AN INTRODUCTION TO GENERAL VIROLOGY

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T.M.B.

PREFACE

THIS book is intended to provide a short up-to-date account of Virology for the undergraduate. It is based on my lectures and tutorials to undergraduates in Medicine and Science and to postgraduates in the class of Public Health. At the request of the Publishers, chapters have been included on the Rickettsia and the Psittacosis-Lymphogranuloma-venereum groups.

In the past this subject has been selectively studied as a part of Microbiology, Medicine, Biochemistry and to a lesser extent Botany, Zoology, Entomology and Genetics. Recently, with the enormous advances in our knowledge, Virology has emerged as a distinct entity, bearing the same relationship to these subjects as they bear to each other. In this respect it resembles Microbiology which was originally studied only as a part of Pathology, Medicine, Chemistry and Botany, and which took its place as a distinct field of study forty years ago. However, the importance of viruses rests on their ability to cause disease and consequently, in this text the accent will tend to be on this aspect of the viruses. Only when they have been obtained from living organisms can we acquire further knowledge of them.

While the student of Virology will be required to be familiar with every aspect of the subject other Science students will find somewhat more clinical detail than they require, as will the student of Medicine with scientific detail. Nevertheless it is important that each should realise that Virology extends beyond the intracellular production of virus particles, or poliomyelitis. These is no reason why the new graduate in associated subjects should not have a broad knowledge of the fields in which he may be likely to find employment. Rarely can knowledge of one field not be used profitably in another and in many instances it is a necessity if obvious connections are to be appreciated and valuable time saved.

The viruses affecting plants and insects have not received attention beyond an illustration of their position in the 'virus kingdom' at this time, but it is hoped that the book will provide an introduction to all aspects of Virology which should be of value to undergraduates in Virology, Microbiology and Medicine. For the benefit of the new graduate commencing post graduate research the text has been liberally supplied with references to original work and, where available, up-to-date review articles. It may also be of use to Junior Lecturers, Physicians, Surgeons and General Practitioners who have an interest in the subject without the desire or the time to read the many excellent volumes written for the specialist.

I now wish to express my gratitude to Dr. Norman R. Grist of the University of Glasgow who taught me general virology as an undergraduate and to Professor Alexander Macdonald of the University of Aberdeen who greatly helped and encouraged me to further my knowledge of the subject.

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CHAPTER ONE

THE HISTORY AND NATURE OF VIRUSES

THE images conjured up by the word "virus" vary from person to person. To some, pictures of crippling or disfiguring diseases like poliomyelitis, yellow fever and smallpox leap to mind. Others remember

FIG. 1.1. Working with tissue cultures under a hood which can be sterilised by ultra-violet light when not in use. (*Photo—L. E. Hewitt.*)

sleepless nights while children suffered the discomforts of measles, mumps, chicken-pox or even the "common cold". Finally, there are those to whom it suggests the fundamental cause of cancer.

To our ancestors the Latin word "virus" meant a slimy substance, poison or venom including the agents responsible for transmissable or infectious diseases. With the advent of the microscope in the nineteenth century microbes were discovered and divided into bacteria, protozoa and fungi. However, the word "virus" still meant "disease-producing" and when some members of these groups were found to be responsible for certain diseases they were, to all practical purposes, "virus" microbes.

Pasteur and Roux knew that rabies was a specific disease and although they could not see the "microbe" causing it they assumed, quite correctly, that this microbe was very small—but it was still a "virus" microbe.

Nowadays, by definition, a virus is a submicroscopic filterable entity capable of self replication only in specific host cells[1]. The "filterable virus" came to light in 1892 when Ivanowsky reported that the juice from tobacco plants with mosaic disease remained infectious after passage through a filter, fine enough to hold back the smallest microbe then known. Nevertheless he too assumed that the tobacco mosaic "virus" was a small microbe. It was not until 1898 when Beijerinck repeated and confirmed this work that the idea of the "filterable virus" was introduced.[2] He also found that the infectivity of the "virus" survived precipitation in ethanol and could diffuse through agar gels. Therefore he stated that this was not a microbe but a "fluid infectious principle". Needless to say most informed people of the time rejected this idea, but with the discovery that similar agents caused foot and mouth disease[3] and yellow fever[4] the way was clear for the "filterable viruses" or "ultraviruses" as they were then called.

Today The Viruses are exclusively the "filterable viruses" and are considered apart from The Microbes. In 1957 Lwoff[5] stated the differences between viruses and microbes and his conclusions have been generally accepted. Using Lwoff's work as a guide, the nature of viruses, their dissimilarity to the microbes and their similarity to genetic material can be simply outlined.

TABLE 1.1

Property	Microbes	Viruses	Genes
Greater than 500 mμ	+	0	0
Types of Nucleic Acid*	2	1	1
Produced only from Nucleic Acid	0	+	+
Multiply by binary fission	+	0	0
Multiply by replication	0	+	+
Presence of enzyme systems†	+	0	0
Obligate intracellular growth	0	+	+
Infectivity	+	+	0

* 2 = DNA *and* RNA; 1 = DNA *or* RNA.
† Some viruses have a few enzymes but none possess complete systems.

With the exception of the last item on the table all the remaining characters are interrelated.

For an organism to possess both DNA (Deoxyribonucleic acid) and RNA (Ribonucleic acid) in addition to a full complement of enzyme systems for growth and free multiplication a certain internal volume is required. The size and obligate intracellular growth are only reflections of the constitution of the different forms of life. It is the actual make

up of a virus which separates it from all other living organisms and which in fact places it nearer the gene in every sense.

So far there is no direct evidence to state that RNA is like DNA in that the latter acts as the genetic messenger among the microbes and higher organisms but this will almost certainly be proved so—ultimately. We have, therefore, the intriguing possibility that viruses are "wild" or "mutinous" genes and ribosomes. This theory postulates that the DNA containing viruses are the result of a gene breaking away from the chromosome of the cell. It then uses its abilities to replicate itself and initiate protein formation to produce an entity capable of surviving the rigours of extracellular existence and of infecting and multiplying within specific host cells. It is but an extension of this theory to postulate that the RNA containing viruses are "mutinous" ribosomes.

In direct opposition to this theory there is the "classical" or degenerative theory which postulates that viruses have evolved from bacteria by losing the enzyme systems necessary for extracellular growth and ultimately one or other of the nucleic acids. The supporters of this theory point to the gradual progression through the Rickettsiales which behave like minute bacteria, Psittacosis—L.G.V.—Trachoma which contain both RNA and DNA, to the largest of the "true viruses", the Poxvirus group.

From a consideration of both these theories a third was naturally advanced. Lwoff stated that the genetic material of the bacteriophage and the genetic material of the bacterium both evolved from a common structure—the genetic material of a primitive bacterium.[5] In the final analysis this must of course be the case, always providing one accepts evolution, because all genetic material evolved from a common structure—the first gene. Probably the viruses of the Poxvirus group evolved by degeneration from bacteria whereas the Picornaviruses and Papovaviruses evolved from mutinous ribosomes and genes respectively.

From being obligate intracellular parasites it automatically follows that they must produce some change in the cell. Viral invasion of a cell may result therefore in the death of the cell or the transformation of the cell into something combining the properties of both cell and virus. Known transformations of host cells occur with the production of lysogenic bacteria or tumour cells.

Destruction of the host cells in a multicellular organism is usually known as an illness, which may be subclinical or result in the death of the host. This type of infection is best demonstrated in humans by diseases such as poliomyelitis, yellow fever, smallpox, measles, chickenpox and the "common cold". It has still to be proved that human cancers are caused by viruses but this seems more than likely when one considers the nature of cancer and the known carcinogenic properties of certain viruses.[6]

Having established our viral diseases the question then arises—how do we cure them? At the present time the old adage is still the best.

Prevention is better than cure. As yet there is no cure for any of the virus diseases, despite the claims of many drug manufacturers—unless one adopts the mind over body position.

To destroy the virus necessarily involves destruction of the infected cells and to do this without irreparably damaging the uninfected cells has so far proved impossible by chemotherapy. A substance, interferon, has been isolated from virus infected cells, which does inhibit infection of other cells, but to date there has been no really successful application of interferon.[7]

Prevention of disease can be effected by the immunisation of susceptible hosts to produce circulating antibodies which neutralise viral infectivity. This vaccination has been successfully applied to most of the diseases mentioned in this chapter and we will discover that the history of virus vaccines antedates the history of Viruses and Virology by exactly one century.

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CHAPTER TWO

GENERAL PROPERTIES OF VIRUSES

As we have seen in the first chapter, a virus is a submicroscopic entity possessing only one nucleic acid and capable of self replication only within specific host cells. Some viruses do possess one or more enzymes but not one has a single complete enzyme system, hence their dependence on host cells.

Two groups of agents which are not quite viruses possess certain characteristics common to viruses. These are the *Rickettsiales*, generally accepted as bacteria, and the Psittacosis—Lymphogranuloma-venereum-Trachoma or PLT group which occupy the no-man's land between viruses and bacteria. Since both of these groups will be dealt with later only the "true viruses"[1] will be considered in this chapter.

Although the viruses superficially appear to form a homogenous group considerable differences exist. Some are obviously of a fundamental nature while others have been artificially made by man. A résumé of these differences with respect to size, physical structure, biochemical structure and growth should illustrate the artificial nature of the man-made divisions into plant, bacterial, vertebrate and arthropod infecting viruses.

Physical Size and Structure

The size and physical structure of the infectious virus particle, or virion, can be considered together because in many ways they are related. The smallest viruses have a simple structure consisting of an inner core of nucleic acid and an outer coat of protein whereas the largest Poxviruses have a very complex structure. Viruses can be divided into four main categories from their appearance in the electron microscope.

(1) *The "spherical" viruses* consist of a central core of nucleic acid surrounded by a layer, or capsid, composed of regular protein sub-units called capsomeres.[2]

(2) *The "rod-shaped" viruses* take the form of a hollow helix with the nucleic acid contained within the helical protein coat which again is made up from regular sub-units.

(3) *The large complex viruses* have an internal structure similar to the *complete* spherical and rod-shaped viruses but they are enclosed within one or more membranes.

(4) *The classical "tadpole-shaped" bacteriophage* or bacterial viruses.

The simple "spherical" viruses range in size from the 25 mμ of the Picornaviruses[3] to the 130 mμ of Tipula Iridescent Virus.[4] These viruses are not in fact spherical, but polyhedral with cubic symmetry. This was postulated in 1956 by Crick and Watson[5] and later confirmed for the Picornaviruses,[6] Papovaviruses,[7] Reoviruses,[8] Adenoviruses,[9] and certain ungrouped plant, insect and bacterial viruses.[10] Most, if not all, of these viruses take the shape of an icosahedron (i.e. a 20 sided polyhedron with each face an equilateral triangle). The number of capsomeres in the capsid can be determined by the formula:—

$10(n-1)^2 + 2$ where n is the number of capsomeres on each edge of the equilateral triangle.[11] The relationship between n, the total number of capsomeres and the size is given in Table 2.1.

TABLE 2.1

The Icosahedral Viruses

Virus	n	No. of capsomeres	Diameter	Reference
Bacteriophage ϕX-174	2	12	25 mμ	12
Papovavirus	3	42	40 mμ	7
Reovirus	4	92	60 mμ	8
Adenovirus	6	252	70 mμ	9
Tipula Iridescent Virus	8	812	130 mμ	4

By the use of extremely sensitive electron microscopy, especially the phosphotungstic acid negative staining technique,[13] the fine structure of the capsomeres is being determined.

The hollow "rod-shaped" viruses have so far been found only among those viruses infecting plants. Again the "rod" is in fact a helical structure made up of regular capsomeres. This structure can best be explained by comparing it with a spiral staircase, having a central well and where each step represents a capsomere. The size and shape of these capsomeres differs with each virus as does the overall diameter, the length of the particle, the size of the central hole and the distance separating each turn. In the case of tobacco mosaic virus (TMV) the distance between each turn is 2·3 mμ and X-ray diffraction has revealed that the diffraction pattern is only the same at every third turn.[14] From this it was determined that for every three turns on the helix there are 49 capsomeres, which, for a length of 300 mμ gives a total of about 2130 capsomeres.[10] Using the analogy of the staircase, if we are standing on the bottom step we should have to ascend three complete turns or 49 steps before the step would be reached which is in the same vertical plane as the bottom step.

Each capsomere is grooved on each side, matching similar grooves on the neighbouring capsomeres. These grooves form a helical tube running throughout the virus particle. The nucleic acid is situated

within this helical tube. There is such a tremendous variation in the length and diameter of the "rod-shaped" plant viruses that the dimen-

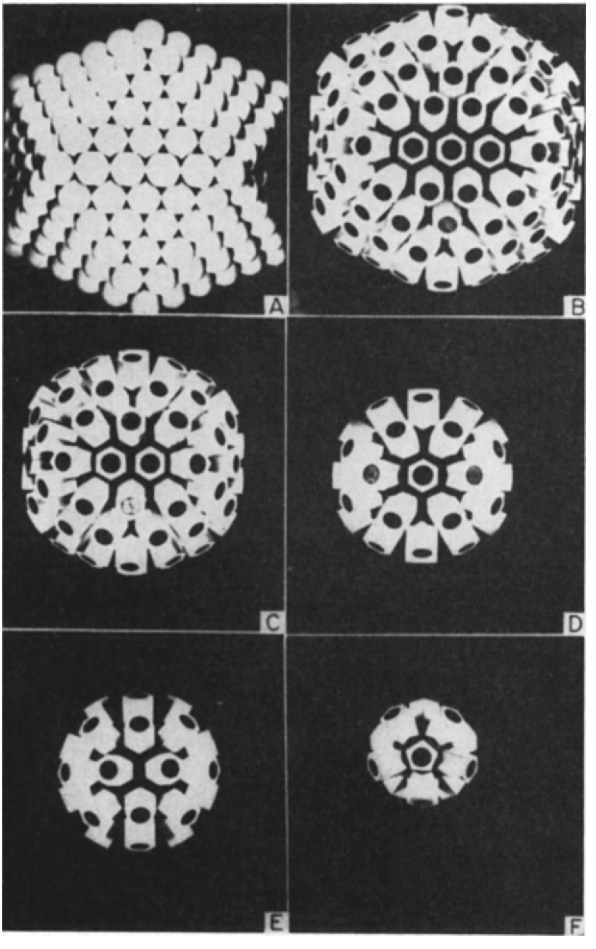

FIG. 2.1. Six models constructed from units arranged in accordance with icosahedral symmetry. A 252; B 162; C 92; D 42; E 32; F 12. It can be seen that the external shapes of the model vary depending on the number of units used.
Note. Model A is composed of solid spherical units which resemble those in the original micrographs of adenovirus. It is likely that these are better represented by hollow prisms. (*Courtesy—Academic Press Inc.*) (R. W. Horne & P. Wildy (1963) *Advances in Virus Research*, **10,** 122.)

sions and number of capsomeres making up each helix could be virtually unlimited.[15]

The structure of the complex viruses such as the Arboviruses,

Herpesviruses, Poxviruses, Myxoviruses and several insect viruses will be found in the relevant chapters but a brief summary is necessary at this point.

Some, including the Herpesviruses have an internal icosahedral structure similar to the "spherical" viruses but this is enclosed within one or more membranes which are often partially destroyed during

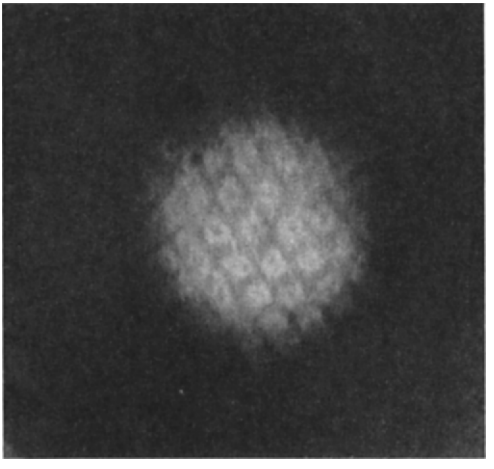

FIG. 2.2. Structure of the capsid of Herpes simplex virus as revealed by negative staining. The hexagonal packing of the polygonal capsomeres is clearly shown. (*Photo—R. W. Horne.*) (*Courtesy—Academic Press Inc., Virology*, **12**, 204; 1960.)

fixation for electron microscopy.[16,17] In the case of Herpesvirus the number of capsomeres in the capsid of the internal structure is 162.

The remaining viruses are even more complex. In each case the internal structure resembles that of TMV but in this instance the hollow helix is coiled on and around itself and is enclosed within several membranes.[18,19,20] These viruses range in size from a diameter of 38 mμ for yellow fever[17] to the brick-shaped vaccinia particles measuring 300 mμ by 375 mμ.[19]

Finally, the classical bacteriophage particle is tadpole-shaped with a polyhedral head and a narrower cylindrical tail.[21–24] All bacteriophage were once thought to have this shape but it is now known that some have the same morphology as the "spherical" viruses. Phage T2, one of the largest of the tadpole-shaped variety, has a head size of 65 × 95 mμ and a tail size of 25 × 100 mμ.

Chemical Structure

All viruses contain one or other of the nucleic acids, either DNA or RNA, and at least one protein. These are the only components of the

spherical, rod-shaped and bacterial viruses. In addition to the nucleic acid and protein the complex viruses have a variety of other components.

With one exception the nucleic acid is composed of nucleotides common to plant, animal and bacterial cells with variations existing only in the proportions. Coliform bacteriophage group (T2, T4 and T6) is the exception, where the cytosine of normal DNA is replaced by hydroxymethylcytosine.[25] The protein components are similar. All those analysed contain those amino acids known in other cells.

In addition to nucleoprotein the complex viruses of group 3 contain many other chemical components. The Arboviruses have large amounts of such lipids as phospholipid, cholesterol and neutral fats;[26] the Myxoviruses have lipid and polysaccharide;[27,28] while the Poxviruses, the most complex, have phospholipid, cholesterol, neutral fat, carbohydrates, copper, biotin and flavin.[29] The membranes of these viruses are composed of the lipid and carbohydrate fractions. Some members of the Myxovirus group have the enzyme neuraminidase which releases the virus from its attachment to the cell wall of erythrocytes after adsorption has occurred.[30] The infectivity of those viruses with an outer layer of essential lipid is destroyed by treatment with ether or chloroform while that of the simple nucleoprotein viruses is not. This is one of the simplest means of differentiating between viruses and only the Pox group is an exception. All Poxviruses contain lipids but only some are ether sensitive,[31,32] suggesting that either the lipid is not essential in this instance, or that it is protected from the ether by some other structural component.

To determine the chemical composition it is first necessary to purify and concentrate the suspension so that only the virus is being analysed and not a mixture of virus and host cell material. Methods of purification and separation vary greatly using both physical and chemical techniques, but only those in frequent use will be mentioned.

The most common method is the use of the high speed or ultracentrifuge which has a force of 150,000g (g = force of gravity) upwards and is able to sediment the smallest viruses. These machines are refrigerated so that the temperature of the virus suspension remains at about freezing point, preventing thermal inactivation. Since the gravitational force required to sediment a particle depends on the size and density of the particle the analyst can therefore use different centrifuge speeds to remove those impurities which are heavier than the virus particles. The virus is then brought down to separate it from the "lighter" impurities. After several cycles of this differential centrifugation many of the viruses can be purified.[33–38]

Attempts at chemical purification must be preceded by testing the reagents to be used, to avoid the use of any which might damage the virus. Once the safety of the reagents is established, standard procedures such as precipitation of impurities with alcohols or solutions of ammonium or magnesium sulphate may be used. A more delicate method

involves the adsorption of the virus onto some material at a specific temperature and pH, from which it can be eluted by washing with a buffered solution of different pH and temperature. Both tobacco mosaic virus and influenza virus were originally purified in this way, by adsorption onto celite at pH 4·5 and eluting at pH 7[39] or by adsorption onto chicken red blood cells at 4°C with subsequent spontaneous elution.[40] More recently, chromatography on DEAE-cellulose has been used and this enables not only the virus particles themselves but also their specific "soluble" antigens to be concentrated and purified.[41] The simplest method, however, is probably the use of fluorocarbons which precipitate impurities while leaving the virus itself in suspension.

If, after chemical purification, it is necessary to concentrate the virus suspension this can be done by dialysis or high speed centrifugation. Once the purified suspension has been obtained and verified as being infectious virus biochemical analysis can be undertaken.

Virus Growth and Multiplication

The living host cell is the common denominator in virus multiplication. Only nucleic acid is required to initiate virus reproduction.[42,43] The protein and other components simply provide a protective covering for extracellular existence and usually enable the nucleic acid to enter the cell.

In general the DNA viruses multiply entirely within the nucleus while those containing RNA multiply in the cytoplasm after an initial stage in the nucleus, but, in all cases the viral nucleic acid "takes over" the host cell metabolism for its own use. The actual mechanisms differ for almost every group of viruses.

Viral multiplication falls into five phases:—Adsorption and penetration; eclipse; replication; maturation; and release. In the first phase the infectious virus particle adsorbs to the wall of the host cell, probably achieved by the mutual attraction of the surface charges of virus and cell, and penetration takes place. The nucleic acid penetrates into the nucleus of the cell where it initiates the commencement of phase two. In the second or eclipse phase no infectious virus is detectable in the cell. The nucleic acid having reached the nucleus "takes over" the host cell metabolism and organises the production of the enzyme systems necessary to form the various components of the infectious virus. The third phase begins with the production of new viral nucleic acid, protein, lipid, etc. In this replication phase each component part of the virus particle is formed separately. Shortly after replication commences the fourth or maturation phase starts and continues until all the component parts have been put together. The virus particles may be completed in the nucleus or in the cytoplasm and in some cases the outermost membranes are not put in place until the virus particle reaches the cell wall. The final or release phase occurs when the host cells burst dramatically as in the case of bacteriophage, or there may be

a gradual extrusion of complete virus from dead or dying cells. Final maturation and release occur simultaneously with those viruses which mature at the cell wall. With this sequence in mind we can now look at the various growth cycles of the different groups of viruses.

The simple DNA viruses like the Adenoviruses and the Papovaviruses[44,45] complete their entire growth cycle within the nucleus. The larger viruses of the Herpes group, containing lipid, undergo the initial stages of multiplication in the nucleus but the lipid membranes are not added until the particle passes into the cytoplasm.[46,47] The Poxviruses, although having DNA, undergo the entire process in the cytoplasm. These are the largest and most complex viruses and may be degenerate bacteria. The essential feature of Poxvirus infection of a cell is the formation of "inclusion bodies" or "Matrix areas" in the cytoplasm[48] and these inclusion bodies are the small "factories" producing new virus.

The Picornaviruses which are simple RNA viruses seem to have the initial stage of RNA replication in the nucleus with the protein component forming in the mitochondrial fraction of the cytoplasm.[49] With the more complex RNA viruses the initial stage in the nucleus is followed by a cytoplasmic stage but final maturation occurs only at the cell wall.[50,51]

In addition to infectious virus particles most viruses cause the infected cells to produce small, non-infectious, virus specific, components. These are soluble antigens and while some are probably excess viral subunits others are distinct from any other components of the virus particle, although specifically produced by the infected cell. Since these soluble antigens have been studied mainly by serological techniques they will be dealt with in serology.

This chapter has been devoted to a résumé of the general properties of the viruses. Their differences have been outlined and it can be seen that they naturally divide into several classes without regard to type of host. These are roughly—an RNA group, a DNA group and a complex group. It is interesting to note that these natural divisions correlate favourably with the evolutionary theories mentioned in the previous chapter.

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CHAPTER THREE

EXPERIMENTAL ANIMALS

SINCE living cells are required for virus multiplication the earliest attempts to grow viruses were made, in the only source of living cells then available—living animals. These animals have now been replaced, for most viral studies, by embryonated hen's eggs and tissue cultures. For the moment we shall deal only with the foundation of virology using such animals as monkeys, ferrets and mice and mention the position of the laboratory animal today.

The rabbit holds the dubious honour of being the first true laboratory animal. Before "The Viruses" were separated from the other disease causing "Microbes" Pasteur and his associates showed in 1881[1] that the intracerebral inoculation of brain homogenate from a rabid animal caused the death of these rabbits, and what was more important, they observed that the virus could be passed serially to other rabbits. This latter observation of continuing passage in a new host resulted in the attenuation of the virus for man and the later use of this material as a vaccine.[2]

In 1909 the rhesus monkey followed the rabbit and was used by Landsteiner and Popper in the growth of Poliovirus. Despite the expense of this monkey, both to obtain and maintain, limited experiments revealed the natural history of poliomyelitis. Again in 1928 the rhesus monkey figured in a new discovery, this time in the cultivation of yellow fever virus.[3] During the next two years the first important steps towards the understanding of the epidemiology of yellow fever were taken before the monkey was replaced by the mouse in 1930.[4] Unlike the rabbit the rhesus monkey is still in common use for determining the potential neuropathogenicity of viruses.

Ferrets were the source of the last major advance associated with an animal, when in 1933 Smith *et al.*[5] discovered that influenza virus could be transmitted intranasally to ferrets using filtrates of gargle material from infected persons. Serial transmission was then maintained by passing extracts of ferret's turbinate tissue. The ferret proved to be a very useful animal because it has a very short lived immunity and can be readily re-infected with the same virus within a few months. Like poliomyelitis and yellow fever in monkeys, the symptoms of influenza in ferrets are similar to those in man.

The Mouse

Swiss white mice[6] are essential at present for the primary isolation of

many viruses from infected humans. Wide use is also made of them in the study of the Arboviruses and the Coxsackie sub-group of the Picornaviruses, as well as the animal pathogens like Foot and Mouth Disease virus. Recently they have been the means of demonstrating

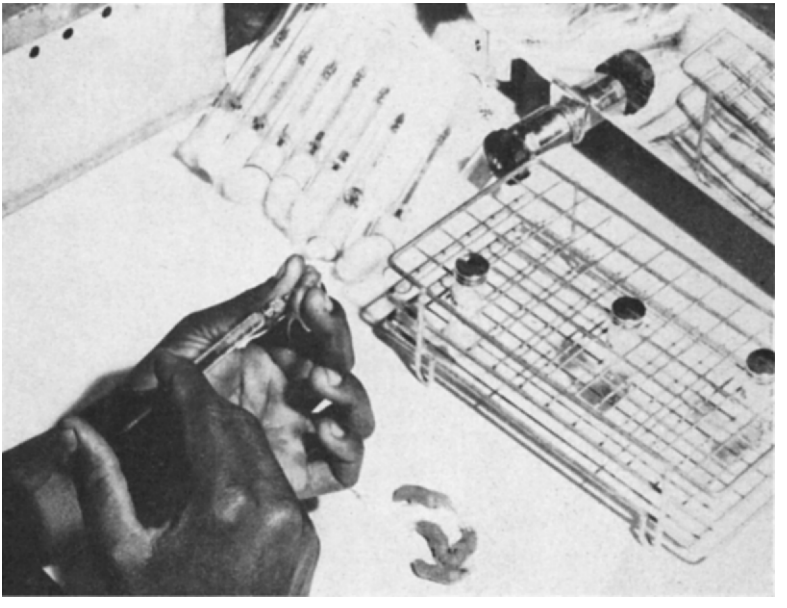

FIG. 3.1. Intracerebral inoculation of day-old mice with an Arbovirus. (*Photo—L. E. Hewitt.*)

the tumour forming properties of the Papovaviruses.[7,8,9] Although adult mice were used originally, today most work is performed on baby or suckling mice. This is largely dictated by ease of handling and cost. Before describing the principle uses of mice certain terms require definition. New-born mice are animals less than three days old. Baby mice are animals over three days old but still being suckled by their mothers. Young mice are those between weaning and adulthood.

Most of the investigations on the Arboviruses are still made using mice for some stage in the work, usually the production of a stock of high titre virus or its initial isolation from clinical material. In general new-born mice are more sensitive and all the known Arboviruses can be propagated by intracerebral inoculation of new-born litters. In many instances satisfactory results follow inoculation by a peripheral route, that is by intramuscular, intraperitoneal, intranasal or sub-cutaneous injection. However, as the mice increase in age their susceptibility to peripheral inoculation decreases and with some types of virus even intracerebral inoculation produces poor and inconsistent results.[10,11]

Finally, in addition to isolation and propagation of the actual virus, new-born mice are used extensively for the detection of antibody in the mouse protection test.[12]

The Coxsackie A viruses are the only other viruses which require mice for reliable propagation and again new-born mice are essential, because

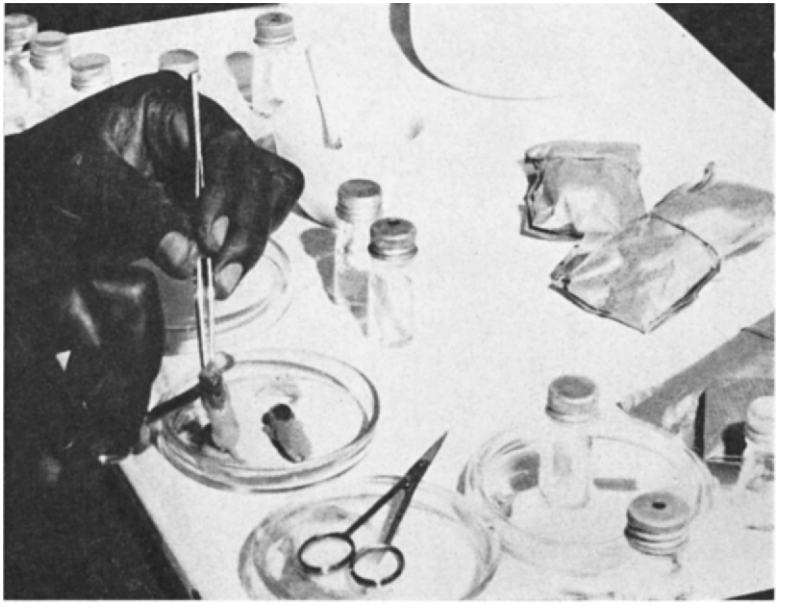

FIG. 3.2. Harvesting brains from mice infected with an Arbovirus. (*Photo—L. E. Hewitt.*)

most strains of these viruses do not produce recognisable illness in adult mice. On the other hand Coxsackie B viruses grow well in tissue cultures, but the type of illness produced in mice is still the only means of determining whether a Coxsackie virus belongs to group A or B.[13]

Other viruses studied in mice include Herpes Simplex, Foot and Mouth Disease, encephalomyocarditis and lymphocytic choriomeningitis viruses. The death of the mouse usually follows infection with any of these.

It is possible to get an idea of the amount (titre) of virus in a given suspension by diluting the suspension in serial tenfold ($\log_{10}$) dilutions and inoculating each dilution into two or more litters of mice. The death of each mouse is recorded until it is obvious that no further deaths will occur. This usually takes about three weeks. The titre of virus can then be calculated as the 50% lethal dose (LD_{50}) of virus, that is, the amount of virus suspension required to kill half the mice which have been inoculated. The simplest and most common method of determining

this is the method of Reed and Muench[14] and an example will now be given.

TABLE 3.1
Calculation of LD_{50}

Dilution of Virus	No. of Mice		Accumulated Values			
					Mortality	
	Died	Survived	Died	Survived	Ratio	Percentage
1/1,000 or 10^{-3} .	12	0	43	0	43/43	100
1/10,000 or 10^{-4} .	12	0	31	0	31/31	100
1/100,000 or 10^{-5} .	10	2	19	2	19/21	90
1/1,000,000 or 10^{-6} .	8	4	9	6	9/15	60
1/10,000,000 or 10^{-7} .	1	11	1	17	1/18	6
1/100,000,000 or 10^{-8} .	0	12	0	29	0/29	0

In the above example, at a dilution of 10^{-6} the mortality is above 50% while at 10^{-7} it is well below 50%. The proportionate distance of the 50% mortality end-point lying between these two dilutions is obtained as follows:—

$$\frac{(\%\text{ mortality at dilution above } 50\%) - (50\%)}{(\%\text{ mortality at dilution above } 50\%) - (\%\text{ mortality at dilution below } 50\%)}$$

$$\textit{i.e.}, \text{ from the above table} = \frac{60 - 50}{60 - 6} = \frac{10}{54} = 0{\cdot}18 = \underline{0{\cdot}2}$$

It is now necessary to multiply this proportionate distance by the logarithm of the dilution factor, in this case $\log_{10}$ of 10 which equals 1·0. This is then subtracted from the logarithm of the dilution above the 50% mortality.

$$\therefore \text{ negative log of } LD_{50} \text{ end-point titre} = -6{\cdot}0 - 0{\cdot}2 = -6{\cdot}2$$
$$\therefore \text{ the } LD_{50} \text{ titre} = 10^{-6{\cdot}2}$$
$$\text{or the titre of the suspension} = 10^{6{\cdot}2}\ LD_{50}$$

Production of Antisera

Although the general principles and techniques of viral serology will be described later the preparation of antisera more naturally belongs here.

When a foreign antigen is introduced into an animal it induces the production of specific antibodies. Since all viruses are antigens of varying potency and are also "foreign" to a vertebrate host, the introduction of any virus results in the production of antibodies. The animal is

then said to be immunised and the serum from this immunised animal is called antiserum. Many species of animals are used in the production of antisera. The most common are monkeys, rabbits, mice, guinea pigs and fowls.

Despite the great variety of immunisation procedures in circulation today only a few are necessary for the production of specific antibodies, the majority depend on the whim of the worker. When a highly specific antiserum is required the special techniques employed are best exemplified by those used to differentiate between different strains of Poliovirus.[15,16,17]

A simple and effective method of producing large volumes of potent and reasonably specific antisera from rabbits has been used by the author for members of the Adenovirus and Picornavirus groups.[18] The virus suspension to be used as antigen is first centrifuged at low speed to remove cell debris and is then injected into the lateral ear veins of two rabbits. This inoculation of 1 ml. of suspension is followed by further inoculations of the same amount every 3 to 4 days so that a total of 8 ml. is administered over a period of 4 weeks. Ten days after the last injection the rabbits are bled from the lateral ear vein when between 20 and 30 ml. of serum can be obtained. (In all attempts at antibody production it is essential to obtain a pre-immunisation sample of blood either from the animal to be used or one of its litter mates.)

Today only the mouse and the hamster[19,20] are used to any great extent in the cultivation and investigation of viruses. The other animals, despite their earlier importance, are now used mainly for the production of antisera.

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CHAPTER FOUR

THE CHICK EMBRYO

THE introduction of the chick embryo in 1931,[1,2] as a useful medium for the growth of viruses, opened the door to the systematic study of viruses and rickettsiae. During the next two decades most of the known viruses were adapted to grow in the chick embryo. With refinements in technique accurate determination of virus identity, growth cycles and antigenic structure became possible. However, in the early 1950's an even more convenient and precise means of studying the multiplication and epidemiology of viruses was developed.[3,4] Tissue cultures replaced the chick embryo—at least as far as most virologists were concerned. This move proved to be premature when T'ang *et al.*, succeeded unequivocally in cultivating the causal agent of Trachoma in the yolk sac of 6–8-day-old chick embryos.[5] Although the Trachoma agent belongs to the Psittacosis group of micro-organisms and is therefore not a true virus it requires virological methods for its cultivation.

It is not my intention to describe in detail the structure of the chick embryo or methods of inoculation but rather to outline those techniques routinely employed, some of which are still the most satisfactory means of studying certain groups of viruses.

When the shell and shell membrane are opened the chorio-allantoic membrane, which surrounds the allantoic cavity, the yolk sac and the amniotic membrane, is uncovered. Within the amniotic membrane is the developing embryo. The virologist has at his disposal the chorio-allantoic membrane (C.A.M.), the allantoic cavity, the yolk sac, the amniotic cavity and the embryo. The allantoic and amniotic cavities are filled with fluid and the importance of these in obtaining suspensions of virus and viral antigens is inestimable.

Before briefly describing the methods of inoculation and their uses a few words on the necessary precautions, when dealing with chick embryos, will not be out of place. Contamination of the embryo with unwanted micro-organisms must be avoided and to that end certain aseptic procedures must be observed. The contaminants fall into three classes, viruses, bacteria and fungi, all of which may result in the death of the embryo. The first are encountered only if the eggs come from an infected flock, the most important being avian Myxoviruses and avian lymphomatosis. Similarly, bacterial contamination can occur from flocks infected with the avian *Salmonella* species. Therefore the flock must be inspected routinely for any sign of disease. Bacterial and fungal contamination can be introduced from the shell. However,

providing the shell is sterilised with 70% alcohol or iodine/alcohol, etc., before use and sterile instruments are used for every manipulation, the chances of contamination are negligible. If these precautions are

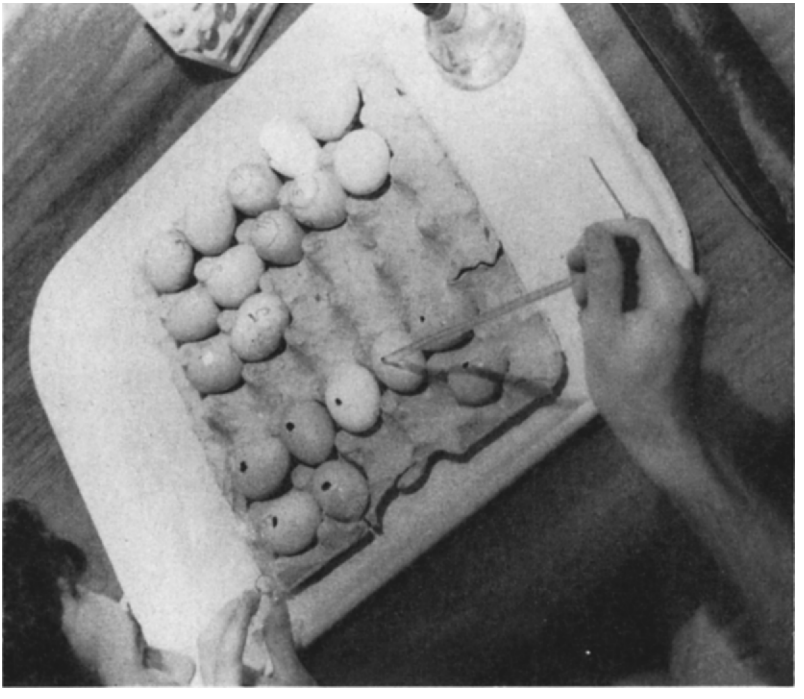

FIG. 4.1. Inoculation of the chorio-allantoic membrane of 10-day-old fertile hen's eggs. (*Photo—L. E. Hewitt.*)

observed the chick embryo becomes a simple and rewarding experimental tool.

Chorio-allantoic Membrane Inoculation

Inoculation of the chorio-allantoic membrane (C.A.M.) with a virus produces focal pock-like lesions, and eggs which have been incubated for 10–12 days are most suited to this method. The area of membrane available for virus growth can be increased by opening a small window in the shell and shell membrane and moving the air space from the end to directly below the window.[6] The virus is then introduced through this window directly onto the C.A.M. The window can be sealed with paraffin wax or a cellulose adhesive tape.

After 2–3 days incubation at 36°C many of the Poxviruses, Herpes simplex and Newcastle Disease Virus (NDV) produce round pocks of varying size and consistency on the C.A.M..[7] The pocks represent focal

proliferation of the ectodermal cells which become surrounded by inflammatory exudate and ultimately show central ulceration. From the size, consistency and colour of the pock it is possible for the experienced

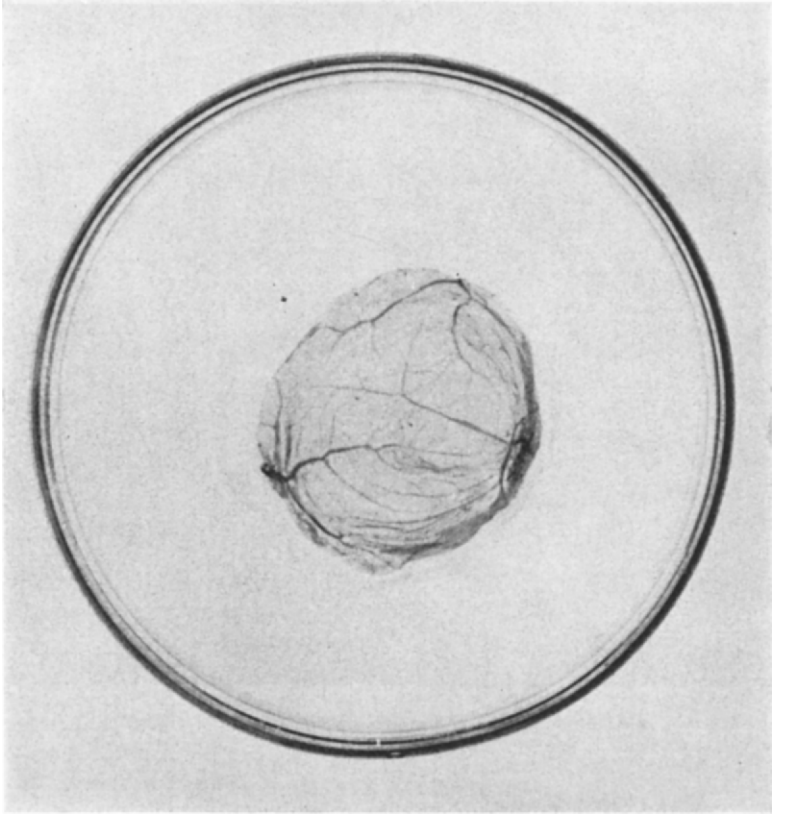

FIG. 4.2. Normal chorio-allantoic membrane. (*Photo—L. E. Hewitt.*)

observer to identify the virus. The main colour variation is between white or non-haemorrhagic and red or haemorrhagic lesions.

If a suitable dilution of virus suspension has been distributed over the C.A.M., the number of pocks can be counted, thereby giving an accurate estimate of the amount of virus present. Each pock originates from a single virus particle. The titre of virus suspension is then obtained by multiplying the number of pocks by the dilution of the suspension and is expressed in pock forming units (PFU).

Allantoic Cavity Inoculation

The entodermal cells found in the allantoic cavity support the multiplication of many of the Myxoviruses in particular influenza A & B,

mumps and NDV.[8] Inoculation is performed in 9–11-day-old eggs by drilling a hole in the shell and injecting the material through the C.A.M. with a hypodermic syringe and needle. The allantoic fluid consists of

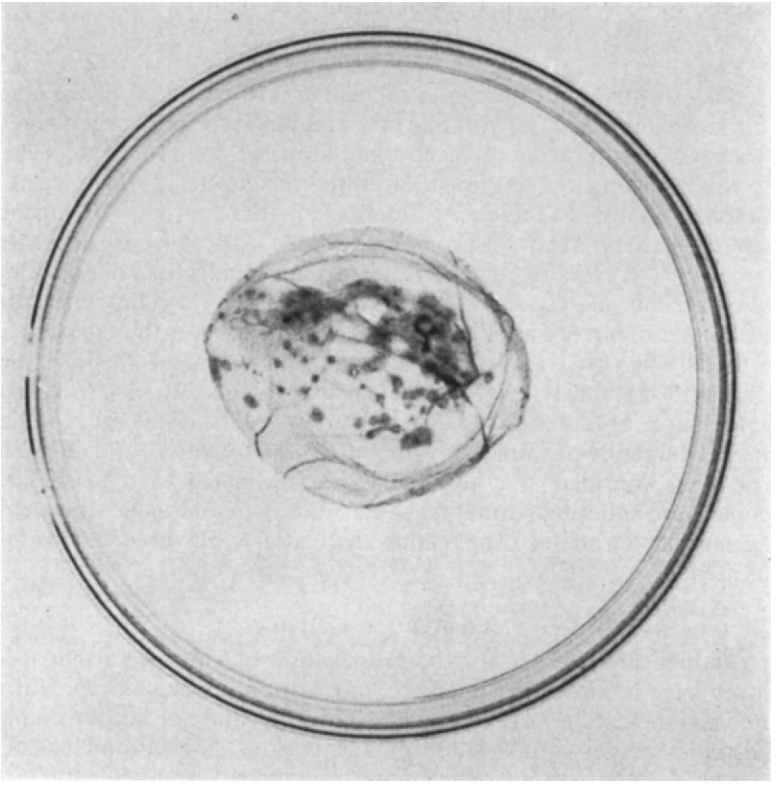

FIG. 4.3. Chorio-allantoic membrane showing the pocks produced by vaccinia virus. (*Photo—L. E. Hewitt.*)

secretions from the entodermal lining and the embryonic kidneys and is about 5 ml. in volume in the 13-day-old egg. After 2–6 days incubation virus is shed into the fluid which can easily be harvested. This is an excellent source of virus for morphological and serological studies and the production of vaccines.

Amniotic Cavity Inoculation

Injection of virus into the amniotic cavity is difficult and necessitates the cutting of a window for accuracy. Several types of cells are in contact with the amniotic fluid. These are the epithelial cells of the amnion, the epidermal epithelium and the cells lining the respiratory and alimentary tracts of the embryo. With most of the Myxoviruses,

inoculation performed on 13–14-day-old eggs followed by 2–4 days incubation, is the most efficient method of isolating and propagating new strains.[9–12] New virus is shed into the amniotic fluid which has a maximum volume of 3–6 ml. in the 13-day-old eggs. Much practice is required to successfully harvest pure amniotic fluid.

Yolk Sac Inoculation

Today inoculation of the yolk sac is the most useful of all the chick embryo techniques. The yolk sac acts as a blood forming organ and as a means of transporting nutrient material to the embryo. The cells of the yolk sac support the growth of all the rickettsiae, the Psittacosis—Lymphogranuloma-venereum—Trachoma (PLT) group of agents, many of the Arboviruses and several Myxoviruses including Rabies[5,7,13,14,15]. Six to eight-day-old eggs are usually used but one-day-old eggs can be used successfully for rabies virus.[16] Inoculation requires much practice but harvesting the yolk sac is much easier than the amniotic fluid. The new virus is present in the actual sac and this is washed clean before emulsifying. Vaccines against typhus, Rocky Mountain spotted fever, Q-fever and rabies are all produced from yolk sac material. This technique is of immense value in the investigation of Trachoma. When one considers that in 1961 it was estimated that 400,000,000 people were suffering from Trachoma[17] the potential value of an easy and efficient means of propagating the causal agent need not be emphasised.

Embryo Inoculation

Although unnecessary for the propagation of viruses, various techniques have been devised for direct inoculation of the embryo. Intracerebral inoculation has been employed in the study of herpes simplex and rabies viruses with success.[18,19] Intravenous inoculation[20] has also been used. However, these techniques are difficult, and while useful to gain specific information they have never become routine.

Death follows inoculation by any of these methods. Usually it is pointless to wait for this to occur, because considerable viral multiplication takes place before death and the presence of new virus can be demonstrated in other ways.

In the case of C.A.M. inoculation the pocks illustrate the growth. By staining the yolk sac emulsion the elementary bodies or "virus particles" of the rickettsiae and PLT groups can be seen, and with the Myxoviruses and Arboviruses in allantoic or amniotic fluids, haemagglutination serves the same purpose. We saw earlier how the Myxoviruses adsorb to erythrocytes. When this occurs the erythrocytes are clumped or agglutinated and this property is called haemagglutination. The presence of these viruses in allantoic or amniotic fluid can be detected by adding an aliquot of fluid to a suspension of susceptible erythrocytes. If virus is present the erythrocytes are agglutinated.

These are the refinements of technique which gave the chick embryo the foremost place in laboratory investigations for many years and which still make it invaluable, despite the introduction of tissue cultures.

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CHAPTER FIVE

TISSUE CULTURES

THE history of tissue cultures is almost as old as Virology itself. Although it is nearly 40 years since Parker and Nye[1] demonstrated viral multiplication in such cultures, it is only in the last fifteen years that they have been widely used. In the early days of tissue culture, before antibiotics were discovered, stringent aseptic procedures were necessary to prevent contamination with bacteria and fungi. This resulted in an atmosphere of awe surrounding anything connected with tissue culture and to this day many people handle tissue cultures as they would a new born baby. In fact, providing established procedures are followed closely, the preparation of tissue cultures is perfectly straightforward.

The earliest successful cultures were grown in 1907 by explanting small pieces of tissue in plasma or lymph clots. The cells then grew out from the tissue into the surrounding clot.[2,3] In 1928 the Maitland cultures followed. This method involves the suspension of small tissue fragments in a fluid capable of supporting the growth of the cells.[4] Nevertheless, the era of tissue culture did not open until 1949 when Enders *et al.*, grew Poliovirus in vitro, in the absence of nerve cells.[5] The use of the enzyme trypsin[6,7] in preparing monolayer cultures set the seal and today the bulk of all virological investigations is carried out using one or other of the above techniques.

In this chapter the methods of preparing tissue cultures will be outlined and reference will be made to their present-day uses. Although plant, insect and cold-blooded animal tissues can be cultivated[8,9,10] only techniques for the growth of mammalian and avian tissue will be mentioned.

Preparation of Material

A smooth glass or plastic surface is required for successful growth of tissue cultures and while disposable plastic tubes and petri dishes are available, they are an unnecessary and expensive source of container. Two types of glass give satisfactory results, namely, the hard, heat resistant borosilicate glass and the soft soda glass. Both may be toxic for cells when new and rigorous cleaning procedures should be followed before their initial use and subsequent re-use. Many cleaning schedules are in use. When choosing, it is wise to bear in mind the purpose of cleaning—to produce consistently good growth of cells. To date the author has found that the cheapest, easiest and most generally efficient

method is the use of a good soap. A cleaning schedule for new glassware which has proved to be of value has six steps.

(1) Soak the glassware for 24–48 hours in concentrated nitric acid.

FIG. 5.1. Roller-drum apparatus (*Matburn Ltd.*) in a water-jacketed, 37°C, incubator (*Laboratory Thermal Equipment Ltd.*). (*Photo—W. Topp.*)

(2) Rinse out the acid in tap water.

(3) Boil in a soap solution for 30–60 minutes and then scrub the inside of tubes and bottles.

(4) Rinse out the soap in at least twelve changes of tap water or running water until all the soap is removed.

(5) Rinse in 3–5 changes of glass distilled, deionised water.
(6) Dry in a hot air oven.

Subsequent cleaning is carried out in exactly the same way but omitting steps 1 and 2. The bungs and liners should preferably be made of silicone or silicone rubber although ordinary rubber can be used. These should be cleaned by boiling in 5% sodium bicarbonate solution and then rinsed and dried as for glass. Finally, before use all glass and rubber ware must be sterilised, either by autoclaving or by dry heat.

Culture Media

The fluid media used for growth are all complex in composition. The earliest were composed almost entirely of biological fluids and tissue extracts such as serum, amniotic fluid, ascitic fluid and chick or bovine embryo extract. Today most media are partially chemically defined and some entirely synthetic solutions have been prepared, which will maintain cells long enough for virus growth to occur. Most of the media for actual cell growth contain whole serum, usually human, calf or horse, a source of amino-acids, a balanced salt solution (BSS) and antibiotics.

The BSS is a buffered solution of inorganic salts, glucose and a pH indicator which has the same osmotic pressure and pH as body fluids. There are many of these solutions but the most important are Hanks', Earle's and Gey's.[11,12]

The source of amino-acids may be an enzymatic hydrolysate of a native protein such as lactalbumin hydrolysate (LAH) or a solution of the amino-acids themselves. The concentration of serum varies from 2% for monkey kidney cells to 20% for human amnion cells, and the only requirement is that it contains no toxic material. The antibiotics must be effective against a wide range of micro-organisms and it is usual to add 100 units or 100 μgms each of penicillin, streptomycin and mycostatin to each ml. of medium. Other antibiotics such as the tetracyclenes and neomycin can be used but those already mentioned are quite satisfactory.

