibility to sulphonamides differentiates the two species of *Chlamydia* (*Table A.2*). The table also presents certain other characteristics which distinguish the species of *Chlamydia*.

Chlamydiae adsorb to the host cell probably by binding to specific cell receptors. Once bound they are capable of activating cellular phagocytosis and endocytosis. The extracellular infectious form of chlamydiae, the so-called elementary bodies,

are metabolically inactive. Shortly after being internalized in the host cells, the elementary bodies develop in cytoplasmic inclusions into the larger so-called initial bodies. Inclusions of *C. trachomatis* contain more glycogen than those of *C. psittaci*. Therefore inclusions of *C. trachomatis*, but not those of *C. psittaci*, stain brown with iodine (*Figure A.3*).

TABLE A.2. Some characteristics differentiating *Chlamydia psittaci* and *Chlamydia trachomatis*

	C. psittaci	*C. trachomatis*
Pathogenicity	Zoonosis	Human disease
Sulphadiazine	Resistant	Sensitive
D-cycloserine	Sensitive	Resistant
Inclusions	Iodine-negative	Iodine-positive

Of the two chlamydial species, *C. psittaci* is mainly responsible for zoonoses in birds and lower mammals: *C. psittaci* may occasionally be transmitted to man. Man is the only natural host for *C. trachomatis*. With both species latent infections are common. *C. psittaci* infections in birds can cause longlasting subclinical infections with continuous shedding of chlamydiae. In man, lymphogranuloma venereum infections may persist for several years.

Chlamydia psittaci infections

Infections with *C. psittaci* are usually spread to man from birds shedding chlamydiae. In this context it should be mentioned that *C. psittaci* can also spread occasionally from man to man after close contact between infected individuals. Laboratory infections with *C. psittaci* may occur among personnel handling infected animals and specimens containing *C. psittaci*. This is a reason why the laboratory should be informed beforehand when samples possibly containing *C. psittaci* are being sent for isolation of the agent. Most of the diagnostic work is performed by means of serology, however, which markedly reduces the risks of laboratory infections.

C. psittaci infection originating from parrots and other birds of the order of Psittaformes is referred to as *psittacosis*, while infection transmitted to man by other birds, for example poultry and pigeons, is usually referred to as *ornithosis*.

In man *pneumonia* is the predominant symptom but the infection might occasionally cause systemic disease including symptoms of CNS infection. The death rate is high in untreated cases of psittacosis, but lower in cases of ornithosis. The therapy is tetracycline (*see below*).

To prevent import of epizootic diseases, many countries have strict quarantine regulations for cage birds – primarily to prevent import of Newcastle disease virus. Outbreaks of psittacosis can often be traced back to the illegal import of birds.

Chlamydia trachomatis infections

C. trachomatis is possibly the most common cause of *sexually transmitted disease* of both the male and female genital tracts (*Table A.3*). *C. trachomatis* may also be spread from the mother to her offspring (*Figure A.1*). As well as being an

TABLE A.3. Isolation of *Chlamydia trachomatis* in men and women attending venereal disease clinics: average percentage of patients culture-positive in 16 studies in different countries

	Men	*Women*
Non-gonococcal urethritis or cervicitis*	41	23
Postgonococcal urethritis**	59	–
Healthy controls	< 1	7

* Urethritis in cases without gonococci being demonstrated
** Persistent signs of urethritis in men successfully treated for gonorrhoea with penicillin

important cause of *urethritis* and *cervicitis* (*Table A.4*), *C. trachomatis* is probably also the most frequent aetiological agent of complicated genital infections, for example *endometritis* and *salpingitis* in women, and *epididymitis* in young men. *C. trachomatis* (particularly immunotypes L1–L3, *see below*) can also infect the rectum, resulting in *proctitis*.

Genital chlamydial infections may induce systemic manifestations, for example *perihepatitis* (or Fitz-Hugh–Curtis syndrome) seen in women with salpingitis. Such infections probably account also for some cases of *endocarditis* and *meningo-encephalitis*. Finnish studies have indicated that up to 90 per cent of all cases of

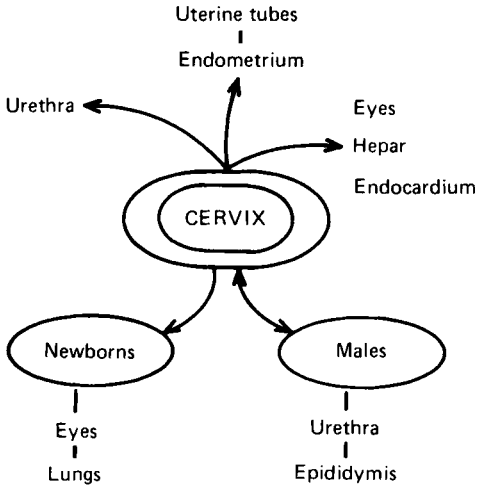

Figure A.1. Transmission and spread of *Chlamydia trachomatis* infection to males, females and newborns

Reiter's disease are associated with chlamydial urethritis. Whether all these manifestations are merely results of an invasion of the liver, the heart, etc., by the chlamydiae or are due to an immunological reaction, remains to be determined.