The maintenance media as distinct from the growth media usually contain much less serum, not more than 2%, or they are completely serum free.[13,14] The serum must be tested for the presence of antibodies to the virus under study. It is usual to sterilise the different components

TABLE 5.1
Tissue Culture Media

Type	Calf serum	Hanks' BSS*	4·0% $NaHCO_3$	1·4% $NaHCO_3$	LAH
A. Maintenance	2%	95%	2·5%	0	0·5%
B. Growth	10%	87%	0	2·5%	0·5%

* The Hanks' BSS is prepared without the sodium bicarbonate.

of the media separately, the BSS by autoclaving and the serum and amino-acids by filtration. Two typical partially defined media used by the author[15] for (A) maintenance of cells and (B) growth of certain embryonic cultures are given in Table 5.1.

10,000 units or μgm each of penicillin, streptomycin and mycostatin are added to each 100 ml.

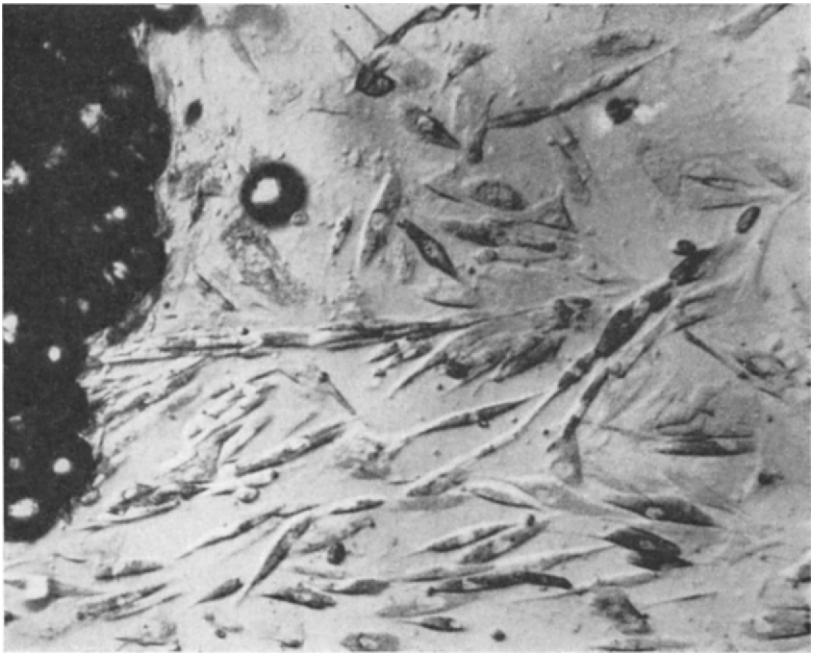

FIG. 5.2. Explant culture from a mesenteric lymph node. Outgrowth of fibroblasts after 18 days incubation. (Unstained preparation ×100.) (*Photo—W. Topp, R. F. Menzies.*)

Primary Explant Cultures

The various explant techniques are all basically similar and their use in modern virology is mainly limited to direct virus isolation from freshly removed tissues. Although more refined techniques using cover slip preparations over cavity slides and perfusion chambers are used in biochemical and physiological studies, most virological explant cultures are prepared in tubes.

While using explant cultures Rowe *et al.*, discovered the Adenoviruses in 1953 in spontaneously degenerating cultures of hypertrophied tonsils and adenoids.[16] This method was used in the isolation of viruses from mesenteric lymph nodes excised from cases of mesenteric adenitis and intussusception.[17] Here the freshly removed tissue is dissected into

small pieces about 1 cumm. Three or four of these pieces are then placed along the lowest two inches of several test tubes in 2 or 3 drops of fowl plasma. The addition of one drop of chick embryo extract clots the plasma and affixes the tissue firmly to the glass. Growth medium containing 1–2% chick embryo extract is added to cover the

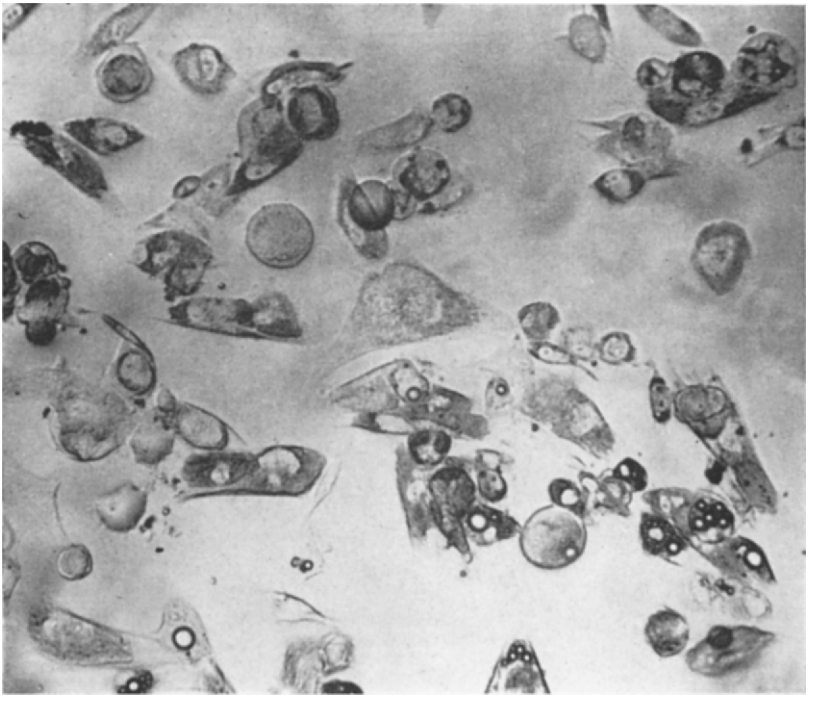

FIG. 5.3. Same culture as Fig. 5.2 three weeks later, showing spontaneous degeneration of cell sheet caused by an Adenovirus present in the tissue which was later identified as type 5. (Unstained preparation ×100.) (*Photo—W. Topp, R. F. Menzies.*)

clots which are then incubated at 34–36°C. During the first week a layer of cells, epithelial from adenoids, fibroblasts from lymph nodes, grows out over the surface of the glass under the plasma clot. Any virus in the tissue advertises its presence by causing degeneration of the cell sheet. This degeneration is called a cytopathic effect (CPE).

Maitland Cultures

This type of culture is used principally for large-scale cultivation of viruses as for Poliovirus vaccine production. Maitland and Maitland in 1928 first applied this technique to cultivate vaccinia virus in suspensions of minced kidney in a mixture of serum and Tyrode's solution.[4] It

was by this method that Enders *et al.* demonstrated the multiplication of Poliovirus.[5]

Fresh monkey kidneys are decapsulated and using scissors the outer cortex is cut into fine pieces. The tissue is then transferred to a 1 litre flask containing synthetic medium 199[13] and incubated at 36°C in a mechanical shaker. After 24 hours the Poliovirus is added and incubation continued for 4 to 7 days when the material is harvested for the manufacture of vaccine.[18] The disadvantage of this method lies in the fact that the cells are not fixed to glass and a CPE cannot be seen.

Monolayer Cultures

As the name implies, these cultures consist of a single layer of cells growing on a glass or plastic surface. This monolayer is obtained by breaking down the tissue into a suspension of single cells and small clumps. Usually this is done by enzymatic digestion or by the use of chelating agents. Trypsin is the most favoured enzyme for this purpose and is also used for the dispersion of established monolayers. Some workers prefer to use the chelating agent versene.

Preparation of Cultures

Youngner's method[7] for preparing primary monolayers of kidney epithelium is the basis of many techniques using solid tissue. After aseptic removal of the kidney the cortex is cut into fragments 4–5 mm in diameter and washed in a phosphate buffered saline solution (PBS)[19] to remove the blood. The tissue fragments are then transferred to 20 ml of a 0·25% solution of trypsin in PBS at 37°C (Bacto-Trypsin 1:250; Difco Corp. U.S.A.). After 10 minutes the trypsin solution is replaced by 20 ml of fresh solution and transferred to a mechanical or magnetic stirring device at 37°C for a further 10 minutes. Then the trypsin solution is passed through three layers of sterile cheese cloth to remove tissue particles and connective fibres. Fresh trypsin is added to the minced fragments in the stirrer and the process is repeated 8–10 times, pooling all the supernatant fluids. The resulting suspension of small clumps and single cells is then centrifuged at 1000 g for 5 minutes and the sediment suspended in approximately 20 volumes of medium (2% horse serum in 199). The cells are then centrifuged out at 600 g, resuspended and centrifuged twice more before final suspension as a 1:50 dilution based on the packed cell volume. The number of cells is determined and the suspension is diluted to give 600,000 to 700,000 cells per ml. From this suspension tube, petri dish and bottle cultures can be prepared by adding to each 0·5 ml, 4·0 ml or 8·0 ml respectively. A monolayer forms within 7 days.

A much simpler and less time consuming method may be used in the preparation of human amnion cells.[15] First strip the amnion from the chorion of a freshly delivered placenta, cut into three or four pieces and wash twice in PBS containing 100 units each of penicillin, streptomycin

and neomycin per ml to remove the blood. Transfer the amnion to a $1\frac{1}{2}$ or 2 litre flask containing 125 ml of 0·2% trypsin solution in Hanks' BSS[11] and incubate for one hour at 37°C without agitating. Transfer the amnion to 125 ml of fresh trypsin and discard the supernatant. After another hour's incubation at 37°C the flask should be shaken vigorously by hand for one minute and incubation continued, shaking every 15 minutes, until the trypsin appears turbid with released cells. This suspension is collected and the amnion is washed in 100 ml PBS. A large number of cells is released and the washing should be added to the trypsin solution which is then centrifuged at 1000 g for 15 minutes. The packed cells are then suspended in 20 ml of growth medium (20% calf, human or horse serum, 0·5% LAH in a BSS) and recentrifuged. Finally the cells are suspended in 20 ml medium, counted and diluted to give 250,000–300,000 cells per ml in 100–150 ml amounts. After overnight incubation at 34–35°C this suspension is dispensed in 1 ml, 6 ml or 15 ml amounts to tubes, petri dishes or bottles respectively and incubation is continued at 34–36°C. The medium should be changed on the third or fourth day to medium A (Table 5.1). Good monolayers form within seven days from trypsinisation.

Many continuous lines of cells have been derived from human and animal tissues. The most common are L cells, a strain of mouse fibroblasts;[20] HeLa cells derived from an epidermoid carcinoma of the cervix;[6] KB, HEp2 and Maben cells established from other human carcinomas[21,22,23] and the FL line of amnion cells.[24] All of these cells are grown in bottles. They can be removed from the glass and dispersed into single cell suspensions by incubation with trypsin or versene for 10–30 minutes. This suspension is then centrifuged, resuspended in medium B (Table 5.1) and seeded into tubes, plates and bottles. After seven days the bottles can be used to prepare a new batch of cultures. Both L cells and HeLa cells have been maintained continuously in some laboratories since 1943 and 1951 respectively. Puck *et al.* reported that it is possible to prepare pure lines or clones of these continuous cell cultures from a single cell using trypsin dispersed suspensions.[25] These clones are extremely useful because their growth characters and susceptibility to viral infection are stable.

Tube Cultures

Monolayer cultures in tubes are prepared to give a confluent cell sheet reaching about two inches up one side of the test tube wall. Providing a reasonably good quality of glass is used, direct observation of the cell sheet is possible under a microscope with 100 × magnification.

When a virus is inoculated into susceptible cultures its growth is seen in changes in the cell sheet. The virus destroys the cells producing the CPE characteristic of that virus. Some of the Myxoviruses form syncytia. For these reasons tube cultures are used for most diagnostic virology.

For virus isolation the material should be inoculated into several tubes of susceptible cells. This material includes throat washings, extracts of throat swabs, low speed centrifuged or filtered suspensions of faeces, C.S.F., and blood. The tubes are then incubated, usually at 36–37°C, either in angled stationary racks or in roller drum machines.[26] In the latter method the drum rotates slowly at an angle of about 5° to the horizontal (Fig. 5.1). Incubation should be continued for up to six weeks, changing the medium when the pH becomes acid. When a CPE is seen the fluid is harvested by storing at temperatures of −20°C or less.

The amount of virus present can be determined by titrations similar to those described in Chapter 3, but by inoculating several tubes with each dilution instead of animals. The end-point of the titration is taken as the last tube to show CPE and the titre is expressed as the 50% tissue culture infectious dose or TCD_{50}. This is calculated in the same manner as the LD_{50}.

The TCD_{50} of a virus suspension can be determined by another method, the metabolic inhibition test.[27] This test uses the knowledge that cells in culture produce large amounts of organic acids during metabolism, which cause the pH to fall below 7·0. When the cells are destroyed by virus infection metabolism ceases and the pH remains higher than 7·0. This difference is readily observed by the change in colour of the phenol red indicator. The end-point is determined by the last tube to show inhibition of cell metabolism. Both the CPE and metabolic inhibition methods may be used for titrations of serum antibodies and serological identification of viruses, but these will be mentioned in the following chapter.

Another type of test tube culture involves the insertion of a small piece of coverslip into the tube when the cells are seeded. A monolayer grows over the surface of the coverslip which can be infected with virus in the same way as ordinary tube cultures. The advantage here is that the coverslips can be removed from the tube and be fixed and stained at various stages of viral multiplication. This method has been successful in the observation of cytological changes following cell infection.

Petri Dish and Bottle Cultures

These cultures are used to accurately determine the amount of virus present. The principle behind the method is to limit the spread of newly-formed infectious virus to neighbouring cells. This is done by solidifying the fluid media with agar. A normal maintenance medium, without the phenol red indicator but with the vital stain neutral red, is mixed with agar dissolved in water. This mixture is allowed to solidify over the cell sheet, after inoculation with virus. Plates treated in this way require a humidified incubator with 3–5% CO_2 in the atmosphere. Sealed bottles can be incubated in ordinary 37°C incubators. The period of incubation varies with the virus used, and colourless areas

are seen in the cell sheet where the cells have been destroyed. Each of these 'plaques' as they are called indicates the presence of one infectious virus particle in the original suspension. This method has been modified in various ways, e.g., the use of a buffering system to obviate the need for CO_2,[28] but the principle remains the same.[29] An accurate estimate of the amount of infectious virus present can be made by counting the number of plaques formed by a given dilution of the virus suspension. This measurement of the infectivity is called the number of plaque forming units (PFU).[19]

As with the test tube cultures, this method can be used for serological testing although its two main uses are accurate determination of the amount of virus produced at each stage of the growth cycle and the production of pure lines of virus. In the latter case plaques are produced as mentioned above. A plaque is then removed from the plate aseptically and the virus present is used as inoculum for fresh cultures. Since each plaque is produced by a single infectious particle, or at worst a small clump, three cycles of picking off, sub-culturing and replaquing a virus suspension, are almost certain to produce a pure culture of virus. This plaque purification method can be used to separate the two components of a mixed virus suspension and should always be performed, if possible, before using any virus strain for the production of antisera.

Finally, the plaque technique is used in the classification of viruses. The morphology of the plaque varies not only between viruses but between different strains of the same virus. The diameter, type of margin and the number of viable cells within the plaque can all be differential characters. In combination with the CPE of tube cultures, plaque production and morphology are useful guides in the identification of newly isolated strains of virus.

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CHAPTER SIX

SEROLOGY

THERE are five main types of serological reaction associated with viral investigations. Three of these, neutralisation, haemagglutination-inhibition (HI) and complement fixation (C-F) are used principally for diagnosis and epidemiology. The other two, agar-gel diffusion and fluorescent antibody staining are primarily research tools, only now well enough understood by all virologists for tentative diagnostic application. The theory behind viral neutralisation and HI tests will be mentioned here but only descriptions of the methods of application of the other three will be given, because they are common to all immunology.

Serological tests are used to investigate both virus diseases and the viruses themselves. They can be divided into diagnostic procedures and biological research methods. The former category involves the rapid, simple and inexpensive tests which are used in laboratory diagnosis, epidemiological investigations and the somewhat more complex work of identifying and typing newly isolated strains. In virus diseases, serology always provides the ultimate diagnosis. Viruses from more than one group often produce identical symptoms and have identical cytopathology in tissue cultures, eggs and animals. Therefore final identification or typing has to be made using mono-specific sera. For example, twenty members of the Picornavirus group cause aseptic meningitis and all produce a similar CPE in tissue cultures. They can be subdivided on their effects in mice and monkeys but all are antigenically different and are readily identified by serology. At the other extreme lies the Russian Tick-Borne Complex of viruses, a sub-group of the Arboviruses, whose members cause a variety of haemorrhagic and encephalitic diseases. These viruses are almost identical antigenically. Serology has shown that there is in fact only one virus with minor antigenic differences between the strains. Thus serology is of immense importance to the epidemiologist in his efforts to trace the natural history of viral infection.

On the other hand the biological research techniques are applied to the antigenic analysis of the products of the virus infected cells, the determination of growth cycles and the site of viral formation within the cell. Used in conjunction with infectivity titrations, biochemical analyses, histological and cytochemical staining, and electron microscopy, serology has given us a comprehensive picture of the virus/host-cell relationship.

Neutralisation

The serum from an animal immunised against a virus has the property of destroying the infectivity of that virus. This neutralisation of infectivity is a specific antigen/antibody reaction and is the basis of all protection or neutralisation tests. The sequence in such tests is as follows. The virus suspension and the serum are first mixed together and incubated to allow the antigen/antibody reaction to take place. The virus-serum mixture is inoculated into a susceptible host and the host is watched for evidence of virus multiplication. If the infectivity of the virus has been destroyed the host will remain unharmed. When the host is an animal this is called a protection test, but when it involves a reduction in the number of pocks formed on the CAM of an egg, the number of plaques in tissue culture, or inhibition of CPE, it is called a neutralisation test.

Before describing the various techniques and applications of the neutralisation test some explanation of the theoretical background will be given.

To the chemist, the neutralisation test is a ternary reaction, i.e., a reaction with three components. In this case two components, antibody and host cell, are competing for the third, the virus particle.[1] When the reaction is divided into two stages one finds that the neutralisation of virus in the first stage follows the Law of Mass Action.[2] Virus and antibody are mixed (neutralisation) in the first stage. An equilibrium is thus set up between virus, antibody, and virus-antibody complex. A proportion of the virus-antibody complex is less easily broken down than the remainder. Several theories have been advanced to account for this phenomenon and all are based on the assumption that after the initial reaction has occurred a secondary reaction takes place which stabilises the union.[2,3,4] The second stage is the addition of the mixture to host cells (detection). The host cell now has to compete with the antibody for the virus particles. If the virus/antibody reaction proceeds at a faster rate than the virus/host-cell reaction, prior incubation will give the antibody an unfair advantage.[1] However, since the purpose of the test in virology is to detect antibody, then the greater the advantage to the antibody the better the test will be. Dilution of the virus/antibody mixture before inoculation of susceptible cells reduces this advantage, i.e., produces dissociation of the virus-antibody complex.[5] Therefore the ideal plan for a neutralisation test is as follows. The virus and serum should be mixed together and incubated under conditions which will allow the equilibrium to become established without thermal inactivation of the virus. This mixture should then be inoculated into the host cells without further dilution. Finally, even when the neutralisation reaction reaches a state of equilibrium there will still be infectious virus present in the mixture. If the detection system involves an all or nothing phenomenon like the death of the host or CPE in a tube culture, excess

antibody must be present to neutralise the new infectious virus produced by the few infectious particles left. This can be overcome in practice by using very small virus inocula such as 100 TCD_{50} so that all the virus is neutralised. Since normal sera often contain heat labile, non-specific virus inhibitors all sera must be heated at 56°C for 30 minutes before use.[1]

The Neutralisation Tests in routine use number seven. The mouse protection test as devised by Sawyer & Lloyd[6] is used for the Arboviruses and Coxsackie A viruses. Modifications have since been made[7] but in general the test is carried out in the following way. An aliquot of virus suspension is mixed with an aliquot of immune serum and incubated for 30 to 60 minutes at 37°C. A similar aliquot of virus is mixed with normal serum and treated in like manner. The virus mixtures are then titrated in infant mice and the deaths are recorded. The titres of each virus mixture can then be calculated and the degree of neutralisation found by dividing the titre of the mixture with normal serum by the titre with immune serum. For example, the titre of virus + normal serum = $10^{-7.9}LD_{50}$, the titre of virus + immune serum = $10^{-4.2}LD_{50}$. The difference between these, the neutralisation index, is $10^{3.7}$ or approximately 5000. In theory this test is far from ideal because the virus/serum mixture undergoes considerable dilution in the body fluids of the animal, but in practice it gives very satisfactory results.

Inhibition of CPE in tube tissue cultures has been applied to a wide variety of viruses. There are several methods in use, one of which is identical with that mentioned above but working in TCD_{50} instead of LD_{50}. One modification of this method is the constant serum-varying virus test. A constant dilution of serum, usually 1/5, is mixed with varying dilutions of virus, usually tenfold steps, from neat to the known end point. A control virus titration is then done and the titre of the serum is expressed as the neutralisation index. This method is expensive in both serum and virus and is identical with the one above, except that there is no dilution of the virus/serum mixture, because titration of the virus is done prior to the addition of serum. Neither of these methods has enjoyed lasting favour and for the Adenoviruses, at least, they are extremely inefficient.[8]

The simplest and least expensive on virus and serum is the constant virus varying serum test.[9] This test is widely applied to most viruses which grow in tissue culture and is the principal method of testing for neutralising antibody in many diagnostic laboratories. The virus is diluted in cell maintenance medium to give 100TCD_{50} per 0·5 ml of suspension and is mixed with equal volumes of serial twofold dilutions of serum, usually starting at 1/4 or 1/5. These mixtures are incubated for 1–6 hours at room temperature or 37°C depending on the virus. Then 1 ml is inoculated into tissue culture tubes which have been drained of medium. A control virus titration is also performed and the end point is read when the virus controls have reached their maximum

expected titre. The titre of the serum is calculated in the same way as TCD_{50} and is expressed either as a dilution of the serum giving 50% inhibition of CPE (i.e., titre = 1/90), or the reciprocal of that dilution (i.e., titre = 90).

In laboratories where hundreds of sera are tested monthly a simple and rapid test is now displacing the above method. This is the metabolic inhibition test.[10,11,12] The preparation of virus/serum mixtures is identical with the previous test but after incubation, a suspension of susceptible cells is added. The cultures are incubated until the pH of the uninfected controls is acid. Neutralisation of the virus is indicated by continued metabolism of the cells and the titre is again calculated as the 50% inhibition of viral growth.

The last of the commonly used methods are the pock reduction and plaque reduction tests. In both cases the constant virus/varying serum method of preparing the serum/virus mixtures is used. These are then inoculated onto the CAM of hens' eggs in the case of the pock reduction test, and the number of pocks produced is counted. In the plaque reduction test, the mixtures are added to bottle or petri dish cultures, overlaid with agar, incubated, and the number of plaques formed is counted.[13,14] The titre of the serum is expressed as that dilution giving a 75% or 90% reduction in pocks or plaques formed. While the CAM method does not give highly reproducible results,[13] the plaque reduction test is probably the most sensitive of all neutralisation tests.

One other test, which has been applied in the study of the Arboviruses, is worth mentioning—Porterfield's method of plaque inhibition.[15] Here the virus dilution is chosen to give almost confluent plaque formation in petri dish cultures of chick embryo fibroblasts. After the cell sheet has been inoculated with virus and overlaid with agar, a porous bead soaked in antiserum is placed on the surface of the agar. As incubation progresses the antiserum diffuses through the agar and its neutralising capacity is measured by the diameter of the zone of plaque inhibition. This test has proved to be more sensitive than mouse protection or tube neutralisation tests for the Arboviruses.[16,17]

The Application of Neutralisation Tests is primarily directed towards the diagnosis and epidemiology of disease. Since virus infection results in the production of antibody, the demonstration of this antibody production indicates that a virus infection has occurred. Fortunately circulating antibody is not present until the second week of illness so that serum specimens collected during the early or acute stage of illness do not neutralise the virus. A rise in antibody titre in the convalescent specimen taken two to three weeks later is considered to be of diagnostic significance. In the constant virus/varying serum test using serial two-fold dilutions of serum, a four-fold rise in antibody usually means that infection by that virus was concurrent with the illness and therefore was the cause of the illness. If only a convalescent specimen of serum is tested the presence of antibody simply tells us that the patient

has been infected with this virus at some time because neutralising antibody can persist for several years. This persistence of antibody is useful to the epidemiologist, who by testing healthy persons in any area can get a good idea of the prevalence of a virus in the community. He can even get an indication of the last occurrence of the virus by testing the sera of infants and children. If no child under the age of three has antibody to the virus then that virus has not been present in the community for three years. Obviously for such work a test must be simple, cheap and rapid. The tube tests, metabolic and plaque inhibition tests are the most popular but all the others have been used at one time or another.

The sensitive plaque reduction test is of great value in the identification of newly isolated viruses. If immune serum is produced in an animal using a plaque purified strain of virus "X" as the antigen, the serum will possess neutralising antibody against virus "X" alone. This is a monotypic serum and can be used to identify all other viruses related to virus "X". If the monotypic antiserum against virus "X" (anti-X) has a titre of 2000 when tested against 100 PFU of virus "X" then we can test new isolates "S" and "T" against this serum. If the serum has a titre of 1500 against "T" but titres less than 20 against "S" we know that virus "T" is the same virus as virus "X" whereas "S" is distinct. Although the plaque reduction test is the most sensitive for this kind of work, the other tests are essential for viruses which do not produce plaques.

Finally, these tests can be used to detect minor antigenic differences. It often happens that the original strains of virus are homogenous antigenically and a "prototype" strain is chosen at random for use in the production of monotypic typing sera. At a later date new strains of this same antigenic type may be isolated which show minor antigenic variations. These are called "prime" strains and antigenic differences can easily be determined by neutralisation.

Haemagglutination-Inhibition

The ability of Influenza virus to agglutinate chicken erythrocytes was discovered in 1941[18,19] and since then members of all but one of the recognised animal virus groups have been found to possess this property of haemagglutination. Not all agglutinate the same erythrocytes and there are several different mechanisms. In some viruses the haemagglutinin or haemagglutinating fraction is an integral part of the virus particle e.g., the Myxoviruses,[20] the Picornaviruses[21] and the Reoviruses.[22] In others it is a soluble antigen or a specific by-product of the virus infected cell e.g., the Poxviruses[23] and the Adenoviruses.[24] However, the haemagglutinin, whether it is virus particle or soluble antigen adsorbs to a specific site on the cell wall of erythrocytes. This erythrocyte-virus complex then agglutinates a second cell by joining it to the original complex. For the Myxoviruses at least, a single virus particle

can cause two erythrocytes to clump together.[20] The types of haemagglutinin vary with the site of attachment to the erythrocyte and the spontaneous disassociation of the virus from the cells. There are several methods of defining the site of attachment.

Cultures of the bacterium *Vibrio cholerae* produce a "Receptor-Destroying-Enzyme" (RDE) which will destroy the sites of attachment of Myxoviruses to erythrocytes.[25] Once treated with RDE the erythrocytes can no longer be agglutinated by the Myxoviruses although they are still agglutinable by Picornaviruses and Reoviruses. On the other hand treatment with potassium periodate renders them useless for agglutination by the latter two groups.[22] It is usual after agglutination for the virus-erythrocyte complex to disassociate. In the case of the Myxoviruses this reaction is enzymatic and the erythrocytes cannot be agglutinated by fresh virus,[26] but with the Reovirus-erythrocyte complex, the cells can be agglutinated with fresh virus after disassociation.[22]

This property of haemagglutination can be destroyed by incubating the virus or soluble antigen with specific immune sera prior to adding the erythrocytes. This is called haemagglutination-inhibition (HI) and can be used to detect the presence of virus or antibody. As a rapid and easy method of detecting antibody the HI test is probably the best. It can be used in place of neutralisation tests for the haemagglutinating viruses.

This test is similar to the constant virus/varying serum neutralisation test. The haemagglutinin is titrated using the most susceptible erythrocytes at the optimum pH and temperature for the virus under study. The end-point of the titration is usually taken as the highest dilution to give recognisable agglutination. This dilution is called one unit of haemagglutinin and it is usual to use four units, i.e., the suspension is four times as concentrated as the end-point dilution. The haemagglutinin is then incubated with dilutions of serum for one hour at room temperature or 37°C, after which the appropriate erythrocytes are added. Incubation is continued to give maximum haemagglutination and the titre of the serum is taken as the highest dilution which just prevents haemagglutination.

Many more sera can be tested in a shorter time using this test. The result of the HI test is known the day the test is performed whereas neutralisation tests do not give an answer in under 48 hours and often it is a week or more. This test can also be used for identification of newly isolated strains but for most viruses the neutralisation tests are preferred.

Complement Fixation

Both the complement fixation test and the agar-gel diffusion test are used to detect the presence of soluble antigens. The history of the C-F test dates back to 1932 and the discovery of the LS antigen of Vaccinia virus.[27] This test invariably involves an 18 hour fixation period at 4–8°C and several refinements have been introduced to reduce the

volumes of serum and antigen originally used. The most common method is that of Fulton and Dumbell,[28,29] which is really a micro-method.

Unlike the antigens detected by the neutralisation and HI tests, which are usually type-specific, the C-F antigens are often group-specific, i.e., all members of a group of viruses produce a common antigen as with the

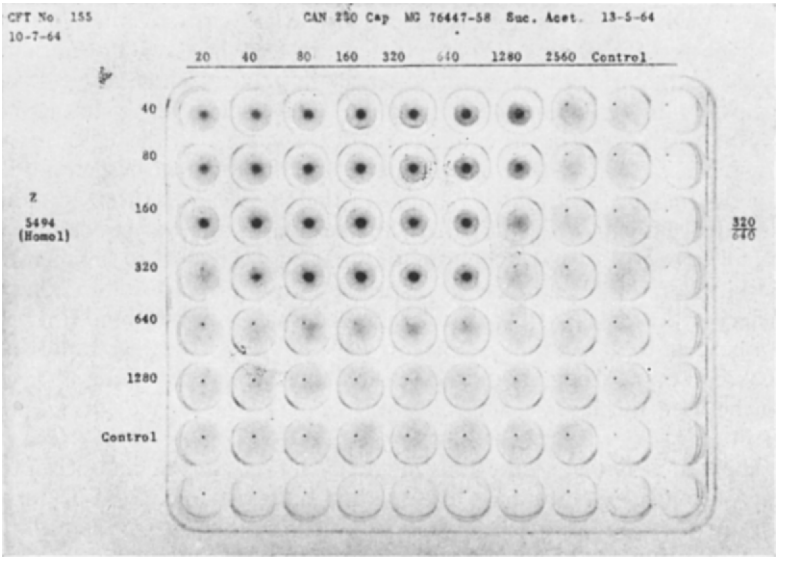

FIG. 6.1. Complement fixation test of a virus—CAN 230—against its homologous antiserum—Z5494. Antigen titre = 1:640; serum titre = 1:320. (*Photo—L. E. Hewitt.*)

Adenoviruses, Reoviruses and Poxviruses.[30,31,32] In the case of the Myxoviruses the C-F antigen is common to all members of each sub-group, i.e., all types of Influenza A share the same C-F antigen.[33] As a result C-F tests are widely used in clinical work to determine whether or not an Adenovirus or Influenza A or B infection has occurred, regardless of antigenic type. In this capacity these tests are invaluable to the epidemiologist in the investigation of an epidemic, when the antigenic type of the causal virus is known. Again the tests are most useful in estimating the efficiency of a homologous vaccine preparation. In the latter case the C-F test is the only useful one, for although vaccinated persons produce high titres of HI and neutralising antibody the C-F antibody levels usually remain low until natural infection occurs.

Agar-gel Diffusion

Agar-gel diffusion techniques visualise the soluble antigens, reveal the presence of more than one antigen and highlight the identity between

two or more antigens or sera. No real attempts have been made to apply this technique to clinical work but its application to antigenic analysis has been attended with considerable success. The most frequently used method involves a layer of agar with a central well, surrounded by several other wells. When immune serum is inserted into the central well and the surrounding wells are filled with homologous and heterologous virus suspensions, the antigen/antibody complex precipitates and forms lines between the serum and virus wells. The number of lines produced with the homologous virus indicates how many soluble antigens are present. Lines formed by the heterologous virus suspensions indicate the presence of antigens common to both viruses. When two antigens are completely identical a continuous line is formed, but when they are slightly different a "spur" is produced. This method has been successfully employed with the Adenoviruses to show the presence of group antigens in viruses derived from different host species.[34,35] It has also been used to demonstrate minor differences between the soluble antigens of strains of Myxoma virus from different parts of the world.[36] Similar information can be obtained using the C-F test in conjunction with chromatography, immune electro-phoresis and absorption techniques, but for most work the agar-gel diffusion technique is much simpler and quicker.[34,37,38]

Further information can be obtained about the structure and composition of the soluble antigens by absorption of virus suspensions or antiserum, by treatment with enzymes, heat or other chemical and physical agents, or by staining the antigen/antibody complex with such stains as acridene orange. In this way the composition and biological properties of each antigen can be determined, as with the Adenovirus group.[34] It is doubtful if there is a more useful tool than agar-gel diffusion for correlating the physical, biochemical and biological properties of the products of the virus-infected cell.

Fluorescent-antibody Techniques

Two types of fluorescent staining are used to study the sites of formation of virus antigens within host cells. These are the direct and the indirect or sandwich techniques. In the direct technique the viral antiserum is conjugated with fluoresceinisothiocyanate or RB200 and then used to stain the virus-infected cells. The antibody combines with the viral antigen *in situ* in the cells. After washing off the excess antibody, the cells are viewed by ultra-violet light. The conjugated antibody fluoresces yellow-green or red. This method is the ideal but it requires high titre antiserum. As a result the sandwich method is more frequently used. In this method an antiserum is prepared against the viral antiserum (i.e., an anti-viral serum antiserum) and is conjugated as above. The viral antiserum is layered over the infected cells, the excess washed off and the anti-viral serum antiserum is added. After washing, the cells are viewed under ultra-violet light as before and fluorescence

is seen. In this case the conjugated serum combines with the viral antiserum which is itself combined with the viral antigens in the cell.

In a detailed, comprehensive study of the growth of Reovirus type 1 in monkey kidney cells Rhim *et al.*[39] combined the use of the direct staining technique with conventional histological staining, electron microscopy and acridene orange staining (with previous nuclease digestion to measure RNA and DNA synthesis). Plaque assays were performed on extracellular and total virus production. From the results of this combination correlations were made using fluorescent-antibody stained cultures harvested at the same time. The complete growth cycle was then determined to a greater degree of accuracy than is possible with fluorescent-antibody studies alone. Similar studies with other viruses have led to a much better understanding of the nature of the various inclusion bodies seen under both the light and electron microscopes.

This technique is now being applied to counting the number of virus particles present in a suspension by adsorbing them to red cells and then staining with fluorescent antibody.[40] The advantage of this method lies in the fact that only the infectious particles are counted, whereas in the electron microscope differentiation has to be made between infectious virus and contaminant particles of similar size which can be present in suspensions.

From this brief account of serological techniques one can see that serology is the corner-stone of Virology. Without it clinical diagnosis would be tedious work with frequent errors, and studies of viral growth and structure, although possible would be much more difficult and less exact.

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CHAPTER SEVEN

VIRAL INTERFERENCE AND ANTI-VIRAL SUBSTANCES

THE discovery of interferon[1] suggested a new approach to the problem of curing virus diseases. Among the most effective chemotherapeutic agents in bacterial infections are the antibiotics which are the byproducts of microbial growth. It is therefore only natural to hope that some byproduct of viral growth might eventually be developed as an effective anti-viral substance. Many enzyme inhibitors can prevent viral multiplication *in vitro* but to date there are no unequivocally successful chemotherapeutic agents. Antibiotics have no effect on viral growth because they are directed against bacterial enzyme systems. Viruses, on the other hand do not have any enzyme systems and rely on those of the host-cell/virus complex. Consequently we are in the delicate position of having to find a substance which will inhibit the enzyme systems engaged in forming virus but which will have no deleterious effect on the normal cell enzymes.

The ability of one virus to interfere with the growth of another was first described by Hoskins in 1935.[2] Since then considerable attention has been directed to this property of viruses, culminating in the discovery of interferon in 1957.[1] A second virus inhibitor, distinct from interferon, has since been found,[3] and it seems likely that the property of viral interference is only an expression of the action of interferon and similar substances. The production of interferon is a property of the virus/host-cell reaction and although it tends to be specific for the cell species from which it was produced, protection of cell cultures from different animals is frequently obtained.

Interference

Viral interference is usually taken to mean mutual exclusion, i.e., once a cell has been infected with one virus a second virus is prevented from entering and multiplying within the cell. Occasionally when two viruses are inoculated simultaneously neither will multiply. Although cells can be successfully infected with a variety of pairs of unrelated viruses this exclusion does occur[4] and can be demonstrated in many ways, using intact living hosts or tissue cultures and live or inactivated viruses. The sequence of events is as follows. The host-cell system is inoculated with the interfering virus and a varying length of time is allowed to elapse before the addition of the second virus. Multiplication of this challenge virus is inhibited, due to the already present association of the

host cell with the original virus.[5] It appears that this interference is brought about by the production of the substance interferon by the infected cell but there is no complete proof of this as yet. Before discussing the production, properties and mode of action of interferon some brief mention will be made of the different ways in which interference phenomena can be observed.

Interference has been demonstrated in the intact animal, the chick embryo and tissue cultures. A good example of this phenomenon in mice is the inhibition of Western Equine Encephalitis (WEE) virus by influenza virus.[6] The influenza strain used in this experiment produces no disease in mice and appears to undergo only one cycle of multiplication in mouse brain.[7] When a large dose of influenza virus is given intracerebrally, either before or at the same time as WEE the mice show no signs of illness. This high degree of protection against WEE lasts for about 7 days after inoculation of the influenza and then it gradually wanes until full susceptibility is restored at about the 21st day. An example of interference by killed virus is the protection of mice with U-V inactivated ectromelia virus. Mice inoculated simultaneously with a lethal dose of ectromelia virus and a massive dose of U-V inactivated ectromelia show little or no signs of infection and live virus cannot be isolated from sacrificed animals.[8]

Many interference effects can be observed in chick embryos infected with the Myxoviruses. In the case of influenza virus the addition of inactivated virus of the same or different strains before or after the inoculation of live virus markedly reduces viral production, and the degree of interference drops the longer the addition of the inactivated virus is delayed.[9] One of the most important of the interference phenomena is the fall in virus yield when mumps virus is serially passed without dilution in the allantoic cavity.[10] This is an example of auto-interference. When undiluted mumps virus is inoculated the titre of the resulting virus is constant for several passages and then drops dramatically. Subsequent undiluted passage of this product results in repeated rises followed by drops in titre. The allantoic fluids of the passage which immediately precedes the fall in titre have a very high interfering capacity when tested against other viruses. Serial passage of diluted virus always results in a good yield of virus. This is important in the preparation of stock virus suspensions and applies to many viruses other than mumps.

Examples of interference between pairs of related and unrelated viruses in tissue cultures have been described.[5] Of these the most interesting is the resistance of various cell cultures, chronically infected with a variety of viruses, to re-infection with active homologous virus. When Newcastle Disease Virus (NDV) is inoculated into cultures of HeLa cells most of the cells are destroyed. The few remaining cells multiply and produce fresh cultures which are chronically infected with NDV. However, only one infectious unit of NDV is produced per 100

cells and the cultures cannot be destroyed by re-infection with active NDV, i.e., the same suspension as was used to infect the initial cultures. It has also been shown by other methods that the challenge virus can enter the cells and will multiply after prolonged incubation.

The interference phenomenon is very common and can be demonstrated in all virus/host-cell systems. Probably interference always occurs but some viruses are more susceptible than others.

Interferon

The substance interferon was first detected in cultures of fragments of allantoic membranes which had been inoculated with heat or U-V inactivated influenza virus. Fresh fragments which were incubated with this substance could not be infected with live influenza virus. Subsequently it was found that cells infected with live influenza virus produce interferon, and that many other (probably all) viruses stimulate its production in most, if not all, cells. Interferon is partially species specific, i.e., interferon produced by infection of cells of one species is most efficient in cells from that species.[11]

Purified interferon has a molecular weight of 60,000 and it appears to be a protein which is a poor antigen. It is stable between pH 1 and pH 11 but is destroyed by such proteolytic enzymes as pepsin and trypsin, and by shaking with ether, although deoxyribonuclease and ribonuclease have no effect on it. The suggestion has been made that it is a normal cell constituent whose production is stimulated by viral infection.[11,12] Evidence of the specific antigenicity of interferon has since been obtained which indicates that it is distinct from any antigenic component of the cells from which it is produced.[13] Interferon would therefore seem to be a product of the virus/cell interaction possibly arising from stimulation of adaptive enzymes present in all cells. Unlike antibody it is non-specific in its action and represents a local defence mechanism.

The inhibitory activity of interferon is usually demonstrated by incubating the cells with the preparation containing interferon for 12 to 24 hours before adding the challenge virus. Cells treated in this way are unable to synthesise infectious virus, viral haemagglutinin or viral nucleic acid, but they produce more interferon. Neither infectious RNA nor complete infectious virus can infect interferon-treated cells indicating that interferon acts within the cell after penetration of the virus.[1,14,15,16]

A clue to the mode of action of interferon was given in the early observations that although interferon was produced by virus-infected cancer cells it did not protect these cells against viral invasion.[17,18] It has since been shown that interferon does protect HeLa cells from viral infection although larger doses are usually required than those which will protect primary cells.[19,20] Since cancer cells are wholly or partially independent of oxidative pathways for their metabolism[21] this suggests

that interferon acts as an inhibitor of an energy providing oxidative process and subsequent work has tended to confirm this hypothesis. Interferon-treated cells show increased glycolysis, increased oxygen uptake, decreased uptake of inorganic phosphate and decreased oxidation of glucose via the pentose cycle. These alterations are similar to those produced by substances which uncouple oxidative phosphorylation.[22] Other observations have indicated that interferon inhibits the formation of viral RNA and when added after the cells have been infected can even prevent RNA replication.[16,23]

In the light of these findings the theory put forward by Isaacs *et al.* in 1961[22] would appear to be valid. This hypothesis states that interferon acts by uncoupling nuclear oxidative phosphorylation. Unfortunately interferon has been found to inhibit the multiplication of at least one virus when it is grown under anaerobic conditions.[24] When HeLa cell cultures are allowed to age before the addition of interferon the protection obtained is as good as that given with primary cell cultures.[25] These results indicate that either the hypothesis of Isaacs *et al.* is incorrect or that interferon may act in more than one way. There is not yet sufficient evidence for a decision as to which is correct but the evidence in favour of the uncoupling of nuclear oxidative phosphorylation certainly suggests that this is at least part of the story.

The influence of interferon on the virulence of a virus and on the recovery from viral infections is being investigated in great detail. Although interferon protects cells from viral infection when it is added before the virus it cannot prevent viral multiplication once infection has occurred. However, it appears to do so if added shortly after infection, but once the replication system has reached an as yet unknown stage of development it cannot be affected by interferon. Similarly the transformation of chick embryo cells by Rous sarcoma virus can be prevented by interferon, but once initiated, the multiplication of transformed cells continues.[26] Nevertheless viral infection of cells results in the production of interferon and this then may be present in sufficient quantities to prevent further infection of other cells.

It has been suggested that the production of interferon may be a more important factor in the recovery from disease than antibody production. Two factors suggest that this is the case. First, and obvious, is the fact that interferon is produced at the same time as the first batch of new virus whereas antibody production does not commence for several days after the onset of illness. The second is that maximum production of interferon occurs at a higher temperature than the optimal for virus growth.[27] Since fever is a common feature of virus infections this may stimulate the production of interferon leading to a more rapid and complete protection of uninfected cells.

The question of why a given strain of virus is more virulent than another is not definitely answered, but some light has been shed on this problem in recent years. Avirulent strains of measles and poliovirus

have been found to produce more interferon than virulent strains.[28,29] Similarly virulent strains are less susceptible to the action of interferon than avirulent strains.[30] In general it would appear that lower production of, and sensitivity to interferon is one of the factors which determine the virulence of a strain of virus.[27]

Antiviral Substances

The discovery of an effective viral chemotherapeutic agent must be the dream of all drug manufacturers. Although the production of substances which will inhibit viral multiplication is relatively simple, to find one which will selectively prevent viral growth and at the same time leave the host intact is much more difficult. Many compounds, both natural and synthetic, will inhibit viral multiplication *in vitro* but almost without exception they have little or no effect in the intact host when administered in doses which are not toxic.

The naturally occurring compounds are similar to the antibacterial antibiotics, while the synthetic compounds are analogues of such essential metabolites as purine and pyrimidene bases and amino acids. The main problem is that most, if not all, of these inhibit the metabolism of normal cells as well as that of infected cells. Therefore to date none of these inhibitors has proved to be of any use in the treatment of viral diseases. The problems underlying the synthesis of an effective chemotherapeutic agent are presented in two excellent reviews to which the interested reader is directed.[31,32]

One compound whose mode of action is unknown has been tested with apparent success against smallpox.[33] The drug, N-methylisatin β-thiosemicarbazone (compound 33T57, "Marboran"), was administered to 1101 house-contacts of smallpox in Madras, India. Only three mild cases of smallpox were seen in this group whereas 78 cases with 12 deaths were recorded from 1126 contacts who were untreated. This drug appeared to be effective even when given late in the incubation period and the results outlined are the first successful application of a synthetic compound in the prophylaxis of a virus disease.

It therefore seems that the long search for antiviral chemotherapeutic agents is at last beginning to show promise. The application of interferon to the treatment of viral infections is just commencing and much can still be expected from this approach, although a great deal will have to be done before antiviral agents are in regular effective use against those diseases caused by viruses.

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CHAPTER EIGHT

CLASSIFICATION

ALTHOUGH there is no universally recognised system of classifying viruses, decisive efforts are being made to bring order to the chaos which reigns at present. We have already seen that Lwoff[1] has removed the Psittacosis-Trachoma-L.G.V. agents from the families of viruses and the system of classification outlined here will be based on the principles now agreed upon by the various sections of virologists. With this in mind an initial division of the viruses into Plant, Bacterial, Vertebrate, and Arthropod groups will be made. The artificial nature of this division will be seen later, but most virologists are agreed that our present state of knowledge does not allow us to adopt a Linnean binomial system of Nomenclature with the attending emphasis on genetic relationships.

In August 1950 agreement was reached at the Fifth International Congress of Microbiology in Rio on the method of viral classification. The eight "Rio" criteria are:[2]

(1) Morphology and methods of reproduction.
(2) Chemical composition and physical properties.
(3) Immunological properties.
(4) Susceptibility to physical and chemical agents.
(5) Natural methods of transmission.
(6) Host, tissue, and cell tropisms.
(7) Pathology, including inclusion body formation.
(8) Symptomatology.

Subsequent work has proved (1) and (2) to be of fundamental importance in dividing viruses into groups, with (3) and (4) of somewhat lesser value. Subdivision within each group can be made using the information in (3), (5), (6), and (7). Symptomatology has been discarded, at least by the majority of "animal" virologists as being of little value in classification.

Consideration of the characteristics which order the viruses into major groups shows that the first division is made by identifying the type of nucleic acid because, by definition,[1] a virus can possess only one type—either DNA or RNA. By determining the number and shape of the capsomeres in the capsid of those viruses which possess an icosahedral form a second division can be made. Other morphological characters such as the number of membranes outside the capsid and of course the size of the virus particle provide further definition. Differentiation

is made between those viruses which are formed entirely within the nucleus, the ones which multiply within the cytoplasm and others which undergo an initial stage in the nucleus and mature in the cytoplasm. In the case of the Myxoviruses and at least one Arbovirus[3] final maturation of the virus particle takes place at the cell surface. The last major characteristic is the sensitivity of the virus to inactivation with di-ethyl ether or bile salts. More recently, work has shown that certain groups of animal viruses possess a common antigen, the Adenoviruses and the Poxviruses being the most adequately investigated groups.[4]

In addition to these main characteristics which apply to all groups of viruses there are special features which serve in the identification of individual groups, such as the production of a characteristic inclusion body by certain insect and animal viruses, transmission by arthropod vector with multiplication of the virus in the insect, and other "biochemical indicators".

With these points in mind we can examine the position of each of the four major divisions of the "virus kingdom" as defined earlier. In each case the classification which is at present recognised will be discussed and in the light of recent discoveries an indication of possible future developments will be given.

Viruses Infecting Vertebrates

At a meeting of the Virus Subcommittee of the International Nomenclature Committee in Montreal in August 1962 agreement was reached on the classification of the animal viruses into major groups, and most of these were officially designated.[5,6,7] Eight major groups were defined and the characteristics identifying them are summarised in Table 8.1.

TABLE 8.1

Classification of the Animal Viruses

Group name	Nucleic acid	Size in mμ	Number of capso-meres	Mem-brane outside capsid	Multipli-cation	Mature at cell surface	Sensitive to ether
Poxvirus	DNA	150–300	?	+	C	0	0 or +
Herpesvirus	DNA	100–200	162	+	N	0	+
Adenovirus	DNA	70	252	0	N	0	0
Papovavirus	DNA	25–45	42 or 92	0	N	0	0
Myxovirus	RNA	80–500	?	+	C or N-C	+	+
Arbovirus	RNA	20–100	?	+	N-C	+	+
Reovirus	RNA	60	92	0	C	0	0
Picornavirus	RNA	20–30	Few	0	C or N-C	0	0

(Note: C = cytoplasm; N = nucleus; N-C = nucleus and cytoplasm.)

Approximately 350 of the viruses known to infect vertebrates are incorporated in this system of classification. Many of those as yet

unclassified may be found to belong to one or other of the groups when all their "Rio" characters are determined. There remain, however, the viruses causing rubella, infectious hepatitis and infectious mononucleosis, which may belong to a separate group. Rabies, lymphocytic-choriomeningitis and a variety of other non-human viruses have properties similar to one or more of the defined groups but it is not yet possible to decide to which, if any, they belong, although rabies virus has now been shown to have a structure similar to the Myxovirus group.[8]

The Insect Viruses

Holmes[9] produced the original scheme of classification of the insect viruses in 1948 on a basis of pathogenicity and host range. He specified two genera but in 1953 by applying the "Rio criteria" Bergold[10,11] was able to define four genera. Since that time considerable advances have been made in morphology, chemical composition and other biological properties, but at the present time this system of classification is still in use. We shall therefore discuss the changes likely to come as a result of increasing basic knowledge of the group.

Genus BORRELINA (Paillot). These are mostly rod-shaped viruses of 20–70 by 200–400 mμ occurring within dense polyhedral-shaped inclusion bodies of about 0·5 to 15 μ in diameter. The virus particles often carry their developmental membranes which are easily shed. The viruses form polyhedral bodies and replicate themselves entirely within the cell nuclei of insect larvae and occasionally insect pupae. The type species is *Borrelina bombycis* and six other species were defined by their natural host range.

Recent data has shown that this genus is probably valid. The viruses possess DNA and probably have a widely spaced helical structure, similar to TMV, inside an intimate membrane.[12] Incomplete serological investigations also show this group to be related antigenically.[13] Strains from different insect species vary directly as the relationship between the host alters and it may prove difficult to substantiate a specific name for each strain.

Genus BERGOLDIA (Steinhaus). This genus consists of mostly rod-shaped viruses of 30–70 by 200–400 mμ without, and 50–100 by 200–500 mμ with the developmental membrane which is not usually lost. They occur within dense ellipsoidal-shaped granules less than 1 μ long in the nuclei or cytoplasm of the cells of insect larvae or pupae. The type species is *Bergoldia calypta* and six other species were defined on the basis of host range.

This genus would also appear to be valid although the fine structure of the virus particles is similar to that of the genus Borrelina.[12] The nucleic acid is DNA and the internal structure is probably a tightly wound helix. Incomplete serological investigations show the members of this group to be antigenically related and that there are few cross-reactions between the species of the *Borrelina* and *Bergoldia* genera.[13,14]

Genus SMITHIA (Bergold). Unlike the above genera these are spherical viruses of 65 mμ diameter occurring in dense polyhedral-shaped inclusion bodies of 0·5 to 15 μ in diameter. The viruses replicate themselves within these cytoplasmic inclusion bodies in insect larvae. The type species is *Smithia rotunda* and no other species has been defined. Recent work on other viruses probably belonging to this group has shown that the nucleic acid is RNA[15] and that they have an icosahedral shape.[16]

Genus MORATOR (Holmes). These spherical viruses are 25 mμ in diameter, are not associated with inclusion bodies, and occur in insect larvae and pupae. The type species is *Morator nudus* and no further species has been defined. Little further work has been carried out on this virus but in size alone it appears to belong to a separate genus.

Several other viruses which do not form inclusion bodies have been described, some outwith the *Insecta* in the *Arachnida*. The most studied of these has been the iridescent virus of *Tipula paludosa* known as *Tipula* Iridescent Virus (T.I.V.). This is a large icosahedral virus (130 mμ in diameter) which multiplies in the cytoplasm. It contains DNA and the capsid is composed of 812 capsomeres. This and the strains from the *Arachnida* are probably the fore-runners of other distinct groups or genera.[12,17]

The Plant Viruses

The classification scheme defined by Holmes[9] of the plant viruses after being almost universally accepted by plant virologists has recently been discarded in at least one important piece of work.[18] Holmes' scheme divided the plant viruses known in 1948 into six families, on the basis of the type of disease they caused in their natural host. Subdivisions into genera and species were made on the pathology and severity of the disease, the insect vector, and the natural host range. This gave rise to 128 species in 16 genera. Smith, in the second edition of his "*Textbook of Plant Virus Diseases*" (1957) used only the English popular names in order that "the controversial question of the nomenclature of plant viruses" would be avoided.[18] Obviously at the present time there is no approved classification of the plant viruses although several workers have suggested schemes of varying scope.

Seven properties have been defined by Knight[19,20] which may be used to differentiate between similar viruses and these appear to be potentially useful at the specific level. In order to state that two viruses are strains of the same species they must show:—(1) Serological cross-reactions; (2) Cross-protection; (3) Similarity in host range; (4) Similarity in method of transmission; (5) Similarity of resistance to destruction of infectivity by chemical and physical agents; (6) Similarity in response to genetic change in the host; (7) Coincidence of specific chemical and physical properties. This last property is of less value than with other groups as all the plant viruses so far studied contain RNA.

Recent attempts to arrange the plant viruses into large rational groups appear to be valid.[17,21] There is an initial division between those transmitted by aerial vectors and those transmitted by other means. The viruses which multiply in insect vectors fall into at least two morphological groups. The larger group consists of near spherical (icosahedral) viruses of about 60 mμ in diameter, while those of the other group superficially resemble the rod-shaped viruses causing nuclear polyhedroses of insects. Harrison[21] has succeeded in dividing some of the soil-borne viruses into seven distinct groups although several unclassified strains remain. Yet another scheme has been drawn up, based on size and appearance as seen in the electron microscope.[22] This gives twelve groups for the rod-shaped viruses and is suggested as a starting point for serological and other determinations. One can see that the problems associated with classifying the plant viruses are being slowly solved.

Bacteriophages

Until a few years ago all bacteriophages were thought to have DNA as the nucleic acid.[23] Now at least one RNA phage has been discovered, f2[24], and it is likely that many more will come to light in due course. Similarly the belief that all had a spherical or polyhedral head and a narrower cylindrical tail has been shattered by the discovery that two have icosahedral form probably with twelve capsomeres in the capsid—ϕX174 and ϕR.[25,26]

Classification into major groups has not been attempted, nor has there been any progress towards a more suitable nomenclature than "coliphage T2" etc. With the discoveries outlined above, the amassing of information on the size and shape of those particles with tails, and biochemical analysis of the nucleic acid component it should be possible to clarify the picture in the not too distant future.

Future Developments

Although the time is not yet ripe to produce a comprehensive system of classification and nomenclature for the "Virus Kingdom" the anomalies of the present "system" are becoming obvious. The multiplication of certain plant and animal viruses in their insect vectors highlights the absurdity of considering these viruses to be "different" in a "fundamental" sense. Recent discoveries have shown that Wound Tumour Virus of plants, which infects three related species of agallion leafhoppers, has a similar morphology and is antigenically related to the Reoviruses.[27] This is the first major step towards the establishment of a family of viruses whose members are pathogenic for at least three of the four host groups.

Finally the objection to the Linnean binomial system is its implication of knowledge of genetic relationships and this will continue as long as two schools of thought exist on the evolution of viruses. However it

should not prevent a start on the extension of the system outlined in Table 8.1 to cover the viruses other than those infecting vertebrates. Probably morphological types such as the classical tadpole-shaped bacteriophages will prove to be specific for their hosts but many of the virus groups will certainly have members specific for each type of host.

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CHAPTER NINE

THE PICORNAVIRUSES

THE Picornaviruses are the smallest viruses yet identified and all have RNA as the nucleic acid.[1] In 1957 the Polio, Coxsackie A, Coxsackie B and ECHO viruses were grouped together as the Enteroviruses[2] and in 1962 the Picornavirus group was named to include all viruses with similar properties. The name was constructed from "pico" meaning "small" and RNA, from their nucleic acid.

The human members are the Enteroviruses, the Rhino or "common cold" viruses and a variety of unclassified strains which will eventually take their place in one or other of the above sub-groups. To date there are approximately 80 distinct human Picornaviruses with many others awaiting full serological identification. During a period of four years in the North East of Scotland the author found no less than six viruses belonging to this group, which he was unable to identify. One has since been classified as ECHO 30 and the remainder will no doubt prove to be identical with other types throughout the world. In fact there is no reason to suppose that we are yet anywhere near total recognition of all the members of this important group.

A similar situation exists among the lower animals. The types affecting animals must already be reaching 100. Enteroviruses, similar to, but antigenically different from the human types, have been demonstrated in monkeys, swine, cattle, cats, mice and fowls. Some of these viruses, like Teschen virus or swine poliomyelitis, cause well-defined disease, while others have been found only in the faeces of healthy animals. The most important are probably those causing Foot and Mouth Disease of Cattle. Finally there are the viruses which cause encephalomyocarditis (EMC) in mice.[3]

All of the sub-groups of the Picornavirus family are sufficiently alike to be considered together.

Biological Properties

The properties of the Picornavirus group have been defined as follows:—[1]

(1) They are small in size—15–30 mμ in diameter.

(2) They are resistant to ether and chloroform.

(3) The nucleic acid is RNA.

(4) Those which have been studied show cubic symmetry, possibly icosahedral, but the number of capsomeres is not yet established.

The particle size of representative strains of each of the sub-groups

has been determined and all fall within the range 23–29 mμ in diameter.[4–9] The Enteroviruses are uniformly 28 mμ;[4] the EMC viruses vary between 25 and 29 mμ;[5,6] the Foot and Mouth Disease viruses are 23 mμ.[7]

Biochemical analysis of the virus particles has only been performed on the Polioviruses and one strain of EMC virus.[10] The polioviruses all contain 20–25% RNA and 75–80% protein, weigh $1{\cdot}1 \times 10^{-17}$ gm. with a molecular weight of $6{\cdot}8 \times 10^6$. Results obtained for EMC virus correspond closely with these, being 30% RNA and 70% protein.[11] The amino-acid composition of the protein of Poliovirus type 1 has been determined and differs only in the proportions from animal proteins.[12] The bases in all three types of Poliovirus are in similar proportions but no other members have been studied in such detail.[13]

In any given suspension of Poliovirus only 1 in 30 to 1 in 260 of the virus particles is infective[14] but most adsorb to susceptible cells at 4°C.[15] On warming to 37°C, 50–60% of the particles are eluted and can no longer adsorb to cells although infectious RNA can be extracted. Possibly minor variations in surface structure account for this, and also for part of the non-infectivity of the majority of the virus particles.[16]

The inactivation of infectivity of the different viruses in acid media varies considerably and is a dividing feature within the group. Each determination has been made using different times and temperatures of exposure and the results are summarised in Table 9.1.

TABLE 9.1

Sensitivity to Acid pH

pH	2	3	4	5	5
Time	3 hours	3 hours	3 hours	1 hour	1 hour
Temperature	37°C	RT	4°C	37°C	4°C
Poliovirus	—	—	—	—	—
Coxsackie A	—	—	—	—	—
Coxsackie B	—	—	—	—	—
ECHO 1–9, 11–27, 29–32	—	—	—	—	—
ECHO 28	+	+	+	A	NT
Rhinovirus	+	+	+	A	NT
FMDV	+	+	+	NT	A
Reference	17, 18, 19, 20	19, 20	19, 20	8	21

Note: — = stable; + = completely inactivated; A = only slight residual infectivity; RT = room temperature; NT = not tested; FMDV = Foot and Mouth Disease Virus.

It is clear from these results that the Enteroviruses, excepting ECHO 28, form a homogenous group while ECHO 28, the Rhinoviruses and FMDV form another. Such results as are available for the other animal viruses indicate that they belong to the former group.

All the Picornaviruses are rapidly inactivated by formalin at all temperatures above freezing point.[19,22] The Enteroviruses are also inactivated by oxidising agents[23] and desiccation,[24] while EMC strains

are destroyed by high concentrations of halogen ions.[25] These properties are probably common to all members of the group. Although heating to 61°C rapidly destroys the infectivity of all these viruses the stability at lower temperatures varies not only between the sub-group but also between individual strains of each antigenic type.[5,6,19,20,26] In general, the Enteroviruses and the EMC viruses are less stable to heating than the Rhinoviruses, FMDV and ECHO 28. The wide range is seen when the half life of the Enteroviruses at 37°C varies from 2·5 to 40 hours.[27] An example of the effects of different temperatures on one strain of FMDV shows that 90% inactivation occurs in 30 seconds at 61°C, in 2 mins. at 55°C, in 21 hours at 37°C but only after 18 weeks at 4°C.[19] A small proportion of any suspension of FMDV has enhanced heat stability and serial passage of this fraction increases the amount of resistant virus.[28] At −70°C all the Picornaviruses can be preserved indefinitely and in most cases they are stable at −20°C for several years. Certain strains become non-infective after only a few months at −20°C and stock strains have to be preserved at lower temperatures, e.g., ECHO 19. (Enteric Cytopathic Human Orphan.)

High concentrations of such cations as Mg^{++}, Ca^{++} and Na^{+} inhibit the thermal inactivation of the Enteroviruses and EMC viruses.[6,29] This property has been called the cationic stabilisation of viruses and occurs with at least 35 of the Enteroviruses, covering all the subdivisions.[4] The EMC viruses are stabilised at 56°C by monovalent cations, even in the presence of large amounts of halogen ions, but they have not yet been tested with the divalent cations.[6] On the other hand, the Enteroviruses are stabilised by the divalent cations at all temperatures between 4°C and 50°C but the monovalent cations are selective in their temperature range. At 50°C, molar $MgCl_2$ or $CaCl_2$ completely prevents inactivation of these viruses for 3 hours whereas 2 molar NaCl only stabilises them for one hour.[4] Preliminary studies with the Rhinoviruses show that molar $MgCl_2$ only partially stabilises them to heating at 50°C.[8] For a human Picornavirus to be accepted into the Enterovirus sub-group it must show complete cationic stabilisation for 3 hours at 50°C.

Soluble antigens have been reported for all strains studied in sufficient detail, but with the exception of FMDV they are not always formed and little is known of their properties.[7,30] The FMDV soluble antigen is present in every suspension of virus. It is non-infectious, has a particle diameter of 8 mμ[7] and is distinct from the infectious particle antigen.[31] This infectious particle is specific for each antigenic type. The soluble antigen is common to all types when tested by the complement fixation test but by agar-gel diffusion it shows type specificity.

Two distinct antibodies react with the soluble antigen one being the type-specific antibody which also combines with the infectious particle.[31] The EMC viruses are all closely related antigenically and vary only in pathogenicity.[32]

By far the greatest amount of work has been done on the Poliovirus

antigens. Two specific antigens are formed in Poliovirus infected cells, the D or native antigen and the C or heated antigen.[33,34] The D antigen is the complete infectious virus particle containing RNA and can be detected by neutralisation, complement fixation and agar-gel diffusion tests.[33,34,35] The C antigen has no core of RNA. The D antigen can be converted into the C antigen by heating at 56°C, by alkaline pH, by phenol and by ultra-violet light.[36] The C antigen reacts with early serum specimens from human infections while the D antigen reacts with late specimens (1 to 3 months). Pure anti-C serum does not neutralise the virus infectivity whereas anti-D serum does.[37]

Group antigens definitely occur which are common to types 1 and 2 and probably also type 3. Heterologous rises in neutralising antibody occur in up to 50% of persons infected with Poliovirus.[38,39,40] Antigenic variation within the same type has been found with many of the Picornaviruses and is a useful feature in distinguishing between virulent and attenuated strains of Poliovirus.[41,42]

The ability to agglutinate erythrocytes of certain animals is not a general character of the group although some members, like Poliovirus, which do not produce a haemagglutinin do adsorb to erythrocytes.[43] Human group 0 cells are agglutinated by some strains of Coxsackie A20, A21, A24, B1, B3 and B5, the ECHO types 3, 6, 7, 11, 12, 13, and 19 and the EMC strains.[44,45,46] Strains of the EMC sub-group also agglutinate guinea-pig and sheep erythrocytes[46] while Coxsackie A7 agglutinates "vaccinia-agglutinable" fowl cells.[47] The optimum temperature for haemagglutination by the Enteroviruses is 37°C although it does occur at 4°C and 25°C.[44,45] The EMC strains only haemagglutinate at the lower temperatures.[46]

The haemagglutinin appears to be associated with the virus particle[45] and in the case of EMC the cells must be disrupted before it is released.[46] Treatment of a suspension of Coxsackie B5 virus with trypsin or receptor destroying enzyme (RDE) does not alter the haemagglutinin. Erythrocytes treated with RDE are likewise unaffected. However potassium periodate in low concentrations rapidly destroys the haemagglutinin.[48]

All the Picornaviruses have an optimal growth temperature of 37°C excepting the Rhinoviruses and ECHO 28 which prefer 33–34°C. This is probably a reflection of the different sites of multiplication of these types which are almost exclusively naso-pharyngeal. The Rhinoviruses obviously form a distinct sub-group with their lower optimum temperature, greater heat resistance and increased susceptibility to acid pH while the Enteroviruses and the EMC strains have virtually identical biological properties, with FMDV coming midway between these two sub-groups.

Growth

The host range is large, but selective, depending not only on the

species from which the virus was isolated but also on the sub-group to which it belongs. Different strains of the same type may vary in susceptible hosts, e.g., some strains of Coxsackie A7 will not grow in tissue culture but only infect mice and monkeys, whereas others will only grow in tissue cultures. A few members have been adapted to grow in eggs, such as the MEF-1 strain of Poliovirus type 2.[49] Tissue cultures are the easiest and most efficient method of growing the Polioviruses and FMDV although they were originally grown in monkeys[50] and mice[51] respectively. When inoculated into monkeys the Polioviruses produce lesions similar to those of poliomyelitis in man. Other members of this group cause paralysis of monkeys, notably Coxsackie A7.[52]

Only Coxsackie A and B, FMDV and the EMC sub-group have been adequately studied in mice. These viruses all produce paralysis and death in mice and each has a distinct pathology.

COXSACKIE A VIRUSES when inoculated into 12-hour-old mice produce a generalised myositis resulting in flaccid paralysis and death after 3 to 21 days, depending on the strain and the size of the inoculum. Microscopic examination reveals widespread lesions of the striated muscle with segmental necrosis of muscle fibre. Occasionally lesions are seen in the liver and lungs.[53] A very large inoculum may produce flaccid paralysis in adult mice but the virus cannot be propagated in this instance.[54]

COXSACKIE B VIRUSES produce lesions in the CNS with spastic paralysis and death after 6 to 21 days in newborn mice. Discrete muscle lesions, necrosis of the brain and fat tissue, lesions of the myocardium, liver and pancreas are seen on microscopic examination and occasionally the ganglion cells of the anterior horn are destroyed.[53] Widespread brain necrosis always follows intracerebral inoculation but with subcutaneous and intraperitoneal inoculation the brain lesions are slight—hyperaemia and scanty perivascular accumulations of inflamed cells.

FMD VIRUSES are highly pathogenic for 7-day-old mice. Flaccid paralysis and slight myocarditis are followed by death in 20 to 30 hours. There is widespread necrosis of striated muscle, similar to that produced by the Coxsackie A viruses. With $3\frac{1}{2}$ to 4-week-old mice there is less muscle damage but myocarditis and necrosis of the pancreas are severe. When these mice die they usually do so within 4 to 6 days. Mice over 4 weeks old do not usually die. With 5-week-old mice muscle damage is not permanent. Necrosis of the suprascapular fat pads is also seen in mice of this age.[55]

EMC VIRUSES kill mice of all ages from 18 hours after infection. The fur is ruffled and the animals become lethargic with progressive flaccid paralysis. Muscle necrosis is followed by focal lesions of the CNS and myocardium.[56,57]

Most of the members of the Picornavirus group can be grown in tissue

cultures. With the exception of the Coxsackie A viruses all the human strains grow well in human embryo kidney (H.E.K.) cells and many will grow in rhesus monkey cells, human amnion (H.A.) cells and the continuous cell lines like HeLa, KB etc. The viruses from other animal

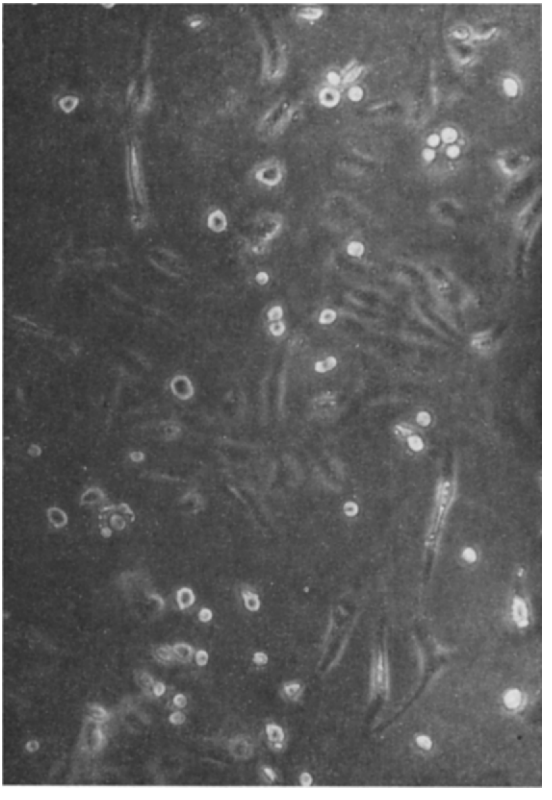

FIG. 9.1. Phase contrast photomicrograph of monkey kidney cells 48 hours after infection with Coxsackie B5 virus showing the small rounded-up infected cells falling from the monolayer. (*Photo—L. E. Hewitt.*)

species usually grow best in cultures of homologous cells but the range varies greatly. The growth of Poliovirus, FMDV and EMC virus in tissue cultures has been studied in detail and they are sufficiently alike for us once more to take Poliovirus as the example.

For successful adsorption of Poliovirus to susceptible cells magnesium or calcium ions should be present at a concentration of about 10^{-3} molar.[58] Once attachment has occurred the adsorbed virus cannot be detected as infectious material and some at least is insensitive to anti-serum.[59] (A lipo-protein, which inactivates Poliovirus, can be extracted from susceptible HeLa cells, but this is not found in cells which are not

susceptible to Poliovirus.)[60] In monolayer cultures the virus remains on the surface of the cell for 30 minutes but in suspension cultures it disappears in 12 minutes.[60,61] Whereas adsorption is independent of temperature, penetration is not and is most rapid at 37°C.[60] Once inside the cell the virus particle appears to be broken down into protein and RNA. This is a property of the cell, not the virus, and may be associated with the lipo-protein fraction.[61]

After penetration, the virus particle initiates a series of cytological and metabolic changes which result in the rapid death of the cell.[62–65] Changes are first seen in the nucleus where there is a loss of chromatin in the centre and a deposition at the periphery. The nucleus becomes distorted and extrudes DNA into the cytoplasm although the nucleolus and the nuclear membranes remain intact.[66] There is an increase in the basophilia of the cytoplasm followed by vacuolation.[65] Hyalinisation of the outer rim of cytoplasm takes place, the cell surface seems to "bubble" and releases pieces of cytoplasm rich in RNA into the medium.[66] Finally the cells round up and become detached from the glass. By the time half of the cells have rounded up 95% of the new virus has been liberated.[63]

Electron microscopy of infected cells shows that the protein first appears as sub-units which aggregate 3 hours after infection into typical icosahedral particles, forming intracytoplasmic crystals.[67,68] The growth cycle takes 6 to 8 hours to complete although another 4 hours may elapse before all the virus is released from the cell. Approximately 100 PFU of virus is produced by each susceptible cell.[69]

The biochemical changes following Poliovirus infection have not yet been fully worked out. Initially there is an increase in glucose utilisation and lactic acid production[70] but the later stages of the growth cycle have only been studied in relation to protein and RNA synthesis. Ackermann *et al.*,[71] using non-multiplying cell cultures found an increase in the production of RNA and protein after infection. On the other hand, Salzman *et al.*,[72] using rapidly multiplying suspension cultures found that Poliovirus infection led to the abrupt cessation of the production of DNA, RNA and protein. These latter findings have been confirmed by other workers.[73] Salzman *et al.* also observed a marked reduction in the incorporation of C^{14} labelled cytidine into the RNA of infected cells.[72] However Maassab *et al.* using non-multiplying cultures found an increase in uptake of P^{32} into the RNA of infected cells.[74] Both groups of workers are agreed that late in infection cellular RNA is broken down.[72,75] These results are more compatible than they appear to be when one considers that new Poliovirus RNA and protein are formed from the acid soluble pools of amino-acids and nucleotides in the cells and not from cellular macromolecules.[76] If the initial stage of viral infection is to stop normal cell RNA synthesis then obviously in rapidly dividing cells there will be a large acid-soluble pool available and little further synthesis of nucleotides will be required. On the other hand, in

non-multiplying cultures the acid-soluble pool will be small and the nucleotides will have to be synthesised in large amounts, resulting in an increase in uptake of P^{32} and RNA synthesis. Synthesis of viral RNA, unlike host cell RNA, is independent of DNA although it appears to be associated with some cell structure, possibly the nucleus.[77,78]

Breakdown of the infecting viral RNA commences 20 minutes after infection and is complete in one hour. The production of viral protein and viral RNA begin 3 to 4 hours after infection (just before infectious virus first appears) and parallel each other until the 6–7th hour when synthesis ceases. This two-hour delay between breakdown and production of new RNA and protein is thought to be due to the time required to set up the intracellular transport system. Maturation of the infectious virus is not complete until the 8–9th hour after infection.[79,80]

As has already been stated, there are two distinct viral antigens produced in Poliovirus infected cells—C, the incomplete and D, the complete virus, the only differences being the absence of RNA in the C antigen, although both are built up at the same time from identical sub-units.[81]

None of the other Enteroviruses have been investigated to the same extent but all the results so far available indicate a similar pattern of growth. The Rhinoviruses require a more acid pH of culture medium and a lower temperature than the other Picornaviruses for their successful cultivation. These properties are paralleled by the site of infection—the nasal mucosa rather than the alimentary tract.[82] In bovine tissue cultures the growth of FMDV is virtually identical to that of Poliovirus in human cell cultures,[83] while EMC virus in mouse cells differs only in that it has been possible to show that the initial stages of RNA production occur in the nucleus.[84] This was done by extracting infectious RNA from both virus particles and infected cells.

Colter *et al.*[85] were the first to extract RNA, from Poliovirus infected tissues, which would infect susceptible animals. Alexander *et al.*,[86] using RNA extracted from partially purified Poliovirus, were able to infect HeLa cell cultures although the infectivity of both of these preparations was much lower than that of the original virus suspension. Similar results were obtained by Brown *et al.*[87] with FMDV. The infectivity of the extracted RNA can be increased if the medium used for adsorption to the cells is alkaline and has a hypertonic salt concentration.[88] Infectious RNA is more stable to heating than complete virus. It adsorbs rapidly and infects cells with the production of normal complete virus.[89] Cells to which complete virus is unable to adsorb can be infected with infectious RNA[90] but the complete virus particles which are produced will still not adsorb to these cells. Thus there is only one growth cycle.

Infectious RNA can be easily obtained from preparations of purified Poliovirus or FMDV but it is much more difficult to obtain from EMC virus particles. However it can be obtained from cells infected with

EMC virus long before complete virus is detectable.[34,91,92] This has been done with FMDV[93] but not as yet with Poliovirus.

Fractionation of EMC virus-infected ascites tumour cells has shown that infectious RNA first appears in the nucleus soon after infection and reaches a maximum titre after $4\frac{1}{2}$ hours. It then decreases in the nucleus and increases in the mitochondrial fraction of the cytoplasm reaching a maximum at $5\frac{1}{2}$ hours. Infectious virus and haemagglutinin first appear at $4\frac{1}{2}$ hours in the mitochondria and reach maximum titre after 8 hours.

From the foregoing account one can see that all the Picornaviruses have a very similar pattern of growth. The protein overcoat of the virus particle determines the ability of a given cell to adsorb the virus to its surface and thereby become infected. The RNA alone is infective for a much wider host range indicating that the actual production of virus can be supported by most tissue cells. The available evidence suggests that growth occurs in the following manner.

Penetration of the virus into the cell is a property of the cell, not the virus, and the initial stages of RNA production are in the nucleus. This is definitely the case with EMC virus and it seems likely that this is so with the other Picornaviruses. Host cell DNA does not mediate in this reaction and the RNA is not only the genetic messenger but also the template for new virus production. The protein appears to be formed in the mitochondria and it is probable that maturation of the complete particle also takes place there.

Classification

The Picornavirus group has not been completely sub-divided yet but the scheme which has been suggested[1] is shown in Table 9.2.

TABLE 9.2

Sub-division of the Picornavirus Group

A. Picornaviruses of Human Origin.
1. Enteroviruses.
   (*a*) Polioviruses.
   (*b*) Coxsackie virus A.
   (*c*) Coxsackie virus B.
   (*d*) Echovirus.
2. Rhinoviruses.
3. Unclassified.

B. Picornaviruses of Lower Animals.

This is very artificial and sub-division A3 will obviously lead to another collection of "orphan" viruses. For example in 1955[94] the then unclassified viruses were provisionally labelled ECHO (Enteric Cytopathic Human Orphan) viruses, and they remain so today. It is also intended to divide the Picornaviruses of the lower animals in a similar manner. Within each of the sub-divisions each antigenic type will be known by a number, as with the human Enteroviruses.

Before considering the Enterovirus sub-group the more important viruses involving the lower animals should be mentioned.

Enteroviruses have been isolated from cattle, swine, monkeys, cats and birds and have been named ECBO, ECSO, ECMO, ECCO, and ECAO respectively.[95] At least one of these has properties similar to the human Rhinoviruses.[8] Apart from FMDV there are the mouse encephalomyelitis viruses (strains, TO, FA and GDVII and the encephalomyocarditis viruses (strains, EMC, ColSK and Mengo). Methods of grouping these together are not yet clear, with the exception of the EMC strains which do appear to have distinct properties.

On examination of the Enterovirus sub-group it becomes evident that the proposed retention of the old names and system will simply confer a distinguished label on a chaotic state of affairs. The Committee on the Enteroviruses[96] suggested that the sub-divisions should be abandoned and all the distinct antigenic types numbered 1 to 58, but this would be cumbersome. Although we are in no position to assign names which will withstand the test of time the unqualified retention of the old names certainly cannot remain much longer. Possibly such properties as haemagglutination and sensitivity to certain chemical compounds may yet lead to a more fundamental classification scheme.

Melnick[4] listed the properties (Table 9.3) to define members of the Enterovirus sub-group and in practically every instance there are

TABLE 9.3

Division of the Enteroviruses

Sub-Group	Growth in Cultures of		Pathogenicity for	
	Monkey Kidney	Human Cells	Mice	Monkeys
Poliovirus . .	+	+	0	+
Coxsackie A . .	0	0	+	0
Coxsackie B . .	+	+	+	0
Echovirus . .	+	0	0	0

exceptions. The criterion for inclusion of a virus in the Poliovirus sub-division was the ability to paralyse monkeys. Coxsackie A7[52] produces severe poliomyelitis in monkeys while other Coxsackie and Echoviruses give rise to milder illnesses. Some Coxsackie types have a very low or non-existent pathogenicity for mice. Some strains of Echoviruses (types 9 and 23) produce typical myositis in suckling mice, but because the prototypes of these viruses were non-pathogenic for mice they were included in the ECHO sub-group. ECHO 9 is frequently called Coxsackie A23. Furthermore some strains of Poliovirus have been adapted to grow in mice.

When one considers the growth of these viruses in tissue cultures further great discrepancies appear. Several Coxsackie A viruses grow well in human tissue culture and type 9 grows in monkey kidney cells. The author has grown all the prototype ECHO strains up to 27 in human amnion cells with little difficulty.[97] Only type 27 required more than one passage for consistently good growth. Nevertheless the situation remains where we shall continue to use the old names and numbers, but rely only on antigenic distinctions to identify the Enteroviruses.

Genetics

The production of live-attenuated Poliovirus vaccine has been the stimulus for much work on the hereditary properties of Poliovirus.[98] Most of the work has been directed towards distinguishing between the virulent "wild" strains and the attenuated "vaccine" strains. Plotkin *et al.*[98] have tabulated the known genetic markers under three headings as described in Table 9.4.

TABLE 9.4

Genetic Markers of the Polioviruses

Group	Marker	Measurement
I. Cell-Virus Relationship	s	Average plaque size after given period of incubation.
	d	Development of plaques under acid and alkaline agar.
	rct/40	Comparative titre at 37°C and 40°C.
	rct/23	Comparative titre at 37°C and 23°C.
	MS	Comparative titre in MS and MK cells.
	H	Comparative titre in HK and MK cells.
II. Reaction to Substance in Medium.	m	Minute plaque size under certain agars.
	cr	Replication in relation to concentration of cystine.
	Ho	Inhibition by equine sera.
	Bo	Inhibition by bovine sera.
	IST	Inhibition by strain-specific hyperimmune sera.
III. Physical Characteristics	T	Heat inactivation rates.
	E	Ease of elution from ion exchange resin.
	$Al(OH)_3$	Ease of elution from $Al(OH)_3$ gel.

Several of these markers show co-variance and it seems likely that each of the associated groups is derived from a common basic property. The associated groups are:—d, rct/40, rct/23, MS and H; cr and T; and E and $Al(OH)_3$. In the case of those markers which have been adequately studied the correlation between the marker and the virulence of the strain has not been complete although it is of a high order.[99,100] However results do show the Polioviruses to be labile, with mutations occurring more frequently than with Bacteriophage.[16]

Attempts have been made to obtain evidence of recombination of Poliovirus by multiple infection of cells with two distinct strains. Ledinko and Hirst[101] found that a large proportion of the progeny from

HeLa cells infected with Poliovirus types 1 and 2 could be neutralised by antiserum against either type. Subsequent growth of these strains produced pure type 1 or type 2. The mixed virus was the result of phenotypic mixing. The RNA belonged to one type while the protein coat was composed of sub-units of both types. More recently Ledinko[102] has shown genetic recombination with two strains of Poliovirus type 1. The frequency of recombinants increases with the time after infection and during the period of RNA synthesis. These results indicate that the RNA initially produced by the cell acts as a template for further RNA production and that recombination may occur at both the initial and secondary stages of RNA synthesis. Further work will be required to elucidate these findings but it might be found from this that the initial RNA is produced in the nucleus and passed to the cytoplasm where further RNA is replicated from it.

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CHAPTER TEN

MANIFESTATIONS OF PICORNAVIRUS INFECTION

THE Picornaviruses probably cause more clinical illness than any other group of viruses. Poliomyelitis, Foot and Mouth Disease and the "common cold" are actual or potential scourges of both man and animals. Vaccines have controlled poliomyelitis and in the case of FMD a combination of vaccine and slaughter is containing the disease. But by far the greatest number of illnesses caused by this group are non-specific respiratory infections or cases of general "malaise". Nevertheless, specific clinical entities are associated with certain sub-groups.

Poliomyelitis was known in the days of the Pharaohs if one can judge from pictures and descriptions. In the early part of this century 80% of the cases of paralytic poliomyelitis in the U.S.A. occurred in children under school age, whereas in 1950 only one third belonged to this group.[1] This change in incidence, attended by an increase in severity with advancing age,[2] is almost certainly due to the improved sanitation and living conditions, which prevent the disease from becoming endemic among the infant population. Today two types of vaccine are in use and have considerably reduced the incidence of the disease. The first vaccine in use was a formalin-inactivated vaccine developed by Salk[3] while the second and more important was the Sabin live-attenuated vaccine.[4]

Although transmission of Poliovirus to an experimental animal was reported in 1909[5] it was not until 1931[6] that the antigenic homogeneity of Poliovirus was questioned. By 1949[7,8] the three antigenic types had been defined and in 1951[9] it was assumed that these were the only viruses causing paralysis. Since then many of the other Enteroviruses have been associated with cases of paralytic poliomyelitis. A future rise in poliomyelitis can therefore be expected with the subsequent addition of more and more antigenic types to the trivalent vaccines rendering them less efficient. In the case of the "common cold" there are already so many distinct Rhinoviruses that any hope of an efficient vaccine must unfortunately be discarded.

Diseases of Humans

The Enteroviruses and Rhinoviruses cause illness ranging from mild upper respiratory tract infections and diarrhoea to paralytic poliomyelitis and fatal myocarditis. Most of the illnesses involve either the respiratory tract or the central nervous system (CNS). Each clinical

entity will be considered in turn, starting with those illnesses caused solely by the Enteroviruses.

ASEPTIC MENINGITIS or non-paralytic polio is the commonest CNS disease associated with the Picornaviruses. Widespread outbreaks occur in the summer and autumn in temperate zones but appear at any time in tropical climates. The majority of the Enteroviruses can and do cause meningitis, but only some give rise to outbreaks.

The onset of illness is sudden after an incubation period of 6 to 20 days. Severe headache is followed by general malaise, fever, vomiting, drowsiness and neck stiffness. When the onset is gradual the first signs are general malaise, loss of appetite and nausea followed by the above symptoms. There is a wide variation in the severity of the illness but all cases show these symptoms. The fever may be anything from 99 to 103°F lasting from 2–14 days, but 4–5 days are more usual. Other symptoms which occur less frequently are photophobia, myalgia, lymphadenopathy, back stiffness, conjunctivitis, diarrhoea and rashes.[10–14] Diagnosis is confirmed by the presence of the Kernig and Brudzinski signs, either or both of which are positive. Usually there is an increase in leucocytes in the cerebro-spinal fluid (CSF) and lymphocytes exceed polymorphs. This increase in white cells ranges from 12 to 2,000 but rarely exceeds 500 per cu. mm. In the initial stages of illness the CSF findings may be reversed.[11,12] The protein content of the CSF is normal or only slightly raised. The illness is never fatal unless it progresses to either of the following two syndromes.

Epidemics of meningitis have been recorded all over the world and involve 14 distinct Enteroviruses, not counting the three Polioviruses. In Europe and America Coxsackie A types 7 and 9,[15,16] Coxsackie B types 1 to 5[17–21] and ECHO types 4, 6, 7, 9, 11, 16 and 30[10–13,22–28] have all caused epidemics. Sporadic cases are associated with Coxsackie A types 2, 3 and 4, Coxsackie B type 6, and ECHO types 1, 2, 3, 5, 13, 14, 15, 17, 18, 19, 20, 22 and 25.[21,29,30,31]

PARALYTIC POLIOMYELITIS has retained its notoriety although it is at present relatively uncommon in Europe and America as a result of vaccination. Like aseptic meningitis it occurs during the summer and autumn in temperate climates causing large epidemics. The initial symptoms are identical with aseptic meningitis and only a variable proportion of cases actually develop paralysis, the remainder passing off as meningitis. The incidence of paralysis depends on the virulence of the strain but mostly on the age of the population at risk with paralysis at its worst in the adolescent age group and among the more active members of the community.[32,33,34] In the tropics where Poliovirus is endemic, danger exists only for the indigenous infant population because all older persons have been infected. Here the ratio of paralytic to non-paralytic infections can be as low as 1:3300.[35]

Between 2 and 4 days after the onset of meningitis, two selected muscle groups can become paralysed. Spinal paralysis, the most common,

involves a flaccid paralysis of the limbs or trunk and is the result of lesions of the anterior horn cells. The other form of paralysis occurs when the medulla is attacked. This bulbar paralysis, as it is called, is manifest in paralysis of the palate, pharynx and larynx and is usually the more severe illness. The fever continues longer and the mortality rate is higher. Direct respiratory failure can occur due either to disturbance of the respiratory control centres in the medulla, or to paralysis of the intercostal and diaphragm muscles. With non-bulbar paralysis the mortality rate is relatively low, but residual paralysis is common.

Widespread outbreaks are caused by Poliovirus types 1, 2 and 3 and small outbreaks by Coxsackie A7.[9,15,36] Sporadic cases have been reported due to Coxsackie A types 2, 4 and 9,[29] Coxsackie B types 2, 3, 4 and 5,[21,37,38,39] and ECHO types 2, 4, 6, 9, 11, 16 and 30.[21,31,38,39,40]

ENCEPHALITIS occurs sometimes in association with and sometimes in the absence of meningeal symptoms. The onset of illness is insidious and the typical clinical picture is one of intense headache, hallucinations, lethargy, mental confusion, paresis of the cranial nerves, blurred vision, papilloedema, hyperacusis and hypertension.[41] Only sporadic cases are seen but there are occasional deaths.

Probably most of the Enteroviruses causing meningitis also cause encephalitis but so far only some have been reported. The three types of Poliovirus are involved, together with Coxsackie A types 2, 5 and 6, Coxsackie B types 2, 3, 4 and 5, and ECHO types 7, 9, 11 and 14.[21,42,43]

RASHES are commonly seen and fall into two distinct groups. The most important is the petechial rash associated with meningitis, which can be confused with a meningococcal septicaemia.[10] Although uncommon, physicians can expect to see it occasionally during outbreaks of meningitis due to ECHO types 7 and 9, and Coxsackie A9.[10,44–47]

The more common rash however, is a maculopapular rash also associated with meningitis. It varies in severity and duration from a scanty rash disappearing in several hours to an almost confluent rash lasting several days. There is no post-exanthematous scaling which is typical of measles and with which it may be confused. In many cases, especially those due to ECHO 16, the rash appears late in the illness and is of very short duration.[48] This rash is a feature of ECHO 9 outbreaks, usually accompanied by generalised lymphadenopathy. Children are more frequently affected than adults and in children the illness is less severe if there is a rash.[49] In outbreaks of Coxsackie B types 2 and 3 and ECHO types 7 and 11 this rash can be expected.[10,14,50,51]

HERPANGINA is a very unpleasant illness characterised by painful lesions of the palate and tonsillar pillars. The palate lesions first appear as vesicles surrounded by an erythematous zone, but soon burst giving a typical appearance of punched out ulcers about 5 mm in diameter. An accompanying systemic illness is usual and the symptoms are fever, vomiting and abdominal pain.[52] The fever lasts from 1 to 4 days and the

lesions continue for a varying length of time but never less than a week. The illness is never fatal and is caused by Coxsackie A types 2, 3, 4, 5, 6, 8, 10.[29,53]

BORNHOLM DISEASE, pleurodynia or epidemic myalgia are the names frequently used to describe a very common and unpleasant illness caused by the Coxsackie B viruses. The patient feels a sudden severe pain in the muscles of the thorax and abdomen or sometimes in the back and limbs. This pain is followed by fever, sweating, headache, anorexia, nausea, sore throat and vomiting.[54] The duration of illness is from 6 to 40 days with relapses occurring up to six months after the initial symptoms. It is never fatal.

It is not uncommon for cases of pleurodynia to be mistaken for acute appendicitis if the pain is localised in the abdominal muscles, or myocardial infarction if it is only thoracic. Only Coxsackie B types 1, 2, 3, 4 and 5 have so far been associated with this illness.[42,50,54–57]

PERICARDITIS is an illness characterised by sudden precordial pain and a loud friction rub. Sometimes pericardial effusion is present. These cases are usually sporadic, occurring during outbreaks of pleurodynia or meningitis. They have been associated with a variety of Coxsackie viruses namely—Coxsackie A type 1, and Coxsackie B types 2, 3, 4 and 5.[42,58–64]

MYOCARDITIS is mainly a disease of newborn infants in nurseries. Shortly after birth the babies have loose stools followed 3–8 days later by fever, tachycardia and the electrocardiographic changes of myocarditis.[65,66] The illness is caused by Coxsackie B types 2, 3 and 4.[66–68] The cases are sporadic and when seen in older children they are usually much less severe. In babies the mortality rate is high.

DIARRHOEA in epidemic form may be caused by the Enteroviruses. Most outbreaks affect young children in closed communities although older persons can be involved. Vomiting and fever are commonly seen in addition to a severe diarrhoea of semi-liquid and mucous stools. This is not usually a particularly serious illness but occasional deaths have been reported. The viruses causing these outbreaks are Poliovirus types 2 and 3, Coxsackie B type 3 and ECHO types 7, 9, 11, 14 and 18.[69–73]

UPPER RESPIRATORY TRACT INFECTIONS can be caused by virtually all of the human Picornaviruses. The most common as its name suggests is the "common cold," characterised by a watery nasal discharge and stuffiness with occasionally, a sore throat and cough.[74] Most "common colds" are caused by the Rhinoviruses and ECHO 28.[75,76] To date the number of Rhinoviruses fully identified is small compared with the rapidly increasing number known.

Other well defined Picornaviruses causing outbreaks of respiratory tract illness are Coxsackie A21 or Coe virus and ECHO types 11 and 20. The illness caused by Coxsackie A21 is more severe than the "common cold" with mild fever and headache as well as the nasopharyngeal

symptoms.[77] This virus frequently features in outbreaks in military camps[78] causing, in this instance, an illness very similar to Adenovirus infection but with milder symptoms. ECHO types 11 and 20 occasionally give rise to mild respiratory illness in children, which can be febrile. The former type has also been associated with croup but it is rarely more than a "common cold" accompanied by a cough.[79] On the other hand, the colds caused by the latter are usually quite severe, with fever, mild pharyngitis and erythema of the eardrums as well as the nasal discharge and stuffiness.[80]

TREATMENT in all of these diseases should be directed towards the alleviation of the symptoms. The use of cortisone is contra-indicated.

THE EPIDEMIOLOGY of all the diseases caused by the Enteroviruses is similar. Infection can occur in the nasopharynx where multiplication takes place, or via the alimentary tract by direct ingestion of contaminated food. In both cases the virus is excreted in large amounts. Although diarrhoea is sometimes seen in young children, adults have a much milder illness, providing there is no further spread to the bloodstream. When this occurs a viraemia develops with the virus gaining access to the CNS, heart or striated muscles.

Infection is spread by contamination of foodstuffs, hands, etc. with infected faeces, or by droplet infection from the nasopharynx. Sporadic cases at the start of an epidemic are probably due to faecal contamination but the majority of later cases are the result of droplet infection.

These clinical entities have an uncertain incubation period ranging from 2 to 40 days. Usually it is in the range of 5–7 days, with the exception of infantile myocarditis where at least some of the infections are intra-uterine. The initial symptom is a very mild sore throat which usually passes unnoticed with the onset of the more commanding symptoms.

On the other hand, the epidemiology of the "common cold" is not yet fully understood. The Rhinoviruses with their lower optimal growth temperatures and acid sensitivity are better suited to nasal infection than the Enteroviruses. Those Enteroviruses which do cause definite outbreaks of respiratory illness appear to do so only under favourable conditions, e.g., schools, orphanages and military camps. The spread of the Rhinoviruses is almost certainly due to droplet infection and this probably accounts for their prevalence in winter, when warm stuffy meeting places are more congested than in summer.

LABORATORY INVESTIGATIONS are necessary to differentiate between the Picornaviruses and other unrelated viruses. The virus may be isolated or alternatively, a rise in antibody may be demonstrated between acute and convalescent specimens of serum. Enteroviruses are most readily isolated from the nasopharynx in the first three days of illness. Up to two weeks after the onset, specimens of faeces provide a good source of virus, but in cases of meningitis, (fatal) paralytic poliomyelitis and herpangina, virus isolation should be attempted from

the CSF, CNS and the lesions respectively. Serological diagnosis by itself can only be done in an outbreak of known aetiology, because there is no common antigen for any of the sub-groups. A rise in antibody titre to a virus isolated from the nasopharynx or faeces usually means that the virus was the cause of illness. Virus isolation from faeces alone is not sufficient because all the Enteroviruses can be readily isolated from the faeces of healthy persons.

The Rhinoviruses can be regularly isolated only from nasal washings and at our present state of knowledge such an isolation from a "common cold" is considered to be significant.

Vaccination

To date there are successful vaccines only against Poliovirus. The efficiency of any vaccine against the Rhinoviruses is limited on two counts. There is an overwhelming multiplicity of distinct serotypes, each of which would have to be incorporated in any vaccine to ensure adequate immunological coverage. Secondly to prevent infection of the nasal mucosa, circulating antibody must be maintained at a sufficient level to be present in the nasal secretions.

However two distinct types of vaccine have proved their usefulness against Poliovirus. The original poliomyelitis vaccine was prepared by separately inactivating suspensions of Poliovirus types 1, 2 and 3 with formaldehyde and pooling them.[81,82,83] For maximum antibody response this trivalent vaccine should be administered in three doses with two to four weeks separating the first two and 6 to 7 months between the last two. Further booster doses are necessary.[3]

Later Koprowski and Sabin independently produced mutant strains of Poliovirus with a greatly reduced neurovirulence in monkeys.[4,84] Although their strains rapidly reverted to neurovirulence after passage through the human alimentary tract,[4,85] successful live-attenuated vaccines were ultimately developed.[86,87] The Sabin-type vaccines have two advantages over the Salk types. They are given orally and their degree of immunity is of a more permanent nature. There is, however, a great deal of controversy still going on over the possible effects on their neurovirulence after multiple human passage.

Non-Human Picornaviruses

All species of vertebrates probably harbour a range of Picornaviruses similar to those of humans, but only two are of veterinary significance as yet. The most important are the viruses causing Foot and Mouth Disease. This disease occurs throughout the world attacking domestic and wild cloven-hoofed animals alike,[88] especially cattle, pigs, sheep and goats, with devastating consequences to agricultural economics.

FOOT AND MOUTH DISEASE is essentially dermotropic in the adult animal, involving mainly the skin and mucous membranes. A primary vesicular lesion develops 18 to 30 hours after injection of the

virus into the mucosa of the mouth or the coronary band of the feet. A viraemia follows, producing secondary lesions of the mouth, muzzle, nares, pudenda, snout, teats and rumen. The epithelium becomes necrotic and sloughs off after a few days, leaving shallow erosions. Fever, malaise, loss of weight, lameness, reduced milk flow and abortion are characteristics of the disease. There is a common legacy of sterility, foot deformities and chronic mastitis and there are occasional deaths. In the young animal the disease is more severe with frequent involvement of the skeletal muscle and myocardium, resulting in a higher mortality rate.[88] The disease is contagious. Each outbreak is invariably caused by one distinct antigenic type and can be controlled by a combination of slaughter and vaccination of the neighbouring herds with a formalin-killed vaccine of the infecting type.[89] All infected animals must be slaughtered because animals which have recovered clinically sometimes excrete the virus for several months.[90]

To date there are seven distinct antigenic types divided into a number of sub-types:—Types 0 and C of three sub-types each, type A of 11 sub-types; SAT 1, SAT 2, SAT 3, and ASIA 1.[88] They differ in their pathogenicity for cattle and there is no cross-immunity. Complete identification of a new isolate is therefore a difficult and tedious process.[91]

TESCHEN DISEASE VIRUS is the other important virus causing disease. It attacks swine in a similar manner to poliomyelitis in man, with inapparent infections being more common than paralysis.[92] The virus multiplies in the alimentary tract and a viraemia follows. During this period most animals show no sign of illness and do not become paralysed. On the other hand, when invasion of the CNS does occur paralysis and death follow. Lesions can be found in the liver, spleen, kidneys and diaphragm muscles.[92]

The spread of infection is probably similar to that of the Enteroviruses and unlike FMDV a policy of slaughter cannot control the disease, owing to the number of infections which pass unnoticed. Effective formalin-killed and live-attenuated vaccines have been produced from infected swine kidney cultures[93,94,95] and as there is only one antigenic type, eradication of the disease is possible.

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CHAPTER ELEVEN

THE ADENOVIRUSES

THE Adenovirus group is a large and widespread family of viruses infecting many species of the animal world. At the present time about 50 distinct antigenic types are known which can be isolated from man, monkeys, cattle, mice, and dogs, and there is every reason to assume that this list is far from complete. With a few exceptions, members of this group do not produce overt disease in small laboratory animals and their discovery was thus delayed until the advent of tissue culture techniques.

Knowledge of the Adenoviruses first came to light in the years 1953–4, when four independent groups of workers isolated agents producing distinctive cytopathic effects in tissue culture. Rowe *et al.* in 1953 reported the isolation of 13 "adenoid degenerative agents" from naturally degenerating cultures of hypertrophied tonsils and adenoids. A few months later Hilleman *et al.*[2] reported the isolation in HeLa cell cultures of five identical agents from cases of acute respiratory disease and primary atypical pneumonia in military recruits. They also showed that some, at least, of the non-influenzal cases of respiratory disease were associated with this new virus which they called "RI-67". The third report of a new agent which was later shown to be an Adenovirus,[3] came in 1954 from Neva and Enders.[4] Their agent was isolated from a case resembling Roseola Infantum in cultures of human kidney cells.

These first three reports all came from the U.S.A. and were followed later in 1954 by one from Scandinavia, when Kjellen[5] announced the isolation of nine agents from cases of pharyngitis, mesenteric lymphadenitis and paralytic poliomyelitis. He showed that these agents were related to RI-67 and the virus isolated by Neva and Enders.[6]

This work was soon followed by comprehensive reports showing that the new viruses were aetiologically associated with upper respiratory tract infections. Parrott *et al.*,[7] investigating an outbreak of febrile pharyngitis and conjunctivitis coined the name pharyngoconjunctival fever. At the same time, the same group classified the known agents into six antigenic types and called them the Adenoidal-Pharyngeal-Conjunctival or A.P.C. viruses.[3]

Other groups of workers in America and Europe published work associating the A.P.C. viruses with large outbreaks of acute respiratory disease in military recruits.[8,9,10] They also showed that the specific disease recognised by the Commission on Acute Respiratory Diseases as ARD[11] was due to these viruses.[12]

The last of these early investigations resulted in the recognition of a new type, from cases of epidemic keratoconjunctivitis, which Jawetz *et al.*[13,14] proved to be the sole type responsible for the widespread outbreaks of this disease.

In 1956 the name A.P.C. given to this group in 1954 was superseded by the name Adenovirus with each member identified by a serotype number, given on the results of neutralisation tests.[15] Latterly haemagglutination—inhibition tests have also been used for identification and at the time of writing there are twenty-eight recognised types infecting humans, numbered 1 to 28.[16] Isolations of members of the Adenovirus group have been made from monkeys,[17] cattle,[18] and mice,[19] as well as man, and the virus of infectious canine hepatitis has been related to this group.[20]

Biological Properties

The basic properties of the Adenoviruses were initially defined as:—[3]

(1) They do not produce illness in small laboratory animals.

(2) They give a characteristic cytopathic effect in HeLa and monkey kidney cell cultures and prefer epithelial cells to fibroblasts.