Lymphogranuloma venereum is one of the classic venereal diseases, predominantly occurring in tropical countries. It is caused by immunotypes L1–L3 of *C. trachomatis*. In typical cases, the disease engages not only the genital mucosa and the underlying tissues, but also the inguinal lymph glands, resulting in abscess formation (bubo). Even the intestinal tract, usually the rectum, may be involved. The disease results in fibrous degeneration with stricture and deformations, which

can result in serious secondary manifestations. For the laboratory diagnosis isolation of the causative agent is valuable, but the diagnosis is obtained more easily by serological tests.

C. trachomatis is the cause of *trachoma*. The immunotypes associated with trachoma are A, B, Ba and C. The pathogenesis of trachoma is complicated and influenced, for example. by immunological reactions against the infectious agent such as cellular immunity reactions, but also by bacterial superinfections in eye

TABLE A.4. Clinical manifestations of *Chlamydia trachomatis* infection

Genital	*Systemic*	*Eye, respiratory tract*
Urethritis	Perihepatitis	Conjunctivitis
Cervicitis	Arthritis (Reiter's)	Keratitis
Endometritis	Endocarditis?	Trachoma
Salpingitis	Meningoencephalitis?	Rhinitis?
Epididymitis		Nasopharyngitis?
Proctitis		Pneumonia

? = documentation is still meagre

lesions caused by the infectious process, and not least by trauma caused by the eyelashes of eyelids inverted by scarring. The eyes of infants living in the so-called 'trachoma belt' (*Figure A.2*) are infected early in life. Flies are effective vectors of chlamydiae. It is estimated that more than 500 million people suffer from trachoma. The disease may result in blindness because of the formation of connective tissue formation in the cornea (micropannus). Trachoma is one of the most important causes of blindness in the world.

Other immunotypes of *C. trachomatis*, for example D – K, may also cause eye infections, usually by autoinoculation from a genital chlamydial infection. Eye infections caused by these immunotypes may range from a comparative mild *conjunctivitis* to intensive longstanding infections and *keratitis punctata*. In the treatment of chlamydial eye infections, general antibiotic therapy should be given. The organism in most cases also infects the nasopharynx. Concomitant local antibiotic therapy is administered to cure bacterial superinfections.

A genital chlamydial infection in women may infect the newborn during the passage through the infected birth canal. The infant may develop *ophthalmia neonatorum*. The neonate can also develop *chlamydial pneumonia*, usually at an age of 2 and 12 weeks. Infections of the upper respiratory tract and the middle ear (*otitis media*) are other possible, but as yet less well recognized, manifestations of chlamydial infection of the newborn.

Laboratory diagnosis

Isolation of chlamydiae

Chlamydiae are strict intracellular parasites, the isolation of which requires living eukaryotic cells. As for isolation of viruses, monolayer cell cultures are employed. In such cultures, chlamydiae form intracytoplasmic inclusions. (*Figure A.3*). The inclusions of *C. trachomatis* are stained brown with iodine, which as mentioned,