(3) They are resistant to ether.

(4) They are filterable.

(5) They are thermolabile.

(6) They produce a soluble antigen which is common to every member of the group.

These properties have since proved to be basically correct and the Adenoviruses are a group of robust viruses, each producing a common soluble antigen when grown in tissue culture or a susceptible animal.

It is almost certain that the virus particle consists of DNA and protein only and has the shape of a regular icosahedron with a diameter of 60–80 mμ[21,22] By electron microscopy, it can be seen that at least some of the particles have a less dense central portion of about 30 mμ in diameter and the capsid is composed of 252 capsomeres.[23,24] Within the nucleus of an infected cell the virus particles form crystals orientated as a body-centred cubic lattice.[21]

The infectivity of the virus particles is not destroyed by proteolytic enzymes or by deoxyribonuclease and most of the types show little decrease in infectivity when held at room temperature for 24 hours at pH 6–10. They are rapidly inactivated at pH 11, but at pH 1·5–3·0 some infectivity remains after 24 hours. At all temperatures up to 36°C the viruses are stable—for at least 7 days at 36°C and indefinitely at −70°C. Inactivation occurs fairly rapidly at 50°C and at 56°C the infectivity is destroyed in 5 minutes.[25]

The soluble antigen, which was originally demonstrated by complement fixation tests, can be separated into three distinct components by agar-gel diffusion, by chromatography on DEAE-cellulose columns or by immuno-electrophoresis.[26,27,28] These components have been

designated antigens A, B and C, of which A and C are composed of protein and DNA, while B is composed of two types of protein, one sensitive and the other resistant to trypsin. The trypsin-resistant component of this antigen is identical with the protein component of antigen C. These antigens differ in size, A being 8 mμ with B and C 17 mμ and 10 mμ respectively. Antigen B shows toxin-like properties—it removes HeLa cells from glass when incubated at 37°C. It also produces the characteristic CPE of rounding and clumping of the cells, but without the production of new virus. Antigen A is common to all Adenovirus types and is therefore the "group or complement-fixing antigen". The trypsin-sensitive component of antigen B also appears to be common but antigen C is type-specific. Antigens A and C are not affected by proteolytic enzymes or deoxyribonuclease.

All but two of the 28 recognised human Adenoviruses agglutinate rat, human group O or rhesus monkey red blood cells.[29] Maximum haemagglutination occurs when the antigens are prepared in HeLa or KB cells and the inoculum is such that the cell sheet is not destroyed in less than 72 hours. Pereira[30] has shown that the haemagglutinin is the trypsin-resistant component of antigens B and C. Many of the Adenoviruses from other species have the ability to agglutinate the erythrocytes of one or more animal species. Only the human types 12 and 18 do not have this property.

Finally, Adenovirus types 1, 2, 4, 5 and 6, but not type 3, produce an inhibitor which is type-specific and which reduces the production of Adenovirus type 5, Poliovirus type 1 and vaccinia virus in tissue cultures. Pereira[31] further showed that this inhibitor is distinct from interferon, is produced only in the cellular phase and is not normally liberated into the surrounding medium. It does not affect the adsorption of virus to the cells but rather, it reduces the number of cells producing virus.

Growth

The early observations of an absence of overt disease in small laboratory animals has since proved to be incorrect, although at the present time there is little evidence to show that the Adenoviruses affecting one species infect members of any other species.

Pereira and Kelly[32] first reported that Adenovirus caused latent infections in rabbits. Type 5, but not types 1, 2, 3 or 4, could be isolated from the spleens of inoculated rabbits for at least two months after infection although there were no overt signs of disease. More recently there has been evidence of types 12 and 18 inducing the formation of tumours when inoculated into the lungs of baby hamsters.[33,34] However, for most work with Adenoviruses tissue cultures are required for growth.

HeLa cell cultures are most frequently used for the growth of the members of this group but any epithelial cell culture can be used. The human types all grow in HeLa, KB, HEp2, human amnion, human

thyroid, human embryo kidney and human embryo liver cell cultures,[35,36,37] but not all types are equally readily cultivated. Types 8, 9, 10, 12, 13, 18 to 28 are more difficult to grow than the others. The poorest-growing type has proved to be type 8, in all cells except HEK and HEL cultures.[38] In this case it is not that infectious virus is not produced in HeLa cells but that only a small proportion of the particles can infect HeLa cells in comparison with the numbers which successfully infect embryonic cells. Most of the human types can be adapted to grow in monkey kidney cell cultures with varying ease, but these cultures are not suitable for the initial isolation of Adenoviruses.

Since their discovery a considerable amount of interest has been directed towards the growth of these viruses. Much of the work has been performed with types 1 to 8 using cover-slip preparations of HeLa cells and occasionally other cells, and plaque-formation tests.

Adenovirus adsorbs slowly to HeLa cells with 50% adsorption occurring after 30–60 minutes for types 4 and 5.[39] New virus first appears intracellularly after 17–18 hours and only 6% at the most is liberated into the medium after 6 days.[40] For the release of all the virus the cells must be disrupted. In this respect type 8 differs from the rest—all the new virus is liberated into the medium by the 5th day.[41] With types 4 and 5 approximately 10,000 PFU of virus are produced per cell.[39] One unusual feature with HeLa cells is the continuation of metabolism which does not cease immediately following the production of new virus. Furthermore cell glycolysis is stimulated resulting in an increase in the amounts of lactic, pyruvic, acetic and α-ketoglutaric acids produced.[42]

The investigations on the cytological changes produced in HeLa cells by types 1 to 7 have shown that there are two distinct patterns, one associated with types 1, 2, 5 and 6 and the other with types 3, 4 and 7. In both groups the changes can be divided into four stages and those characteristic of type 5 Adenovirus will be described:

Stage 1. This stage is reached 12 to 20 hours after infection and is characterised by the presence of acidophilic granules in the nucleus. Specific staining of these granules with fluorescent antibody is given but no infectious virus is found.

Stage 2. This occurs 20 to 26 hours after infection when the acidophilic granules coalesce. The nuclei become enlarged with a reduction in the amount of chromatin and the number of mitotic figures to about half that of the controls. Fluorescent antibody stains the acidophilic masses but infectious virus is not found.

Stage 3. In this stage, occurring 24 to 30 hours after infection, small basophilic granules which increase in number can be seen within acidophilic cords. The nuclei become more enlarged and the chromatin more distorted. Refractile material begins to form in the acidophilic mass and has been called the hyaline matrix. New virus is first observed at this stage and fluorescent antibody stains the cytoplasm of some cells.

Stage 4. From 30 hours onwards the nuclei are greatly enlarged and long slender protein crystals are found in the hyaline matrix. The basophilic granules coalesce into a mass surrounded by the acidophilic material. About twenty times as much infectious virus is associated with this stage as the previous stage. Specific fluorescence can be seen in the cytoplasm of about half of the cells but nuclear fluorescent staining is seen in most.

There is considerable asynchrony between individual cells in a single culture and the times of the stages given above are those when the majority of the cells show the characteristic changes. Type 5 is the only virus which gives the protein crystal described is stage 4. Types 3, 4 and 7 also give a series of changes which can less readily be divided into four stages. Once again infectious virus is associated with stages 3 and 4 only and about five times as much is given with stage 4 as with stage 3. These types also give rise to the production of crystals, composed of virus particles, in the nucleus of the cell, a phenomenon which is rarely observed with type 5 etc. From these results it can be concluded that protein synthesis occurs before DNA is formed.[43,44,45] Cytochemical studies have shown that RNA is not present at any stage of synthesis and that neither lipid nor carbohydrate are present in the basophilic granules which are composed of DNA and protein. The protein of the virus particles differs from that of the protein crystal, both being of a non-histone form.[43] Some of the biochemical changes associated with Adenovirus infection of HeLa cells have been mentioned above but the intracellular activity has also been of great interest.

From the ninth hour after infection the uptake of P^{32} into infected cells increases and from the 27th hour decreases. Phosphorus incorporation into DNA rises to 3·5 times that of the controls but incorporation into RNA is only raised 2·5 times. This activity is associated with an increase in the proportion of guanylic and cytidylic acids produced in infected cells when compared with adenylic acid. These observations were made in cells infected with Adenovirus type 2.[46,47] Similar changes have been found occurring in cells infected with types 4 and 5, although with type 5 there is a much greater stimulation of RNA synthesis than with the other types.

Classification

The Adenoviruses are classified principally on the basis of serological tests showing antigenic individuality. For identification purposes they are also divided into species groups, human, simian, mouse, etc. according to the animal from which they were isolated. It is not yet certain that all the non-human types are distinct from each other and those from heterologous species as antibodies for many types are in fact found in heterologous hosts. In addition to this simple classification scheme it has been possible to divide the human strains into sub-groups by means of cytopathic effects as described earlier, by the quantitative

neutralisation test carried out in tubes, and by the pattern of haemagglutination using rhesus and rat red blood cells. Using the quantitative neutralisation test Ginsberg[48] showed that types 1 and 2 gave a slope of two for the neutralisation curve whereas types 3 and 4 gave a slope of unity. This followed the same pattern as the cytological changes. Other workers have found that these slopes are not constant, and it would be expected that the results would depend on the cell line used for the tests.

Haemagglutination of rat and rhesus monkey red cells gives a somewhat different series of results and it is possible to divide the human types into four groups by this method.[16,29]

Group 1. This group consists of types 3, 7, 11, 14, 16, 20, 21, 25, and 28 which agglutinate rhesus cells only. By haemagglutination-inhibition (HI) tests cross-reactions are seen between types 7, 11 and 14. By neutralisation tests it is possible to distinguish two varieties of type 7, but these are identical by HI tests.

Group 2. This group consists of types 8, 9, 10, 13, 15, 17, 19, 22, 23, 24, 26 and 27 which agglutinate rat cells completely. Of these, types 9, 13 and 15 agglutinate rhesus cells to a lower titre. Cross-reactions by both HI and neutralisation tests are seen between types 8 and 9. Cross-reactions by neutralisation tests are also observed between types 26 and 27, and types 15 and 25.

Group 3. This group consists of types 1, 2, 4, 5, and 6 which partially agglutinate rat cells. In the presence of antiserum to any of the heterologous types, except type 4, complete agglutination of the cells is given by all the types except type 4. No cross-reactions are seen by HI or neutralisation tests.

Group 4. Only types 12 and 18 belong to this group. These types do not haemagglutinate any cells and show no cross-reactions by neutralisation tests.

Clinical Manifestations of Adenovirus Infection

The two major characteristics of illness due to Adenovirus infection are pharyngitis and regional lymph gland enlargement. Each of types 1 to 6 can cause clinical illness when swabbed onto the conjunctiva of susceptible individuals but only certain types have so far been associated with epidemics.[49] Types 1, 2, and 5 are most commonly found as latent infections of tonsils and adenoids in children, while the majority would appear to cause sporadic cases. Of the four most important epidemic diseases due to Adenovirus, three occur under natural conditions but the fourth, acute respiratory disease (ARD) of military recruits, is the result of an artificial environment. The three "natural" syndromes are really different manifestations of the same disease in different age-groups. Pharyngoconjunctival fever is a generalised infection of the respiratory tract, with conjunctivitis as one symptom and it occurs mainly in children. When adults become infected there is usually only

conjunctivitis with or without keratitis, the proportion of keratitis depends on the virus type causing the outbreak. ARD of military recruits, however, is the result of bringing together in close proximity a group of susceptible young adults during the initial period of military training. It is thought that the combination of the large mass of susceptible individuals with a marked change in their environmental conditions leads to the large outbreaks of ARD during the winter months. The incubation period for each of these diseases is 4 to 10 days.

Latent Infections of tonsils and adenoids are most frequently associated with types 1, 2, and 5 but types 3, 6, and 7 are also found. The former types are endemic in pre-school children and although they are probably the cause of much undifferentiated pharyngitis they are never associated with epidemic disease. By the age of five about 60% of all children have acquired antibody to these types. When types 3, 4, 6, and 7 infect children of this age they usually give a much milder disease than in the older children and it appears that Adenovirus infection in the very young, with the exceptions detailed later, is relatively mild and non-specific.[50]

Pharyngoconjunctival Fever, the first disease to be specifically associated with Adenoviruses, was described by J. A. Bell.[51] It can occur in all age groups but is predominant in children. Outbreaks involving type 3 have been noted in school children but types 4, 7, and 14 also occur in epidemic form with other types giving sporadic cases.[52,53]

The clinical picture is one of fever, frontal headache, conjunctivitis, rhinitis, sore throat and enlarged cervical lymph glands. Migration of lymphocytes into the submucosal tissue is a characteristic of the conjunctivitis. Abdominal symptoms such as nausea, vomiting, diarrhoea, and abdominal pain are often found and accumulating evidence shows that about 2% of all cases develop a maculopapular rash on the trunk.[54] The disease is not severe and has a duration of from 1 to 10 days with 5 days being the average.

Follicular Conjunctivitis is probably a form of pharyngoconjunctival fever affecting adults without the pharyngitis and fever. Adenovirus types 3 and 7 appear to be responsible for such cases, but types 2, 4, 6, and 9 have been associated.[55] Keratitis is an uncommon complication of the disease and permanent corneal damage is rare.

Epidemic Keratoconjunctivitis has been known as a single entity since 1894 when it was reported from Japan. In the West it has occurred in widespread epidemics associated with swimming baths and industries carrying a high incidence of eye injury. It is now known that Adenovirus type 8 is the sole cause of epidemic keratoconjunctivitis (EKC) although sporadic cases may be caused by other types.

The clinical picture varies with the age of the patient. In children it is virtually identical with pharyngoconjunctival fever with little or no keratitis. In the adult there are usually no signs other than conjunctivitis,

keratitis and pre-auricular lymph gland enlargement. An initial development of conjunctivitis with oedema of the eyelids and conjunctiva is followed 2 to 8 days later by punctate keratitis. Pain, severe photophobia and the presence of a pseudomembrane occur in a varying number of cases. Bilateral conjunctivitis is found in 20% with the second eye less severely affected. The conjunctivitis lasts about two weeks but residual corneal opacity may remain for at least 10 years.[56,57]

The epidemiology of this group of diseases is similar. The epidemic Adenovirus types are those which are not endemic in young children—types 3, 4, 7, and 8. When one of these viruses is introduced into the school child population it spreads giving outbreaks of pharyngoconjunctival fever. This is then passed to the adults who develop conjunctivitis or keratoconjunctivitis if type 8 is the offending source of infection. Swimming pools are important in the spread of disease. Although actual dissemination may be due to droplet infection, by faecal contamination or even presence of virus in the water supply, an irritated conjunctival membrane is the most obvious and convenient mode of entry for members of the Adenovirus group.[49] Similarly EKC epidemics are usually associated, in the West, with industries which have a high incidence of eye injury, because Adenovirus is sufficiently resistant to remain infective while being transmitted from patient to patient by contaminated instruments or even anaesthetic solutions![58]

Acute Respiratory Disease (ARD) or febrile catarrh has been recognised as a major cause of winter illness among military recruits in both America and Europe for some years. During surveys carried out in U.S. training camps between 1942 and 1945 a very high proportion of the recruits suffered from ARD whereas "seasoned" men were not affected to the same extent.[11] Adenovirus types 4 and 7 have since been established as the major causes of ARD although type 3 is an occasional cause and, in Europe, types 14 and 21 have also been implicated.[10,59,60,61]

The clinical picture here is one of sore throat, fever, chills, rhinitis, cough, hoarseness and headache. Conjunctivitis and lymphadenitis are also observed, while a varying number of the cases present as primary atypical pneumonia. The severity of the disease ranges from a mild "common cold" lasting one day to several weeks hospitalisation with pneumonia, sometimes leading to discharge from the force! In the U.S.A. from October to April, 80% of all new recruits become infected during their first week of training. Of these 25% require to be hospitalised, 25% have a milder illness, while the remaining 50% do not report any illness. In summer only 10% of the recruits become infected, although the proportions of the illness remain the same.[62]

Other Diseases associated with Adenoviruses are pneumonia, diarrhoea, aseptic meningitis, mesenteric lymphadenitis and intussusception. Pneumonia has occurred during epidemics of ARD in military recruits but sporadic cases and small outbreaks among children also occur. In infants the disease is often fatal and so far types 2, 3, 4, and 7 have

TABLE 11.1

Association of Adenovirus Types with Clinical Illness

Disease	Epidemic Types	Sporadic Types
Febrile pharyngitis	—	1, 2, 3, 5, 7
Pharyngoconjunctival fever	3, 4, 7, 14, (8)	1, 2, 5, 6, 11
Follicular conjunctivitis	3, 7	1, 2, 5, 6, 9, 10
Keratoconjunctivitis	8	3, 7
ARD	3, 4, 7, 14, 21	—
Pneumonia	—	2, 3, 4, 7
Meningitis	—	2, 3, 7
Mesenteric adenitis & Intussusception	—	1, 2, 3, 4, 5, 6, 7

been implicated.[63] Less severe are the cases of aseptic meningitis associated with Adenovirus. Although no outbreaks of central nervous system disease have been caused by Adenovirus there is accumulating evidence to show that sporadic cases of meningitis are caused by Adenovirus type 2.[38] Other types will almost certainly follow as causes of this disease.

Abdominal symptoms have been frequently reported but it appears that diarrhoea and mesenteric lymphadenitis may be due to Adenovirus types 1, 2, 3, 4, 5, 6 and 7, in the absence of any other symptoms.[64,65] Acute idiopathic intussusception is a very different problem. There is an association between these cases and Adenovirus infection but it is too early yet to say that this association is of definite aetiological significance, there being little doubt that many factors play their parts in what is a mechanical defect.[65,66,67]

TREATMENT must always be directed to alleviation of the symtoms. If there is streptococcal infection prevalent in the community prophylactic treatment may be initiated with penicillin to prevent secondary infection.

LABORATORY INVESTIGATIONS are required to give differential diagnoses of Adenovirus infections. This may be done by demonstrating a rise in the group C-F antibody which will show that an Adenovirus infection has occurred, or by isolating the virus which will identify the actual type. In all the manifestations of Adenovirus infection, virus may be isolated from specimens of faeces and, in the earlier stages, from the naso-pharynx. In the author's experience a throat swab gives better results than throat washings for the isolation of members of this group. When conjunctivitis is present swabs or preferably scrapings of the inflamed conjunctiva should be collected for virus isolation. It is also possible to isolate Adenoviruses from the CSF of cases of meningitis and from the lymph nodes of cases of mesenteric adenitis and intussusception. In all cases the isolation should be confirmed by demonstrating the rise of antibody between acute and convalescent specimens of serum.

Vaccination

In the civilian population Adenovirus infections account for only a very small proportion of the respiratory illness although they are of great importance in military recruits. While it is true that there are widespread outbreaks of pharyngoconjunctival fever among school children and epidemic keratoconjunctivitis among adults they are not sufficiently important to warrant mass production of a vaccine. The importance of EKC can be minimised by efficient sterilisation of optical instruments and vaccines. In the case of military recruits vaccination is imperative since Adenovirus infection causes the loss of the greatest number of man-hours during the winter months.

Several vaccines have been produced combining types 3, 4 and 7 or only types 4 and 7. The virus for these preparations is grown in cultures of monkey cells and is then inactivated with formalin. Antibody response to vaccination is variable but only a low level of antibody is necessary to prevent clinical illness.[68] Type 3 is not required in the vaccine due to heterologous antibody response elicited by types 4 and 7. It has been postulated by Van der Veen that this is due to the "recall phenomenon" where antibody to a type which has infected the person in childhood is "recalled" in response to infection or vaccination with another type.[69] When recruits are vaccinated within a week of arrival at training centres, all respiratory disease is reduced by up to 60%. However, reduction in diseases caused by Adenovirus may be as much as 90% with the greatest improvement in the more severe forms of illness.

Non-Human Adenoviruses

Viruses related to the human Adenoviruses have been recovered from many species of the animal world. Most show complete identity of the group complement-fixing antigen with that of the human viruses, but infectious canine hepatitis virus (ICHV) shows only a one-way cross-reaction. The soluble antigen of ICHV fixes complement with human convalescent sera, but human Adenovirus antigen does not fix complement with convalescent dog sera. The other properties of ICHV are similar to those of Adenovirus types 3, 4 and 7 and this virus has been admitted to the Adenovirus group as type D1.[20,70]

INFECTIOUS CANINE HEPATITIS virus produces two entirely different types of illness after an incubation period of 2–6 days.[71] In foxes it causes a severe encephalitis with damage to the brain and spinal cord. There is a mortality rate of 10–25%.[72] However in dogs it takes the form of hepatitis which varies widely in severity.[71,73,74] In the most severe cases, an apparently healthy dog suddenly collapses and dies within 12–24 hours. When the illness is less severe, with full recovery after 4 to 6 days, the symptoms are high fever (up to 106°F), vomiting, apathy and general malaise. There is a reduced white cell count. Signs of an encephalitis have been recorded but they are rare.

The mortality rate may be as high as 30% but mild or inapparent infections are common.

Histological examination reveals intranuclear inclusions in the liver cord and endothelial cells.

Monkeys and chimpanzees have been the source of about 16 distinct types of Adenovirus. They have been isolated from kidney cell cultures, faeces of normal monkeys and monkeys suffering from diarrhoea. It is possible that they may cause diarrhoea and other diseases in monkeys but the evidence is not yet complete. The chimpanzee strain is now Adenovirus type C1, while the monkey strains so far classified are types M1 to M11.[17,75,76]

The three remaining Adenoviruses have been isolated from cattle and mice.[18,19] Two strains (types B1 and B2) were isolated from the faeces of normal cattle and one from laboratory mice, type Mo1. No disease has yet been associated with the bovine strains but the mouse strain produces a fatal disease when inoculated intranasally into suckling mice.

Antibody to many of these non-human types can be demonstrated in human sera but few, if any, can be serially passed in cultures of human tissues. It remains to be seen whether any of these viruses are related to the human strains or if there is a pool of Adenoviruses which produces natural infections in several animal species comparable with the Reo-, Myxo-, and Arboviruses.

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CHAPTER TWELVE

THE REOVIRUSES

THE Reoviruses are the smallest distinct group of viruses so far recognised officially. Only three types are known and their relationship to clinical disease is still questionable. The prototype strain was isolated from the faeces of healthy children in the U.S.A.[1] and was originally classified as ECHO 10. Differences between this type and the other ECHO viruses led the Enterovirus Committee of the National Foundation to remove ECHO type 10 from the Enterovirus group and reclassify it as Reovirus type 1[2]—the name being chosen because it conveys the idea of association with both respiratory and enteric tracts.

Although only three serological types have been identified strains have been isolated from monkeys,[3] cattle,[4] mice,[5] and dogs,[6] which have proved to be identical with those of human origin. In addition antibodies have been found in the sera of guinea pigs, cats, swine, rabbits and horses.[7] Despite this widespread distribution throughout the animal kingdom, which is reflected in an equally widespread geographical incidence, the association of the Reoviruses with any recognised disease is still doubtful.

Recent studies on the structure and immunology of certain plant viruses have shown that two insect-vectored plant pathogens are almost certainly Reoviruses.[8–12] One of these plant pathogens, wound tumour virus (WTV), and Reovirus type 3 both have double-stranded RNA.[13,14] They are the only two viruses so far shown to possess this type of nucleic acid.

Biological Properties

The properties of the Reoviruses were defined as follows:—[2]

(1) They are ether resistant.

(2) They are 72 mμ in diameter as determined by filtration.

(3) They give a characteristic C.P.E. in monkey kidney cells with intracytoplasmic inclusions and intact nuclei.

(4) They grow in tissue cultures from a wide range of hosts.

(5) They grow in suckling mice but only sometimes produce clinical disease.

(6) Some strains grow in eggs.

(7) They occasionally kill monkeys when inoculated intracerebrally.

(8) They all agglutinate human group O cells and the erythrocyte receptors are not destroyed by R.D.E. but by potassium periodate.

(9) All strains produce a common complement-fixing antigen.

Electron microscopy shows that the Reovirus particle has an icosahedral form with a diameter of 60 mμ and 92 capsomeres in the capsid.[8,9] The nucleic acid is RNA[15] and in any given preparation of virus this central core is missing from at least one quarter of the particles.[16] By X-ray diffraction this RNA appears to be double-stranded resembling DNA in its structure,[13,14] and this has been confirmed by analysis of the

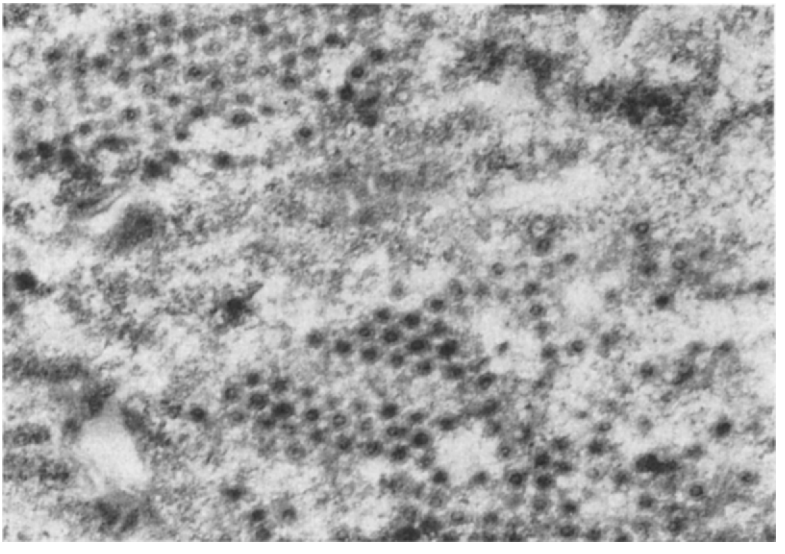

FIG. 12.1. Electron micrograph of a thin section of monkey kidney cells infected with Reovirus type 3. Preparation fixed in glutaraldehyde and osmic acid and stained with uranyl acetate. The arrangement of the particles in a crystalline lattice is well demonstrated. (*Photo—E. Griffin, Wellcome Trust electron microscopy unit, Dept. of Anatomy, Makerere University College.*)

base composition of the RNA of Reovirus type 3 and WTV.[17,18] Ultrasonic disintegration of a suspension of Reovirus does not increase the yield of PFU and aggregates can be seen in these preparations. The ratio of the particle count to the number of PFU demonstrable is about 11:1 but when aggregates and coreless forms are taken into account this approaches very nearly to a 1:1 ratio.[16]

The viruses are all resistant to ether and some strains show remarkable heat resistance. Using a human strain of type 1 Rhim *et al.*[16] found that at PH 7·5 the infectivity was lost in 3·7 days at 4°C, 2·0 days at 24°C and 19 hours at 37°C. At 56°C they found that a suspension containing 7×10^7 PFU/ml was completely inactivated in 45 minutes but not 30 minutes. On the other hand, certain strains have been found which are not completely destroyed after 2 hours at 56°C.[19] Treatment of suspensions with trypsin does not destroy infectivity but actually increases it, probably by dispersing the aggregated particles.[20] The infectivity is

markedly destroyed by treatment for one hour at 37°C with potassium periodate (M/180,000 of KIO_4).

The Reoviruses possess three demonstrable antigens. One is a common group antigen which is observed by complement fixation tests and which is probably soluble. The other two are demonstrated by neutralisation and haemagglutination-inhibition tests. The haemagglutinin is associated with the virus particle and its destruction is accompanied by a loss of infectivity. Only human group O cells have so far been agglutinated by all members of this group[20] although type 3 has been reported as agglutinating bovine erythrocytes.[21] Haemagglutination occurs equally at 4°C, 20°C and 37°C although the reactions require twice as long for completion at 37°C as at 4°C. After 48 hours, types 1 and 3 are eluted from the erythrocytes although type 2 is not, and the cells can be agglutinated with fresh virus of all three types, indicating that the reaction is not enzymatic. Treatment of the erythrocytes with trypsin or potassium periodate decreases their agglutinability by Reoviruses, but RDE has no effect. The mechanism of haemagglutination is therefore distinct from that of the Myxoviruses but similar to that of the Picornaviruses.

Growth

These viruses have a very wide host range and natural infections of man, monkeys, dogs, mice and cattle have been observed.

Inoculation of mice less than three days old with large amounts of any strain will produce disease. Types 1 and 2 give lesions of the liver, heart and brain not unlike those of the Coxsackie B viruses.[22] Type 3 strains however give rise to jaundice, peritoneal exudate, the fur of the mice becomes "oily" and if the animal survives long enough it develops alopecia.[23,24] Occasionally Reoviruses grow in fertile hen's eggs and produce pocks on the chorio-allantoic membrane, but serial propagation in eggs is difficult.[22]

Most strains of Reovirus have been isolated in tissue cultures and the very wide range of host tissues in which they grow was the first indication that they were not typical ECHO viruses. Hsuing[25] first grew ECHO 10 in the kidney cells of Rhesus, Patas and Capuchin monkeys, swine, cats, dogs, calves, rabbits, and guinea pigs. They also grow in many human cells including HeLa and KB. Plaque formation under agar is produced by these viruses only when a serum free overlay is used and neutral red is not added until after 48 hours incubation.[26]

Several studies have been made of the growth of Reoviruses in HeLa, KB, L and primary monkey kidney cells.[16,27,28,29] These all show that the virus multiplies slowly in the cytoplasm with an eclipse period of about 6 hours. The occurrence of the maximum concentration of cell-associated virus varies from 15 to 54 hours and most of the virus remains within the cytoplasm unless artificially ruptured. Using fluorescent-antibody staining with type 1 in HeLa cells, virus antigen first appears

in particulate form 6 to 8 hours after infection.[27] These particles increase in size and coalesce to form a reticulum throughout the cytoplasm by the 12th hour. At 14 to 16 hours the reticulum is concentrated in a perinuclear position, sometimes completely surrounding the nucleus. In the final stages of development the perinuclear reticulum becomes less well defined and the antigen is spread throughout the cytoplasm which has a "bubbling' appearance. Antigen is released at this point by the pinching off of extended cell processes. Viral antigen and infectious virus reach a maximum concentration 24 hours after infection. Under the electron microscope type 3 grown in KB cells appears as small crystalline-like structures within an intracytoplasmic matrix.[28] Long filaments of about 35 mμ were also observed among the virus particles.

The movement of viral antigen with spindle formation is an unusual feature of Reovirus multiplication in FL cells.[30] When spindle formation, and therefore mitosis, is prevented the virus antigen forms into spherical globules and does not show the movements described above.

Classification and Natural Hosts

Although Reoviruses have been isolated from many different animal species all those tested so far belong to three antigenic types. However, type 2 strains show great heterogeneity. Serological identification can be performed by either neutralisation or haemagglutination-inhibition tests.[2,31] All three strains have been identified from man and cattle, while types 1 and 2 have been found in monkeys[2,7] and type 2 has been isolated from wild town and country mice.[5] Type 1 has also been found in the lung of a dog which died following a respiratory illness.[6] In addition to these three types, two other viruses from monkeys show properties similar to the Reoviruses, but have a narrower host range in tissue culture and agglutinate erythrocytes from Macaca monkeys but not human group O cells.[7] It is possible that these two simian viruses will prove to be Reoviruses,

There are also the two plant pathogens, wound tumour virus and rice dwarf virus which infect a wide range of plants and are transmitted by leafhoppers.[10,12,32,33] The virus multiplies within the insect and can readily be demonstrated in the cells of the vector.[12] One other virus may belong to this group—BGA virus whose presence has recently been demonstrated in blue-green agae.[34]

Clinical Illness

The exact relationship of Reoviruses to disease in man and animals is not yet certain. Two definite outbreaks of Reovirus infection in children have been recorded. In the first,[35] the symptoms were low fever, rhinitis, pharyngitis and diarrhoea, while in the second[36] there was a maculopapular rash associated with fever, pharyngitis and cervical lymphadenopathy. Reoviruses have been isolated from sporadic cases of diarrhoea[37] and upper respiratory tract illness of children.[38] Aseptic

meningitis has also been associated with types 1 and 2 but such cases appear to be infrequent.[39,40]

Reovirus type 3 has been isolated from two other syndromes. In Australia Stanley *et al.*[19,41] isolated this virus from a native child with steatorrhoea and pneumonitis who later developed alopecia areata. This finding is of great interest in view of the known symptoms induced by this virus following oral or intranasal infection of mice.[24] The other isolation, by Bell *et al.*[42] in Uganda, was from a case of Burkitt's Tumour. Once again this is of interest considering the relationship between this virus and wound tumour virus of plants.

Overt disease in the animal world has seldom been noticed. Fatal respiratory infections due to types 1 and 2 respectively have been observed in a single dog and a monkey[3,6] while an outbreak of rhinitis in chimpanzees has been associated with type 2.[43] Most of the simian strains have been isolated from degenerating cultures of kidneys from healthy monkeys. In cattle most animals in the U.S.A. have been infected by one or more types when they are 12 months old with no overt disease.[44]

Antibodies to Reovirus have been found in both human and animal sera, not only in Europe and the U.S.A. but also in the South Pacific islands and the jungles of Panama.[45] We have, therefore, in this group of viruses a paradoxical situation, where one of the most widespread of viruses, easily transmitted to both humans and animals produces little evidence of disease.[46] It is possible however that infection at a certain age or under certain conditions, for example, neonatal infection or the introduction of the virus directly into the blood-stream by mosquito bite, may give rise to occasional serious and unexpected illnesses. For the most part, the Reoviruses appear to be perfect obligate parasites with an almost unlimited number of hosts who will be entirely unaware of their presence.

Non-Animal Reoviruses

The morphological similarity between wound tumour virus of plants and the Reoviruses has been observed recently[8,9] and has been followed by a report of Reovirus complement-fixing antibody in the sera of animals immunised against wound tumour virus.[11] This virus is not only a plant pathogen, infecting several species of plants in different families but is also transmitted by insects. It can produce lesions in at least three related species of leafhoppers and infects a small proportion of the offspring of these insect vectors.[32,33]

Another plant virus which would appear to be a Reovirus is rice dwarf virus which also multiplies in its leafhopper vectors.[12] Both of these viruses are important plant pathogens and their transmission by insects leads one to expect that the animal viruses may also be arthropod vectored. Finally the demonstration of what is probably a Reovirus in blue-green algae[34] has extended the host range of this group even further.

It therefore seems that the Reoviruses are the most widespread group in nature and that their future study may produce far reaching and unexpected results.

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CHAPTER THIRTEEN

THE ARBOVIRUSES

THE Arbovirus group is the largest of the recognised virus groups and comes second only to the Picornaviruses in order of clinical significance. It is not a homogenous group and will probably be divided when the basic properties are adequately determined. An Arbovirus has been defined as "one, which in nature, can infect haemophagous arthropods by their ingestion of infected vertebrate blood. It multiplies in their tissue and is transmitted by bite to susceptible vertebrates."[1] At the moment this is the only property recognised for inclusion in the group. The original name was arthropod-borne virus, now shortened to arborvirus or Arbovirus.[2] There are approximately 170 known "types" divided into about 21 sub-groups. However, the word *type* in connection with the Arboviruses does not have the same exact connotation that it has in the groups with which we have just dealt. Despite the abundance of literature published on these viruses there is great poverty of fundamental detail.

The most important member of the group is Yellow Fever Virus which was first recognised in 1901[3]. In the tropics this virus causes more disease and distress than all the other viruses put together. Like all Arboviruses, Yellow Fever Virus is not only transmitted by an arthropod but also multiplies within the insect. Although the Arboviruses are restricted to the areas of their vectors, no part of the world is entirely free of them because the vectors can be mosquitoes, ticks, sandflies or midges. As one would expect they are most prevalent in tropical and sub-tropical countries.

Beyond the fact that they are arthropod-vectored comparatively little is known of their biological properties although information is now being obtained. The next few years should see a marked change in the classification of this group. Since 1954 some advances have been made towards grouping these viruses on an antigenic basis rather than the crude grouping on clinical symptoms.[4,5] A similar sort of pattern to that of the Picornaviruses is emerging, although the insect vector must always be taken into account with the Arboviruses.

In all previous descriptions of this group each virus has been treated individually, regardless of natural order. This is both a lengthy and pointless system since the Arboviruses, apart from transmission, are in no way different from any other group of viruses. We shall therefore depart from this practice and proceed to the biological properties, as far as they are known.

Biological Properties

The biological properties of the Arboviruses can be defined as:—

(1) They can infect and multiply within haemophagous arthropods.
(2) They are sensitive to 20% di-ethyl ether and 1/1000 deoxycholate.
(3) The nucleic acid is RNA.
(4) They agglutinate chicken and goose erythrocytes.
(5) They are heat labile.
(6) They are spherical or rod-shaped viruses with diameters between 20 and 100 mμ.

Electron microscopy has shown that at least two, possibly three, distinct groups can be defined. The first group is composed of Yellow Fever (YF), Semliki Forest Virus (SFV), Venezuelan Equine Encephalitis (VEE), West Nile (WN) and Western Equine Encephalitis (WEE). These viruses appear to be spherical, ranging in size from 35–50 mμ.[6–10] The virus particle is composed of an electron-dense central core within an outer lipid membrane. This central core has a visible substructure which may be polyhedral.

The second group consists of Rift Valley Fever (RVF) which is a spherical virus particle about 60–75 mμ in diameter.[11] The surface is covered with hollow cylinders but there is no real evidence of an external lipid membrane.

These may not be two groups because using a different method of preparation for electron microscopy VEE is shown to have a diameter of 65–75 mμ although the inner core is only 45 mμ.[12] Smaller particles of 16–22 mμ have been observed occasionally, which may be viral sub-units.[7,10,12]

The third group consists of Vesicular Stomatitis Virus (VSV). This virus is rod-shaped—a bullet-shaped particle 175 mμ long by 68 mμ diameter.[13] One end is rounded while the other is flat with a central hole (17 mμ in diameter) extending half-way up the particle. Around the periphery are fine projections 10 mμ long. The internal structure appears to be a tightly wound helix with 4·5 mμ separating each turn and the overall dimensions are 160 × 48 mμ.

By filtration, the size of several other viruses, in addition to some of those already mentioned, has been determined within the range of 15 to 122 mμ.[14] Many of the lower figures appear to be too low when compared with the available electron microscopy determinations. Nevertheless several morphological types appear to exist within the Arbovirus group and three have been suggested here. The ratio of particle counts to infectious virus has been estimated as 1·1:1 for SFV[7] and 40:1 for VSV,[13] which represent two of the morphological types.

Several of the Arboviruses have been chemically analysed and all are of similar composition. In addition to RNA and protein, phospholipid and cholesterol are present.[15–18] The RNA is about 3–6% of the total mass while the ratio of protein to lipid varies with the virus. In Eastern

Equine Encephalitis (EEE) the total lipid is equal in amount to the protein. With Sindbis and VEE it is only half and with VSV it accounts for about three quarters of the virus particle. Sindbis and VSV have the nucleotides of the RNA the same as the host cell, but in very different proportions.[17,18]

All the Arboviruses are sensitive to 1 : 1000 dilution of sodium deoxycholate[19] or 20% di-ethyl ether.[1] They are readily inactivated by heat and as a group are sensitive to an unfavourable environment. Using a diluent of medium 199 and 5% calf serum buffered to pH 7·0–7·4 the thermal inactivation of seven types at 37°C was compared.[20] All were completely inactivated after 80 hours and the drop in titre after 24 hours was as follows:—EEE, WEE, SLE (St. Louis Encephalitis), WN, = 2·5 logs; Bwamba = 3·4 logs; Ntaya = 4·1 logs; Sindbis = 4·5 logs. Inactivation occurs also at 4°C and −20°C although not as rapidly, while at −70°C these viruses can be preserved indefinitely. The viruses can be stabilised at room temperature for titrations by adding protein—such as 0·5% LAH, 10% calf serum or 0·75% bovine plasma albumin.

The majority of the Arboviruses possess antigens which can be demonstrated by neutralisation, complement fixation, haemagglutination-inhibition and agar-gel diffusion tests.[21]

The haemagglutinin of SFV is associated with the infectious virus particle which also contains most of the C-F antigen.[22] Although both infectivity and haemagglutinin are inactivated simultaneously at 37°C the haemagglutinin is resistant to ether at 3°C whereas the infectivity is not. As infectious RNA can be obtained from SFV-infected mouse brain with ether extraction[23] this inactivation of infectivity of the whole particle by ether does not involve the RNA. The C-F and HA antigens are also distinct. Treatment with protamine enhances the HA activity but decreases complement fixation.[22] Thus it appears that the SFV particle contains several antigens.

Other workers have reported the separation of the haemagglutinin of several Arboviruses into two fractions by chromatography.[24] One fraction, which had a large size, contained most of the infectivity and C-F activity. The other, which was smaller, was non-infectious. Antisera prepared against one fraction reacted equally well with the other and vice versa, suggesting that the smaller particle is a sub-unit of the infectious particle. This corresponds with the 16–22 mμ particles seen in the electron microscope. For at least one other virus, Japanese B Encephalitis (JBE), the haemagglutinin and C-F antigen can be separated from the infectious virus particle by differential centrifugation. They appear to be 10 mμ particles and are probably identical.[25] Again these may be sub-units of the complete virus particle. Virus specific soluble antigens appear to be only produced occasionally by the Arboviruses.

Although haemagglutinin can be prepared in tissue cultures by using the supernatant fluid from completely degenerated cultures[26] it is more

usual to prepare it from infected mouse brain.[27] The infected mouse brains are extracted with ether and stored at pH 9·0. On incubation at 37°C the haemagglutinin can be demonstrated using day-old chick or adult goose erythrocytes. The optimal pH for haemagglutination varies from virus to virus, but it is usually within the range pH 6 to ph 7.

Growth

The host range of the Arboviruses is the widest of all the viruses, encompassing mammals, birds and arthropods. Much information has been accumulated at the macroscopic level on their growth in vertebrate and arthropod hosts although little is known of the actual formation within the cell. What information there is will be mentioned after a general consideration of their growth in living hosts.

THE VERTEBRATE HOSTS include virtually every known mammal and bird, although naturally not all the viruses infect all of them. Every member can be grown in either adult or suckling mice. The most consistent results follow intracerebral inoculation but peripheral routes can be used for many of these viruses. Infection in suckling mice is followed by a fatal encephalitis and vast amounts of virus are present in the brain and blood. This viraemia is common to all Arbovirus infections of vertebrates even in the absence of apparent disease.

When an inoculum of 10 LD_{50} of RVF is administered to adult mice either intravenously or intracerebrally, death occurs in two to three days, after only two to three hours of illness.[28] The blood has a titre of $10^{10}LD_{50}$ per ml. while the brain titre varies from 10^{8} to $10^{9}LD_{50}$. Little or no virus is excreted in the milk, urine or faeces, but some is found in the nasal mucosa.

During the first hour after inoculation, the titre of virus in the blood drops. This is followed by a stationary phase, the eclipse phase, lasting until the fifth to ninth hour when the virus titre rises. The virus is liberated into the blood stream almost as soon as it is formed and the titre continues to rise, depending on the size of the original inoculum, until a peak is reached just before death. When the inoculum is huge, only one growth cycle is required and death follows from six hours after infection. Inoculation of most of the other Arboviruses into mice produces a typical meningo-encephalitis and death, with higher titres of virus in the brain than in the blood.

One recent observation has widened the host range still further. Garter snakes can be infected with WEE from the bites of infected *Culex tarsalis*.[29] The bites of these insects produces a viraemia in the snakes sufficiently high to be passed back to fresh insects at a later stage. If the snakes are infected just before hibernation the virus also hibernates and produces the viraemia when the snake emerges in the Spring.[30]

THE ARTHROPOD HOSTS include members of the three families —Culicidae, Psychodidae and Ixodidae or to use the common names—

mosquitoes, midges (Culicoides), sandflies (Phlebotomus), ticks and mites. Some Arboviruses have a very wide range of arthropod hosts whereas others appear to infect only one or two species. After the arthropod has taken in infected blood the virus multiplies and then passes to the salivary glands. From there it is injected into a new vertebrate when the arthropod next makes a meal. This multiplication within the arthropod produces no disease and continues for a considerable time. In some cases transovarian transmission to the offspring of the insect occurs. A few Arboviruses have been isolated solely from wild arthropods but they still cause disease in mice and are therefore considered to be Arboviruses, rather than viruses solely infecting arthropods.

Several experiments on transmission are worth mentioning. The mosquito *Culex tritaeniorhynchus* will transmit JBE virus from chickens and pigs to a wide range of birds and animals. As little as 3 mouse LD_{50} of virus in 0·04 ml. of blood will infect the mosquito which can then transmit the virus to fresh mice in about two weeks.[31] This delay between infection and ability to transmit the virus indicates viral multiplication within the mosquito. In support of this is the drop in titre of SFV in *Aedes aegypti* (L) during the first 24–48 hours after infection, which demonstrates the occurrence of an eclipse phase before viral multiplication begins.[32] Similar experiments with *Aedes aegypti* (L) mosquitoes showed that three days after infection with VSV they were able to transmit that virus to mice. Again the virus multiplies in the mosquito and although naturally occurring infected mosquitoes have not been found, VSV has been accepted into the Arbovirus group.[33]

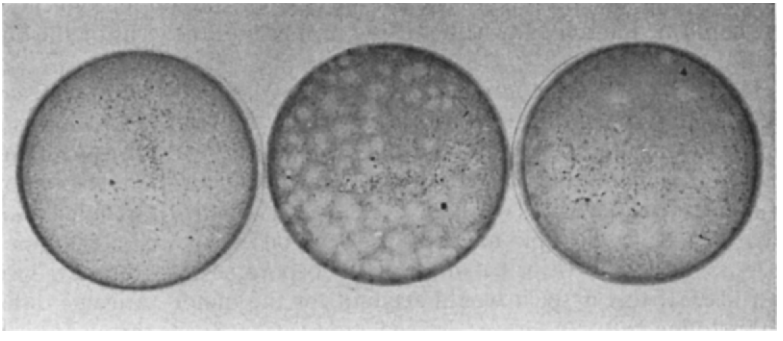
Transovarian transmission occurs with the tick-borne agents. In this instance the female ticks lay infected eggs which automatically produce a brood of infected adults. Colorado Tick Fever (CTF), when ingested by nymphs of the tick *Dermacentor andersoni*, remains dormant until metamorphosis to the adult stage takes place when the virus increases in amount. Ticks observed for 10 months thereafter show no drop in virus titre and the females produce infected eggs.[34,35]

In addition to these natural hosts it is possible to infect other arthropods of different families.[36] In no case however is transovarian infection observed, although artificial serial passage succeeds and the insect remains infectious for up to 10 months. Grasshoppers, bedbugs, black carpet beetles and houseflies have all been infected.

TISSUE CULTURES have been used to study growth cycles of several Arboviruses. Most of these studies produced patterns similar to that of the Myxoviruses. Cultures of mouse, rat, hamster and guinea pig cells[37] have been used as well as HeLa,[38] KB[9] and L[39] continuous cell lines and chick embryo fibroblasts.[40] Since this is a very diverse group the time sequence of events varies considerably between viruses, but we shall look closely at some of the well documented studies.

The classic study is that of Dulbecco & Vogt[41] with WEE in chick

embryo fibroblasts. The virus adsorbs more rapidly to a monolayer of cells than to a suspension, 15% of the virus being adsorbed to the monolayer in 3·3 minutes. Adsorption is followed by the eclipse phase of 2–3½ hours, being shorter when a large inoculum is used. Virus multiplication then commences and continues for 4 to 6 hours. Release of virus is gradual and each cell produces about 200–1000 new particles. Similar experiments have been performed using VEE in KB[8] and L[42]



(a) (b) (c)

FIG. 13.1. Plaques formed in chick embryo fibroblasts under agar by Sindbis virus. (a) uninoculated plate, (b) plate inoculated with 0·5 ml of 10^{-4} dilution of a suspension of Sindbis-infected mouse brain, (c) as (b) but using a dilution of 10^{-5}.

cell cultures and VSV in chick embryo fibroblasts[43,44] and L cell cultures.[45] The eclipse phase varies from 1 to 5 hours depending on the size of inoculum and all the results are similar. Using hamster kidney cell cultures infected with JBE it was noted that the production of haemagglutinin exactly parallels that of infectious virus.[22]

For all the viruses mentioned so far production and release of new virus occur simultaneously and continue over a long period for each cell. With one exception there is always more extracellular than intracellular infectious virus, unlike those groups described previously where most of the virus is retained within the cell. Rift Valley Fever (RVF) is more like the Adenoviruses and Picornaviruses. Using Ehrlich ascites tumour cells the newly formed RVF virus is retained within the cells until it is all abruptly liberated into the medium, seven to eight hours after infection.[46]

Using conventional histological staining, fluorescent-antibody staining and electron microscopy all but one of the Arboviruses studied multiply in the cytoplasm. These include YF,[6,47] Junin virus,[48,49] VEE,[8] WEE,[50] and VSV.[13] After infection basophilic granules form in the cytoplasm and can be stained with specific fluorescent antibody, showing that at least viral antigen is being formed. The viruses appear

to mature at either the cell wall[13,50] or at the membranes of cytoplasmic vacuoles.[8] In the case of VSV the whole particle is formed as an extrusion of the cytoplasm. This formation at the cell or vacuole membrane is similar to the Myxoviruses. The time between maturation of the virus and its release is very short, about 20 seconds in the case of VEE.[8]

On the other hand, WN virus multiplies in the nuclei of KB cells[9] with completion of the virus particle at the membrane surface, but in this instance it is the nuclear membrane. Since WN is antigenically related to YF it will be interesting to learn if the different sites of multiplication are confirmed and if so, whether it is a property of the virus or the host cell.

Some biochemical studies have been made with Sindbis virus-infected chick embryo fibroblasts.[51] In this instance the viral RNA does not appear from the nucleotide pool but is synthesised freshly. Although the synthesis of phospholipid is reduced by infection, the phospholipid content of the virus is largely derived from pre-existing cellular phosphatides. Since the lipid membrane is "put on" at the cell membrane it is possible that host cell material is incorporated into the new virus particle. If this is so, it would account for the minor antigenic differences found between viruses derived from various hosts throughout the world.

CHICK EMBRYOS have been used for growing the Arboviruses but they have never replaced the suckling mouse in routine work. Most of the viruses will grow when inoculated into the yolk sac, while some will grow irrespective of the route of inoculation.[52,53] Infected embryos are rapidly killed and the new virus can be obtained by harvesting the allantoic or amniotic fluids, the yolk sac or the embryo itself.

Classification and Natural Hosts

The present classification scheme is based entirely upon antigenic relationships[4,5,54] and before discussing it we shall see how our knowledge at the present time suggests natural divisions. Using only morphology and growth cycles we can get three groups:—

I. These are small (35–50 mμ in diameter) spherical viruses with an outer lipid membrane, which multiply in the cytoplasm, maturing at the surface of cytoplasmic membranes. New virus is released by the cell as soon as it is formed and this release continues for several hours.

II. These are larger (60–75 mμ in diameter) spherical viruses without an obvious outer membrane. New virus is liberated in a single burst after multiplication is complete.

III. These are rod or bullet-shaped viruses 70 × 175 mμ which multiply in the cytoplasm in a manner identical with group I.

If further work shows these divisions to be valid, then the Arbovirus group cannot stand in the same way as the other groups of viruses. Each of these divisions will be a group in its own right, sharing only the property of being arthropod-borne. Arthropod-borne Group I will

then be composed of WEE, VEE, SFV, YF, WN, JBE and Junin viruses and presumably all those antigenically related to them. Group II will contain RVF and possibly the others which have a large diameter as shown by filtration. Group III at the moment is only composed of the VSV strains but the morphology of many of the Arboviruses is not yet known and other bullet-shaped types may yet be found. The antigenic classification will still be valid as no two related viruses have yet been found to have radically different biological properties and this scheme is based on shared antigens as demonstrated by HI, C-F or neutralisation tests.

At first two sub-groups were defined, A and B.[4] The members of each shared antigens to a greater or lesser extent with each other, but were completely distinct from the members of the other sub-group. A third sub-group was added later, C,[5] and to date there are 21 sub-groups, the last 18 being known by name instead of by letter.[54] It has been suggested that within each sub-group the members share a mosaic of several common antigens but that one or two are present to a greater extent than the rest in each type. An excellent exposition of this hypothesis is given by Porterfield[21] and the interested student is advised to consult his paper. The definition of a type in this group is very arbitrary and in the ensuing tables the registered "types" will be listed. These must not be confused with the "type" associated with the other virus groups, for in many cases several of the "types" are virtually indistinguishable antigenically.

In order to simplify the following chapter it is necessary to tabulate all the Arboviruses to indicate their natural vertebrate and arthropod hosts and to give their recognised abbreviations which will be used exclusively from now on. The references which will be given refer to their first isolation.

SUB-GROUP A consists of 17 "types" of which five have been isolated only from mosquitoes. Antibodies to some of these five are present in human sera but this may be due entirely to a sharing of antigens with human pathogens. All but one of the rest have been isolated from mosquitoes, which appear to be their only arthropod host.

TABLE 13.1

Sub-group A

Name	Distribution	Arthropod Hosts	Vertebrate Hosts	Ref.
Chikungunya (Chik)	East, West, South Africa, Thailand	Aedes africanus, Ae. aegypti.	Man.	55
O'nyong-nyong (ONN)	East Africa.	Anopheles funestus, An. gambiae.	Man.	56
Semliki Forest (SFV)	East & West Africa.	Ae. abnormalis, Eretmapodites, Ae. argenteopunctatus.	None known.	57
Mayaro	West Indies, South America.	Mansonia venezuelensis.	Man, monkey.	58
Getah	Malaya, Japan, Australia.	Culex tritaeniorhynchus, Cul. gelidus, An. amictus.	None known.	1
Bebaru	Malaya.	Cul. lophoceratomyia.	None known.	1

TABLE 13.1 (*contd.*)—

Name	Distribution	Arthropod Hosts	Vertebrate Hosts	Ref.
Eastern Equine Encephalitis (EEE)	North, Central, South America, West Indies.	Man. perturbans, An. crucians, Culiseta melanura, Culicoides, Ae. mitchellae.	Man, horse, squirrel deer, birds.	59
Western Equine Encephalitis (WEE)	North, Central, & South America.	Aedes sp., Anopheles sp., Culiseta sp., Culex tarsalis.	Man, horse, deer, birds, squirrel.	60
Highlands J	North America.	None known.	Bird.	54
Sindbis	Egypt, India, East & South Africa, Malaya, Australia.	Cul. univittatus, Man. fuscopennata, Cul. tritaeniorhynchus, Cul. bitaeniorhynchus, Cul. annulirostris.	Cattle, man, birds.	61
Venezuelan Equine Enceph. (VEE)	South America, West Indies.	Man. titillans, Ae. taeniorhynchus.	Horses, man.	62
Be An 8	South America.	As for VEE.	Man, monkey.	54
Pixuna	South America.	An. nimbus.	Man.	54
Aura	South America.	Culex sp.	None known.	1
Una	South America, West Indies.	Psorophora ferox.	None known.	1
Middelburg (MID)	South Africa.	Aedes sp.	Man, (Sheep)	63
Ndumu	South Africa.	Ae. circumluteolus.	None known.	64

SUB-GROUP B consists of 35 "types", of which 24 are transmitted by mosquitoes and 8 by ticks. No vector has yet been found for the other three. Within this sub-group there are several "complexes" of "types" which are closely related antigenically. These are the Tick-borne viruses, and the SLE, JBE, MVE, WN and Ilheus complex of viruses. Altogether nine "types" have been isolated only from mosquitoes or ticks, but antibodies to several have been found in human sera.

TABLE 13.2

Sub-group B

Name	Distribution	Arthropod Hosts	Vertebrate Hosts	Ref.
Yellow Fever (YF)	Africa, Central & South America, West Indies.	Aedes sp., Haemagogus sp., Sabethes chloropterus.	Man, monkeys.	65
Uganda S (US)	East Africa.	Aedes sp.	None known.	66
H 336	South Africa.	Culex rubinotus.	Man.	67
Dengue type 1	Pacific, Greece.	Aedes sp.	Man.	68
Dengue type 2	S.E. Asia, West Indies.	Aedes sp.	Man.	69
Dengue type 3	S.E. Asia.	Aedes sp.	Man.	70
Dengue type 4	S.E. Asia.	Aedes sp.	Man.	70
Spondweni	South & West Africa.	Taeniorhynchus uniformis.	Man.	71
Wesselsbron	South Africa.	Ae. circumluteolus, Ae. caballus Taen. uniformis.	Man, sheep.	72
Zika	East Africa.	Aedes africanus.	Man, monkey.	73
St. Louis Encephalitis (SLE)	North & Central America, West Indies.	Culex sp., Aedes dorsalis, Psorophora ferox, Sabethes sp.	Man, birds.	74
Japanese B Encephalitis (JBE)	East Asia.	Culex tritaeniorhynchus, Culex gelidus, Culex vishnui.	Man, birds.	75
West Nile (WN)	Africa, India.	Culex sp.	Man, birds.	76
Murray Valley Encephalitis (MVE)	Australia, New Guinea.	(Culex annulirostris)	Man.	77

TABLE 13.2 (*contd.*)—

Name	Distribution	Arthropod Hosts	Vetebrate Hosts	Ref.
Ilheus	South America, West Indies.	Psorophora sp., Aedes sp.	Man.	78
Usutu	South Africa.	Culex univittatus.	None known.	54
Turkey Meningo-encephalitis	Israel.	None known.	Turkeys.	79
Tembusu	Malaya.	Culex tritaeniorhynchus.	None known.	1
Ntaya	Uganda.	Aedes aegypti.	None known.	80
Bussuquara	South America.	Culex sp.	Monkey, Rodents.	81
Kunjin	Australia.	Culex annulirostris.	Not known.	82
Kokobera	Australia.	Culex annulirostris.	Not known.	82
Edge Hill	Australia.	Aedes vigilax.	Not known.	82
Stratford	Australia.	Aedes vigilax.	Not known.	82
Russian Spring Summer Encephalitis (RSSE)	U.S.S.R., Central Europe.	Ixodes persulcatus, Ixodes ricinus.	Man, birds, rodents.	83
Central Europ. Enceph. (CEE)	Central Europe.	Ixodes ricinus.	Man, birds, rodents.	84
Omsk haemorr-hagic fever (OHF)	U.S.S.R.	Dermacentor pictus, Dermacentor marginatus.	Man, rodents, birds.	85
Louping Ill (LI)	Great Britain.	Ixodes ricinus.	Man, cattle, sheep, grouse.	86
Kyasanur Forest Dis. (KFD)	India.	Haemaphysalis spinigera, Haemaphysalis turturis, Haemaphysalis papuana.	Man, monkeys.	87
Negishi	Japan.	None known.	Man.	88
Powassan	North America,	Dermacentor andersoni.	Man.	89
Langat	Malaya.	Ixodes granulatus.	None known.	90
Rio Bravo	U.S.A.	None known.	Bats.	91
Entebbe bat	East Africa.	None known.	Bats.	92
Modoc	U.S.A.	None known.	Rodents.	1

THE OTHER SUB-GROUPS have over 70 "types". There are some serological cross-reactions between members of different sub-groups. New relationships among the 50 or so ungrouped agents will no doubt bring about the expansion of existing sub-groups and the creation of new ones. Although only a few of these viruses are important in human disease several involve domestic animals. A total of 15 "types" have never been isolated from arthropods and a further 24 have been isolated only from this kind of host, although antibodies to several of the latter are found in vertebrate blood.

TABLE 13.3

Sub-groups C and Bunyamwera

Name	Distribution	Arthropod Hosts	Vertebrate Hosts	Ref.
		SUB-GROUP C		
Oriboca	Brazil.	Mansonia sp., Psorophora sp. Sebethini sp., Culex sp.	Man, monkey, rodent.	93
Itaqui	Brazil.	None known.	Man, monkey, rodent.	94
Apeu	Brazil.	(Aedes aegypti)	Man, monkey, mouse.	93
Caraparu	Brazil, West Indies.	Mosquitoes.	Man, monkey, mouse.	93
Marituba	Brazil.	(Aedes aegypti)	Man, monkey, mouse.	93
Murutucu	Brazil.	(Aedes aegypti)	Man, sloth, rodent, monkey.	93
Nepuyo	Brazil, West Indies.	Culex sp.	Mouse.	93 54

TABLE 13.3 (*contd.*)—

Name	Distribution	Arthropod Hosts	Vertebrate Hosts	Ref.
		BUNYAMWERA SUB-GROUP		
Bunyamwera	Africa.	Aedes circumluteolus.	Man.	95
Ilesha	East & West Africa.	None known.	Man.	96
Cache Valley	U.S.A., Brazil, West Indies.	Culiseta inornata, Ae. scapularis, An. crucians.	None known.	97
A9 171b	U.S.A.	Culiseta inornata.	None known.	54
Germiston	South Africa.	Culex sp.	Man.	98
Batai	Malaya, India, Czechoslovakia.	Culex gelidus, An. maculipennis, An. barbirostris.	None known.	1
Guaroa	South America.	Anopheles sp.	Man.	99
Sororoca	Brazil.	Sabethini sp.	None known.	54
Kairi	Brazil, West Indies.	Aedes scapularis.	None known.	100
Wyeomyia	South America, West Indies.	Wyeomyia melanocephala, Sabethini sp.	None known.	101

TABLE 13.4

Other Sub-groups

Name	Distribution	Arthropod Hosts	Vertebrate Hosts	Ref.
		CALIFORNIA SUB-GROUP		
California Encephalitis	U.S.A.	Culex tarsalis.	None known.	102
Travittatus	North America.	Aedes trivattatus.	None known.	1
Melao	Brazil, West Indies.	Aedes scapularis.	None known.	93
Tahyna	Central Europe	Aedes caspids, Ae. vexans.	Man.	103
Lumbo	Mozambique.	Aedes pembaensis.	None known.	54
		GUAMA SUB-GROUP		
Guama	Brazil, West Indies.	Limatus durhami, Culex sp.	Man, rodents Monkeys.	93
Catu	Brazil, West Indies.	Culex mojuensis.	Man, rodents, monkeys.	93
Moju	Brazil.	Culex sp.	Mouse.	54
Bimiti	West Indies.	Culex sp.	None known.	104
Be An 20525	Brazil.	None known.	Mouse.	54
		BWAMBA SUB-GROUP		
Bwamba	East Africa.	(Aedes aegypti)	Man.	105
Pongola	South & East Africa.	Ae. circumluteolus, Mansonia sp,. Culex univittatus.	None known.	106
		SIMBU SUB-GROUP		
Simbu	South Africa.	Aedes circumluteolus.	None known.	107
Oropouche	Brazil, West Indies.	Mansonia venezuelensis.	Man, monkeys, sloth.	108
Sathuperi	India.	Culex vishnui.	None known.	1
Ingwavuma	South Africa.	Culex univittatus.	Birds.	54
Manzanilla	West Indies.	None known.	Monkey.	109
Akabane	Japan.	Aedes vexans.	None known.	110

TABLE 13.4 (*contd.*)—

Name	Distribution	Arthropod Hosts	Vertebrate Hosts	Ref.
		CAPIM SUB-GROUP		
Capim	Brazil.	None known.	Marsupial.	54
Guajara	Brazil.	None known.	Mouse.	54
Bush-Bush	Brazil, West Indies.	Culex accelerans.	None known.	54
		TACARIBE SUB-GROUP		
Tacaribe	West Indies.	Mosquitoes.	Bats.	54
Junin	Argentine.	Echinolaelaps echidninus.	Man, mouse.	111
		NAPLES PHLEBOTOMUS FEVER (NPF) SUB-GROUP		
NPF	Italy.	Phlebotomus papatasi.	Man.	112
Icoaraci	Brazil.	None known.	Rat.	54
JW 10	Panama.	None known.	Man.	54
		EPIZOOTIC HAEMORRHAGIC DISEASE (EHD) SUB-GROUP		
EHD NJ	U.S.A.	None known.	Deer.	113
EHD SD	U.S.A.	None known.	Deer.	113
		VESICULAR STOMATITIS VIRUS (VSV) SUB-GROUP		
VSV Indiana	U.S.A.	(Aedes aegypti.)	Cattle, man.	114
Cocal	Brazil, West Indies.	Gigantolaelaps.	None known.	54
VSV New Jersey	U.S.A.	(Aedes aegypti.)	Cattle.	115
		IRITUIA SUB-GROUP		
Irituia	Brazil.	None known.	Rodents.	54
BT 436	Panama.	Phlebotomus sp.	None known.	54
Be Ar 35646	Brazil.	Phlebotomus sp.	None known.	54
		ANOPHELES A SUB-GROUP		
Anopheles A	North & South America.	Anopheles boliviensis, Culex tarsalis.	None known.	101
Lukuni	Brazil, West Indies.	Aedes scapularis, Anopheles nimbus.	None known.	1
		TURLOCK SUB-GROUP		
Turlock	North & South America.	Culex sp.	Birds.	116
Umbre	India.	Culex vishnui.	None known.	54
		TRINITI SUB-GROUP		
Triniti	West Indies.	Trichoprosopon sp.	None known.	1
Ieri	West Indies.	Psorophora albipes, Psor. ferox.	None known.	1
Aruac	West Indies.	Psorophora sp., Culex sp.	None known.	1

TABLE 13.4 (*contd.*)—

Name	Distribution	Arthropod Hosts	Vertebrate Hosts	Ref.
		BAKAU SUB-GROUP		
Bakau	Malaya.	Culex lophoceratomyia.	None known.	1
Ketapah	Malaya.	Culex lophoceratomyia.	None known.	1
		KONGOOL SUB-GROUP		
Kongool	Australia.	Culex annulirostris.	None known.	82
Wongal	Australia.	Culex annulirostris.	None known.	82
		AFRICAN HORSE SICKNESS SUB-GROUP		
African Horse Sickness (AHS) (8 serological types)	Africa, Asia.	(Culicoides sp.)	Horse.	117
		BLUE TONGUE OF SHEEP (BTS) SUB-GROUP		
BTS (Many serological types	U.S.A., Europe, Africa.	Culicoides sp.	Sheep.	118

THE UNGROUPED VIRUSES number at least 50 and only those which are important to Medicine or Veterinary Medicine will be mentioned. Some, such as Rift Valley Fever, have been well studied and are antigenically distinct, whereas others may yet be found to belong to one or other of the recognised sub-groups. The present intense examination of arthropods for known Arboviruses will lead to an increase in the number of new "types". Certain other agents, like the Semunya complex may not be Arboviruses because of their large size. Nevertheless those viruses presently recognised as Arboviruses will be included in Table 13.5.

TABLE 13.5

Ungrouped Viruses

Name	Distribution	Arthropod Hosts	Vertebrate Hosts	Ref.
Rift Valley Fever (RVF)	Africa.	Eretmapodites sp., Aedes sp., Culex sp.	Man, sheep, cattle.	119
Sicilian Phlebotomus Fever (SPF)	Italy, Egypt.	Phlebotomus papatasi.	Man.	112
Colorado Tick Fever (CTF)	U.S.A.	Dermacentor sp.	Man, squirrels, chipmunks, mice.	120
Nairobi Sheep Disease (NSD)	Africa.	Rhipicephalus sp.	Sheep.	121
Crimean Haem. Fever (CHF)	U.S.S.R.	Hyalomma plumbeum, Hyalomma anatolicum	Man.	122
Semunya	East Africa.	None known.	Man.	123

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CHAPTER FOURTEEN

MANIFESTATIONS OF ARBOVIRUS INFECTION

THE Arboviruses cause a wide range of diseases in both man and animals. Yellow Fever is probably the best known and was the scourge of Africa and the Americas from the seventeenth century[1] until recent times, when a combination of vaccination[2,3] and mosquito eradication brought the disease under control.

Encephalitis and haemorrhagic fevers are severe and often fatal in man, while among the lower animals Arbovirus infections cause diseases with picturesque names like "Louping Ill" and "Blue Tongue Disease". Many non-specific, generalised illnesses with a high morbidity rate also occur although they are rarely fatal.

Since the Arboviruses, unlike any other group, are usually arthropod-vectored, epidemics occur all the year round in tropical climates where mosquitoes abound, whereas they are seen only during the summer months in cooler climates. This is also true of the tick-borne viruses of Europe and Asia.

Although transovarian transmission occurs with certain arthropods the majority require a reservoir of virus to infect each new brood. As is the case with Yellow Fever this natural reservoir may be man himself but the wild or domestic animal or bird is the usual source of virus. Man is usually a "dead end" host, i.e., he does not infect fresh mosquitoes.

This reliance on the arthropod vector restricts each virus to the area of its vector and this is fortunate because, as the tables in the previous chapter illustrate, the arthropod host range varies considerably. As a result some of these viruses are widespread, while others are limited to a relatively small area of the world. On the other hand, some viruses whose vectors occur widely have not yet crossed the intervening seas!

With this localised incidence of each virus the common practice has been to consider each "type" separately even although several produce the same illness. While it is true that symptoms and mortality vary, the same can be said for the Enteroviruses but the different types causing meningitis are always considered together. Therefore each *syndrome* will be mentioned and as usual any major differences will be noted.

Diseases of Man

In addition to Yellow Fever, Encephalitis and Haemorrhagic Fevers, the Arboviruses cause dengue-like or 'flu-like systemic illnesses. All

Arbovirus infections are necessarily systemic due to direct injection of virus into the blood-stream.

YELLOW FEVER, or yellow jack as it used to be called, is a severe hepatic infection endemic in Africa and South America. There are two varieties, "urban" and "jungle" yellow fever, due to different transmission cycles although clinically there is no difference.[4]

The onset is sudden after an incubation period of 2–6 days.[5] Initial symptoms are fever, rigors, headache, and backache. The temperature rises rapidly, rarely exceeding 103·5°F, drops on the 3rd or 4th day and rises again later. During the early or congestion stage the patient's face is flushed, the lips are swollen, the tongue is red and the eyes injected. After the drop in temperature, the flush is replaced by a dusky pallor, the forerunner of the jaundice which becomes most noticeable in the convalescent stage. In this second stage the gums swell and bleed easily. Nausea usually occurs and degraded blood in the vomit gives it its characteristic black appearance. The pulse rate slows (Faget's sign) and the blood pressure drops. Albuminuria becomes very marked as does leucopenia which reaches a maximum about the 6th day.[6]

The disease ranges from a mild or sub-clinical infection to a short severe illness and death, which usually occurs at the 6th or 7th day and rarely later than 10 days after the onset. The mortality rate varies but is probably about 5% of all infections. When the patient survives, recovery is usually rapid and complete.[7]

Pathology reveals widespread necrosis of the midzone of the liver with normal cells at the periphery and around the central areas; lower nephron necrosis; degenerative changes of the spleen and heart; haemorrhages of the pyloric mucosa and jaundice.[8,9,10,11]

Treatment of all cases, however mild, is bed rest, plenty of fluids and no solids. Persistent vomiting can be treated with dextrose-saline intravenously and the intense headache by analgesics.

ENCEPHALITIS AND MENINGO-ENCEPHALITIS are the most common severe illnesses caused by these viruses. Only in Western Europe are such cases rare. There is a wide variation in the severity of the disease but the basic clinical picture is the same.

The onset is sudden, after an incubation period of 2–10 days, with fever, headache, malaise, nausea and vomiting. These symptoms may be accompanied by other typical encephalitic or meningitic symptoms—stiff neck, drowsiness, irritability, convulsions, photophobia and mental disorientation, leading to coma and sometimes death. Many cases show only low fever and mild headache. In the worst cases temperatures up to 106°F, severe occipital headache and spastic paralysis are found. Other symptoms are backache, lethargy, general muscle pains and anorexia. The disease is often biphasic with an initial mild stage followed by a period of remission before the onset of the true encephalitis. Therefore the duration is variable, being as short as one day to as long

as 30 days. In several epidemics the disease has been more severe in children than in adults although there is usually no difference.

In all cases there is an increase in the protein and leucocyte content of the CSF with an initial preponderance of polymorphs, followed by a typical lymphocytic picture. Cell counts vary from 10 to 1,000 per cu. mm. The picture is similar to that of the Enterovirus meningitis syndrome, apart from the leucopenia which is not a feature of Enterovirus infection.

Permanent damage occurs frequently. The most common sequelae are paralysis (which is often indistinguishable from that of poliomyelitis), personality changes and mental impairment.

Eight different Arboviruses have been implicated in these outbreaks. These are WEE,[12,13] EEE,[14,15] VEE,[16,17] JBE,[18,19] MVE,[20,21] SLE,[22,23,24] RSSE and CEE.[25–27] Another three give rise to sporadic cases:—LI,[28] CTF[29] and CE (Californian Encephalitis).[30] The major differences in severity of the disease are tabulated in Table 14.1.

TABLE 14.1

Arbovirus Encephalitis

Virus	Mortality Rate	Permanent Sequelae			Duration	Biphasic Course
		Frequency	Paralysis	Psycho-logical		
WEE	5–15%	Rare	+	0	7–10 days	0
EEE	80%	Usual	+	+	7 days	±
VEE	Low	Nil	0	0	1–4 days	0
JBE	10%	Frequent	+	+	7–8 days	±
MVE	40%	50%	0	+	variable	0
SLE	20%	Rare	+	+	3–10 days	0
RSSE	Up to 30%	3–5%	+	0	5–30 days	±
CEE	2%	Rare	+	0	5–30 days	+
LI	Nil	Nil	0	0	5–30 days	±

The pathology of the CNS in these diseases is very similar. The cord is only occasionally involved. There is slight lymphocytic infiltration of the meninges, widespread lesions of the grey matter, scattered lesions of the white matter and occasional thrombosis of blood vessels. With JBE, MVE and RSSE destruction of the Purkinje cells of the cerebellum often occurs.[31]

Treatment is palliative.

HAEMORRHAGIC FEVERS caused by seven distinct Arboviruses are endemic in various parts of Asia and South America. They vary in symptoms and severity but the basic pattern is the same.

The onset is sudden after an incubation period of up to 5 days. Fever and headache are followed by back pain, severe pain of the extremities, gastro-intestinal upset and prostration. The fever rarely exceeds 103°F

and lasts for 5 to 10 days. There is usually a drop on the 2nd or 3rd day of illness. Inflammation of the conjunctiva and enlarged lymph glands (cervical, inguinal, axillary and epitrochlear) are commonly found. In severe cases vomiting, diarrhoea, bleeding from the gums, nose, intestines, stomach and uterus occur. Frequently the fever returns about 3 weeks after the onset, and may last for a further 2–10 days. Occasionally there is a meningitis but this is usually mild.

All cases show severe thrombocytopenia and most, a marked leucopenia. Leucocytosis occurs with CHF. In severe cases there is a distinct proteinuria often accompanied by leucocytes and casts in the urine.

The mortality rate varies from about 5–10%. Convalescence is very slow although recovery is complete.

The viruses causing haemorrhagic fevers are KFD;[32,33] D3, D4, Chik;[34] OHF;[35] CHF[36,37] and Junin (Argentinian Haemorrhagic Fever).[38,39] OHF tends to produce a milder illness than the rest.

The pathological picture is one of haemorrhagic lesions of the kidney, liver, spleen, pituitary, and right auricle. In the case of KFD there is also haemorrhagic consolidation of the pulmonary parenchyma[40] and it has been suggested that this might be due to viral toxaemia.

Treatment is complete bed rest. If diarrhoea and vomiting lead to dehydration, large amounts of fluid should be administered. Blood transfusions are necessary when there is severe haemorrhage.

DENGUE-like illnesses have been associated with many Arboviruses. All cases of haemorrhagic fever, where the haemorrhagic signs are minimal or absent, could be classed as Dengue-like. True Dengue as defined by Lumsden[41] differs only from the rest in that lymphadenopathy is invariably present.

The onset is usually sudden, after an incubation period of 3–15 days, with fever and headache, retro-orbital, back and muscle pains. The temperature may rise as high as 105°F and lasts from 5–7 days, often recurring after an afebrile period of 1–3 days. Generalised lymphadenopathy follows. A characteristic rash appears up to 7 days after the onset. Usually it is maculopapular but is seen occasionally as a scarletini-form rash, and lasts for up to a week. Other symptoms which may occur are anorexia, constipation, nausea, vomiting, coryza, cough, sore throat and epistaxis.

At least seven viruses cause this type of illness:—D1, D2, D3, and D4 (which were named after the disease),[34,42,43] WN[44,45] Chik,[46,47] and ONN.[48,49] Occasionally there are signs of meningitis, especially with WN. All cases show a marked leucopenia. Bradycardia, although uncommon with ONN, is seen sometimes late in the illness. Most of the illnesses due to Chik have a biphasic course with the rash occurring in the second phase.

The mortality rate is negligible and complete recovery is made after one or two weeks. Joint pains may recur up to 4 months afterwards.

Treatment is palliative.

OTHER DISEASES of man include the sandfly or Phlebotomus fevers (NPF and SPF), Colorado Tick Fever (CTF) and a host of non-specific illnesses. All are similar to the Dengue-like illnesses.

The principal symptoms are fever, headache and generalised aches and pains. Photophobia, conjunctival injection, slight jaundice, slight neck stiffness, epistaxis, nausea and vomiting may also occur. There is usually a marked leucopenia but definite evidence of meningeal irritation is rare.

These illnesses may last from 1–14 days and recovery is complete.

Outbreaks of this type of illness have been associated with Mayaro,[50] and Uruma[51] viruses of sub-group A; Wesselsbron[52] and Zika[53] viruses of sub-group B; most strains of sub-groups C,[54,55] Bunyamwera[56,57] and Guama;[54] Bwamba,[58] NPF and SPF,[59] CTF[60] and RVF[61] viruses.

Epidemiology

With the exception of RSSE and CEE viruses the spread of disease is, in all cases, by arthropod vector. In the case of RSSE and CEE, virus is present in the milk of infected animals, especially goats, and infection can spread to humans who drink the milk.[62,63]

The insect vector is not essential, as is shown by the high incidence of laboratory infections, but infectious virus is rarely excreted by an infected person so that man to man spread is uncommon.

An outline of the distribution of the Arboviruses is given in Tables 1 to 5 of the previous chapter together with a list of the known arthropod hosts, so that only the two distinct transmission cycles of Yellow Fever will be mentioned here. They will serve as models for all the other Arboviruses, irrespective of whether they are transmitted by mosquitoes, sandflies or ticks.

In "urban" Yellow Fever the reservoir of infectious virus is man himself. During the acute stage of illness, while there is a viraemia, the *Aedes aegypti* mosquito bites the infected person making a meal of blood well laced with YF virus. After a period of 12 days, during which time the virus multiplies, the mosquito can infect the next susceptible person whom it bites. Thus the cycle of infection is Man—*Aedes aegypti*—Man.

The *Aedes aegypti* mosquito has become domesticated in the New World and epidemics of Yellow Fever are associated only with areas of dense population where there is a continual supply of susceptible humans. Epidemics of "urban" Yellow Fever are now unknown in the Americas because eradication of *Aedes aegypti* from urban areas has broken the transmission cycle.

In Africa *Aedes aegpyti* is found in the forests as well as the towns and cases of "jungle" or "sylvan" Yellow Fever occur, although they are usually sporadic. Probably monkeys and other vertebrates act as the reservoir. In the Americas *Haemagogus sp.* mosquitoes transmit "jungle" Yellow Fever between vertebrate hosts, and man becomes a

secondary host. Therefore the cycle in this instance is Vertebrate—*Haemagogus sp.*—Man.

The latter type of transmission where man is a secondary or "dead end" vertebrate host is the most common cycle of infection with the Arboviruses where the main reservoir of infection is a vertebrate, often a bird or a rodent. Infection of man is only incidental to a mosquito bite, and plays no part in the maintenance of the virus in the area. For this reason the Arboviruses are common in rural areas unlike the Adeno- and Picornaviruses which are most prevalent in urban areas.

LABORATORY INVESTIGATIONS are usually necessary for a final diagnosis. Considerable difficulties are encountered in identification due to the many cross-reactions found within this group. As usual both isolation and serology can be used, but when testing acute and convalescent sera many heterologous rises in antibody are found, even by the neutralisation test.

In general, virus isolations are successful only from serum taken during the first 4 days of illness or from post mortem CNS specimens. Since these viruses are very heat labile, specimens intended for virus isolation should be delivered to the laboratory as soon as possible after collection. If possible they should be refrigerated, and in the case of CNS material and separated serum, frozen in solid CO_2. The usual method of isolation is by intracerebral inoculation of baby mice. When the mice die, virus can be extracted from the brains and identified by neutralisation, C-F and HI tests.

Serological diagnosis is made using paired acute and convalescent sera. The neutralisation test is the most selective test and picks up the fewest heterologous antibody rises but C-F and HI tests have the advantage of speed and cheapness and can at least indicate the subgroup to which the virus belongs.

Vaccination

Successful vaccines have been produced against Yellow Fever[2,3] and several others have been tried including Dengue types 1 and 2, SLE, JBE, RSSE, KFD, and CTF.[64–67]

Two types of Yellow Fever vaccine are in use today, the 17D strain[2] and the Dakar neurotropic strain.[68] Both are live attenuated strains of Yellow Fever Virus and although there is a poorer antibody production with the 17D strain, a significant number of complications arise from the Dakar strain. This latter vaccine is prepared from a mouse brain-passaged strain of YF by grinding freeze/dried infected mouse brains with infusorial earth. This material is then inoculated by scarrifying the arm. About 50% of the persons vaccinated have a viraemia and about 10% of the total develop mild fever, headache and backache. In a controlled vaccination scheme in Nigeria 29 deaths were recorded among 20,000 children given this Dakar vaccine.[69]

The 17D vaccine is a tissue culture-passaged strain of YF from which

the vaccine is produced in chick embryos. The whole infected embryo is pulped, centrifuged and the resulting suspension is freeze/dried. This is reconstituted when required for use. Vaccination is done either by scarrification or injection. Fewer ill effects result from the use of this vaccine, but the antibody production is not as good as with the Dakar vaccine.[70]

The efficiency of these vaccines has been proved in YF endemic areas with the result that Yellow Fever is a relatively minor problem apart, of course, from the work for Public Health officials involved in vaccinating all persons entering YF zones.

Non-human Arbovirus Infections

There are almost as many Arbovirus diseases of animals as there are of man. Several viruses infecting animals also infect man, producing similar symptoms in all hosts. Brief mention will now be made of the principal diseases of domestic animals.

ENCEPHALITIS due to EEE, WEE, and VEE is common in horses as their names suggest.[71–73] Louping ill virus produces encephalitis in sheep[74] and Turkey meningoencephalitis virus, CNS disease in turkeys.[75] These diseases are all similar to the human diseases and there is a significant mortality rate.

HAEMORRHAGIC FEVERS similar to those in man are produced in sheep and goats infected with NSD,[76,77] in cattle and sheep infected with RVF,[78,79] and in deer infected with EHD.[80] RVF produces a more severe illness in sheep than in cattle and abortion is common in both. The main sites of haemorrhage are in the gastro-intestinal tract and a bloody diarrhoea usually signifies the imminent death of the animal.

Mortality varies with the breed of sheep but is higher in young animals, reaching 95% in new-born lambs. Usually it is around 10% in cattle and 20% in sheep infected with RVF. With NSD the mortality rate is 70% in Masai sheep, 30% in Merinos and 10% in goats.

AFRICAN HORSE SICKNESS AND BLUE TONGUE OF SHEEP are similar diseases produced by different viruses in horses and sheep respectively. There are several antigenic types of each and the AHS types appear to be specific for horses but BTS can infect other ruminants besides sheep.[81–84] The disease varies in severity in all animals but there is always a pyrexia. In severe cases there is pulmonary oedema with nasal discharge, or a generalised oedema. Gastro-intestinal haemorrhagic signs are not uncommon and the tongue becomes swollen and dark blue in colour. With BTS foot lesions are sometimes seen. Both viruses give paralysis of the oesophagus in the most severe cases

The mortality rate varies from 2 to 30% with BTS in sheep and from 25 to 95% with AHS in horses. Infection of cattle with BTS is rarely fatal.

VESICULAR STOMATITIS is principally a disease of horses and

cattle although pigs are also susceptible.[85] The symptoms are similar to those of Foot and Mouth Disease but FMDV does not infect horses. Two distinct antigenic types of VSV have been associated with outbreaks. The illness varies in severity. In the most serious cases there are widespread lesions of the tongue, feet and teats. The mortality rate is less than 0·5% but the morbidity rate is high with loss of weight and cessation of milk production in cattle. Recovery, however, is rapid and complete.

OTHER DISEASES probably due to Arboviruses are found in sheep in South Africa. Wesselsbron virus causes abortion of ewes and death of pregnant ewes and their lambs.[86] Another virus, Middleburg, was isolated during a febrile epizootic of sheep but a relationship with the illness was not fully established.[87]

Of these diseases of animals due to Arboviruses, infection with Vesicular Stomatitis Virus is probably the most important on account of the difficulties in differentiating between it and Foot and Mouth Disease. The vaccines which have been prepared against many of these viruses have been highly successful in reducing the morbidity in large herds of animals.

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CHAPTER FIFTEEN

THE MYXOVIRUSES

THE Myxoviruses appear to be a heterogenous collection of ether-sensitive viruses causing a variety of respiratory and other illnesses of man and animals. In fact, all members of the group have a similar morphology by electron microscopy and their multiplication within the cell follows the same basic course. The group was first defined to include the Influenza viruses (A, B and C), fowl plague virus (FPV), Newcastle disease virus (NDV) and mumps virus.[1] To these were added the Parainfluenza viruses, when they were discovered,[2] and more recently the viruses of measles, canine distemper and rinderpest.[3] Probably rabies virus and the avian tumour viruses[3] are also Myxoviruses because their morphology and growth cycles fall within the range of this group.

There are now approximately 20 distinct Myxoviruses and with the exception of mumps, measles and rabies viruses the human pathogens cause respiratory illness.

The best known disease is undoubtedly influenza, which has ravished the world twice this century. The ability of influenza A virus to change its antigenic structure results in pandemics. Thus the strain which caused the minor outbreaks of 1946–1957 was completely distinct from the new "Asian" strain which appeared during 1956–57. Since this new strain was not neutralised by antibodies to the previous A strains the entire population of the world was susceptible and the subsequent outbreak of "Asian" influenza swept across the world from China to America.

Biological Properties

The properties of the Myxovirus group were originally defined as follows in 1955.[1]

(1) They are spherical viruses ranging from 60–200 mμ in diameter.

(2) Filaments of similar diameter to the spheres but several microns long are also present in viral suspensions.

(3) They are ether sensitive.

(4) They are very stable at −70°C but less so at −10°C.

(5) They agglutinate chick and certain vertebrate erythrocytes and then spontaneously elute, producing an enzyme-like irreversible change on the mucoprotein receptors on the cell surface.

(6) Erythrocytes treated with the receptor destroying enzyme (RDE) of *Vibrio cholerae* are not agglutinaged.

(7) A soluble antigen is produced which is less specific than the virus particle.

(8) The nucleic acid is RNA.

Although our knowledge of these viruses has been extended by later work this outline of their properties remains unchanged.

The structure of most members of the group has been determined. The virus particle consists of an outer diffuse layer of rod-like structures

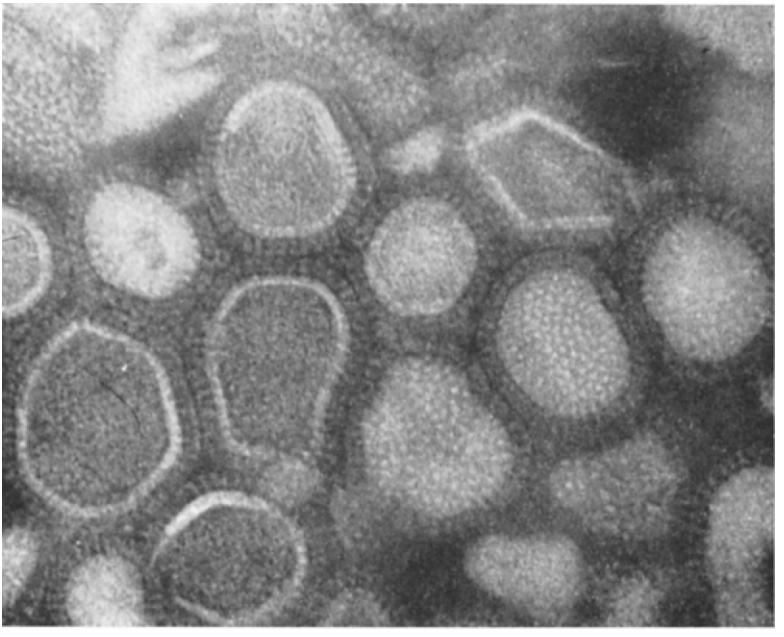

FIG. 15.1. Electron micrograph from part of a spray droplet of influenza virus embedded in phosphotungstate. The intact particles are of variable shape and size and show well-defined surface projections. (*Photo—R. W. Horne.*) (*Courtesy—Academic Press Inc., Virology*, **13**, 448; 1961.)

8–12 mμ long, projecting from the surface of a lipo-protein membrane which encloses the central core of nucleoprotein.[5–8] This lipo-protein membrane is 10–15 mμ thick and is probably of host cell origin, while the central core consists of a coiled helix composed of nucleoprotein. The outer diameter of the helix is 17–18 mμ for the Parainfluenza and Measles sub-groups[7,9,10,11] and 9 mμ for the Influenza sub-group.[12] The internal diameter is approximately 4–5 mμ. Rabies virus has an identical internal structure although the external diameter of the helix has not been definitely calculated.[4] Evidence shows that this structure is in fact a double helix, each composed of regular capsomeres. Overall diameters of the virus particles vary, ranging from 80–180 mμ for influenza A to

120–750 mμ for rinderpest. Filamentous forms, several microns long have been seen in preparations of some Myxoviruses. These particles usually have a low infectivity although they haemagglutinate.

The chemical composition is very complex. In addition to RNA and

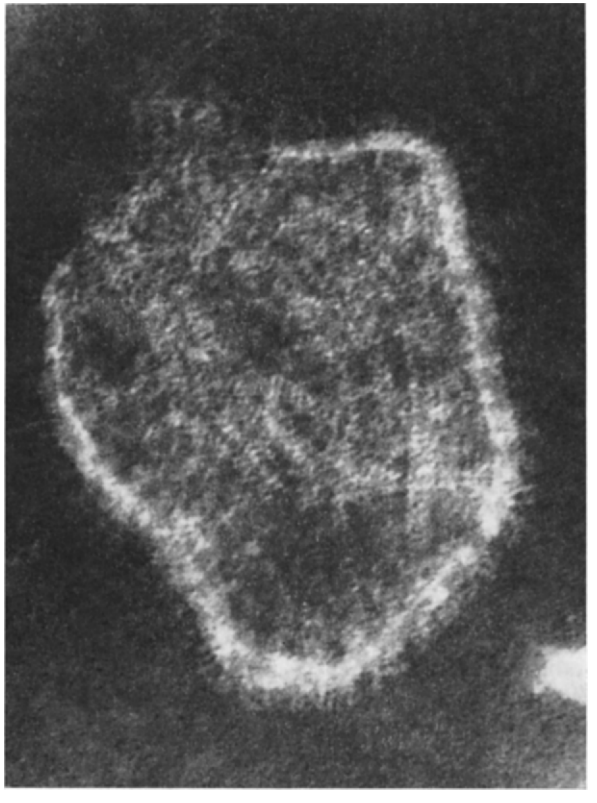

FIG. 15.2. Electron micrograph of a partially disrupted particle of parainfluenza type 3. A large proportion of the helical internal component is visible. (*Photo—Professor A. P. Waterson*) (*Courtesy—Academic Press Inc., Virology*, **14,** 374, 1961.)

protein large amounts of lipid and polysaccharide are present and most strains possess the enzyme neuraminidase. In purified suspensions RNA accounts for about 1% of the total. Deoxyribonucleic acid has been found in some preparations.[13] The opinion that this was an impurity has been confirmed by the extraction of infectious RNA from influenza-infected cells.[14] This infectious RNA differs from host cell RNA only in the proportions of the bases.[15] The proportions of the non-nucleoprotein components of the Influenza sub-group are roughly: total lipid 17–20% and 5–7% polysaccharide. The lipid is composed

of the phospholipids—cephalin, lecithin and sphingomyelin; a glycolipid and cholesterol. Galactose, mannose, fucose and amino-sugar make up the polysaccharide. These figures were obtained for virus purified from the allantoic fluid of infected eggs and the proportions of lipid and polysaccharide were identical with those found in normal allantoic membranes from uninfected eggs.[16] From this it would appear that much of the non-nucleoprotein component of the virus is host cell material, incorporated into the virus particle on maturation.

All the Myxoviruses are very labile and are readily inactivated by heat, formalin, ether, trypsin, deoxycholate or U-V light. The stability of different strains of the same type varies greatly but rabies virus seems to be the most readily inactivated. For the remainder, a slightly alkaline medium (pH7–9) with a high protein content is best for preservation. Lyophilised preparations preserve well although there is an initial drop in titre when the suspension is freeze/dried.[17,18,19]

The inactivation of influenza virus by U-V light is a "one hit" process, i.e., the U-V light has only to hit the virus particle once to effect inactivation of infectivity. When U-V irradiated influenza virus is inoculated into eggs with a high ratio of virus particles to cells, infectious virus is produced.[20] This "multiplicity reactivation" as it is called is due to a genetic recombination of the damaged RNA from the different infecting particles.[21] There would appear to be several sites on the RNA molecule, any one of which when damaged by U-V irradiation renders the particle non-infectious. Multiplicity reactivation occurs when the RNA from two or more damaged particles combines to give fully infectious RNA.

Antigens are produced by Myxovirus-infected cells which can be detected by haemagglutination, complement fixation or neutralisation tests. At least two particles are produced, the virus or V antigen and the soluble or S antigen,[22] and in the case of measles there is evidence of a third particle.[23] The S antigen is detectable by the agar-gel diffusion or the C-F test and has a size of about 15 mμ.[1,24] This antigen is common, not throughout the group as with the Adenovirus group, but each subgroup has a common antigen. The soluble antigen is composed of nucleoprotein and can be extracted from infectious virus particles by treatment with ether.[25] In untreated suspensions it is almost certainly excess nucleoprotein which has not been incorporated into virus particles.

All but three of the Myxoviruses possess a haemagglutinin. These are rabies, rinderpest and respiratory syncytial virus (RSV). The haemagglutinin is a mucoprotein and can be separated from the C-F antigen by treatment with ether.[25] This non-infectious haemagglutinin appears to be the outer layers of the virus particle and it has been suggested that the rod-like projections, or some of them, are the haemagglutinin.[26,27,28] In untreated suspensions the haemagglutinin, is entirely associated with the infectious virus particle, is the V antigen and is type or even strain

specific. These V antigens can be detected by neutralisation tests and even by C-F tests if a specific anti-V serum is used.

The antigenic variation which occurs among the influenza A and B strains is thought to be due to each virus having a mosaic of antigens similar to that described for the Arboviruses.

Amino-acid analysis of the various antigenic components of influenza A virus indicates that the various proteins are distinct.[29] The haemagglutinin and membrane proteins appear to be almost identical and are very similar to the protein of normal cell membrane, apart from a much higher histidine content. The protein of the soluble antigen is quite distinct and possesses twice the amount of arginine of any of the other virus or cell membrane proteins.

Enzymes form an integral part of most, if not all, of the Myxoviruses. These are neuraminidase, which is common to all those tested and a haemolysin which is not possessed by the Influenza sub-group. Since haemagglutination, neuraminidase activity and haemolysis are all connected, their activities can be considered together.

The Myxoviruses haemagglutinate over a wide range of temperatures (from 4°C to 37°C), although some types have high titres only over a narrow range. The types of agglutinable erythrocytes also vary. Influenza A2 strains will agglutinate erythrocytes from at least 17 different species of animal, but chick, human group O and guinea pig cells are the most frequently agglutinated by members of this group.[30] The site of adsorption onto the erythrocyte receptor is probably a complex of polypeptide and oligosaccharides. At any rate one indispensible component of the receptor is N-acetylneuraminic acid. It is this component which is destroyed by the enzyme neuraminidase, thereby eluting the virus from the erythrocytes.[31] This spontaneous elution of virus from red cells is most rapid at 37°C and the erythrocytes cannot be agglutinated again by the same strain of Myxovirus.[32] Eluted virus can, however, be used to agglutinate fresh erythrocytes and this property is used to purify suspensions of influenza virus.[33] Although the haemagglutinin and neuraminidase are closely linked they are nevertheless distinct components of the virus.[34,35]

Haemolysis of erythrocytes is a property common to all the Myxovirus sub-groups except Influenza.[28,36] Haemolysis occurs at 37°C but not at 4°C and acts on the same erythrocyte receptors as the haemagglutinin.[37] Although the full mechanism of haemolysis is not yet understood, work has shown that calcium ions inhibit haemolysis but have no effect on haemagglutination.[38] The haemolysis of erythrocytes by Newcastle disease virus (NDV) is preceded by a release of potassium ions from the cell, due to increased cation permeability of the cell membrane.[39]

These three factors, haemagglutinin, neuraminidase and haemolysin all appear to be distinct. The haemagglutinin is not destroyed by heating at 56°C for 30 minutes whereas the enzymes are readily

inactivated.[40,41] Formaldehyde and U-V irradiation selectively destroy the haemolysin[40,41,42] indicating that the two enzymes are not the same.

The degree of destruction of the receptor sites on the erythrocytes varies with the virus; some strains are more efficient than others. Thus, if erythrocytes from which mumps virus has been eluted are treated with influenza A or B they will agglutinate. This feature led Burnet *et al.*[43] to introduce the "receptor gradient". A virus can agglutinate erythrocytes from which any strain appearing before it on the gradient has been eluted, but it is unable to agglutinate erythrocytes treated with a strain coming after. Influenza C and mumps viruses come first with influenza A (porcine) and some strains of influenza A2 coming last.

The property of haemadsorption is a characteristic of this group. When monolayer tissue cultures are infected with a haemagglutinating Myxovirus, erythrocytes added to the monolayer will adsorb to the cell surface.[44] This technique was employed in the original discovery of the parainfluenza viruses.[45]

The final antigenic character associated with a Myxovirus is the presence of the human blood group substance "B" in the human DA strains of Simian Myxovirus SV5[46]. Preliminary results show that the "B" substance forms an integral part of the virus particle.

Growth

Influenza virus was the first Myxovirus to be grown and this was done in 1933 using ferrets.[47] Some strains were soon adapted to grow in mice[48,49] but it was not until 1935 and the introduction of the fertile hen's egg[50] that large-scale studies were made on this group. Most of the Myxoviruses can be cultivated in eggs but some of the newer types of Parainfluenza require tissue cultures. Nevertheless the egg is still the method of choice for most workers.

FERRETS infected intranasally with influenza A and B viruses develop a typical respiratory illness, similar to influenza in man. The severity of the infection varies widely with the strain but the animals recover rapidly after 4–5 days of illness. The virus remains confined to the nasopharynx.[47] If the inoculation is done under ether anaesthesia the illness is often a fatal pneumonia and virus can be recovered from the lungs.[49] Serial passage of the virus in ferrets can be maintained using extracts of infected turbinate tissue. Infection with B strains tends to be milder than with A, while influenza C gives virtually no disease, despite multiplication of the virus and good antibody production. The Sendai strains of parainfluenza type 1 are often fatal, producing consolidation of the lungs.[51]

There is a good antibody response with all strains when the animal recovers but immunity is short lived and reinfection with the same strain a few months later will result in another illness. As one would expect, the ferret is also susceptible to canine distemper virus (CDV).

MICE anaesthetised with ether, can be infected by intranasal instillation of influenza A and B and the Sendai strains.[48,49,52] Serial passage every 2–3 days of suspensions of lungs of inoculated mice adapts the virus to mice. Once the virus is well adapted, death due to pneumonia occurs between 2 and 14 days, depending on the dosage. Viral production in the lungs reaches a maximum 48 hours after inoculation and precedes the development of pulmonary symptoms.[53] Highly adapted strains inoculated intraperitoneally may produce pulmonary lesions, suggesting that a viraemia occurs.[54] Influenza C multiplies in the turbinate tissue but it cannot be adapted to lung tissue. Intracerebral inoculation of mice with the NWS strain of influenza A results in death but most of the other viruses do not multiply intracerebrally.[55] Nevertheless other strains of influenza A do give a single growth cycle in mouse brain, although they do not continue to multiply.[56]

CHICK-EMBRYO TECHNIQUES are the most satisfactory for the cultivation of most of the Myxoviruses, especially for their initial isolation from clinical material. Amniotic inoculation of 13–14-day-old eggs is usually the most sensitive for isolation but most strains will multiply after allantoic inoculation of 9–10-day-old eggs, once they have been adapted by the amniotic route. Some strains can even be isolated directly in the allantoic cavity.[57–60] The virus multiplies in the embryonic tissue cells and is liberated in high titre into the amniotic and allantoic fluids after 2–4 days incubation.

All members of the group grow well in the chick embryo, except the Parainfluenza sub-group and RSV. Some strains of the Parainfluenza types will multiply in eggs but no strain of RSV has yet been found to do so. Influenza A, B and C, mumps, NDV, measles, rinderpest, CDV and rabies viruses can all be isolated by amniotic inoculation and most strains will subsequently multiply following allantoic inoculation.[1,57,58,59,61–67] Mumps and rabies virus usually grow better with yolk sac inoculation of 6–8-day-old eggs,[61,67] while rabies can be successfully cultivated in one-day-old eggs.[68]

Most of our knowledge on the growth cycles of the Influenza, mumps and NDV viruses has been accumulated from experiments using chick embryos. However, since a large amount of complementary information has resulted from tissue culture studies a brief mention will be made of tissue culture before considering the method of replication.

TISSUE CULTURES are essential for the growth of Parainfluenza and RSV although all the other members can be cultivated in susceptible cells. Growth and multiplication of the Myxoviruses can be supported by a wide variety of cells—monkey kidney, human amnion, chick embryo fibroblasts as well as a variety of continuous cell lines. The viruses of the Parainfluenza and Measles sub-groups have been best studied in tissue cultures where most give a typical syncytial formation although those of the Influenza sub-group do not.[69]

This syncytial effect is best seen in the human continuous cell lines but

it does occur with some strains in primary monkey cell cultures. The syncytia appear to be formed by a "toxin" rather than the actual viral infection and this has been shown to be the haemolysin in the case of measles virus.[70,71] A similar result has been obtained for Parainfluenza, where syncytia are produced within 30 minutes of viral inoculation and long before any viral multiplication has occurred.[72]

Many of these viruses produce intracellular inclusions. The Measles sub-group and the SF strains of parainfluenza type 3 both produce intranuclear eosinophilic inclusions,[19] while these and many others produce intra-cytoplasmic eosinophilic inclusions.[69] The connection of these inclusions with viral synthesis is still vague, but there is no doubt that this will be clarified in the near future. Although the nucleoprotein of the Influenza viruses appears to be formed in the nucleus and that of the remainder in the cytoplasm[73] the growth cycles will be considered together because we have, with the Myxoviruses, a similar situation to that with the Picornaviruses.

The adsorption and penetration stage of influenza virus is probably nearer to being understood than for any other virus. Virus particles first attach themselves to the cell wall in a lock and key fashion—the rod-like projections of the virus particle interlock with similar projections on the cell surface.[74,75] Within 20 minutes of infection many of the attached virus particles have been enclosed within cytoplasmic vesicles by phagocytosis.[75] Breakdown of the particles within these vesicles then occurs with the disruption of the viral membrane and the release of free lipid from the lipoprotein.[74] This stage appears to be completed in 2 hours. Influenza virus therefore does not enter the cell as an intact particle, but is broken down within the intracytoplasmic vesicles from which the RNA can enter the cell.