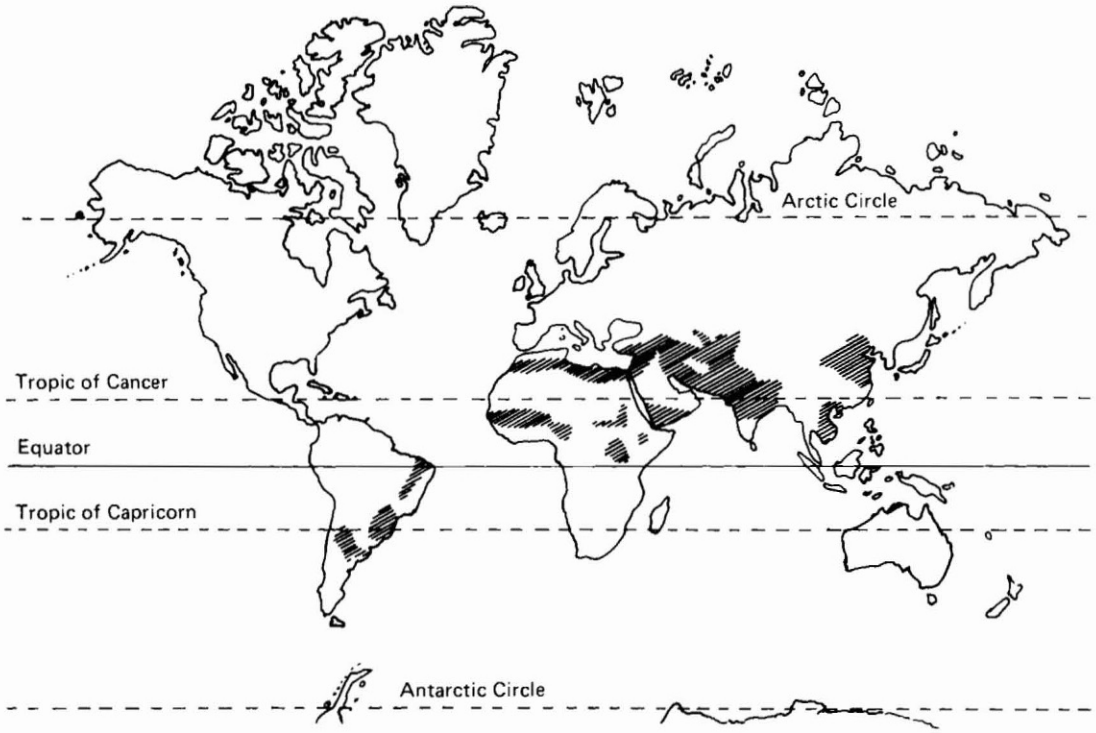

Figure A.2. **The 'trachoma belt'. Trachoma is an eye infection caused by *Chlamydia trachomatis* which affects more than 500 million people. Several millions have become blind due to trachoma**

differentiate them from inclusions of *C. psittaci*. The demonstration of inclusions is thus used as a marker for the presence of *C. trachomatis*.

For a successful isolation of *C. trachomatis* a number of prerequisites must be fulfilled.

1. Use of a Chlamydia-susceptible cell line. The following cells have proved particularly useful for the isolation of *C. trachomatis:* McCoy cells, certain strains of HeLa (229), and baby hamster kidney (BHK_{21}) cells. The McCoy cell line, primarily known to be human synovial cells, seems at some point to have become contaminated with murine cells and now displays the characteristics of mouse fibroblasts (L cells). McCoy cells are today the most widely used cell line for isolation of *C. trachomatis*.
2. Reduction of the metabolic activity of cells to be used for isolation of chlamydiae. Such a reduction can be achieved by irradiation or treatment with cytostatics. Among cytostatic compounds, IUDR, cytochalasin B, and more recently cycloheximide, have been used. The cycloheximide treatment of the cell monolayer can be performed in association with the inoculation of the sample on to the cell culture. Using McCoy cells, cycloheximide treatment has proved to be more effective than other means in rendering cells susceptible to the *C. trachomatis* infection.
3. Facilitating the contact between chlamydiae and host cells and to increase endocytotic activity of the host cells by centrifugation. Inoculated cultures are centrifuged at 3000 rpm for 60 minutes at 36°C. Certain immunotypes of *C. trachomatis*, viz. L1–L3, can, however, infect McCoy cells without centrifugation. Using HeLa 229 cells, centrifugation can be excluded provided the cells are treated with diethylaminoethyl (DEAE)-dextran.

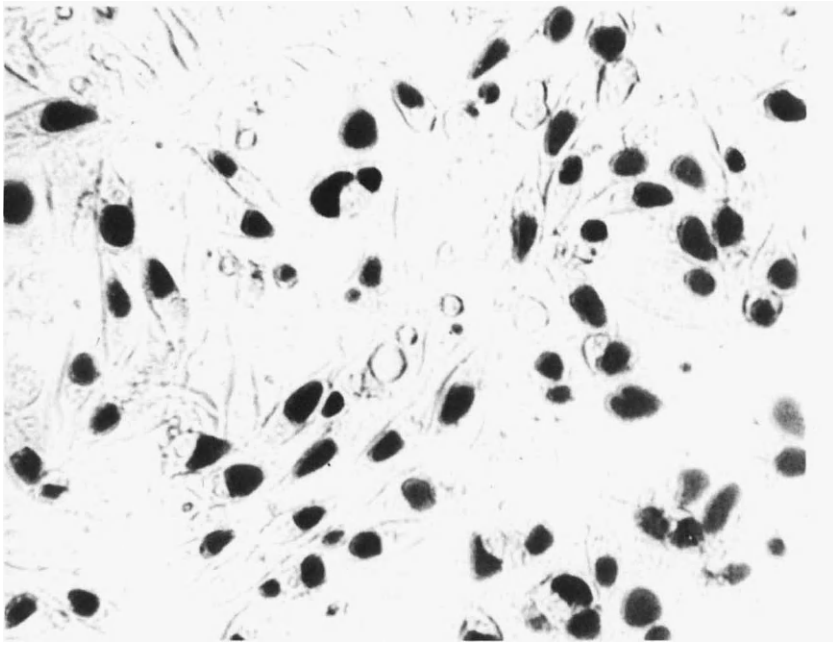

Figure A.3. Intracytoplasmic inclusions of *Chlamydia trachomatis* in McCoy cells

The inoculated cell cultures are observed after 72 hours of incubation at 36°C to detect chlamydial inclusions. The inclusions (*Figure A.3*) dislocate the cell nucleus and can partly surround it. Chlamydial inclusions may be demonstrated early after inoculation by immunofluorescence tests, but this method is not routinely applied. Other laboratories prefer to stain inoculated cells with Giemsa to detect chlamydial inclusions.