The eclipse phase lasts from 4 to 12 hours depending on the virus, although morphological changes are usually visible by electron microscopy 3 hours after infection. With influenza and rinderpest viruses these take the form of small and irregular osmiophilic bodies in the nucleus and cytoplasm respectively.[76,77] The actual sites of viral multiplication in the cell are still under discussion. For some viruses no intranuclear stage can be observed whereas for others the initial stage is undoubtedly in the nucleus. Evidence from fluorescent antibody staining and electron microscopy on tissue cultures infected with NDV,[78] RSV,[79] rinderpest[76] and parainfluenza[73] viruses shows that the nucleoprotein is formed entirely in the cytoplasm with these viruses. In the case of rinderpest the nucleoprotein core is formed within or in close association with the mitochondria.[76]

The number of new infectious particles produced by each infected cell, irrespective of site of multiplication, is low, numbering less than 40.[80,81]

The nucleoprotein of the Influenza sub-group is formed in the nucleus although the nucleolus does appear to be involved.[73,77] When a small

virus inoculum is used in chick embryos, the soluble antigen can first be detected by fluorescent antibody staining in the cytoplasm. In this instance a large number of new infectious particles is produced per cell.[82] When the inoculum is increased the amount of new virus produced per cell is much lower and the new soluble antigen can first be detected in the nucleus. With fowl plague virus which is an avian type of influenza A, the soluble antigen is first seen in the nucleus although the haemagglutinin is confined to the cytoplasm.[83] However for all the viruses studied the final maturation of the particle occurs at the membrane either of the cell or a cytoplasmic vacuole. This appears to be where the lipid membrane is added to the otherwise complete virus particle.[76,84] It has been suggested that during the course of viral synthesis the composition of the cell membrane alters so that it contains the necessary viral components with which to "coat" the incomplete virus particle as it leaves the cell.[84]

It is possible to summarise the probable course of events in the growth of all the Myxoviruses. The virus particle adsorbs to the cell surface by an interlocking device. A vacuole is formed round the particle, which is then broken down within the vacuole, and the RNA enters the cell and passes to the nucleus. There it initiates the production of a series of enzyme systems to form new nucleoprotein within the nucleus. The nucleoprotein diffuses out into the cytoplasm, probably to the mitochondria where the haemagglutinin is produced and added to the nucleoprotein. The incomplete particle then passes to the cytoplasmic membranes which have been altered to have the structure and composition of the viral membranes. The particle matures by passing through the cytoplasmic membrane, coating itself as it does with its essential lipid membrane.

Working from this hypothesis the seemingly contradictory results already described can be explained, on the assumption that the infected cell is relatively inefficient at producing new nucleoprotein in the nucleus, and that this diffuses into the cytoplasm so rapidly that it cannot be detected in the nucleus by any of the available techniques. Consequently when the infected cell produces large amounts of nucleoprotein, sufficient remains within the nucleus to be detected. Although the whole of this hypothesis is not yet confirmed for all the Myxoviruses, it would seem likely that it is correct. In which case the growth cycle of this group is very similar to that of the Picornaviruses and probably the Arboviruses.

There is also still some doubt about the nature of the inclusions seen in cells infected with some Myxoviruses. Electron microscopy of the intracytoplasmic inclusions produced by measles and canine distemper viruses shows them to be crystalline in structure, possibly protein toxins which do not form an integral part of the virus particle but which are specifically produced by the infected cell, like those produced by Adenovirus type 5.[85,86] On the other hand, the intranuclear inclusions

of these viruses are composed of filaments of high electron density in random array, the nature of which is still unknown.[86,87]

The filamentous rod-shaped forms mentioned earlier, which are seen in some virus suspensions are probably non-infectious. These are produced at the cell membrane in the same manner as the spherical infectious particles.[88] Some workers have suggested that the spherical particles may in some cases be formed by the segmentation of the filaments,[89] whereas others claim that they are non-infectious aberrant forms of virus.[88] While the former theory may be correct in some cases, the bulk of the evidence certainly suggests that they are in fact non-infectious.

The growth cycle of Rous sarcoma virus, one of the avian tumour viruses, is very similar to that described for the Myxoviruses.[90]

Some of the biochemical changes which occur in the influenza virus-infected cell have been determined. There is an initial increase in aerobic glycolysis which is raised 50–100% 1½ hours after infection, when C-F antigen first appears. Three hours after infection, when the haemagglutinin first appears, the direct oxidation of glucose by the pentose cycle is also raised by 30–50%.[91] There is an increase in the size of the acid soluble nucleotide pool[92] and in the production of amino-acids[93] in cells infected with influenza virus. The production of two, possibly as many as four viral specific proteins has been reported.[94] Unfortunately there are few details of similar studies with any of the other Myxoviruses, but the available details are similar to the Picorna-viruses. The main difference between the two groups involves the structure, and the essential lipid membrane of the Myxoviruses which is absent in the Picornaviruses.

Classification

The original classification scheme of this group in 1955 dealt only with six viruses—Influenza A, B, C, Newcastle disease virus (NDV), fowl plague virus (FPV) and mumps virus.[1] At that time it was decided to adopt what were called "non-Linnean binomials" for members of the group. This practice was continued in 1959 when the parainfluenza viruses were incorporated, but most workers have never used these names and today they have been virtually discarded.[2,3] The latest additions to the group have not been given binomial titles and continue to be known by their original common names.

The Myxoviruses can easily be divided into four sub-groups—the Influenza, Parainfluenza, Measles and Avian Tumour viruses. Two viruses cannot be readily assigned to groups—respiratory syncytial virus (RSV) and rabies virus, which has not yet been officially classified as a Myxovirus but has the typical morphological characters of one. The Avian tumour viruses will be discussed in Chapter 20 so they will be excluded from further discussion here, and the division of the remainder into three main sub-groups and RSV is summarised in Table 15.1.

TABLE 15.1

Division of the Myxoviruses

Group	Size	Syncytia	Inclusion Bodies		Haemagg.	Haemolysin	Diameter of Helix
			Nucleus	Cytoplasm			
Influenza	80–180 mμ	−	−	∓	+	−	9 mμ
Parainfluenza	90–300 mμ	+	∓	±	+	±	17 mμ
Measles	120–300 mμ	+	+	+	+	+	17·5 mμ
RSV	90–130 mμ	+	−	+	−	−	ND

∓ = usually not present; ± = usually present; ND = not done.

The Influenza sub-group is composed of 11 antigenic types. These are listed in Table 15.2.

TABLE 15.2

Influenza Sub-group

Antigenic Type	Natural Host	Non-Linnean Binomial
Influenza A (Classic)	Man (1933–1946)	Myxovirus influenzae A
Influenza A1 (Prime)	Man (1947–1957)	,, ,, ,,
Influenza A2 (Asian)	Man (1956– ?)	,, ,, ,,
Influenza A porcine	Swine (Swine flu)	None given
Influenza A equi	Horse (Horse flu)	,, ,,
Influenza A anatis	Duck (Duck flu)	,, ,,
Influenza A galli	Fowls (Fowl plague)	Myxovirus pestis-galli
Influenza B (Lee)	Man (1940–1948)	Myxovirus influenzae B
Influenza B (Great Lakes)	Man (1950– ?)	,, ,, ,,
Influenza B (Japan/7/56)	Man (1956– ?)	,, ,, ,,
Influenza C	Man	Myxovirus influenzae C

The human strains of Influenza A all have the same C-F antigen but differ by haemagglutination-inhibition. They do not fall exactly into three serological types but rather into three families. Each family represents a major antigenic variation, within which the strains show minor variations. The Influenza B strains are similar to the A strains but the antigenic variation is not so marked. So far only one antigenic type of Influenza C has been encountered.

The Parainfluenza sub-group is much less complex. Many of the viruses share antigens with other members of the group but there is no common antigen. Only with parainfluenza type 1 is there any marked variation. Within this type there are two distinct sub-types, the haemadsorption virus type 2 (HA2) strains and the Sendai strains. The seven members of this sub-group are listed in Table 15.3.

The Measles sub-group comprises measles virus, canine distemper virus and rinderpest virus.

Respiratory syncytial virus is probably an aberrant member of either the Parainfluenza or Measles sub-groups which has lost its

TABLE 15.3

Parainfluenza Sub-group

Antigenic Type	Host	Strains	Non-Linnean Binomial
Mumps . . .	Man		Myxovirus parotidis
Newcastle Disease (NDV) . . .	Fowls		Myxovirus multiforme
Parainfluenza type 1 .	Man	Sendai, HA2	Myxovirus parainfluenzae 1
Parainfluenza type 2 .	Man	CA	Myxovirus parainfluenzae 2
Parainfluenza type 3 .	Man, Cattle	HA1, SF	Myxovirus parainfluenzae 3
Parainfluenza type 4 .	Man		None given
Simian Myxovirus .	Monkeys, Man	SV5, SA, DA	,, ,,

ability to haemagglutinate. Alternatively it may be the first member of a distinct sub-group.

The position of Rabies is less certain. While it is nearer in size to the Influenza sub-group it is the most labile of the Myxoviruses[4] and may not in fact be a member of this group.

Genetics

Earlier in this chapter the phenomenon of multiplicity reactivation was mentioned. This is in effect a form of genetic recombination but between two or more inactive particles of the same strain. Recombination between two entirely different strains can be demonstrated but most of the work has been performed on the various Influenza A strains and genetic recombination has been obtained between types A, A1, A2 and A swine.

The initial observations were made using the mouse neurotropic NWS strain of type A crossed with a variety of other non-neurotropic type A and A1 strains.[95,96] The first series of tests were performed using mouse brain inoculation, while the second[96] were carried out by allantoic inoculation of eggs. In both cases it was possible to obtain the transference of the mouse neuropathic character to the non-neurotropic strains which retained their original antigenic characters. Several other changes in genetic make-up were also noted often giving strains of virus intermediate in character between the parents. These included susceptibility to inhibitors of haemagglutination, enzyme activity, position in the receptor gradient and heat resistance.[95–98] The newly acquired characters of the recombinants were then stable over several passages. However these earlier experiments only demonstrated a one-way transfer of characters from NWS to the other strains. Later work has shown that two-way transfers readily occur, providing sufficient care is taken to detect all possible recombinants.[99]

Using the NWS × MEL cross in mouse brain, recombinants first appear 12 hours after inoculation.[100] These N-M recombinants when

again inoculated into mice with NWS shows a stepwise increment in neuro-virulence with each inoculation.[101] When the neurovirulent strains are serially propagated at terminal dilution, less virulent or completely avirulent clones can be obtained.

To date a wide variety of genetic characters of the Influenza A viruses have been exchanged between two different strains. These include morphology, serotype, plaque type, infectivity, enzyme activity, inhibition of haemagglutination, position in the receptor gradient and heat resistance.[99,102] The number of different recombinants obtained out of the theoretically possible total has varied from 3 out of 56 to 38 out of 40.[99] These results indicate that with the characters under study there is little or no evidence of linkage groups and each of these genes is distinct and independent.

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CHAPTER SIXTEEN

MANIFESTATIONS OF MYXOVIRUS INFECTION

SEVERAL of the illnesses due to Myxoviruses have been known as distinct clinical entities from early times. Recognised epidemics of influenza and mumps in man and rinderpest in cattle have occurred for well over a thousand years, while rabies has been recognised as a disease of dogs and of man for just under two thousand years. Although the greater proportion of the diseases caused by this group are respiratory; exanthematous fevers, encephalitis and generalised systemic illnesses also occur.

Influenza, measles, mumps, rinderpest, fowl plague and Newcastle disease occur regularly in epidemic form. The morbidity rate is very high but the mortality is usually low, unless the virus is introduced into a completely virgin population. When this occurs mortality can be quite appreciable. With the exception of influenza each of these diseases represents a single antigenic type and immunity to infection is lifelong. Thus mumps and measles are predominantly diseases of children throughout the civilised world.

The position regarding influenza is different because the most virulent are the various influenza A strains which show marked antigenic variation. Therefore although immunity to one strain is good, a person may in the course of a lifetime suffer several attacks of influenza as each antigenic type appears. Persons in the 50–60 age-group today have probably suffered four distinct influenza A infections starting with the 1918–19 pandemic type and ending with the 1957 Asian type. Because of its rapid spread and high morbidity, influenza is most important as a cause of general dislocation of services.

Vaccination against influenza, mumps, measles and rabies in man, and rinderpest, Newcastle disease and canine distemper in animals, enjoys varying success.[1–7] The main problem, which arises with influenza, stems from this antigenic variation of both the A and B strains, and makes production of an effective vaccine against the current virus difficult.

Diseases of Man

The diseases caused by the Influenza sub-group, the Parainfluenza and respiratory syncytial viruses are all respiratory in man. While the majority have a low mortality rate, fatal pneumonias can result. Mumps and measles viruses cause distinct clinical entities which can be complicated by encephalitis or pneumonia. Aseptic meningitis,

indistinguishable from that caused by the Enteroviruses has been associated with mumps virus infection, in the absence of any other symptoms.[8] Finally, there are the human infections with rabies virus and those of NDV which may range from a mild conjunctivitis to a severe influenza.[9]

INFLUENZA is by far the most important Myxovirus disease. The symptoms associated with Influenza virus infection are produced by many other viruses, although they occur only as a part of the syndrome. Fatal pneumonia, due entirely to the virus has been observed but much of the mortality during epidemics follows secondary bacterial infection.

The onset of illness is sudden, after an incubation period of 1–2 days with chilliness, fatigue, headache, and generalised aches and pains. The temperature rises to 101–104°F within 24 hours. Other common symptoms include a flushed appearance, ocular tenderness, conjunctival injection, watery eyes, sneezing, mild nasal discharge, laryngitis, hoarseness and an unproductive cough. The generalised muscle pains are not so common in children, but in adults they may be very severe. Symptoms which are *not* usually seen but which are useful in a differential diagnosis are photophobia, diarrhoea, a particularly sore, as distinct to an irritated throat, vomiting and a genuine conjunctivitis. Occasionally encephalitic symptoms may be present.[10] The nasal mucous membranes are often bright red and epistaxis is not uncommon. The nasopharynx and soft palate are red and covered with a shiny mucoid coating. Leucopenia is found in about one third of the cases. The fever lasts 3–4 days and a rapid recovery is made in the uncomplicated cases. After apparent recovery there is a prolonged period of fatigue which is most noticeable in the older patient.

Although influenza is a respiratory illness the major signs and symptoms are constitutional and are probably the result of a toxic reaction to the large amounts of complete and incomplete virus produced. The only serious complications are pulmonary and range from bronchitis to a fatal pneumonia. Much of this pneumonia is due to secondary bacterial infection although a genuine fatal pneumonia due to the virus does occur.[11,12] The very high percentage of cases of pneumonia, 5% and 20–30% recorded in the 1889–90 and 1918–19 pandemics[13,14] with up to 30% mortality were probably the result of secondary bacterial infection.

There is some evidence[15,16] that infection with influenza virus during pregnancy may give rise to foetal deformities.

At post mortem the lungs of fatal cases of non-bacterial pneumonia show interstitial inflammation with necrosis and haemorrhages of the bronchiolar and alveolar epithelium.

Epidemic influenza, sometimes reaching pandemic proportions, is caused by the various influenza A and B serotypes. Although types A and B may occur simultaneously the individual types of each occur over a restricted period of time and are then replaced by a new antigenic

type. A list of the known A and B types and the periods in which they appeared is given in Table 16.1.

TABLE 16.1
Epidemic Influenza

Type	Epidemic Periods	Major Pandemics	References
Influenza A	1933–1946	none	17, 18
Influenza A1	1946–1957	none	19
Influenza A2	188?–189?	1889–1892	20, 21
	1957– ?	1957–1958	20, 21
Influenza A (porcine)	19? –1928	1918–1919	22
Influenza B (Lee)	1936–1948	none	23, 24
Influenza B (Great Lakes)	1950– ?	none	25

In addition to these epidemics, small outbreaks and sporadic cases of influenza have been associated with influenza C[26,27] and the Sendai strains of parainfluenza type 1.[28,29] The incidence of pneumonia appears to be relatively high with Sendai virus.

Treatment is purely symptomatic unless the fever goes on beyond the 5th day or if pneumonia sets in before this time. Antibiotics should be given when the first signs of pneumonia are observed.

CROUP or ACUTE LARYNGOTRACHEOBRONCHITIS was originally associated with parainfluenza type 2 virus, which was initially called croup associated (CA) virus.[30,31] It is now recognised that parainfluenza types 1, 2 and 3 and influenza A can all cause croup in children under 3 years of age.[32,33,34]

The illness takes the form of an upper respiratory tract infection characterised by a croupy cough, stridor, dyspnoea and hoarseness. The temperature is variable and bears no relation to the severity of the illness. Although the disease may be little more than a mild respiratory infection it can also be severe, with oedema of the subglottic regions and bronchi. In the most severe cases tracheotomy may be essential. Prompt treatment with cool moist air and tracheotomy if necessary usually ensures recovery.[35]

BRONCHOPNEUMONIA and BRONCHIOLITIS of infants are also caused by parainfluenza types 1 and 3[36] although RSV appears to be the most important[37,38,39] The clinical picture of acute bronchiolitis is usually confined to children under 6 months of age. They become pale and have a continuous cough. Breathing is rapid and shallow. The chest is emphysematous and crepitations are always present.[40] There is a tendency towards pneumonia in older children as well as the infants. In both groups X-ray examination reveals foci of collapse and consolidation of the lungs.

OTHER UPPER RESPIRATORY TRACT INFECTIONS are associated with many of the Myxoviruses. They range in severity from

a typical "common cold" to a moderately severe influenza-like illness with systemic as well as respiratory symptoms. In children these are mild forms of infection with parainfluenza types 1, 2, 3 and 4 and RSV, while in adults they are usually second infections with the same agents.[41,42] The typical symptoms in the adult are sore throat, nasal congestion and malaise.[43]

MUMPS or epidemic parotitis, is an acute disease with a high morbidity rate although the mortality is low. The illness is much more severe in the adult than in the child and it is probably one of the most uncomfortable of the common infections.[44]

After an incubation period of 18–21 days a low fever is followed 12–24 hours later by swelling of the parotid glands. The degree of pain usually varies with the size of the swelling and can be severe. One gland is usually affected first, the second becoming swollen 1–5 days later. The fever subsides in 1–3 days but the parotitis usually lasts for 7–10 days. In addition to parotitis, or even in its absence, orchitis, meningitis, pancreatitis, mastitis, epididymitis, prostatitis and ovaritis may occur. The incidence of these complications varies from epidemic to epidemic but meningitis and orchitis are probably the most common, and occur quite frequently in the absence of parotitis.[8,44,45] Up to 40% of the infected adult males have orchitis, which is bilateral in about one third of these cases. Nevertheless sterility only occasionally results. With the exception of the meningitis, the complications are found more frequently and are more severe in adults. An increase in serum amylase is usual in patients suffering from mumps but the pancreas need not necessarily be involved.[46]

It has been reported that mumps infection during the first trimester of pregnancy increases the chance of abortion or congenital abnormalities in the infant. Our present information indicates that this risk, although present, is not high.[47,48]

Mumps is caused by a single serological type which was named after the illness.[49] The infection rate is high, being up to 85% of the population at risk, of whom 75% have clinical symptoms. The majority of the patients recover completely and very few deaths have been recorded.

Knowledge of the pathology of mumps infection is limited but typical lesions of the parotid glands show serofibrinous exudate with leucocytes in the connective tissue and degeneration of the duct cells.[50] There is also marked destruction of the epithelium of the seminiferous tubules.[51]

Treatment is purely symptomatic.

MEASLES is one of the commonest of the childhood diseases in modern civilisation. Serious complications can arise, some of which are entirely due to the virus while others follow secondary infection.

After an incubation period of 5–9 days there is a rapid onset of respiratory symptoms. Chilliness is followed by sneezing, a watery nasal discharge, redness of the eyes, sore throat, cough and fever. A faint scattered rash may be present but the characteristic lesions at this

stage are the Koplik spots, which are found on the mucous membranes of the lower lips and round the papilla of the parotid ducts. They are 1–3 mm in diameter and appear bluish white on a scarlet base.[52] The fever and cough progressively worsen until 3–5 days when the rash appears, first on the forehead and behind the ears. It spreads over the rest of the body in the course of the next 24–48 hours and when it is fully developed the fever drops. This rash may be macular or maculopapular and after 3–4 days it fades, leaving a brownish staining which is followed by desquamation. In the most severe cases conjunctivitis and photophobia may occur. Myocarditis can be demonstrated by electrocardiograms but it appears to be transient in most cases.[53] The most severe forms of the disease are found with a haemorrhagic rash or when pneumonia or encephalitis occur. Thrombocytopenia is common with the haemorrhagic rash and leucopenia is present in the majority of cases at the height of the illness.[54] Encephalitis is relatively uncommon, being most severe in the youngest children. The mortality rate is about 15% when there is an encephalitis and permanent physical and mental sequelae are frequent.[55] In addition to the secondary bacterial infections which may complicate the illness, a true virus pneumonia, which can be fatal, also occurs.[55,56]

Although measles virus occurs as a single antigenic type it is related serologically to the viruses of canine distemper and rinderpest.[57]

The Koplik spots appear as shallow ulcers with infiltration of leucocytes and necrosis of the basal epithelium. The pneumonia is interstitial with infiltration of macrocytes into the alveolar and bronchial walls. Giant multinucleated cells are found in the bronchial epithelium, in the Koplik spots and in lymphatic tissue.[58]

The severity of the illness can be reduced during the early stages of the incubation period by the administration of gamma-globulin. Antibiotic prophylaxis reduces the risk of secondary bacterial infection.[59,60]

The epidemiology of this group of diseases, all of which are highly infectious, is basically similar. Dissemination of the virus is by droplet infection. Studies conducted on outbreaks of mumps and measles viruses in "virgin" communities show that virtually the whole population becomes infected. The great influenza pandemics require no further comment.

The distinctive features which divide these viruses into three epidemiological groups are, firstly, the antigenic variability of influenza A and B and secondly, the ability of measles and mumps to produce a viraemia. These latter two viruses therefore produce systemic illnesses with viral multiplication in the tissues. The others rarely, if ever, penetrate beyond the respiratory tract and although they give rise to such lower respiratory diseases as pneumonia and bronchitis their severe symptoms appear to be due to a toxic effect rather than viral multiplication.

With the exception of influenza they are, in the main, childhood diseases. Epidemics occur as each new population of non-immune

children arise. The length of time between epidemics varies with the virus but they tend to occur at regular intervals. Second infection in the adult is localised in the nasopharynx and its spread is rapidly curtailed by the production of high levels of circulating antibody.

The picture with influenza is very different, because of its ability to change its antigenic structure. There is some evidence to support the theory that there are only a limited number of antigenic variants of influenza A with the same type occurring only once in 60 to 70 years. Whatever the validity of this theory, we still have the situation where a previously unknown antigenic type appears, spreads rapidly causing huge epidemics and then settles down to produce smaller epidemics in a new susceptible population, until it is replaced by another distinct antigenic type after about 20 years.

Laboratory Investigations

Although a clinical diagnosis is sufficient for classical mumps and measles; meningitis or pneumonia, in the absence of any other symptoms, require laboratory studies for diagnosis. The presence of the Warthin-Finkeldey giant syncytial cells is diagnostic of measles infection[53] but for all other Myxovirus infections the final diagnosis must be made virologically. Mumps virus can be isolated from the CSF in cases of meningitis; measles virus from the blood and all can be isolated from the nasopharynx or saliva during the acute stage of illness.[39,61–64] Throat washings or cough swabs are the best source of material for isolation of the Myxoviruses and they should be inoculated into eggs or tissue cultures with minimum delay. Storage at −70°C will usually preserve the infectivity except for RSV where immediate inoculation into tissue cultures is essential.

In most cases serological tests are more convenient and a rise in antibody titre between acute and convalescent sera may be demonstrated by C-F, HI or neutralisation tests. The HI tests are best for influenza virus because the C-F test only gives information on whether the infecting virus is A, B or C, whereas with the HI test the exact type can be determined. For the remainder the C-F test is probably the most convenient although cross-reactions do occur with the parainfluenza and mumps types.[65]

Vaccination

Vaccines have been produced against most of the human Myxoviruses but so far only those against influenza have been used routinely. Although live-attenuated vaccines are now being produced the most usual preparation is the formalin-inactivated virus grown in the allantoic cavity.

The influenza vaccines contain strains of types A1, A2 and B. It is essential to have the same antigenic strain in the vaccine as that which is causing the outbreak, because vaccination against related strains has

much less effect.[1,66,67] When administered before an epidemic homologous vaccines have 60–70% efficiency, but the protection is short lived.

Recommendations have been made that all doctors, nurses, hospital employees and sufferers from chronic heart and chest complaints should be regularly vaccinated. For the general population such vaccination is of dubious value due to the great antigenic variations and the necessity of keeping the vaccine up to date. The live-attenuated strains which have been developed appear to be promising although they are still at the trial stage.[2]

Formalin-inactivated and live-attenuated vaccines against mumps virus have been successful but so far there has been little practical application of them.[3,68] When the incidence of serious complications is so low vaccination seems to be pointless, except in closed communities which have had no previous experience of the virus.

Measles, on the other hand, presents some difficulty owing to the more frequent occurrence of complications and a variety of vaccination procedures have been developed using live-attenuated virus alone, or with gamma-globulin, and formalin-inactivated virus. The original live virus vaccine produced a severe reaction with temperatures rising to 103·5°F.[4,69] A less vicious attenuated strain is now available which appears to be efficient without the unpleasant reaction, and a formalin-inactivated vaccine is also showing some promise.[70,71]

Non-human Myxoviruses

There are six important diseases of animals caused by the Myxoviruses. At first sight the most important are rabies and canine distemper, but Newcastle disease, fowl plague, rinderpest and shipping fever can be highly destructive when they reach epidemic form.

Rabies is maintained within the community of wild animals and only accidentally infects man or the domesticated animals. The virus is present in the salivary glands of infected animals and is transmitted by bite. It can infect all mammals although clinical disease is only regularly produced in some.

Evidence is now coming to hand which indicates that animals which are healthy carriers of rabies virus can develop and succumb to rabies after the administration of adrenocorticotrophic hormone (ACTH).[72] This appears to disturb the virus/host relationship allowing the virus to enter the CNS and multiply, thereby causing the animal to become rabid. Since ACTH secretion is stimulated by any physiological stress, an animal which is a healthy carrier may become rabid and bite any other creature with which it comes in contact.

Rabies

Although the disease is principally a disease of animals it can be passed to man by the bite of a rabid dog, wolf, skunk, or bat. The

manifestations of rabies infection are similar in both man and dogs and those in man will be described.

The incubation period varies with the site of the bite and the amount of virus present in the animal's saliva, ranging from as short as 6 days to as long as 12 months, but 30–60 days is the more usual. The first symptoms are headache, malaise, anorexia, nausea, sore throat and fever (100–102°F). In about 80% of the cases there is some discomfort at the site of the bite and this indicates the onset of rabies. There is often a feeling of apprehension and sensitivity to stimulation. This is followed by an excitation phase where the patient has increasingly frequent paroxysms of convulsions interspersed with quiet periods. Difficulty in swallowing fluids (hydrophobia) appears early in the illness and develops into not only an inability to swallow fluids, but the mere sight of fluid will usually induce characteristic spasms of the throat muscles, which violently expel the fluid if an attempt is made to swallow. The patient invariably dies during or just after a convulsion, but occasionally delirium and coma occur first. Sometimes a flaccid paralysis replaces the hydrophobia, but in all cases the mortality in untreated rabies is 100% in man. A man bitten by a dog either dies or he does not become infected at all. In dogs hydrophobia is not seen although swallowing is difficult.

The lesions produced by rabies virus are similar to those found in other forms of fatal encephalitis. However, eosinophilic inclusion bodies are found in the cytoplasm of large neurones especially in the hypocampus. These Negri bodies, as they are called, are diagnostic for rabies.[73]

Treatment is possible by vaccination because of the long incubation period. Local treatment of the bite is also recommended. It should be washed thoroughly, cauterised with concentrated nitric acid and antiserum or powdered gamma-globulin should be applied.[74] Various vaccines have been devised using phenol or U-V-inactivated virus grown in rabbit brain, or egg-passaged live-attenuated vaccine.[74]

NEWCASTLE DISEASE of FOWLS is an acute, highly infectious pneumo-encephalitis with an incubation period of 4–11 days. The disease varies in severity with a mortality rate ranging from 2–100%. The commonest symptoms are a watery offensive diarrhoea, mucous discharge from the nostrils and mouth and severe respiratory distress. At post mortem a haemorrhagic encephalitis is found.[76,77] This disease is caused by a single antigenic type[79] (Newcastle disease virus) and infects a wide range of domestic and other birds.

FOWL PLAGUE or FOWL PEST is another acute disease of fowls. The incubation period is 3–5 days and the principal symptoms are depression and dullness, darkening of the comb and wattles and inflammation of the buccal mucosa. The disease lasts 2–4 days and has a high mortality rate.[79,80] It is caused by influenza A galli[81] but it does not have as wide a host range as NDV.

SHIPPING FEVER is a respiratory disease of cattle which varies in severity from a febrile upper respiratory infection to a fatal pneumonia. It is associated with the SF-4 strains of parainfluenza type 3 although bacterial pathogens are usually also present. It would appear to be severe only when cattle are subjected to stress such as movement over long distances.[82]

CANINE DISTEMPER is an acute infectious illness of dogs. The incubation period is 3–8 days and the main symptoms are coryza, conjunctivitis, severe gastro-enteritis and a vesicular or pustular dermatitis. The central nervous system may become involved and in such cases the disease is usually fatal. A giant cell pneumonia similar to that in man often occurs. The mortality rate is high, but several vaccines have been developed and they give good protection.[7]

RINDERPEST is a severe infection of domestic cattle, sheep, goats and a variety of other animals. The incubation period is 3–4 days and the predominant symptoms are inflammation of the buccal mucosa, gastro-enteritis, necrotic stomatitis, dermatitis, leucopenia and degenerative changes of the lymphoid tissue. The severity of the disease varies with the strain of virus and the immunological state of the herd. Mortality can be as high as 90% of a herd. A variety of efficient vaccines are in everyday use.[83]

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CHAPTER SEVENTEEN

THE HERPESVIRUSES

THE Herpesvirus group is a collection of ether sensitive, DNA viruses which have a similar, if not identical, morphology. Members of the group infect a wide range of birds and mammals and many cause persistent infections in their natural hosts. In 1954[1] the group was defined to include herpes simplex (from which the group was named), varicella and three other viruses of monkeys, swine and rabbits. Since then the Cytomegaloviruses[2] and several avian and mammalian types have been added bringing the total number up to about 20.[3]

The clinical manifestations of these viruses are very variable, especially herpes simplex which usually occurs as a mild febrile illness on initial infection in children. Subsequently the virus lies dormant, breaking out from time to time in characteristic herpetic lesions or 'cold sores' as they are commonly called. Occasionally the infection is more serious and results in an encephalitis or keratitis. Several members share this ability to remain dormant for years and the Cytomegaloviruses are often carried as latent infections which never manifest their presence.

The relationship between chicken pox and herpes zoster (shingles) interested and puzzled the medical profession for many years. We now know that the two diseases are caused by the same virus, varicella, which has the ability to lie dormant in the tissues of the immune host.

Biological Properties

The characters of the group may be defined as follows:—[3]

(1) They are about 100–200 mμ in diameter.

(2) They are complex viruses with DNA as the nucleic acid.

(3) They are sensitive to ether.

(4) They have a central icosahedral nucleoprotein core with 162 capsomeres in the capsid and an outer membrane.

(5) They multiply entirely within the nucleus, giving rise to Cowdry type A inclusions.

The early electron microscopy work, using osmic acid fixation and ultra-thin sectioning, showed that herpes simplex was a complex particle with an overall diameter of 110–210 mμ.[4] Typical particles, observed in the cytoplasm of infected cells, had a central electron-dense core of 40–50 mμ; a membrane enclosing the central core, of about 6–8 mμ thickness with an external diameter of 70–100 mμ; and an outer membrane of 120–130 mμ in diameter. Intranuclear particles sometimes appeared in crystalline array, possessed only one membrane and

had a diameter of about 90 mμ.[5] When examined by the phosphotungstic acid negative staining technique,[6] the central core was found to be icosahedral with an average diameter of 77·5 mμ and surrounded by an icosahedral capsid composed of 162 capsomeres, with an average diameter of 105 mμ. The capsomeres are hollow polygonal prisms,

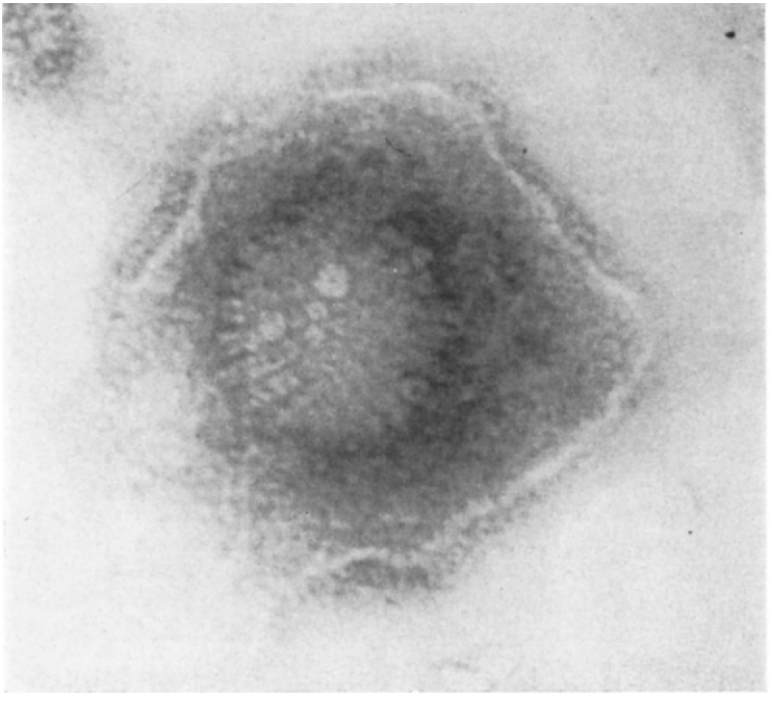

FIG. 17.1. Electron micrograph of herpes simplex virus embedded in phosphotungstate showing the outer envelope surrounding the capsid. (*Photo—R. W. Horne.*) (*Courtesy Academic Press Inc., Virology*, **12**, 204; 1960.)

mostly hexagonal but a few are pentagonal, and measure 9·5 × 12·5 mμ. The central core is about 4 mμ in diameter.

It was suggested that the hexagonal capsomeres are composed of six, and the pentagonal of five sub-units, each with a molecular weight of about 80,000. The envelope surrounding the capsid is of varying size and shape, with the mean diameter ranging from 145–205 mμ although the majority are 180 mμ. The periphery of the envelope appears denser than the rest giving the appearance of a membrane 4–10 mμ thick, and the surface is covered with projections 8–10 mμ long, spaced at intervals of about 5mμ.

Similar studies have been undertaken on five other members all of which have an identical basic structure, although the dimensions of each

part of the substructure may differ slightly. These viruses are varicella;[7] pseudorabies;[8] infectious laryngotracheitis of chickens (ILT);[9] and two equine strains, the LK strain isolated from a horse with catarrh, and a strain of equine abortion or rhinopneumonitis virus.[10]

Particles similar to those described for herpes simplex in ultra-thin sections have been demonstrated in cultures of virus B of monkeys,[11] infectious bovine rhinotracheitis (IBR)[12] and the Cytomegaloviruses.[13] In addition to these typical particles, other structures have been seen in the nuclei of cells infected with ILT and the Cytomegaloviruses.[13,14] Filaments varying in length from 90–900 mμ, but with a uniform thickness of 65 mμ, are present in ILT infected cells. They have an electron-dense core of 22 mμ extending the whole length of the filament and are enclosed within a membrane 8–10 mμ thick.[14] Large spherical bodies of 300–500 mμ in diameter, occasionally exhibiting an internal structure, are found in cells infected with the Cytomegaloviruses but the relationship of any of these forms to the virus particles is not yet known.

The chemical composition of the Herpesviruses is not fully determined. However, the ether sensitivity of the members of the group suggests the presence of lipid, which is probably in the outer membrane. The capsid is almost certainly composed of protein with the DNA in the central core, although it seems likely that other substances are present in the core.[6,15]

Analysis of the DNA and protein content of herpes simplex and pseudorabies viruses has shown that the nucleic acid of both is virtually identical. Each virus particle contains about 25 times as much protein as DNA.[16] The base composition of the DNA is unusual, with 68–74% guanine and cytosine.[17] Equine abortion virus also has a high (56%) guanine + cytosine value. The quantity of DNA is also different from that found for herpes simplex, being about 9% of the particle.[18]

All the Herpesviruses are relatively labile although there is wide variation between the different members. They are rapidly inactivated by ether, both at 4°C and 37°C. At the latter temperature herpes simplex and pseudorabies viruses are completely inactivated in 30 minutes. They are all very heat labile and require temperatures of −60°C for preservation. Incubation for 5 hours at 44°C has inactivated all but 0·014% of one strain of herpes simplex. Pseudorabies virus, however, proved to be more stable with 28% of the infectivity remaining after similar treatment.[19] Inactivation also readily occurs when these viruses are treated with such agents as chloroform, urea and formalin.[20] They are also very sensitive to change in pH and are rapidly inactivated outwith the range pH 6·8–7·4.[21]

The antigens of this group have received little attention as yet but they can be demonstrated by neutralisation and complement fixation. The complement-fixing antigen is partly soluble and partly associated with the virus particles, and is retained within the cell and liberated when the cell is disrupted. The amount of soluble antigen produced seems to

depend on the host cell system. No member of the group has been found to agglutinate the erythrocytes of any animal. This is the only defined group in which haemagglutination does not occur.[22,23,24,25]

Growth

The host range of these viruses is extremely variable but the majority can be most easily cultivated in tissue cultures derived from the natural hosts. Some members, in particular, herpes simplex, herpes B, pseudorabies and ILT viruses produce pocks on the CAM. Suckling mice can also be used for herpes simplex and herpes B viruses. A fatal encephalitis follows intracerebral inoculation of mice with either of these viruses and this is probably the most sensitive test for the presence of herpes simplex virus.[26] In general, tissue cultures are undoubtedly the best means for detecting and growing the members of this group. Most of the viruses prefer epithelial cells to fibroblasts but the Cytomegaloviruses are exceptional in that they grow well only in fibroblasts.[27]

One of the distinguishing features of the Herpesviruses is the production within the nucleus of a typical eosinophilic inclusion body called the type A inclusion by Cowdry.[28] This inclusion was redefined by Pereira as follows:—[29] "Type A inclusions are represented by single homogenous eosinophilic bodies occupying most of the central area of the nucleus and clearly separated from the marginated chromatin". All members of the group produce this type of inclusion which, although not confined to the Herpesviruses, is nevertheless a useful distinguishing feature.

The CPE produced in tissue cultures varies with the strain of virus and the size of the inoculum used. The typical CPE of herpes simplex is foci of rounded granular cells showing intranuclear inclusions and amitotic divisions.[29] Some strains of herpes simplex, herpes B and pseudorabies viruses produce multinucleated giant cells which subsequently lyse.[30,31,32] Small inocula of feline rhinotracheitis virus give a mixture of focal granulations and giant cell formation.[33] Demonstration of the intranuclear inclusions depends on the method of fixing and staining and they cannot be observed by phase contrast microscopy, or in the electron microscope in osmic acid fixed preparations.[11,34] It therefore appears that the inclusion body formation is a combination of the effects of the virus and the fixing and staining procedures.

The various stages of the growth cycle have been observed for several members of the group by conventional staining, fluorescent-antibody staining, electron microscopy and infectivity titrations. Although the early stages of the growth cycle have not yet been determined, it seems likely that adsorption of the virus particles to the host cell is accomplished by the interlocking of the projections on the outer membrane with complementary projections on the cell surface. This is, of course, a reasonable assumption since the Herpesviruses have a similar external structure to the Myxoviruses.

The adsorption of herpes simplex is not temperature dependent and although it occurs more rapidly at 37°C than at 4°C the final quantity of virus adsorbed is the same. At 37°C, 50% of the inoculum has adsorbed to HeLa cells within one hour and for successful adsorption electrolytes must be present. On the other hand, penetration of the virus markedly depends on the temperature and occurs most rapidly and to the greatest degree at 37°C. Most of the adsorbed virus has penetrated into the cell 2 hours after inoculation.[35]

Following penetration there is a long latent period before any cytological changes become visible. This period varies from about 9–10 hours for herpes simplex and varicella to 18–24 hours for the Cytomegaloviruses.[36,37,38] The first noticeable changes occur in the nucleus at 9–10 hours with enlargement of the nucleoli, distortion of the nucleus and granulation of the chromatinic reticulum.[29,39] In human fibroblasts infected with varicella virus small eosinophilic granules appear, which ultimately coalesce to form the type A inclusions.[37] With herpes simplex, however, the early stages of these inclusions are basophilic.[40] The Cytomegaloviruses appear to be exceptions to this sequence because the first changes appear at about 18–24 hours and occur in a cytoplasmic zone adjacent to the nucleus.[38] This zone is rich in RNA and contains lipid and protein. Similar cytoplasmic changes are present at the same time in FL cells infected with herpes simplex virus.[36]

The initial appearance of viral antigen has been reported as occurring in the cytoplasm in a perinuclear position for herpes simplex and varicella viruses.[36,41] These observations were made on preparations 18–24 hours after infection. Another group of workers[37] have observed viral antigen first in the nuclei of varicella-infected cells only 10 hours after infection. This group also found that the antigen left the nucleus and entered the cytoplasm after 48 hours and that by 96 hours there was very little nuclear antigen. Viral antigen, however, appears in both the nucleus and the cytoplasm of Cytomegalovirus-infected cells between 24 and 48 hours after infection.[38] Before attempting to correlate these very different findings, the changes as seen by conventional staining, electron microscopy and infectivity titrations should be considered.

Although it has not been possible to definitely observe the Cowdry type A inclusions by electron microscopy, viral synthesis appears to occur within these bodies.[4] The virus particle then passes to the cytoplasm and the fully developed eosinophilic inclusion is then devoid of virus. Therefore it would appear that the inclusion bodies when ultimately seen are the remnants of the production areas for the virus which has moved into the cytoplasm. The development of herpes simplex particles is associated with granular areas at the margin of the nucleus. The first formation which can be observed is a dense object 30–40 mμ in diameter (the core), which then acquires a membrane 70–100 mμ in diameter. At this stage the core becomes less dense and slightly larger 40–50 mμ. The maturation of the virus particle occurs

at the nuclear membrane and the second membrane is probably acquired as the particles pass through into the cytoplasm, because all particles in the cytoplasm have two membranes and are found within vacuoles.[4,39] Some strains produce crystalline formations of particles with single membranes in the nucleus but for most, synthesis of virus particles occurs at random within the granular areas. IBR virus has also been found to have a similar growth pattern but inner membranes without a central core are seen. This suggests that either the inner membrane forms first or that the core and membrane form simultaneously.[12]

Early infectivity determinations have shown that infectious herpes simplex virus does not occur in the nucleus.[42] Intracellular infectious virus first appears about 12 hours after infection, when virus particles are present at least in the nucleus and probably also in the cytoplasm.[43,44] These observations support the theory that to be infectious the virus particles require the outer membrane which is only added to the intranuclear particle at the nuclear membrane. The exit of the virus particles is accomplished without rupture of the nuclear membrane, by deposition of the membrane behind the particles as they pass through.

The ambiguous results obtained by fluorescent-antibody staining with regard to the site of formation of viral antigen can now be considered. In the first instance we do not know the specificity of the antibody or for that matter with how many antigens we are dealing. There are therefore two explanations which have one part in common. The components of the lipid-containing membrane are synthesised in the cytoplasm in a perinuclear position while the protein which forms the inner membrane, or capsid, may either be synthesised in the nucleus, or in the cytoplasm, but whichever is the case the icosahedral capsid is built up within the nucleus.

Metabolic changes following Herpesvirus infection have been studied for herpes simplex and pseudorabies viruses. The most noticeable alteration is a massive increase in the synthesis of DNA by the cells.[45,46] This synthesis commences about 4 hours after infection and is preceded by protein synthesis.[47] Synthesis of cellular DNA is suppressed following infection and this increase in DNA is purely viral in origin.[46,48] Uptake of thymidine into the DNA is initially depressed when the cells are rapidly multiplying before infection whereas it is markedly increased in non-multiplying cultures.[49] The viral DNA is thus synthesised from a nucleotide precursor pool which is large in multiplying cells but small in non-multiplying cells. In addition some breakdown of host DNA also occurs which will contribute to the pool.[45] The quantity of DNA produced per cell is far in excess of that required for the formation of new virus particles and only about 20% is ultimately incorporated in the mature virus.[46] Any estimation of the amount of new virus produced per cell is difficult due to its rapid inactivation at 37°C but about 1 in 10

of the particles seem to be infectious. Synthesis of DNA takes place in the following manner:—

Protein formation commences first, between 2 and 4 hours after infection and includes the production of the enzyme systems necessary for the incorporation of thymidine into DNA. When DNA synthesis starts, the formation of these enzymes ceases and it appears that the synthesis of DNA only begins when one or more of the DNA precursors reaches a critical concentration.[47]

In cells infected with herpes simplex there is an increase in the amount of organic acids produced,[51] as a result of an alteration in the carbohydrate metabolism whereby glycolysis remains unaltered but the pentose shunt pathway is stimulated.[52]

The growth cycle of the Herpesviruses appears to be similar for all members (although the Cytomegaloviruses are much slower than the others) and can be summarised. Following attachment and penetration of the virus, the DNA sets in motion a series of metabolic changes in both the nucleus and the cytoplasm. Synthesis of host cell DNA is inhibited and existing DNA is broken down to fill the nucleotide pool. Viral DNA is synthesised entirely within the nucleus and in much greater quantity than is necessary for the production of new virus particles. Within the areas of synthesis, the DNA forms into units which are coated with protein. The protein capsid may be synthesised either in the cytoplasm or the nucleus but further study is required to establish the exact site of protein formation. The immature particles then pass through the nuclear membrane into the cytoplasm acquiring the lipid-containing outer membrane on the way. The constituents of this layer are almost certainly synthesised in the cytoplasm and are passed to the nuclear membrane where maturation takes place. The complete particles then pass through the cytoplasm and are liberated into the medium. At this stage stained cells have the typical Cowdry type A inclusions which are the remains of the intranuclear factories where the virus was formed. When the inclusions become visible, most, if not all, of the new virus has left the nucleus.

There are several other interesting phenomena connected with the intracellular cycle of Herpesvirus synthesis. Treatment of herpes simplex-infected HeLa cells with cortisone increases the carbohydrate metabolism via the pentose shunt. It also delays the appearance of a CPE suggesting that the cells are protected in some way from damage by the viral multiplication and can continue metabolising for a longer period.[51,52] Maben cells infected with the Z strain of herpes simplex virus and incubated at 31°C become persistently infected. The cells are not normal and multiply slowly, releasing large amounts of virus, but they do not show a typical CPE. A very high proportion of the cells carry the virus and incubation with medium containing antiserum for at least three weeks has no effect. When the temperature is raised to 34°C however, the typical CPE of herpes becomes obvious; the amount

of virus produced is raised and the cultures are destroyed. At the lower temperature cell metabolism proceeds very slowly and this may account in part for what is an unusual phenomenon.[53]

As has already been mentioned syncytia are formed by certain strains of Herpesviruses. Studies with one strain of herpes simplex have shown that even when the cell sheet is inoculated with a small amount of virus and then incubated in the presence of immune serum it is totally destroyed. Similar treatment using a non-syncytia forming strain usually results in the recovery of the cell sheet.[36] These strains therefore are able to infect adjacent cells without passing through the medium. Fluorescent-antibody staining demonstrates the presence of viral antigen in cytoplasmic processes connecting the syncytia with each other and non-infected cells. These processes are not observed in the non-syncytia forming strains and this would consequently appear to be the means by which the virus passes from cell to cell. Similar cytoplasmic strands have been seen in cultures infected with varicella virus.[41]

The last interesting observation concerning the growth of herpes simplex is the effect of different strains on multiplying HeLa cells. One strain apparently has the property of increasing the multiplication rate[54] whereas another prevents multiplication.[45] When HeLa cells are infected with the latter strain those cells which are in interphase exhibit the type of reaction described above. However, when a cell in mitosis becomes infected the normal mitotic process is inhibited. The exact mechanism of this inhibition is unknown but the virus appears to be able to act at several stages, namely—by destroying the nucleus, by preventing spindle formation and by breaking down host cell DNA early in the growth cycle.

Classification

Andrewes has recently[3] divided the Herpesviruses into four sub-groups, the last of which is a collection of miscellaneous viruses waiting to be fitted into one of the other three sub-groups, or even to be defined in a new sub-group. The sub-groups are:—

(1) The "Classical" Herpesviruses.
(2) The Cytomegaloviruses.
(3) The Avian Herpesviruses.

It seems reasonable at this stage to place two of Andrewes' miscellaneous viruses (IBR and equine abortion) and a new one (LK) in the Classical sub-group which now brings the total to eight. Non-Linnean binomials have been applied to some of these viruses, but they have never found favour. The viruses contained in sub-group 1 are listed in Table 17.1.

Herpes simplex and B viruses are antigenically related[55] although the presence of neutralising antibodies to herpes simplex in man does not prevent a fatal infection with B virus.[56] There is also a minor antigenic relationship between herpes simplex and pseudorabies viruses.

TABLE 17.1

The Classical Herpesviruses

Virus	Natural Host
Herpes simplex	Man
(Herpes) B virus	Monkeys
Pseudorabies	Swine
Virus III	Rabbits
Varicella-Zoster	Man
Infectious Bovine Rhinotracheitis (IBR)	Cattle
Equine abortion or Rhinopneumonitis	Horses
LK strain or Equine Herpesvirus 2	Horses

Members of the Cytomegalovirus sub-group have been isolated from man, guinea pigs and mice. There are probably many more occurring in other animals as histological evidence suggests.[3] The human strains belong to several distinct immunological types and three have so far been defined.[57] All the strains isolated from mice and guinea pigs fall into two distinct immunological types each associated only with one species of host.

The Avian sub-group is composed of Infectious Laryngotracheitis (ILT), Pacheco parrot disease, pigeon disease and cormorant disease viruses. Little is known of their antigenic relationships and only ILT has been shown to have the typical Herpesvirus structure.

Finally, there are the several viruses from a variety of animals which will probably prove to be Herpesviruses. These include inclusion body rhinitis of pigs, feline rhinotracheitis and the Atherton and Orphan viruses isolated from lumpy skin disease.

Clinical Manifestations of Herpesvirus Infection

There are three human pathogens in the Herpesvirus group—herpes simplex, the Cytomegaloviruses and herpes zoster or varicella. In order to avoid confusion the latter virus will be referred to only as varicella. Human infections with some of the other members do occur occasionally and those due to B virus are extremely serious. For the moment we shall deal only with the various syndromes associated with herpes simplex, varicella and the Cytomegaloviruses.

Both herpes simplex and varicella cause aseptic meningitis and encephalitis, usually in association with the typical skin lesions, but as is the case with mumps virus, there may be no other sign.[58–61] However, the majority of illnesses involve lesions of the skin or mucous membranes. Herpetic keratoconjunctivitis is not uncommon. Although most are unpleasant only some of the illnesses can be termed serious and death is a very rare occurrence.

CHICKEN POX or VARICELLA is a relatively mild febrile illness characterised by a vesicular rash and is most frequently seen in children.

There is an incubation period of 14–21 days. A fever is followed 24 hours later by the appearance of papules and vesicles on the face and trunk. These spread to the mucous membranes and extremities but the distribution is unlike that of smallpox where the rash is extensive on the face and extremities. The varicella rash is predominantly on the trunk. Lymphadenopathy is very common. Pneumonia is an occasional complication which is not due to secondary bacterial infection and is more common in adults.[62] If contracted during the neonatal period or as an intrauterine infection the disease is very serious with a mortality rate as high as 20%.[63]

The pathology of chicken pox is quite distinct from that of smallpox. Large multinucleated cells with Cowdry type A inclusions are present within the vesicles which form in the lower layer of the epidermis.[64] Lesions are found in the trachea, lungs, pleura, myocardium, spleen, kidneys, pancreas, adrenals, ovaries and the gastric and intestinal mucosa. These lesions are focal with Cowdry type A inclusions present in the cells of the affected areas.[63,64]

Treatment should be directed towards the alleviation of the symptoms. Since the administration of cortisone is contra-indicated any patient who is receiving cortisone therapy should be taken off this immediately, because a number of fatalities have resulted from continued therapy.[65]

SHINGLES or HERPES ZOSTER is primarily a disease of adults caused by varicella virus and appears to originate in two ways. It can occur in the person who is partially immune to varicella when exposed to contact with the virus or it can be caused by the stimulation of varicella virus which has been lying dormant in the sensory ganglia.[64]

The illness is characterised by a localised crop of papules and vesicles identical with those of chicken pox, severe pain, fever and malaise. These vesicles are seen over an area of skin or mucous membrane innervated by one or more of the spinal nerves or by sensory divisions of the cranial nerves. The pain corresponds with the distribution of the rash, which it frequently precedes. In the older patient the pain often remains after the skin eruption has healed.[64] A slightly raised white cell count in the CSF is often found. The illness usually lasts about 2 weeks and it is never fatal.

Although shingles itself is never fatal the typical lesions have been studied at post mortem examinations of patients who have died from other causes, while suffering an attack of shingles. The lesions are confined to the skin and nerve tissue. Necrosis of the dorsal root ganglia, with occasional degeneration of the related motor and sensory nerves, is the essential feature, while the skin lesions are identical with those of chicken pox.

The virus causing both chicken pox and shingles is varicella or herpes zoster virus. When the identity was established by various serological techniques it was suggested that this virus should be called either varicella or zoster to avoid confusion with the other Herpesviruses.[66–70]

HERPETIC KERATOCONJUNCTIVITIS varies greatly in severity, from a follicular conjunctivitis to a very severe keratitis resulting in blindness.[71,72,73] The follicular conjunctivitis, which frequently progresses to a superficial keratitis is a common manifestation of primary herpes simplex infection in children. The severe deep keratitis is a chronic illness which may occur in several forms. It invariably leaves deep scarring when the lesions ultimately heal. This may take up to a year and recurrences are frequent.

It is important to differentiate between herpetic keratoconjunctivitis and adenovirus keratoconjunctivitis. A previous history of the illness immediately rules out Adenovirus, as does the presence of "dendritic" ulcers on the cornea and vesicles on the eyelids. Finally the adenovirus lesions tend to be painful whereas in most cases due to herpes simplex there is relative anaesthesia of the cornea.

Treatment is limited to cauterisation of the corneal lesions to prevent spread and chemotherapy to prevent secondary bacterial infection. Steroids should on no account be used because they increase the severity of the lesions. When scarring is advanced a corneal transplant is the only means of restoring the sight.

Diseases of the Skin and Mucous Membranes

Herpes simplex causes a variety of lesions of the skin and mucous membranes. The primary infection often presents as an acute gingivostomatitis in children between 1 and 3 years of age.[74] When a baby suffering from eczema becomes infected with herpes simplex virus a severe eczematous disease follows.[75]

The symptoms of *acute herpetic gingivostomatitis* are fever, irritability, red swollen gums, lymphadenopathy and a vesicular eruption of the oral mucous membranes. These mouth lesions rapidly become macerated and appear as shiny greyish-yellow plaques. Although the disease varies in severity with temperatures of up to 104–105°F there is always a constitutional reaction. This illness lasts from 7 to 14 days and recurrences are uncommon.

Eczema herpeticum or Kaposi's varicelliform eruption is an unpleasant complication of eczema characterised by high fever (104°F or more), numerous vesicles and restlessness. In very severe cases the vesicles continue to appear for up to 9 days and large areas of epithelium may be lost. Prognosis is usually good but death can occur as a result of untreated dehydration, due to loss of fluids, electrolytes and protein. Occasional recurrences are seen.

The "*cold sore*" is the most common recurrent herpetic lesion. There is irritation at the site of recurrence followed by papules which rapidly vesiculate, become purulent, scab and heal. The commonest site is at the junction of the skin and mucous membrane of the mouth although eruptions do appear on the chin and genitalia. Primary herpetic infections of the female genitalia occur but they are rare in the male.[76,77]

Neonatal infections with herpes simplex and varicella viruses are sometimes seen. Vesicles are found on the skin and there is a stomatitis and conjunctivitis. These symptoms are followed by high fever, dyspnoea, jaundice, encephalitis and death. At post mortem there is necrosis of the liver.[78]

The Epidemiology of herpes simplex and varicella viruses is similar. Both are highly infectious and primary infection usually occurs early in childhood. With herpes simplex virus only about 10% of the cases show any clinical symptoms whereas with varicella some illness is usual.[79] Again both viruses can lie dormant in the tissues after primary infection, herpes simplex in the skin, varicella in the nervous tissue. Recurrences follow physiological or environmental changes whereupon local lesions appear without a systemic illness. The presence of antibody probably prevents dissemination of the virus and reduces the systemic symptoms in patients who are subject to recurrences.

CYTOMEGALOVIRUS infections appear to occur frequently *in utero* although infection in later life also occurs. The resulting illness has been known as cytomegalic inclusion disease because of the characteristic histological picture in infected tissues. The cells in these tissues have basophilic nuclear and cytoplasmic inclusions and may be as large as 40 μ in diameter. These cells are also present in the urine and from them the virus can be readily isolated.[80,81] Probably most of the infections are subclinical but when illness occurs it is often fatal.

In the neonate death usually follows after 2 weeks of progressive illness characterised by jaundice, petechial haemorrhages, hepatosplenomegaly, anuria and sometimes broncho-pneumonia and calcification of the brain.[81] In older babies, 3 weeks to 3 months, it takes the form of diarrhoea, vomiting, respiratory illness, hepatic and cerebral lesions which invariably result in death. When adults become infected a fatal pneumonia is common.[82]

LABORATORY DIAGNOSIS of varicella is usually made by examining smears from the vesicles and is only necessary to differentiate severe chicken pox from mild smallpox. The smears contain giant multinucleated cells with typical Cowdry type A inclusions. Herpes simplex vesicles show the same picture but this virus will grow readily on the CAM or in mice after intracerebral inoculation, whereas varicella can only be grown in tissue cultures. Serological diagnosis by antibody rise can also be performed for herpes simplex and this is most useful in cases of meningitis.

Non-human Herpesviruses

Although herpes B virus of monkeys produces a fatal encephalitis and ascending paralysis in man,[83] for the most part, the Herpesviruses infect only their natural hosts. In monkeys, B virus infection resembles that of herpes simplex in man and it would appear to be the simian variety of this virus.[84]

The viruses affecting the lower animals mainly cause respiratory illnesses of varying severity, occasionally associated with abortion.[10,85–88] The two most important are probably equine abortion and infectious laryngotracheitis of fowls. The former is usually associated with respiratory disease of horses but abortion of pregnant mares is common, with considerable damage to the foetal liver.[10] The Cytomegaloviruses of the lower animals cause a disease similar to that in man.[89]

INFECTIOUS LARYNGOTRACHEITIS affects fowls of all ages and has a morbidity rate of 50–90%. After an incubation period of 7–12 days the birds show respiratory distress with extended necks and elevated heads. Violent paroxysms of coughing occur on expiration and masses of clotted blood and mucous are expelled. On the average the mortality rate is about 20% but may range from 5 to 90%. This disease is highly infectious and chronic carriers of the virus appear to be the cause of each new outbreak.[90]

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CHAPTER EIGHTEEN

THE POXVIRUSES

THE history of smallpox, or variola, is lost in antiquity; Chinese literature records epidemics prior to 1000 B.C. It is one of the most infectious and unpleasant diseases known to man. With a mortality rate which can be as high as 50% and disfigurement for life for those who do survive, it is hardly surprising that drastic methods were adopted to achieve protection.

Early attempts to control smallpox involved the inoculation of susceptible individuals with vesicular fluid from mild cases. This variolation, as it was called, produced a modified form of smallpox with a greatly reduced mortality rate. At the same time it helped the virus to become endemic in Europe. In 1798 Jenner reported that cowpox vaccination gave as good protection as variolation and from this observation developed the first standard use of a live virus vaccine.

Smallpox virus or Poxvirus variolae is one of the largest "true" viruses and is the principal human pathogen in a large group of related viruses affecting many animal species.[1] All Poxviruses possess a common nucleoprotein antigen which is demonstrable by the complement fixation test[2] and they can be divided into sub-groups on the basis of their antigenic relationship to smallpox. The majority give rise to skin lesions, usually of the "pock" type, in their natural hosts and occasionally in other species. As a rule they do not infect more than two or three heterologous species despite their close antigenic relationship.

The Poxviruses, owing to their size, can be seen under the oil-immersion lens of the ordinary microscope. Tiny dots which are seen in stained fluid from the vesicles of smallpox patients are called elementary bodies, to distinguish them from the much larger inclusion bodies.[3] Electron microscopy reveals that these elementary bodies have an organised structure[4] unlike that of the other true viruses[5] and peptic digestion of vaccinia virus and certain bacteria suggests that the Poxviruses have a similar structure to bacterial cells.[6] It does seem likely that the Poxviruses are degenerate bacteria which have lost the ability to carry their own RNA and have therefore to rely on the host cell for their replication.

Biological Properties

The original definition of the properties of the Poxviruses was:—[1]

(1) In the hydrated state they are large ovoid bodies which, when prepared for dried films for electron microscopy, appear "brick-shaped".

(2) In size they range from 250 to 330 mμ long by 200 to 250 mμ broad.

(3) They are relatively resistant to inactivation by heat.

(4) They are very stable at −70°C but less so at −10°C.

(5) Most are resistant to ether but the Myxoma sub-group is sensitive.

(6) They are closely related antigenically within sub-groups.

(7) They produce a variety of antigens smaller than the virus particle.

Subsequent investigations have confirmed that the properties of all those Poxviruses which have been adequately studied fall approximately within these limits. Two other characters are now known. The Poxviruses possess a common nucleoprotein antigen[2] and all participate in a non-genetic reactivation phenomenon.

Despite the quantitative differences which exist between the Poxviruses, all have a complex structure and are composed of DNA, protein, phospholipid, carbohydrate, neutral fat and cholesterol.[8] Most vaccinia particles contain only double-stranded DNA, but a proportion have a single-stranded DNA molecule[9] although the significance of this is not yet known. The individual virus particles are rectangular in shape with rounded corners. They vary in size from a minimum of 150 × 220 mμ for Contagious Pustular Dermatitis virus (CPD) to a maximum of 300 × 375 mμ for Vaccinia.[5]

The virus particle is composed of an outer membrane which encloses an arrangement of electron-dense tubules. The membrane of vaccinia appears to be made up from regular round sub-units 15 mμ in diameter at the base, 10 mμ long and 6·5 mμ in diameter at the top,[10] while the arrangement of the tubules depends on the sub-group to which the virus belongs. Those of vaccinia are about 20 mμ in diameter and are wound to form a double helix 50 mμ in diameter with a central hollow core of 10 mμ. There are three of these double helices in each particle.[11] CPD and bovine papular stomatitis have the tubules wound onto the inside of the membrane.[5,12] These tubules are believed to be composed of nucleoprotein and estimates of their size vary from 3 to 20 mμ.[11,12,13,14] Virus particles form within the dense intracytoplasmic inclusion bodies which are weakly eosinophilic.[1]

The chemical composition of a strain of purified vaccinia particles is: carbon, 33·7%; nitrogen, 15·3%; phosphorus, 0·57%; copper, 0·05%; cholesterol, 1·4%; phospholipid, 2·2%; neutral fat, 2·2%; reducing sugars, 2·8%; DNA, 5·6%. In addition, biotin and flavin appear to be integral components of the virus particle. The composition of the other members of the group has not been investigated to the same extent.

All members of the group are relatively stable and can be preserved at −70°C indefinitely, although they are inactivated at varying rates at −20°C. Suspensions of vaccinia, cowpox and rabbitpox prepared from chorio-allantoic membranes (CAM) show a drop in titre of only 10 to 100-fold when heated to 55°C for 40 minutes. Some strains are

inactivated more rapidly, as are those of the Myxoma sub-group.[1,16] If the extraneous protein is removed heat inactivation proceeds more rapidly. Storage of concentrated suspensions of vaccinia virus at 4°C for one week or more results in a small fraction of the virus becoming highly resistant.[17] Freshly prepared and partially purified CAM suspensions are completely inactivated at 55°C in $2\frac{1}{2}$ hours, but after

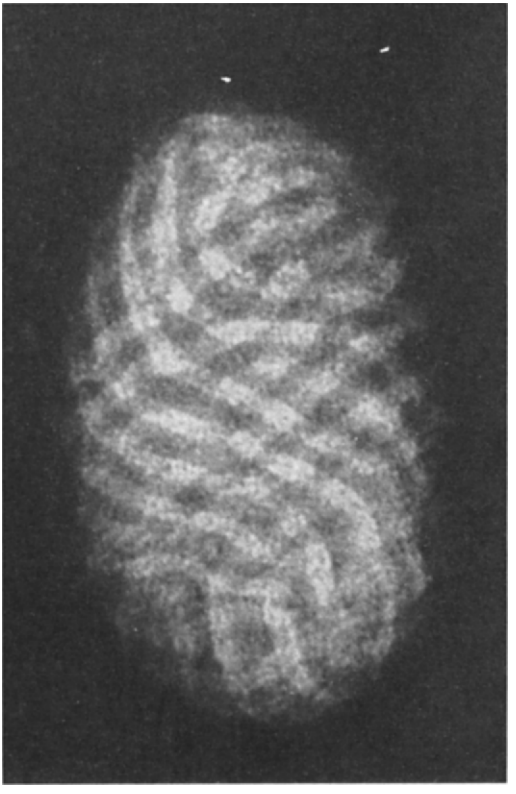

FIG. 18.1. Electron micrograph of Orf (C.P.D.) virus particle embedded in phosphotungstate showing the woven pattern of tubules. (*Photo—R. W. Horne.*) (*Courtesy Academic Press Inc., Virology*, **16**, 248; 1962.)

storage for one week at 4°C a small number of particles is resistant. This does not occur if the suspension is stored at −6°C or if it is diluted 1:100 before storage. Heat resistant virus appears rapidly on storage at 4°C reaching a constant level after 2 days. There seems to be some impurity in the suspension which mediates a physical or chemical change in a small proportion of virus particles, rendering them remarkably heat resistant.

The Poxviruses vary in their sensitivity to 20% ether. The "classical"

types, vaccinia, smallpox, cowpox, ectromelia and the fowlpox viruses are all resistant, whereas the Myxoma and Fibroma sub-group is inactivated.[18,19] Sheeppox, CPD, bovine papular stomatitis and bovine lumpy skin disease occupy a position of intermediate sensitivity. They are neither completely resistant nor completely inactivated.[20] These differences are of obvious value in classification.

In 1936 Berry and Dedrick[21] demonstrated the recovery of active myxoma virus from rabbits inoculated with a mixture of heat-inactivated myxoma and live fibroma virus. A heat-inactivated Poxvirus when mixed with another active member of the group and inoculated into susceptible cells will be reactivated and grow. This is now accepted as another property of the Poxviruses.[7,22,23,24,25] Using inactivated rabbitpox and live ectromelia inoculated into mouse brain, 1 in 600 particles of inactivated rabbitpox were reactivated. Similar results were obtained using CAM inoculation. Reactivation was also demonstrated in tissue cultures and rabbit skin. This reactivation of the inactivated virus occurs even when it is added to the cells 3 days before or 24 hours after the live virus. However, the maximum reactivation occurs when the live and inactivated viruses are added together. Reactivated virus has all the properties of the heat-inactivated virus and it is not a genetic transformation. All members of the group will reactivate inactivated vaccinia and rabbitpox to a greater or lesser extent. This does not happen with members of the Arbovirus, Myxovirus, Herpesvirus, Psittacosis or Rickettsia groups.

Heat or urea-inactivated rabbitpox and myxoma can be reactivated by a Poxvirus in which the DNA has been destroyed. It is therefore the protein component which has to be supplied and no genetic recombination is necessary for reactivation. Finally, the DNA of these inactivated viruses has been found to be protected by a protein coat. The reactivation phenomenon therefore occurs when the intact outer membrane of one member combines with the intact nucleoprotein core of another.

Soluble antigens are produced as well as the virus particle. Up to 9 distinct antigens have been found using agar-gel diffusion techniques.[26,27] Only two of these occur regularly and in large amounts. When the others appear, they do so in small amounts. None of the soluble antigens reacts with the neutralising antibody. Vaccinia virus has been studied in the greatest detail and can be used as a model, although not all the Poxviruses possess all the antigens.

The six major antigens produced by vaccinia virus-infected cells are: (1) the *LS* soluble antigen;[28] (2) the soluble haemagglutinin; (3) the particle-associated haemagglutinin; (4) the NP (nucleoprotein) antigen;[29] (5) the X antigen;[30] (6) the antigen demonstrated by the neutralisation test, which is distinct from the others.

The LS antigen is an elongated protein molecule with a molecular weight of about 240,000 and an axis ratio of approximately 30:1.[31] The

name LS was given because this protein has two distinct antigenic components, a heat labile and a heat stable fraction. It can be demonstrated by complement fixation or precipitation tests. Pure antiserum can be prepared to each of the components. The untreated antigen reacts with either antibody although only anti-S serum reacts with the antigen after heating at 70°C for 30 minutes. On the other hand, if the antigen is digested with chymotrypsin it only reacts with anti-L serum.[32] This LS antigen also forms part of the surface structure of the virus particle and antibody to either component will agglutinate infectious particles without neutralising the infectivity.

The NP antigen comprises about half the mass of the virus particle and can be extracted by treatment with dilute alkali.[29] It is stable to heating at 56°C for 30 minutes and contains about 6% DNA. It is best demonstrated by complement fixation and precipitation tests. Recently[2] the NP antigen has been shown to be a group antigen common to all the Poxviruses so far tested.

The X antigen is an integral part of the virus particle and can be demonstrated by agglutination tests. It is distinct from the LS and NP antigens and probably also from the others.[30,32]

Haemagglutination of certain chick and mouse erythrocytes is a property common to the members of the Vaccinia and Avianpox subgroups.[33,34] The haemagglutinin can be separated into a soluble, heat labile fraction and a heat stable fraction associated with the virus particle.[35,36] This separation of the haemagglutinin into two fractions has been confirmed recently by Youngner and his coworkers.[37,38] In these studies the soluble antigen was at first believed to be a normal tissue lipoprotein[37] but it has now been established that it is a virus specific antigen and is a very complex lipoprotein. The lipid fraction consists of 58% glycerides, 21% phosphatidylcholine and a variety of sterols, sterol esters and other phospholipids.

Only certain chick cells are agglutinated by the Poxviruses and are referred to as vaccinia-agglutinable erythrocytes. Although haemagglutination occurs at all temperatures above freezing point to about 52°C, the optimal temperature for vaccinia virus haemagglutinin is 40°C.[39] Some strains of vaccinia virus do not produce a haemagglutinin and passage of those strains which do, in Erhlich's ascites tumour cells results in the loss of the haemagglutinin.[40] This loss occurs by selection of non-haemagglutinating mutants, due to the presence of a haemagglutinin inhibitor in cell-free fluids from ascites tumour cells.[41]

Growth

With the exception of vaccinia virus the host range of individual Poxviruses is usually limited to a few animal species. However, the group as a whole infects a very wide range of mammalian and avian species. All members of the Vaccinia, Myxoma and Avianpox subgroups grow well on the CAM of fertile hens' eggs.[1] The viruses of

CPD, bovine papular stomatitis and paravaccinia (Milker's nodes) infect only sheep or cattle and occasionally man. They do not grow on the CAM although they can be grown satisfactorily in tissue cultures of ovine, bovine or human origin.[42–45] Those members which grow on the CAM can also be cultivated in a wide range of tissue cultures, but CAM inoculation remains the method of choice for initial isolation.

CAM inoculation followed by 2 to 3 days incubation at 35–36°C with members of the Vaccinia, Myxoma and Avianpox sub-groups results in the formation of well-defined characteristic pocks. The experienced worker can identify the virus type from the appearance of the pocks. For this reason CAM inoculation is the ideal method of isolation. When the pocks are sectioned and stained by haematoxylin and eosin the cells are seen to contain eosinophilic intracytoplasmic inclusions. The macroscopic appearance of the pocks and the microscopic appearance of the inclusions are summarised in Table 18.1.[1,16,46]

TABLE 18.1

Appearance of Pocks on CAM

Virus	Appearance of Pock	Intracytoplasmic Inclusions
Smallpox	Small, opaque, grey-white	Weakly eosinophilic, irregular
Vaccinia	Large, opaque, white or haemorrhagic ulcer	Weakly eosinophilic, irregular
Cowpox	Haemorrhagic ulcer	Strongly eosinophilic, well defined
Ectromelia	Small, opaque, white	Strongly eosinophilic
Rabbitpox	Haemorrhagic ulcer	Weakly eosinophilic, irregular
Monkeypox	Small, opaque, grey-white	Weakly eosinophilic
Fowlpox	Large, opaque, white	Strongly eosinophilic
Myxoma	Small, opaque, white	Diffuse
Fibroma	Very small	None.

Vaccinia virus produces two distinct types of pock. The large white pocks are the result of the dermotropic strains while the haemorrhagic pocks are formed by the neurotropic strains. All the types producing haemorrhagic ulcers give rise to white mutants which never revert to the haemorrhagic form and about 1 in every 100 pocks is a white mutant.

None of the other Poxviruses regularly produce pocks on the CAM although ill-defined lesions occur occasionally on primary inoculation. Continuous passage of these viruses on the CAM is unsuccessful. CPD is probably the best example of this group.[47,48]

The Growth Cycle of the Poxviruses appears to be very similar and as most work has been done on vaccinia it can once more be the model. The entire growth cycle usually takes place within the intracytoplasmic inclusions, which resemble miniature factories, set up for the multiplication of new virus. Some members do not form inclusions although they complete their growth cycle in the cytoplasm.[49]

Adsorption of virus to the cells is rapid. When the multiplicity of infection is 2 PFU per cell, 50% of the virus is adsorbed to KB cells in 30 minutes. Penetration is also rapid, 50% of the virus allowed to adsorb for 30 minutes at 22°C has entered the cell after a further 40 minutes incubation at 37°C.[50] When a much higher multiplicity of infection is used with L cells some of the virus which has adsorbed to the cells is rapidly eluted. However, a fixed fraction (about 40%) of the

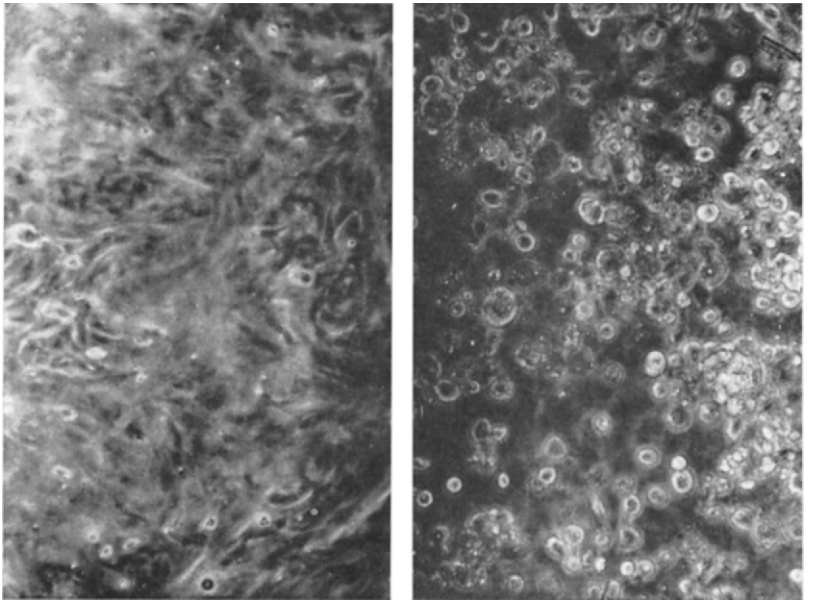

FIG. 18.2. Phase contrast photomicrograph of monkey kidney cells. *Left* an uninoculated confluent cell sheet. *Right* a similar preparation 48 hours after infection with vaccinia virus showing the highly vacuolated cytoplasm of the infected cells. (*Photo—L. E. Hewitt.*)

inoculated virus "disappears" and presumably enters the cell where it initiates viral multiplication.[51] Once inside the cell the virus is broken down to liberate the DNA and only then is viral synthesis initiated. This appears to be a property of the infectious virus particle and the host cell. By 6 hours after infection at least 50% of the virus particles have been broken down.[52]

The length of the eclipse phase varies with the multiplicity of infection. Virus antigen first appears in the cells 3 hours after infection with 20 PFU per cell and 4 to 5 hours after infection with 2 PFU per cell.[50] Kinetic studies[53] show that DNA synthesis precedes protein synthesis by about 2 hours, and has already started 2 hours after infection. There is also an early rise in RNA synthesis in the cytoplasm which is first

demonstrable by acridene orange staining 2 hours after infection of HeLa cells.[54] Still using this stain centres of DNA synthesis are first demonstrable 4 hours after infection. At the same time LS antigen can be demonstrated by immuno-fluorescence. The nucleoprotein antigen first appears at 5 to 6 hours and is produced in distinct centres, almost certainly the inclusion bodies in which the DNA is synthesised. The LS antigen, on the other hand, is formed in the remainder of the cytoplasm and is not combined with the nucleoprotein until about 8 to 10 hours after infection, when new virus first appears. At this stage marginal fragmentation of the inclusion bodies occurs. Haemagglutinin, however, does not appear until the 10th hour, i.e., only after the production of new virus.[54]

Several studies[55,56,57] have shown that the new virus particles are formed within these intracytoplasmic inclusion bodies. It would therefore seem likely that the nucleoprotein is formed within the inclusion bodies and the LS antigen is brought in from the cytoplasm to form the complete virus.

The yield of new virus from each cell may be as high as 10,000 particles which was 3% of the volume of the L cells which were used in the study.[58] The new virus is mostly retained within the infected cell until late in the growth cycle when maturation is complete.[50,59] The amount of virus produced per cell depends on the multiplicity of infection, and maximum yield is obtained when 40 particles per cell are used, dropping off to virtually nil when as many as 400 particles per cell are used.[58] In the latter case, there is a rapid and complete CPE.

The CPE produced by rabbitpox in ERK 1 cells has two stages. The first is a rounding of the cells which appears to be associated with the production of soluble antigens and the second is fusion of the cells associated with the production of new virus.[60] Since vaccinia virus only occasionally produces fusion of cells[61] it seems likely that the CPE produced by large doses of vaccinia virus[58] and that produced by U-V inactivated rabbitpox[60] are the same and are due to a mobilisation of the cytoplasm to form new viral protein.

Some of the biochemical changes associated with Poxvirus infection are now known. These are concerned principally with DNA and lipid synthesis. An increase in the enzymes synthesising DNA with an increase in the uptake of thymidine into the infected cells commences 2 to 3 hours after infection. An increased activity of DNA polymerase also occurs at this time and is raised to three times the level in uninfected cells $5\frac{1}{2}$ hours after infection.[62,63] The increase in thymidine kinase activity reaches a maximum level 8 to 9 hours after infection and remains constant for at least a further 14 hours. It therefore appears that the viral DNA initiates the production of the viral DNA synthesising enzyme systems and then inhibits their production when they have reached the required level.[64] The site of production of these enzymes has, however, not yet been established.

Initial studies on the changes in lipid metabolism induced by vaccinia virus suggest that there is an increase in synthesis of phosphatidylinositol and triglyceride at the expense of choline and ethanolamine phosphatides.[38]

The growth cycle of the Poxviruses can be summarised briefly. Adsorption of the virus particle to the cell is followed by its penetration into the cytoplasm of the cell where it is broken down to liberate the DNA. This is a property of the virus as well as the cell. The DNA then initiates the production of viral DNA and the protein of the nucleoprotein antigen within intracytoplasmic inclusion bodies. Synthesis of the LS antigen and haemagglutinin takes place in the cytoplasm, outwith the inclusion bodies. The complete virus particles are finally built up by coating the nucleoprotein tubules with an external membrane which is drawn into the inclusion body from the cytoplasm. The inclusion bodies therefore appear to be the factories for the production of viral nucleoprotein and the assembly lines for the maturation of complete virus particles. The whole process seems to take place entirely within the cytoplasm which strengthens the hypothesis that the Poxviruses are in fact degenerate bacteria.

There are two final points concerning the growth of vaccinia virus which are of interest. It appears that the virus can spread from one infected cell to the neighbouring cells during the early stages of infection without being liberated into the medium.[65] Secondly when vaccinia-infected L cells are treated with hydrocortisone they show a more severe and extensive cytopathic effect and a marked increase in the release of infectious virus when compared with untreated cells.[66]

Classification

At first sight the classification of the Poxviruses appears to be established on a very arbitrary basis. To a very great extent the sub-divisions into types are made with reference to host range and symptomatology, but antigenic distinction is of little value because within each sub-group the types share antigens to a greater or lesser extent. Each sub-group, however, is antigenically distinct with only the nucleoprotein antigen being common to the group as a whole. Other characters which are useful for dividing the viruses into sub-groups are sensitivity to ether, production of haemagglutinin and serial passage on the CAM. Using these properties four sub-groups are defined as shown in Table 18.2.

Non-Linnean binomials were applied to several members in 1957 but they have never come into common usage. The Avian Poxviruses were all grouped together as one type called Poxvirus avium. There are several of these strains which infect a variety of birds and which show some degree of cross-neutralisation, therefore the exact position of this group in uncertain but at least two types are recognised—fowlpox and canarypox.

The members of the Vaccinia and Myxoma sub-groups are virtually

TABLE 18.2
Sub-division of the Poxviruses

Sub-group	Ether Sensitivity	Haemagglutinin	Grow on CAM
Vaccinia	Resistant	+	+
CPD	Partially inactivated	−	−
Avianpox	Resistant	+	+
Myxoma	Sensitive	−	+

indistinguishable from each other antigenically and are divided on symptomatology and host range. The antigenic differences are no greater than one finds with the different strains of the Enterovirus types but their pathogenicity is distinct and only varies very slightly within each type. This stability of pathogenicity is very important and although its use in the classification of the Poxviruses places them apart from the other viruses it is a valid means of sub-division. The antigenic relationships among the members of the CPD sub-group are not as marked but some cross-reactions do occur.[20] In addition, there are three other viruses which are probably members of the Poxvirus group but not sufficient is known yet to assign them to sub-groups. The members of these three sub-groups are listed in Table 18.3.[1,20,46,67,68,69]

TABLE 18.3
Mammalian Poxviruses

Vaccinia sub-group	CPD sub-group	Myxoma sub-group
Variola major (smallpox)	Contagious Pustular Dermatitis (Orf)	Myxoma
Variola minor (Alastrim)		Rabbit fibroma
Vaccinia	Bovine Papular Stomatitis	Squirrel fibroma[68]
Cowpox	Horsepox	
Monkeypox (Von Magnus[46])	Goatpox	Miscellaneous
Ectromelia (Mousepox)	Sheeppox	Molluscum contagiosum
Rabbitpox	Swinepox	Paravaccinia[45]
	Camelpox	Monkeypox (Yaba)[69]
	Lumpy Skin Disease (Neethling[67])	

The differences between Variola major and Variola minor will be discussed in detail later and there is good reason to consider them separately. There is not yet sufficient known about the viruses of molluscum contagiosum, paravaccinia or Yaba monkeypox to state unequivocally that they are Poxviruses, but in the electron microscope they have a very similar appearance to vaccinia.[45,69,70]

Genetic recombination has been demonstrated between strains of

vaccina and rabbitpox viruses.[71,72] These two viruses are very similar and it has been suggested that rabbitpox is a recent evolutionary development from vaccinia.[34] Vaccinia virus itself would also appear to be a laboratory-bred type originating either from cowpox or smallpox virus. There seems little doubt that all the members of the Vaccinia sub-group have evolved from a common parent and that this process is continuing today, at least with laboratory-maintained viruses.

Clinical Manifestations of Poxvirus Infection

Although man can become infected with many of the animal Poxviruses there are only two specific Poxvirus infections of man. The first is smallpox, probably the most infectious, most devastating and most unpleasant virus disease yet known. The second is molluscum contagiosum which is a mild, although chronic, superficial disease of children and young adults.

Smallpox has deservedly received more attention than any other virus disease due to the high mortality rate (25%) of the "classic" form.[73,74] During the present century a milder form of smallpox called alastrim has appeared in Africa, Western Europe and America with a mortality rate of less than 1%.[75] The table below will demonstrate the importance of smallpox in the world today.

TABLE 18.4

Yearly Incidence of Smallpox[76]

Continent	1958	1959	1960	1961	1962
Africa	14,403	14,155	15,851	24,146	23,984
America	4,334	4,899	3,090	1,923	3,029
Asia	227,229	58,487	39,241	52,342	46,629
Europe	12	14	47	25	136
Total	245,978	77,555	58,230	78,430	73,778

The two forms of smallpox will now be discussed, together with their epidemiology and laboratory diagnosis before considering the other Poxvirus infections of man and animals.

Smallpox

The clinical picture of classical smallpox was first described in detail in 1908 and little has been added since that time.[77]

The incubation period varies from 6–22 days although it is usually about 12 days. The onset is marked by a toxaemic illness of about 4 days duration with fever, systemic symptoms and prostration, before the typical rash appears. During this stage it is usual to find severe

headache, backache, pains in the limbs and there is sometimes vomiting. The temperature rises to 104–105°F and there is often a toxaemic rash of the axillae, groin and flanks, which may be erythematous or petechial depending on its severity. Petechial haemorrhages occur in the most severe cases. The typical focal rash appears on the third or fourth day usually involving first the buccal and pharyngeal mucosa, the face and the forearms. It then spreads to the trunk and lower limbs but at all times it is more extensive on the face, forearms and lower legs. This centrifugal distribution of the rash is useful in the differential diagnosis between modified smallpox and chicken pox.

One or two days after the appearance of the rash the temperature falls nearly to normal and the patient feels much better. At first the rash is macular but quickly becomes papular and after 2–3 days develops into vesicles. Pustulation follows rapidly and 8–9 days after its first appearance, crusts begin to form and are complete by the 16th day of illness. The crusts have usually separated by the end of the third week. A second rise in temperature usually accompanies the pustulation but returns to normal as the lesions dry up.

Death may occur at several stages. The most severe cases die before the appearance of the focal rash, within a week of the onset of fever. There is haemorrhage of the skin and bleeding from the mouth, nose, vagina and bowel. This is called purpura variolosa or haemorrhagic smallpox and such cases invariably result in death. Haemorrhages may also occur after the rash has appeared—secondary haemorrhagic smallpox, and the mortality rate is very high—about 80%. Prognosis is better if there are no haemorrhages although the mortality rate is still about 45% if the lesions on the face and arms are confluent. The discrete type of smallpox where each lesion is separate, has a relatively low mortality rate—about 6%.[73]

The symptoms of alastrim are very similar to those of classic smallpox in the pre-eruptive stage but haemorrhages are very rare. The focal rash is more superficial and develops more rapidly so that the eruptive stage is therefore shorter and only rarely does the rash become confluent. Many cases of alastrim show no secondary fever.[78]

A detailed description of the development of the skin lesions may be found elsewhere.[75] The pitting is apparently caused by destruction of the sebaceous glands followed by organisation and subsequent shrinking of granulation tissue.[79] Typical cytoplasmic inclusions (Guarnieri bodies) are found in the epithelial cells of the affected skin and mucous membranes. Hyperaemia of the lungs, enlarged liver and spleen and focal necrosis of the testes are commonly found in fatal cases.

Treatment

Since nothing can be done for the patient efforts are principally directed to the protection of possible contacts and this is done either by vaccination or by the administration of vaccinial gamma-globulin.

When gamma-globulin from recently vaccinated persons is administered to close contacts there is a marked reduction in the incidence of smallpox.[80] Although this treatment is impracticable in endemic areas due to the scarcity of gamma-globulin, it is of value in outbreaks in non-endemic areas.

Epidemiology

The epidemiology of smallpox is naturally of profound importance. The disease can be spread from person to person in a variety of ways. During the first few days of the eruptive phase large amounts of virus are discharged into the mouth from the mucosal lesions.[81] Up to this period the patient is usually non-infectious and isolation of all cases during the pre-eruptive phase or within 2 days of the rash appearing prevents the spread of the disease by droplet infection.

Since the virus is present in the skin lesions and crusts, transmission can occur from contaminated bedding[82] and even from imported contaminated cotton.[83] The virus is extremely resistant. It can be recovered from crusts wrapped in cotton, after storage at 20–25°C for at least 17 months.[83] The smallpox patient is therefore infective from the time the rash appears until all the crusts have dropped off. This is most important in cases where the disease is modified by previous vaccination and is so mild as to resemble chicken pox.

In all cases the virus enters via the respiratory tract where it multiplies. A viraemia then develops before the virus finally becomes localised in the skin.

Laboratory Investigations

Diagnosis in typical cases of either classical smallpox or alastrim can usually be made by the clinician. In the vaccinated person or if the patient dies before the focal rash appears clinical diagnosis alone may not be sufficient. A laboratory diagnosis can be readily made and should always be attempted if a suspected case of smallpox occurs in a non-endemic area. When there is even the remotest possibility of smallpox it is better to feel safe than to feel responsible.

There are four different laboratory procedures for the diagnosis of smallpox.[85] The poorest method is the demonstration of a rise in antibody which is a "better late than never" diagnosis, because antibody does not appear until late in the eruptive stage. Vaccinia virus is used to detect the rise since it is virtually identical with smallpox virus by serology. The most usual methods are directed towards detecting the presence of virus. This can be done by isolating the virus on the CAM or by demonstrating the presence of C-F antigen, from the blood in the pre-eruptive stage and from lesions throughout the eruptive stage. The skilled worker can also diagnose smallpox in 30 minutes by direct microscopic examination of stained smears from the lesions. In this instance the typical elementary bodies of smallpox can be seen. The

detection of C-F antigen takes 24 hours and isolation of the virus takes 2 to 3 days.

Finally, differentiation can be made in the laboratory between Variola major and Variola minor. While it is obvious in a large outbreak which type is present it is impossible to tell from one or two cases. Although there are three tests which can be used, the most efficient and exact is the differential temperature test. Two series of eggs are inoculated with the virus and incubated at 35°C and 38·5°C. Variola major will grow on the CAM and produce pocks at both temperatures whereas Variola minor produces pocks only at the lower temperature.[86,87] The other two methods rely on the greater pathogenicity of smallpox for eggs[86,88] and its greater resistance to prolonged incubation on the CAM.[86,89]

Vaccination

The various strains of vaccinia virus in use today for vaccination against smallpox have been derived from cowpox virus or perhaps in some cases smallpox virus. Vaccinia virus is an artificially produced and maintained Poxvirus which has been serially passed by dermal inoculation of cattle, sheep or rabbits.

Vaccination is performed by introducing live vaccinia virus into the deeper layers of the epidermis. A variety of techniques have been used but the linear scratch method using a strong Hagedorn needle has been recommended as the most efficient.[90] Primary vaccination should be undertaken for all children during the first two years of life. There is much controversy over the ideal age but it should be at least over 3 months, probably six.

Most primary vaccinations result in a typical localised lesion which appears as a papule about the 4th day and rapidly vesiculates. The vesicle enlarges and a zone of erythema develops around it. By the 8th to 9th day the centre of the vesicle becomes depressed, the contents become turbid and there is often a slight fever and axillary adenitis. A scab begins to form about the 10th day and the vesicle dries up. About one week later the scab falls off.

Complications are relatively infrequent. Generalised vaccinia has a very low incidence[91] although post-vaccinal encephalitis varies and can be as high as 1 in 20,000.[92]

The duration of immunity is variable but in general re-vaccination is advisable every year for persons who are liable to come in close contact with smallpox, and every three years for persons living in an endemic area. Immunity to smallpox appears by the 10th day after vaccination and contacts of cases vaccinated within a few days of contact can be prevented from developing typical smallpox. In vaccinated persons the disease is usually mild and there is often no eruption at all.

Smallpox vaccination has proved to be the most efficient means of controlling the disease. Its widespread use in Europe and North

America, in conjunction with public health measures has resulted in the virtual eradication of smallpox in these areas.

Other Poxvirus Infections

With the exception of myxomatosis, ectromelia and the avian poxes all the other Poxvirus diseases are relatively mild. In man, molluscum contagiosum, cowpox, contagious pustular dermatitis or Orf and paravaccinia all produce localised but painful lesions. In animals cowpox, CPD, bovine papular stomatitis, paravaccinia, and most of the other pox diseases are mild and are only of academic interest. The Avianpoxes and myxomatosis, however, can be very destructive. These other infections of man will now be mentioned with a closer consideration of myxomatosis.

Molluscum Contagiosum

This chronic disease is characterised by the formation of multiple, white, painless nodules about 2 mm in diameter. They are limited to the epidermal layer and may appear on the face, arms, legs, buttocks, genitalia or scalp but never on the palms of the hands or the soles of the feet. There is a localised proliferation of the epidermis forming a lobulated and umbelicated papule.[70] A secondary bacterial infection of the lesions may arise but infected lesions tend to heal up more rapidly than uninfected lesions. Although typical Poxvirus particles can be seen in specimens from the lesions[70,93] the virus has not yet been successfully cultivated. Limited growth does occur in HeLa cells but so far man is the only satisfactory host.[94]

Cowpox

As the name implies this is primarily a disease of cattle but it can be transmitted to man—especially milkers. In cows the disease produces vesicular lesions on the teats and udder which are readily ruptured at milking. The milker then becomes infected, usually on the hands, but the virus can enter through any cut or abrasion. In man the disease resembles primary vaccination with local oedema, lymphangitis, lymphadenopathy and fever. Some slight differences can be detected but the virus is antigenically almost identical with vaccinia.[8,26] Reinfection can occur but again it is similar to a re-vaccination with milder symptoms and no fever. Vaccinated persons contracting the disease also have this milder illness. The lesions always remain localised and in unvaccinated persons the scabs take 4–5 weeks to separate. Vaccinia virus can be transmitted to cows and from them to man giving an identical clinical picture.[75]

Paravaccinia

Paravaccinia, pseudo-cowpox or milker's nodes are the names given to a disease similar to cowpox which involves cattle and man in

America.[95,96] In cattle the disease is virtually indistinguishable from true cowpox, but in man it is quite different. Following an incubation period of 1–4 weeks nodules develop on the hands, wrists, neck or face. The lesions are smooth and wart-like, becoming pustular and lasting for up to 2 months. There is usually a mild fever and lymphadenitis. The virus is antigenically distinct from cowpox and is readily distinguishable because it does not grow on the CAM.[45,96]

Contagious Pustular Dermatitis

Contagious pustular dermatitis or Orf is primarily a disease of sheep although it can be transmitted to man.[97] Sheep develop a vesiculopapular eruption which is usually confined to the lips and surrounding tissue. Occasionally it may also occur on the oral mucosa. When transmitted to man Orf produces a local vesiculo-papular lesion which is usually painless. A central ulcer develops which takes about two months to heal. Again the virus is unrelated to vaccinia and cannot be propagated on the CAM.[48]

Myxomatosis

Myxomatosis is a naturally occurring infection of the South American rabbit *Sylvilagous brasiliensis*. In this rabbit it is a very mild disease characterised by a localised skin lesion.[98] When introduced into the European rabbit *Oryctolagous cuniculus*, an epizootic results with a mortality rate of about 99·5%.

The initial lesion is similar to that produced in the South American rabbit but this is followed by a viraemia with widespread secondary lesions of the skin and lungs.[99] Myxoma virus was released into the rabbits of Europe and Australia in 1952 and 1950 respectively and vast numbers of rabbits died. In Australia the changing character of the virus has been followed carefully. A less virulent strain of virus has arisen and the rabbit population is more resistant.

The disease can be transmitted mechanically by mosquitoes, which remain infective for up to 17 days, or by direct contact.[100] In the early epizootics in Australia the mortality rate was 99·5% but a new and less virulent varient appeared with a mortality rate of only 90%. The mean survival time of the infected rabbits increased from 10–12 days to 23 days.[101] Similarly rabbits which came from colonies which had survived 5 epizootics were more resistant and the new virus only killed 50% of these. By the time 7 epizootics had been survived the mortality rate was down to 25%.[102]

This history of myxomatosis in Australia is very important because it demonstrates the development of a host/virus relationship which is best suited to the survival of both host and virus. The less virulent strains which appeared leave a sufficiently large population of rabbits to produce a new susceptible population in time for the next mosquito season. The virus spreads again and the increased survival time

increases the possibility of the virus being transmitted to a new host. The selected breeding from the animals which survive has resulted in the selection of a more resistant host. It would seem likely that the modified myxoma virus will ultimately produce the same mild endemic disease in the Oryctolagous rabbit as it does in the Sylvilagous rabbits.

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CHAPTER NINETEEN

UNGROUPED VIRUSES

THERE are several viruses which are not yet classified and all are associated with disease. Lymphocytic choriomenigitis (LCM) which was first associated with meningitis in man has been known for 30 years and although a great deal of work has been done on the growth of this virus in mice very little of it is of value for classification.[1,2] We know next to nothing about its biological properties and nothing at all about its growth cycle.

The other two viruses infecting man are those which cause Rubella (German measles) and hepatitis. Rubella virus has only recently been cultivated[3] while the agent or agents of serum and infectious hepatitis may or may not have been grown in the laboratory.[4] Finally there is the collection of viruses causing swine fever (hog cholera) and diarrhoeal or mucosal disease of cattle. These viruses appear to be antigenically related.[5,6] Apart from the clinical details comparatively little is known of any of these viruses and we shall only look briefly at each in turn.

Lymphocytic Choriomeningitis

Although this virus was originally isolated from a monkey and then from man there is little doubt that its natural hosts are rodents.[1,2,7] In man it causes a variety of symptoms ranging from an influenza-like illness to a fatal systemic illness, but meningitis is most common.[8] In mice it can produce persistent tolerant infections when passed on by an infected mother *in utero*, or by inoculation of newborn mice.[9,10]

The known properties of the virus can be summarised as follows:[11]

(1) It is 37–60 mμ in diameter as determined by ultrafiltration and centrifugation.

(2) It is readily inactivated by heat.

(3) It multiplies in the chick embryo following CAM and yolk sac inoculation.

(4) It produces a soluble complement-fixing antigen.

Most cases of LCM illness in man are similar to influenza or they are typical of aseptic meningitis. A more severe illness resulting in death occasionally occurs and the virus can be isolated from the CNS and lungs.

The virus has been found in naturally infected mice, guinea-pigs, monkeys and dogs but most of the experimental work has been done using mice and the outcome of infection in mice depends largely on the

age at which the animal becomes infected.[12] When mice are inoculated during the first 24 hours of life the mortality rate is very low. The exact age at which the mortality begins to rise depends more on the strain of virus than on the strain of mouse. Inoculation of 6–7-day-old mice results in a mortality of about 90% while in the intervening period many mice show an intermediate response with a large number of "runts". Mortality is also determined by the passage history of the virus.[13] Virus which has been previously passed by intracerebral inoculation gives a high mortality rate whereas the same virus passed by the intraperitoneal route results in a low mortality rate. The animals which survive infection become persistent tolerant carriers of the virus and the greater the amount of virus used as the inoculum the greater is the proportion of tolerant animals. It would be expected that the greatest number of persistent tolerant infections would be produced by intraperitoneal inoculation but in practice when the same virus suspension is used, intracerebral inoculation inexplicably gives the greatest number. As intracerebral passage does not occur in nature, most wild strains of LCM are "docile", i.e., they give rise principally to these persistent tolerant infections.

The pathological picture produced by LCM in mice suggested that the persistent tolerant infections were a form of immunological tolerance.[14] Thus congenital or neonatal infections are characterised by the persistence of the virus in the tissues, a chronic viraemia and a failure of the host to develop specific antibodies.[15] On the other hand infection of adult mice results in death, characterised by a lymphocytic infiltration of the meninges and choroid plexus or a massive pleural effusion. Adult mice are protected by X-ray irradiation and cortisone therapy which suggests that death is due not to the effects of the virus but to an immune reaction on the part of the host.[16,17] This theory has been confirmed by recent reports of the protection of mice, thymectomised at birth, from the lethal effects of LCM virus when inoculated 3 to 9 weeks later.[18,19,20] These mice develop a persistent viraemia and the recovered virus does not show an increased ability to cause persistent infections as it does when it is recovered from immunologically intact animals with persistent infections.[20]

Lymphocytic choriomeningitis virus therefore appears to be different from all other viruses which are known to infect mice. With LCM virus the actual infection is not in itself lethal but an immune reaction occurs in the immunologically competent animal resulting in its death. With such viruses as the Arboviruses and the Coxsackie sub-group of the Picornaviruses successful lethal infections can often be obtained only in new-born mice suggesting that death results from the unhindered multiplication of the virus in the immunologically immature host. Other factors almost certainly affect the age response of mice to viral infection but immunological maturity is probably the most important.

Rubella or German Measles

This is a common mild disease of childhood and is only important when it occurs in the adult female during the first trimester of pregnancy. Infection during this period results in the development of defects of the foetus in 50–80% of such cases.[21,22]

Although the causal agent was shown to be filterable over twenty years ago[23] the virus has only recently been cultivated successfully in tissue cultures.[3] Considerable progress is now being made and a reasonably comprehensive picture of its biological properties and growth can be given.

The Biological Properties of Rubella virus can be summarised as follows:[24,25,26]

(1) It is an irregularly shaped particle with an overall size of 120–280 mμ and a dense brick-shaped core.

(2) It multiplies in a wide range of tissue cultures but produces a CPE only in human amnion.

(3) It is sensitive to ether and deoxycholate.

Electron microscopic examination of concentrated and partially purified rubella virus reveals particles of irregular shape and size varying from 120–280 mμ in overall diameter with a dense rounded or brick-shaped central core of 80–220 mμ in diameter. Neither the outer envelope nor the core show any surface structure, but the outer envelope which is about 10 mμ thick is ruptured by treatment with ether. As this treatment also destroys the infectivity it is probable that the outer envelope is required for adsorption to susceptible cells and contains lipid. The internal structure of the core has not yet been determined.

The infectivity of the virus is destroyed by incubation at 5°C for 24 hours with 50% di-ethyl ether and also by treatment with deoxycholate, chloroform and other organic solvents. Inactivation occurs when the virus is heated at 56°C for 1–2 hours and at 37°C the addition of 1% bovine plasma albumin (BPA) markedly reduces the inactivation. In the presence of BPA the infectivity drops only 0·9 logs after 3 hours whereas in the absence of BPA it drops 1·7 logs. The virus remains viable for several weeks at 4°C and for a year at −50°C or less. However at −20°C the titre drops about 0·5 logs in four weeks.

So far it has not been possible to demonstrate haemagglutination or haemadsorption for any of the isolated strains nor has a complement fixing antigen been found.