Samples for isolation of chlamydiae are usually collected by a cotton-tipped swab. Many commonly used sampling swabs are toxic to chlamydiae; both the cotton and the stick may possess antichlamydial activity. The samples should be transported in sucrose phosphate buffer to which antimicrobials (amphotericin B, gentamicin and/or vancomycin) are added to suppress contaminating microbes. Specimens should preferably be inoculated onto tissue cell cultures on the same day as that of the sampling but not more than two days later.

Immunotyping and antibody tests

On the basis of microimmunofluorescence (micro-IF) tests *C. trachomatis* can be divided into 15 immunotypes (*Table A.1*).

The micro-IF test can also be used for determination of antibodies to *C. trachomatis* in patients' sera.

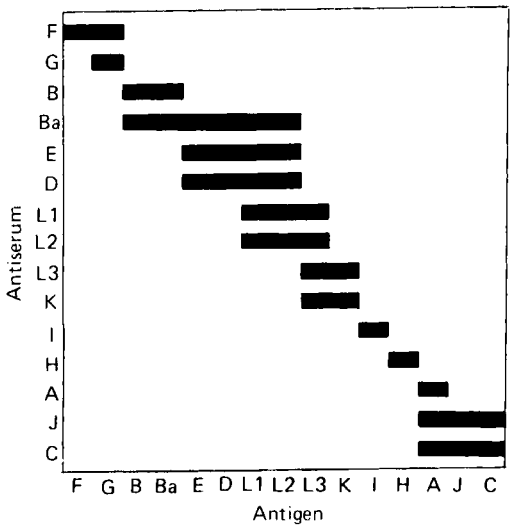

Figure A.4. Cross-reactions in microimmunofluorescence between different immunotypes of *Chlamydia trachomatis*

Cross-reactions between different immunotypes do frequently occur (*Figure A.4*). On the basis of the fluorescence pattern, the immunotype of an isolate can be determined. The test procedure is very time- and labour-consuming and cannot be performed in routine diagnostic work.

In order to simplify the procedure of antibody detection, pools of antigens have been used, either on the basis of the biological properties of the immunotypes, viz. A–C, D–K (except J), and L1–L3, or pools composed on the basis of antigenic cross-reactions. The use of only one broad-reacting antigen, e.g. L2, has also been

recommended, although it means that some percentage of the patients will appear as falsely chlamydia-negative. Such modified micro-IF tests can be applied in routine diagnostic work in studies on selected sera.

The micro-IF test allows determination of Ig class-specific antibodies. In this indirect immunofluorescence test, isothiofluorescein-labelled human anti-IgM, -IgG and -IgA antibodies can be applied. Not only sera but also tears and genital secretions can be tested by the micro-IF test for chlamydial antibodies.

While the micro-IF test detects antibodies specific to one or more immunotypes, complement-fixation (CF) tests can be used to detect antibodies directed to a group- (or genus-) specific antigen; viz. an antigen common for both *C. trachomatis* and *C. psittaci.*

Recently a number of other means of detecting antibodies to *C. trachomatis* have been devised, for example RIA and ELISA techniques, including the use of monoclonal antibodies. The introduction of these techniques in routine diagnostic work will probably simplify the sero-diagnosis of *C. trachomatis* infections in the future.

The CF test is widely applied for the diagnosis of *C. psittaci* infections, i.e. psittacosis and ornithosis. For the diagnosis of these diseases, the CF test has an acceptable sensitivity.

In cases of lymphogranuloma venereum, not only micro-IF but also CF tests are of diagnostic value. Extremely high antibody titres may often be demonstrated with both tests.

Susceptibility of chlamydiae to antibiotics

Like other bacteria, chlamydiae are susceptible to antibiotics. The drugs of choice for treatment of chlamydial infections are tetracyclines and erythromycin. The latter drug is used for treatment of infections in, for example, pregnant and lactating women as well as in newborns and children below 12 years of age. Some strains of *C. trachomatis* have been reported to have a decreased susceptibility to erythromycin. So far, corresponding observations have not been made for the tetracycline drugs. Penicillins are not effective.

As yet there are no standardized procedures for testing the susceptibility of chlamydiae to antibiotics. Both minimum inhibitory concentrations and minimum chlamydicidal concentrations have been determined, however.

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