The growth of rubella virus is apparently restricted to man, monkeys and tissue cultures. Transmission of tissue culture-grown virus to rhesus and grivet (African green) monkeys has been successful although only the latter developed typical rubella.[27,28]

The first description of the growth of rubella virus came from Parkman *et al*,[29,30] who used grivet monkey kidney (GMK) cells. They

noted that rubella virus-infected cells would not support the multiplication and subsequent production of the haemagglutinin of ECHO 11 virus. Rubella virus has since been shown to interfere with the growth of Sindbis; Coxsackie A9, B1 and B4; Poliovirus types 1 and 2 and SV-4 viruses although it does not affect the production of influenza A2 haemagglutinin.[25,27,28] The early passage interfering titres (InD_{50})

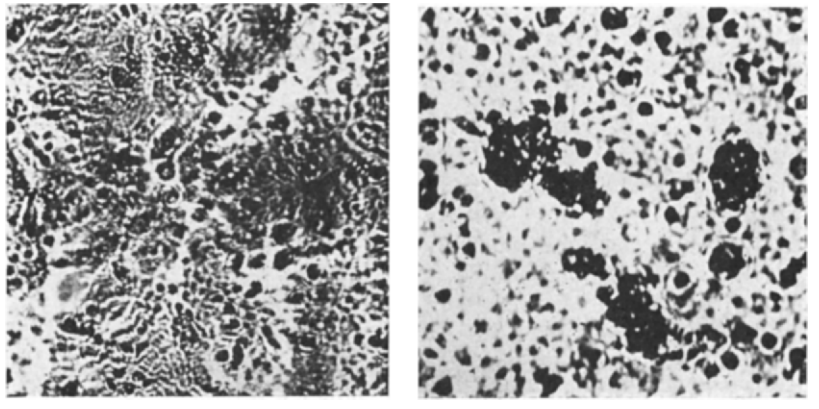

FIG. 19.1. Phase contrast micrograph of RK13 cells, *left* uninoculated cell sheet; *right* cell sheet showing CPE of Rubella virus. (*Photo—R. West.*) (*Courtesy—Glaxo Laboratories Ltd.*)

were about $10^{-4 \cdot 0}$ per 0·1 ml and were detected only after the infected cells had been incubated for 10–15 days before the addition of the ECHO 11 challenge virus.[26] In these early passages of the virus 1 InD_{50} was equivalent to 100–1000 infectious doses (ID_{50}) but after adaption of the virus to GMK cells interference could be demonstrated earlier and with a much smaller amount of infectious virus. Thus with 17th passage rubella virus 1 InD_{50} measured 5 days after inoculation was equivalent to only 10 ID_{50}. These results indicate that on passage this virus becomes adapted to GMK cells and multiplies rapidly so that a smaller dose of virus will render a culture incapable of supporting the growth of the challenge virus.

Multiplication of the virus has been reported in cultures of primary and continuous rhesus and grivet monkey kidney, human embryo kidney, primary human amnion, FL, Chang's liver and HeLa cells. A CPE can be observed only in primary human amnion cell cultures. With all the other cells viral growth can only be demonstrated by interference effects.[25,26]

In primary human amnion cell cultures the CPE can be seen 17–34 days after inoculation with early passage virus and progresses slowly for several weeks. As the virus becomes adapted initial changes are seen after 5 days and 90% destruction of the cell sheet occurs after

3–5 weeks. Infectivity titres calculated as TCD_{50} 28 days after inoculation are $10^{-2\cdot0}$–$10^{-3\cdot7}$ per 0·2 ml.[25]

At any given time only a small number of cells show cytological changes. In haematoxylin and eosin-stained preparations both nuclear and cytoplasmic changes are seen. Within the nucleus there is an aggregation of basophilic material into small, and later, large clumps while within the cytoplasm eosinophilic bodies occur, apparently in close association with the nuclear basophilic masses.

The Classification of rubella virus is not yet possible. From its known properties it does not belong to any of the defined groups. All the strains so far isolated appear to be antigenically similar if not identical and are unrelated to a wide range of ether-sensitive viruses.[26,31] There is little doubt that this virus is the cause of German measles. Isolations have been made from many cases of the illness and there is a very good correlation between clinical rubella and a rise in neutralising antibody to rubella virus.[3,25,26,31]

The Clinical Manifestation of this virus is a mild febrile illness associated with a rash and lymphadenopathy. Following an incubation period of 14–23 days there is a short prodromal period of fever, catarrh and malaise. The disease can be differentiated from measles at this stage by the absence of Koplik spots. A rash then appears on the head and face, spreads to the neck and trunk in 24 hours and is accompanied by lymphadenopathy. In the early stage the rash resembles that of measles and consists of round, pink, slightly raised macules. On rare occasions it may be papular. While this rash and fever last for only 2–3 days, the lymphadenopathy may persist for up to 3 weeks. The cervical and occipital nodes are affected most frequently and may be tender.

Complications which are rare and more common in adults than in children include arthritis, neuritis and encephalitis. Encephalitis may appear 3–4 days after the rash and with these cases the mortality rate may be as high as 20%. Otherwise a full and rapid recovery is made.[32,33]

The most important aspect of this disease is its effect on the patient who is also pregnant. Infection during the early stages of pregnancy results in a high incidence of such foetal deformities as deafness and secondary mutism, microcephaly, eye defects—especially cataracts, cardiac malformations and dental abnormalities. Varying estimates (25–80%) have been made but the greatest incidence of defects occurs as a result of infection during the first trimester.[21,22,34]

The virus is present in the urine, blood and nasopharynx of patients during the early stages of the illness and spread is probably by droplet infection.[3]

The illness is generally so mild as not to involve even bed-rest and in view of the foetal deformities which accompany infection during pregnancy every attempt should be made to bring girls into contact with the disease early in life. When a patient who is pregnant and has no

history of rubella has come in contact with the illness the administration of gamma-globulin is recommended. The efficiency of this treatment depends on the batch of gamma-globulin and even if the disease is prevented in the mother, the foetus may still become infected.[3]

Hepatitis

There are two recognised types of hepatitis—infectious hepatitis and serum hepatitis. It has been variously suggested that these two similar diseases may be due to the same or different viruses. Infectious hepatitis is an acute or sub-acute illness of children and young adults. Spread is probably by faecal contamination and it usually has a shorter incubation period (15–40 days) followed by a sudden onset. The symptoms of serum hepatitis are identical with those of infectious hepatitis but the incubation period is very long (60–160 days), the onset is insidious and transmission of the disease is by inoculation of infected blood—usually from normal individuals.[35]

Volunteer studies have resulted in the accumulation of a considerable amount of information on the properties of the virus or viruses causing hepatitis. These studies indicate that these viruses are small, ether and heat resistant and are easily preserved at temperatures below 0°C. Recently three very different groups of viruses have been isolated from cases of hepatitis and these may or may not be the cause of either or both forms of the disease.[4,36,37] We shall consider each of these in turn and review the evidence in favour of an aetiological relationship.

DA Virus

The DA strain of Simian Myxovirus SV5 was isolated from whole blood from a fatal case of infectious hepatitis.[36,38] Chicken erythrocytes were agglutinated by the virus and it was allowed to elute. These erythrocytes were then agglutinated by sera from animals immunised against the virus.[39] Similarly erythrocytes treated with RDE could be agglutinated by these sera. Two different antibodies were shown to be involved, one of which was specific for the DA virus. Antibody studies on sera from hepatitis patients demonstrated that in an outbreak of hepatitis a significant number of patients showed a rise in antibody by direct haemagglutination-inhibition tests.[40] On the other hand no such response was found in the sera from volunteers infected with material containing hepatitis virus. All the sera were able to agglutinate DA virus-treated erythrocytes. The significance of these findings is questionable because a Myxovirus is ether and heat-sensitive and does not preserve well unless kept at temperatures well below freezing point, but the DA virus contains the human blood-group substance B and this may account for some of the serological results.[41] Therefore, although this virus may cause some of the cases of hepatitis it does not appear to be the major cause of either infectious or serum hepatitis.

Hemoviruses

A group of viruses tentatively called Hemoviruses was isolated by Taylor *et al*,[4] from serum specimens collected from cases of hepatitis and normal individuals. Three serotypes have so far been identified of which two appear to cause hepatitis.

The known properties of these viruses may be summarised as follows:

(1) They are spherical or polyhedral with a diameter of 15 mμ.

(2) They are ether resistant.

(3) They survive heating at 60°C for 4–10 minutes.

(4) The nucleic acid is probably RNA.

They can be grown only in a limited range of tissue cultures in which they produce a characteristic CPE. Initial observations show that the site of multiplication is associated with the ribosomes although the mitochondria are destroyed and this cytoplasmic growth is in accordance with the suggestion that the nucleic acid is RNA.

A survey has shown that these viruses are present in the serum of 5–35% of normal people and more than 95% of people with hepatitis. These findings taken in conjunction with their biological properties suggest that the Hemoviruses are the aetiological agents of a high proportion of cases of hepatitis. The presence of the virus in serum of normal persons would explain the incidence of serum hepatitis in persons who have received blood from healthy individuals and would suggest that infectious and serum hepatitis are different manifestations of the same virus disease. Of the three groups of viruses so far associated with hepatitis the Hemoviruses have the best claim to being the causal agents.

Lipovirus

Chang[37] isolated a single strain of virus and considerable efforts have been made to identify this virus which has been provisionally named lipovirus,[42] and to associate it with infectious hepatitis.[43] The biological properties are extremely unusual.[42,44]

(1) It is very sensitive to ether and chloroform but less so to deoxycholate.

(2) The nucleic acid is probably DNA.

(3) It is resistant to treatment with proteolytic enzymes as well as deoxyribonuclease and ribonuclease.

(4) The infectivity is destroyed by freezing and thawing but it can be preserved for at least 8 months at 4°C.

(5) It is slowly inactivated by formalin.

(6) It has a complex structure and is apparently made up from aggregates of rings, 15 mμ in diameter.

Multiplication of the virus appears to take place in the nucleus where large numbers of rings or spheres 15 mμ in diameter with a central core of 10 mμ are formed. These spheres aggregate into large spongiform bodies up to 100 mμ in diameter, which then pass through the

cytoplasm to the cell surface. It has been suggested that the virus is composed of DNA contained within a surface coat, rich in lipid.

When grown in cultures of human, murine or avian cells there is a striking metabolic change in the rapid degradation of DNA and thymine. There has been a further suggestion that the degradation enzymes are not normally present in the cell and that the genetic information for their production is supplied by the viral genome.[45] Cells infected with lipovirus produce a soluble toxin which increases the total fatty acid content of cultured cells and changes the major constituent fatty acids.[46]

In early studies on the immunology of lipovirus it proved impossible to detect any antigens. Specific neutralisation of the virus by the sera from hyper-immunised animals was not successful although heat-labile inhibitors were present in many animal sera.[42] Recently it has been found that a C-F antigen can be prepared and that sheep erythrocytes treated with the virus are agglutinated by specific antisera.[47] Using these tests[43] antibody to lipovirus has been demonstrated in 59% of the sera from a sample of normal individuals. A higher incidence of antibody was found in sera from cases of infectious hepatitis in two outbreaks but not in another.

The position regarding the aetiological relationship between lipovirus and infectious hepatitis is therefore similar to that of the DA virus. It is possible that some cases of hepatitis are caused by this virus but its properties do not correspond exactly with the known properties of the hepatitis viruses. While the Hemoviruses appear to correspond very well with the properties of both infectious and serum hepatitis as obtained from volunteer studies, it is likely that some cases of infectious hepatitis are caused by the DA virus and the lipovirus. Infectious hepatitis would therefore seem to have a diverse aetiology and like aseptic meningitis may be caused by many viruses belonging to several distinct groups.

The Clinical Syndrome of infectious hepatitis may be divided into two stages—the pre-icteric and the icteric stages. The former may last for 1 to 21 days and in a large number of cases the icteric or jaundiced stage never appears. The disease is much shorter and milder in children than in adults and in the very young it may be sub-clinical.[48]

The incubation period ranges from 15–50 days and the onset of illness, which may be sudden or insidious, is marked by such symptoms as anorexia, nausea, abdominal discomfort and diarrhoea and chilliness. Cervical lymphadenopathy and splenomegaly are also common and leucopenia is a characteristic of this stage of the illness. The icteric phase may last from 1 to 10 weeks in adults and is accompanied by more severe gastro-intestinal symptoms, vomiting and enlargement of the liver. Jaundice reaches its maximum in about 2 weeks, whereupon there is an improvement in the other symptoms. A rapid and complete recovery is usually made. The mortality rate varies from 1 to 3 per

thousand but increases with the age of the patient as does the incidence of such complications as pneumonia and meningitis.[49]

At post-mortem the principal lesions are confined to the liver but other sites are also affected. Inflammation and haemorrhages of the stomach and intestinal walls and enlargement of the spleen with follicular hyperplasia occur regularly. In the liver, swelling and irregularity of shape of the hepatic cells and numerous mitotic figures are followed by destruction of the parenchyma which is uniformly and completely destroyed in fatal cases.[50,51]

The disease is probably transmitted by faecal contamination or possibly by droplet infection.

Serum Hepatitis

The clinical symptoms of serum hepatitis are identical with those of infectious hepatitis apart from a longer incubation period (60–160 days), less fever and a more insidious onset.[52] The mortality rate tends to be higher probably because the disease tends to occur in those who are already ill.

The principal difference between the two forms of hepatitis is the mode of transmission. Serum hepatitis is transmitted by the accidental inoculation of the virus directly into the blood stream, either from blood and plasma transfusions or from the use of inadequately sterilised needles and syringes.

Treatment of both forms of the disease is purely symptomatic although a high calory intake should be maintained.[53] Prophylaxis with gamma-globulin varies in efficiency and some batches which give good protection against infectious hepatitis cause serum hepatitis.[54]

Virus Mucosal-disease Complex

Recently an antigenic relationship has been shown to exist between the viruses causing hog cholera, virus diarrhoea of cattle and mucosal disease of cattle.[5] As yet little is known of these viruses except that they are filterable, are readily preserved at $-40°C$ but are inactivated at $-20°C$.[55]

Most strains can be cultivated in tissue cultures but only some produce a CPE.[6] There are several distinct immunological types which probably possess a common soluble antigen demonstrable by agar-gel diffusion tests.[5,6] It is impossible to say at this stage whether this group of agents is related to any of the known virus groups or whether it will prove to be a new and distinct group. The nomenclature is also uncertain and priority would demand that the name cholera should be incorporated in the name, but by the time more information about the properties becomes available a name descriptive of the virus itself would seem the more likely choice.

Hog Cholera or Swine Fever has been known as a disease of swine for many years. As early as 1903[56] the causal agent was known to be a

filterable virus probably transmitted by ingestion of material contaminated with infected urine.

The pathological picture of hog cholera is one of haemorrhages of the pleura, peritoneum, intestinal mucosa, larynx, skin and subcutaneous tissue. The disease is often fatal and during epidemics mortality may be very high.

Virus Diarrhoea of Cattle was first reported in 1946 in the U.S.A. and has since been recognised in many parts of the world.[55,57] Rabbits, sheep and young swine have all been infected artificially but the natural disease appears to occur only in cattle.

The principal symptoms are fever (often diphasic), nasal discharge, cough, diarrhoea, dehydration, lymphadenopathy, leucopenia and erosions of the mucous membranes of the alimentary canal.[55,58] The disease occurs in varying forms and three have been defined. In the acute form lesions may be found on the mucous membranes of the oral mucosa, muzzle and vulva while lameness and abortion have been recorded. The mortality rate is about 10% and virtually the whole herd becomes infected. The other two are a chronic form which affects about 10–20% of the cattle and has a mortality rate of 20–50% and a mild form with a low mortality rate.

Mucosal Disease of Cattle was first observed in 1951[59] and is characterised by fever, anorexia, a mucoid nasal discharge, haemorrhagic diarrhoea and dehydration. Erosions and ulcerations of the nostrils, muzzle, gums, lips, tongue and oral cavity are striking features of the disease. The morbidity is as high as 60% with a mortality rate of about 40%.[60,61] Once again three forms are recognised—acute, mild and chronic.[62] This disease can also be transmitted to sheep and goats.[61]

Recently evidence has shown that this disease and virus diarrhoea of cattle are one and the same, probably representing opposite extremes of a variable clinical picture which has one common symptom—diarrhoea. It is therefore suggested that this complex should be called virus diarrhoea of cattle.[63]

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CHAPTER TWENTY

TUMOUR VIRUSES

IT is many years since the first virus-induced tumour was recognised. In 1908 Ellermann and Bang[1,2] demonstrated the viral aetiology of certain fowl leukaemias and in 1911 Rous[3] showed that a solid tumour, fowl sarcoma, was also caused by a virus. Discoveries of tumour viruses of vertebrates have continued since that time and today there are many viruses which are known to cause a wide variety of neoplastic diseases in fowls and rodents.[4,5,6] So far no human cancer has yet been aetiologically related to any virus although two human adenoviruses (types 12 and 18) have been found to produce tumours in hamsters and mice.[7,8]

The mechanism of tumour induction by viruses is not fully understood but it appears that in some cases at least, the virus is incorporated into the genetic make-up of the host cell, transforming it into a malignant cell. Several hypotheses regarding the origin of human cancers have been advanced but we shall only consider those which suggest a viral aetiology. Burnet[9] considers that from the age incidence of the human cancers, only leukaemia is caused by a virus. Andrewes[5] on the other hand says "It would seem unlikely that cancer should have a different cause in different areas of the animal kingdom." The evidence of a viral aetiology of cancer in birds and rodents is much too strong for us to seriously consider Burnet's positive denial any further. While we are in no position to definitely state that all cancers are ultimately due to a virus we are in an even poorer position to adopt the opposite point of view. Zilber[10] presents some evidence which supports his theory that a non-viral carcinogen acts as a stimulant on a host-cell/virus symbiotic state and converts it into a malignant cell by the incorporation of the nucleic acid of the virus into the genome of the cell and we shall discuss this hypothesis in greater detail after we have considered the types of virus which cause tumours.

The known tumour-inducing viruses do not belong to a single homogenous group nor do they even possess the same nucleic acid. It is possible in our present state of knowledge to assign at last some of these to three major groups. The Adenoviruses have already been discussed and the remaining DNA viruses belong to the Papovavirus group.[11] The RNA viruses are in many ways similar to the Myxoviruses[12] and are probably non-haemagglutinating members of this group. There is therefore no single "Group of Tumour Viruses" and it would seem quite possible that tumour induction is a property of some members of

all the virus groups. However before any discussion of tumour formation by any of these viruses the Papovaviruses will be described.

The Papovaviruses

This group was defined in 1962 by Melnick[11] to include SV_{40}, polyoma and the papilloma or wart viruses. Most of these have been known for some time although SV_{40} (simian vacuolating virus) has only recently

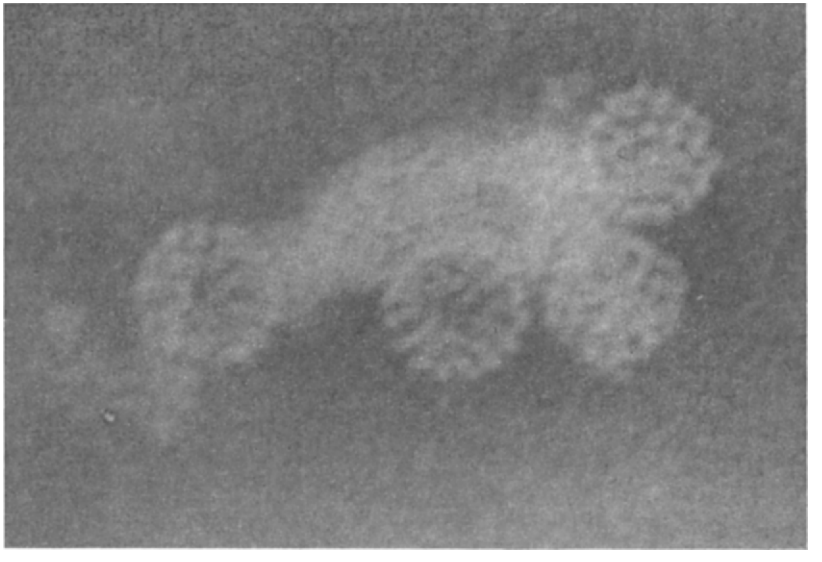

FIG. 20.1. Electron micrograph of a clump of polyoma virus particles embedded in phosphotungstate. (*Photo—R. W. Horne.*) (*Courtesy—Academic Press Inc., Advances in Virus Research*, **10**, 126; 1963.)

been proved to be oncogenic.[13] The original papilloma virus of rabbits was discovered in 1933 by Shope[14] and three years later it was shown that the viruses producing warts in man and dogs were similar although antigenically distinct.[15] Polyoma virus and SV_{40} are also antigenically distinct and the name Papovavirus was chosen to denote papilloma (PA), polyoma (PO) and vacuolating (VA) viruses.

Biological Properties

The biological properties of the Papovaviruses were defined as:

(1) They are icosahedral viruses with a diameter of 40–50 mμ and a capsid of 42 capsomeres.

(2) Filamentous forms with a cross-section the same diameter as the spherical forms are present.

(3) The nucleic acid is DNA and is double-stranded.

(4) They are ether resistant.

(5) They are relatively stable to heating in water at 50°C but are rapidly inactivated in the presence of $MgCl_2$.

In purified suspensions of Shope papilloma, polyoma and SV_{40} viruses two different types of particle are found.[16,17,18] One particle is the complete virus particle composed of DNA and protein while the other is the non-infectious empty capsid devoid of DNA.[19] The size of the complete particle as determined by negative staining varies from 42–55 mμ according to the virus. The smallest is K virus with a diameter of 42 mμ,[20] polyoma and SV_{40} have a diameter of 45 mμ[19,21] while the papilloma viruses (Shope, bovine, canine and human) are 54–55 mμ.[21,22] Most groups of workers agree that all these viruses have 42 capsomeres in the capsid[11,20,23] but one group has suggested that SV_{40}, polyoma and K viruses have 92 or possibly 72.[24] However 42 is the most likely number to be correct.[20] The size of the individual capsomeres is about 8–9 mμ in diameter and it has been suggested that 12 of these are made up from 5 protein sub-units and the other 30 from 6 sub-units with each sub-unit of a molecular weight of 160,000.[16,18,24] In addition to these icosahedral particles filamentous forms can also be seen in preparations of most, if not all, of these viruses.[11,16,17,25]

Infectious DNA has been extracted from polyoma virus-infected tissue cultures indicating that the DNA core is the genetic messenger.[26,27] The particle is composed entirely of protein and DNA and estimates of the proportion of DNA range from 7 to 13·4%.[18,24,28] The molecular weight of the DNA of those viruses which have been tested falls within the range 4–7·5 $\times$ 10^6.[17,18,29] The DNA is double-stranded and appears to occur naturally in the form of linear and cyclic double-stranded helices of equal molecular weight.[30,31] The base composition of the DNA of five Papovaviruses has been determined and the guanine + cytosine value varies from 41% in human papilloma to 48% in polyoma viruses.[21,32]

These viruses are all remarkably stable to heating.[11,33] The infectivity of polyoma virus is only partially destroyed by heating at 60°C for 60 minutes or 70°C for 30 minutes. At 4°C infectivity is preserved for at least 24 weeks and at −20°C it can be preserved indefinitely. All members of the group are resistant to ether and desiccation but they are inactivated by formaldehyde. Unlike the Enterovirus sub-group of the Picornaviruses the addition of high concentrations of $MgCl_2$ markedly increases the thermal inactivation of at least two of these viruses, namely polyoma and SV_{40}.

The antigens of the Papovavirus group appear to be confined to the virus particles. Polyoma and K viruses agglutinate the erythrocytes from several animals while polyoma virus can be detected by C-F tests.[33,34,35] The haemagglutinin of polyoma virus is entirely associated with the capsid of the virus and is present in both complete and coreless particles.[19,36] So far K virus has been found to agglutinate only sheep

erythrocytes but polyoma virus agglutinates many others including guinea pig, human group 0, dog, rat, mice and chicken erythrocytes.[37] Agglutination occurs at 4°C for both viruses but at 37°C only K virus haemagglutinates. Polyoma virus is readily eluted on heating to 37°C and some strains apparently have lost the ability to haemagglutinate. However when suspensions of such strains are treated with RDE at

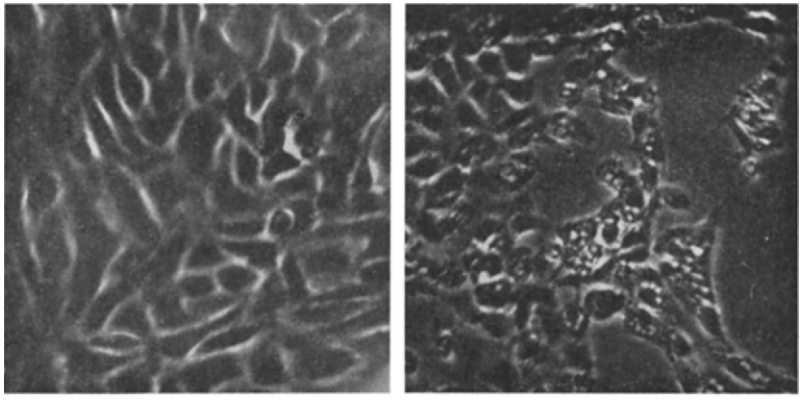

FIG. 20.2. Phase contrast micrograph of primary grey monkey kidney cells, *left* normal cell sheet; *right* cell sheet showing CPE of SV_{40} virus. (*Photo—R. West.*) (*Courtesy—Glaxo Laboratories Ltd.*)

22°C, heated to 37°C for 2 hours or 56°C for 30 minutes the haemagglutinin is demonstrable.[33,34] Similar treatment of poorly haemagglutinating suspensions of K virus also enhances its activity.[35] By HI and neutralisation tests all the Papovaviruses are antigenically distinct. As one would expect minor antigenic differences occur between different strains of the same type.[38]

Growth

With the exception of K virus, all the recognised members of the Papovavirus group produce tumours in at least one species of host. In the case of SV_{40} virus, tumour induction in monkeys, the natural host species, has not yet been successful but all the rest give rise to warts or malignant neoplasms in their natural hosts. On the other hand K virus has not yet shown itself to be oncogenic and in young mice it produces an unusual type of pneumonitis.[39]

Infection of tissue cultures with a Papovavirus results either in the transformation of some of the cells into tumour cells or a productive lytic growth cycle. In some cases only transformation has so far been accomplished[40] but investigations using biopsy material have shown that the wart viruses have a very similar if not identical growth cycle to polyoma, SV_{40} and K viruses. All grow relatively slowly with an eclipse

phase of about 24 hours before new virus can be detected. The typical lytic growth cycle is similar to that of the Adenoviruses in that it occurs entirely within the nucleus with the production of Feulgen-positive inclusions.[41]

Fifty per cent adsorption of polyoma virus to mouse embryo cells occurs in 30 minutes and is followed by an eclipse phase of 20 hours before viral antigen can be detected in the nuclei. At the 22nd hour new infectious virus can be detected within the cell and at 24 hours some is liberated into the medium. Most of the virus produced remains cell-associated and may be as much as 800–1000 PFU per cell.[42] Phase contrast microscopy of polyoma virus-infected mouse embryo cells has shown that two distinct types of inclusions occur in the nucleus.[43]

The first observable change is an increase in the size of the nucleoli and the appearance of dense bodies of varying size and shape in the nucleus. These bodies become more dense and the cytoplasm more granular until the cell dies, following retraction of the cytoplasm. This sequence takes about 3 hours from the first signs to the death of the cell and in the system which was used in these experiments was usually seen 3 to 4 days after infection of the cultures. The other type of inclusion which has been observed first appears as a darkening of the nucleoplasm which develops into a network with margination of the chromatin. Again cell death follows shrinking of the cytoplasm but the sequence is longer—between 5 and 13 hours.

Studies with SV_{40} and polyoma viruses, using fluorescent antibody and acridene orange staining have confirmed that synthesis of both DNA and protein occurs in the nucleus.[44,45,46] Electron microscopy of polyoma, rabbit papilloma, SV_{40} and K viruses also confirms that virus particles first appear in the nucleus and that the nucleolus is associated with the initial stages of viral development.[11,41,47]

Transformation of cells "in vitro" into "tumour" cells which have lost the property of contact-inhibition has been recorded for several members of this group but as this property is shared with other tumour viruses we shall leave its discussion until later.

Classification

The Papovaviruses fall naturally into two sub-groups on the basis of particle size.[21] The smaller sub-group is composed of polyoma, SV_{40} and K viruses with diameters of 40–45 mμ. Melnick[11] has called polyoma virus Papovavirus type 3 and SV_{40} Papovavirus type 4.

The second sub-group contains the Papilloma viruses which number not less than ten. Melnick called the Shope rabbit papilloma virus Papovavirus type 1 and human wart virus type 2. Bovine and canine papilloma viruses have been shown to have identical morphology.[21] Infectious papillomas are also to be found in horses, goats, and monkeys.[48] There is a second antigenic type of papilloma virus causing

oral as distinct to cutaneous warts and is associated with dogs and rabbits, while there are at least two types infecting man.

Andrewes[12] has suggested that the Rat virus of Kilham[49] may be a Papovavirus. This virus is one member of a group of viruses variously isolated from rats and human tumour tissues.[50] To date two antigenic types have been identified although their relationship to the tumours from which they were isolated is unknown. They have many properties similar to the Papovaviruses—intranuclear growth, DNA as the nucleic acid and a similar haemagglutinin but they are very much smaller. Electron microscopy shows them to be about 13–16 mμ in diameter.[41,51] It is possible that these viruses might be considered members of a third sub-group of Papovaviruses but it would seem more reasonable to create a new group for them.

Tumours Induced by Viruses

Most of the known tumour viruses have been found in mice and chickens. Beard has stated:[52] "There is good evidence to indicate that the great proportion of the growths in the chicken are of viral aetiology and that indeed, with the exception of an insignificant minority, most of the pathologic processes observed in nature can be induced directly under proper conditions of virus transmission in the laboratory or may occur in association with the neoplasms thus transmitted." Similarly Huebner has said:[53] "Present evidence suggests that virtually all leukaemias observed in these two different classes of animals (chickens and mice) may be caused by viruses." It is now virtually certain that all varieties of leukaemia occurring in mice and chickens are caused by viruses.

The leukaemia viruses are all similar in morphology and resemble the Myxoviruses in their growth cycle. Virus particles are always associated with the tumour cells in leukaemia and often large numbers of particles are produced by actively growing cells. Particles similar to those seen in chicken and murine leukaemias can also be observed in purified preparations from human and bovine leukaemias.[53] Recently a virus similar to these has been isolated from cases of human leukaemia although an aetiological relationship has still to be proved.[54,55]

Antigenically and morphologically related to the chicken leukaemia viruses are the fowl lymphosarcoma viruses such as Rous sarcoma virus.

The host range of all these viruses is not restricted to the species from which they were originally isolated. The Gross, Maloney, Graffi and Friend leukaemia viruses of mice will also produce leukaemias in rats while several strains of Rous sarcoma virus produce tumours in rats, rabbits and monkeys.[53,56–63] In most tumours in the natural host infectious virus may be demonstrated easily in the cells although this is more difficult when tumours of the heterologous hosts are tested. According to Rubin[64] the RNA of Rous sarcoma virus is not associated with the genome of the infected cell and therefore it is probable that the

continued presence of the virus itself is necessary for continued tumour formation. If this should prove to be the case then the leukaemias, sarcomas and other chicken tumours will fall into a group quite distinct from the tumours induced by the Papova- and Adenoviruses.

It is only within the last two years that the carcinogenic properties of human Adenovirus types 12 and 18 have been discovered. These two types produce sarcomas locally after intraperitoneal, subcutaneous, intracerebral and intrapulmonary injection of newborn hamsters;[65,66] after intraperitoneal injection of newborn rats[8] and after intrapulmonary injection of newborn mice.[7] Efforts to detect infectious virus in these tumours have been unsuccessful but specific viral antigens are present and specific antibodies are produced in the tumourous animal. These antigens can be detected by the complement-fixation test and appear to be similar to the type-specific nucleoprotein soluble antigen "C". This is perhaps surprising since the protein component of this antigen of types 1, 2, 4, 5 and 6 is part of the haemagglutinin[67] and Adenovirus types 12 and 18 are the only two human types which do not haemagglutinate. Whether there is any connection between tumour-formation and non-haemagglutination has still to be discovered. Finally, when high levels of C-F antibody are present in tumourous hamsters, neutralising antibodies can also be detected.

High-titred Adenovirus type 12 produces visible tumours in hamsters as early as 30 days after inoculation; in mice after 155 days and in rats after 130 to 145 days. Type 18 virus has proved to be somewhat slower and requires at least 60 days to produce visible tumours in hamsters. The titre of C-F antibody present is related to the size of the tumour in each animal and not the length of time after inoculation. Thus the larger the tumour the higher is the antibody titre which continues to rise as the tumour enlarges.

The other major group of tumour viruses is that of the Papovaviruses which may be divided into two sub-groups—the papilloma or wart viruses and the genuine tumour-viruses. The papilloma viruses produce a benign neoplastic disease of the skin, warts,[48] which only occasionally progress to a carcinoma[68] whereas polyoma and SV_{40} viruses produce widespread and rapidly fatal tumours in mice and hamsters. The pattern of viral development in the warts is very interesting. It is impossible to demonstrate the presence of virus antigen, infectious virus or virus-like particles in the germinal or basal cells of the wart where one would expect the virus to be exerting its oncogenic effect.[48,69,70] Fully infectious, antigenic virus particles are present however in the keratinising epithelium. It has therefore been suggested[69] that in the germinal cells the virus is present as the DNA and mature virus is only formed in the keratinising epithelial cells. This hypothesis has received a measure of confirmation by the isolation of infectious DNA, capable of producing warts, from papilloma tissue of domestic rabbits in which complete virus could not be demonstrated.[71] It is therefore quite

possible that some at least of the papilloma viruses may under favourable circumstances induce malignant neoplasms similar to those induced by SV_{40} and polyoma viruses.

The tumours induced by polyoma virus are numerous, diverse and occur in a wide range of rodent and non-rodent hosts. This virus is a naturally occurring virus of mice but only rarely does it appear to produce tumours under natural conditions.[53] When inoculated into newborn mice, hamsters and rats rapidly fatal tumours of various types are produced.[72] In mice the most diverse forms are found and appear as early as 6 weeks to as late as 1 year after inoculation. All strains of mice which have been tested so far are susceptible with a tumour incidence of 80–100% of inoculated mice.[72,73] Parotid tumours occur most frequently but other sites include salivary and mucous glands, the kidney, thymus, mammary glands, hair follicles and bone.

Tumours may be produced in hamsters as late as 24 days after birth but the best results are obtained with newborn animals.[72,74,75] Renal sarcomas are the most common tumours of hamsters and appear within 7 days of inoculation. Other tissues are affected later but the range is much smaller than with mice. Again in rats these renal sarcomas are the most common and about 30% of newborn rats inoculated with polyoma virus produce these tumours.[76] Two other species in which polyoma virus produces tumours of newborn animals are rabbits and ferrets.[72,74,77] In rabbits at least, these tumours are not malignant and regress after 1–4 months.

Polyoma virus can be readily isolated from primary tumours in the different hosts but tumours originally induced by polyoma virus and subsequently transplanted into fresh animals frequently do not contain virus.[78,79] Even in primary tumours the number of cells containing viral antigen detectable by fluorescent antibody is relatively small.[80]

A new antigen specific for polyoma virus-induced tumour cells can be demonstrated in all these tumours whether infectious virus or viral antigen are present or not.[81,82] This antigen appears to be specific for the tumour cells and is not a viral antigen like those produced by Adenovirus-induced tumour cells.

The last of the oncogenic Papovaviruses is SV_{40}. This is a naturally occurring virus of various monkeys, in particular rhesus and cynomolgus monkeys.[83] When inoculated into newborn hamsters a variety of sarcomas are produced with the first appearing about 100 days after inoculation.[84,85] Although virus can be isolated readily from these tumours antiviral antibodies are only rarely produced in the affected animals.[84] However, antibodies are produced against antigens in the tumour cells which, although not apparently found in SV_{40} virus, are specific for SV_{40} virus-induced tumours.[8] This is therefore similar to polyoma virus but the SV_{40} antigen is much more readily demonstrable. So far SV_{40} has not proved to be carcinogenic in any species of monkey.

Before discussing the "in vitro" transformation of cells, there are

several viruses which have been isolated from human tumours which should be mentioned, as should the position of the Poxviruses. Within the Poxvirus group there are several viruses which produce benign tumours of the skin in different animal species. In man there is molluscum contagiosum, in rabbits myxoma and fibroma and in monkeys there is the "Yaba" monkeypox virus. This latter is a most interesting virus.[86] When inoculated intradermally or subcutaneously into man or monkeys it produces benign local histiocytomas which subsequently regress, but intravenous inoculation of rhesus monkeys produces widespread tumours of a variety of tissues and occasionally results in the death of the monkey. It therefore appears most likely that the Poxviruses should be added to the ever increasing list of tumour viruses.

We have already mentioned the isolation of viruses from cases of human leukaemia.[54,55] The relationship of these viruses, which all appear to be antigenically identical, to leukaemia has still to be established but the initial reports do suggest that the search for the cause of leukaemia is beginning to bear fruit. Secondly there are those viruses related to the rat virus of Kilham which have been isolated from human tumour tissue.[50] They appear to be present quite frequently in neoplastic tissue but whether they are aetiologically related to the tumours or whether they simply have a preference for tumour tissue has still to be determined. The latest series of observations have been made on a rapidly fatal tumour syndrome known as Burkitt's tumour.[87] This tumour occurs over large areas of tropical Africa and so far two identified and one unidentified group of viruses has been isolated from biopsy material. Herpes simplex has been demonstrated several times[88,89,90] but there is no evidence to suggest that this is anything other than the isolation of a common latent virus. Another group of workers have isolated six strains of a virus which may be related to the syndrome but the virus has still to be identified.[91] Finally a single strain of Reovirus type 3 has been isolated and a preliminary serological survey shows a significantly higher incidence of antibody to Reovirus type 3 in lymphoma patients than in normal children and those suffering from other forms of cancer.[92]

Transformation of Cells

Many of the tumour viruses are capable of transforming cells in culture. It is difficult to decide with any certainty whether such a transformation is malignant or not and the only certain method is to demonstrate that they will produce progressively growing tumours in animals. Nevertheless a fairly accurate "in vitro" test for malignancy is the loss of contact inhibition by tumour cells.[93]

The best examples of transformations are those induced by Rous sarcoma virus and the Papovaviruses. Rous sarcoma virus produces several morphological types of transformed chick embryo and turkey fibroblasts depending on the environmental conditions.[94,95] When

low multiplicities of infection are used with chick embryo fibroblasts clones of malignant cells are formed which do not produce infectious virus.[96] This result is very similar to those obtained for the Papovaviruses.

Although transformation of bovine cells by bovine papilloma virus as recently been reported[40] there is no evidence of malignancy in the

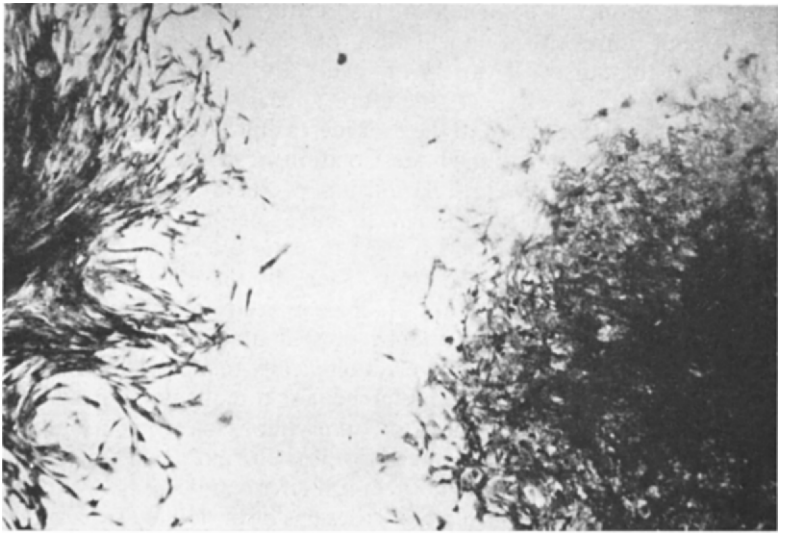

FIG. 20.3. Photograph showing *left* a colony of normal hamster cells, and *right* a colony of hamster cells transformed by polyoma virus. (M. G. P. Stoker (1963) *Cold Spring Harbour Symposium on Quant. Biol.*, **27**, 377.)

resulting cells. However, genuine malignant transformation of hamster cells by polyoma and SV_{40} viruses occurs readily while the former virus will also transform mouse cells and the latter human cells.[93,97,98] Much work has been done on the polyoma virus transformation of hamster cells and a little is now known of the mechanism.

Only a relatively small proportion of the infected cells are transformed by polyoma virus (0·15–0·6%) and a variety of morphological types are produced. It seems likely that the rate of transformation and the morphology of the cells are a function of selective physical factors rather than the actual virus/host-cell interaction.[99–102] Although transformation can occur immediately after infection of the cell, it may be delayed for several generations.[103] Fluorescent antibody studies have suggested a possible explanation for this delay.[104] Hamster cells infected with polyoma virus rapidly adsorb the virus which can then be detected in the cytoplasm. As the cells divide the virus may remain in the cytoplasm of the daughter cells for several generations. Finally,

the cells transformed "in vitro" are similar to the cells of induced tumours in that they possess virus-specific antigens.[105] It appears from these studies that after the absorption of the virus into the cell some environmental factor is required for the nucleic acid of the virus to become incorporated into the genome of the cell thus rendering it malignant.

The Aetiology of Cancer

There is little doubt that the aetiology of cancer is highly complex and requires the correct combination of a number of physical and biological conditions. Under laboratory conditions a wide range of chemical substances have tumourogenic properties while X-rays and the various nuclear radiations are known carcinogens. Other factors which may be involved in tumour induction are chronic inflammation, hormonal defects and genetic characteristics. However, the virus-induced tumours are the only naturally occurring neoplasms of known and specific aetiology.[6] The chicken tumours appear to be virtually all caused by viruses and their epizootiology is similar to that of other infectious diseases. Indeed the incidence of lymphomatosis has increased with the overcrowding which has resulted from intensification of poultry husbandry.[52] As for the mammals, there seems little doubt that leukaemia is caused by a virus—possibly one or more serotypes specific for each species of animal. Even the most sceptical workers are agreed on a viral aetiology of leukaemia but with the solid tumours of later life differences of opinion arise. At one end of the scale are those who say that a virus cannot be the cause of cancer in man because the age incidence is wrong[9] while at the other are those who claim that the ultimate cause of all cancers are viruses.[10]

On considering the conclusion that human cancer cannot be caused by a virus it becomes obvious that this is based on the assumption that the sequence of events is: viral infection–immediate cell transformation —tumour growth. If this were the case then one would naturally expect a higher incidence of tumours in the younger age-group than there is. Therefore on this hypothesis human cancers cannot be caused by viruses. The supporters of this theory have forgotten or prefer to ignore the latency of many viruses which can lie dormant in the tissue for months or years. The best known examples are the Adenoviruses, the Cytomegaloviruses and the classical Herpesviruses. In the case of the latter group, herpes simplex and varicella viruses are both able to remain dormant for years until a change in environmental conditions causes the cold sores of herpes simplex or the shingles of varicella virus to erupt. The Adenoviruses present a different problem—two human serotypes are known to cause tumours in several species of rodents and although they do not belong to the latent sub-group it is possible that they are not the only Adenoviruses which are carcinogenic. It is therefore natural to consider the Adenoviruses at least, as a potential

cause of some of the mammalian cancers. How many of the other viruses can remain dormant is not yet known but it is not impossible that just as latent herpes simplex and varicella viruses respond to environmental change by producing skin eruptions other viruses may respond by causing a cell transformation.

It is on this assumption that the opposite theory is based and the sequence of events thus envisaged is: viral infection—symbiotic state of virus and host cell—environmental change resulting in transformation of the host cell—tumour growth. The length of time which may elapse before a suitable environmental change occurs could easily account for the age incidence of human cancer. This change may take the form of a dose of ionising radiation, the ingestion of a carcinogenic chemical or a change in hormone production. The former are known to produce tumours while steroid hormones are known to stimulate latent rabies virus. This theory of Zilber maintains that a cell can only become malignant when viral nucleic acid is incorporated into the genome of the cell. That this does occur is easily seen from the presence of specific antigens in virus-free tumours induced by Adenoviruses, Papovaviruses and Rous sarcoma virus.[8] The final piece of evidence in support of this theory is the discovery that the "in vitro" transformation of hamster cells by polyoma virus does not necessarily occur at once but may be delayed for several cell divisions.[103]

However it seems possible that a cell may become malignant in the way that Zilber suggests or equally well by the loss of a portion of the genome. In the latter case a chemical or physical carcinogen could act by breaking off a section (a gene perhaps) of the host cell chromosome thus rendering it malignant. The portion which has been detached may then of itself become a virus capable of infecting new cells and even subsequently transforming them. At any rate there seems to be little doubt that it is only a question of time before at least some types of human cancer are aetiologically related to viruses with a combination of hereditary characteristics and environmental factors combining to produce a tumour.

Before leaving this fascinating question a brief mention of the problems confronting cancer research should be made. There are two main difficulties in these investigations. Firstly it is obvious that most virus-induced tumours which have reached a sufficient size to be noticed are most unlikely to contain infectious virus. That an occasional isolation may be made is possible but by and large a mature tumour will probably be a poor source of virus. This difficulty is to some extent mitigated by the discovery that in most if not all virus-induced tumours specific antigens are present which in some cases are the same as the soluble antigens produced by the virus itself. The second difficulty arises from the investigations on chemical and physical carcinogens in laboratory animals. It is impossible to know whether the tumour is due to the carcinogen alone or to the carcinogen stimulating an unsuspected

virus. The road to the discovery of the aetiology of cancer is liberally sprinkled with obstacles, by no means the least being man's unwillingness to believe that cancer may be caused by viruses although there is every reason to suspect that all viruses may be potential carcinogens under the right conditions.

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CHAPTER TWENTY-ONE

BACTERIOPHAGE

ALTHOUGH there are many bacteriophages infecting a wide range of bacteria the most fully studied are the three strains called the T-even coliphages. These strains T2, T4 and T6 infect *Escherichia coli*, are closely related and have an unusual form of double-stranded DNA. The cytosine is replaced by 5-hydroxymethyl-cytosine.[1] Consequently these phages are useful in biochemical studies because their DNA is readily differentiated from that of the host cell. The T-even coliphages have the classical "tadpole" shape which was once considered to be universal among the bacterial viruses. Icosahedral phages have now been discovered, some of which contain single-stranded DNA (S13 and ϕX 174) while others contain RNA (f2)[2,3,4] and at least two rod-shaped DNA phages (f1 and fd) are also known.[5]

The host range of each individual phage is variable—some are highly specific while others can infect a relatively wide range of bacteria. The specificity of a phage for a bacterial cell is usually much more exact than detectable antigenic differences between the cells. This specificity has been applied with great success in the epidemiology of *Staphylococcus aureus* by typing the organisms on the basis of their ability to support the growth of a range of phages.

Differentiation between phages can also be made by their life cycles. Infection of a cell with a virulent phage always results in the replication of new virus particles with the accompanying death of the cell in much the same manner as the animal viruses. The so-called temperate phages however do not always produce this "lytic" growth cycle but often exhibit lysogeny.[6] Here the phage DNA becomes integrated with the host cell chromosome and replicates as a part of the genetic make-up of the host cell. Only occasionally does one of the daughter cells produce a lytic infection with subsequent production of new phages and cell death. Lysogeny has been compared with the cell transformation described in the previous chapter but although similarities do exist it is not yet known whether the transformation of vertebrate cells is equivalent to lysogeny of bacterial cells.

It is not possible in the space of this chapter to discuss in detail the various structures and growth cycles of the bacteriophages but we shall look at the structure of the tadpole-shaped phages, outline the lytic growth cycle and lysogeny and consider the phenomenon of transduction and the practical applications of phage-typing. For those who

wish to study this branch of Virology further there are many recent reviews which cover all aspects of the bacteriophages.[3,6–14]

Structure

The physical and chemical composition of the classical tadpole-shaped bacteriophage particle is remarkably complex.[3,12] A diagram of the

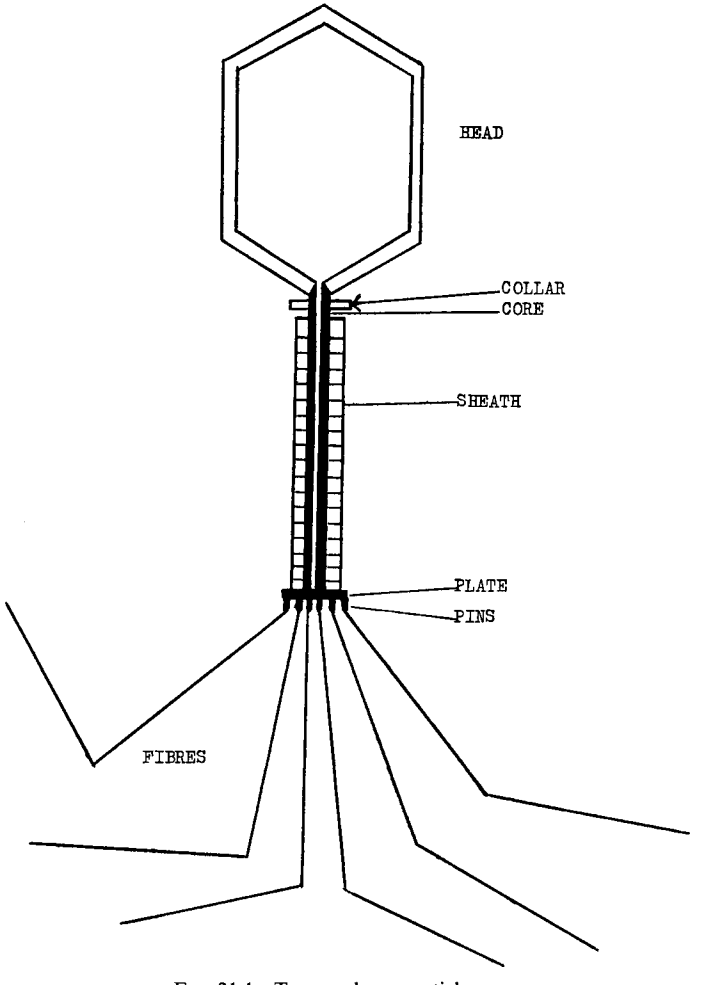

FIG. 21.1. T-even phage particle.

T-even phage particle (Fig. 21.1) should demonstrate the complexity of the tail element. The overall length of the particle excluding the tail fibres is about 200 mμ of which 95 mμ is the tail. About 60% of the mass of the particle is DNA and 40% protein.

The head of the particle appears to be a bipyramidal hexagonal prism composed of about 1000 protein sub-units enclosing the DNA. Each sub-unit has a molecular weight of about 80,000 and the protein of the head amounts to about 90% of the total. Also incorporated into the head is an internal protein component which is intimately connected with the DNA. This is composed of several different proteins and amounts to between 3 and 7% of the total. Its function however is not yet clear. The tail complex is attached to one of the pyramidal vertices of the head.

All together there are six different tail components. The innermost is the hollow core, about 8 mμ in diameter with a central hole of about 2·5 mμ. Coaxially with this is the sheath which is 80 mμ long and 16 mμ in diameter. Between the sheath and the head is the collar while to the other end of the sheath is attached the baseplate. This baseplate is hexagonal and has six pins protruding from it to the ends of which are attached the tail fibres. The sheath is composed of 140–200 sub-units of a molecular weight of 54,000. Under certain circumstances it can contract to 35 mμ while increasing in diameter at the same time to 25 mμ. This sheath appears to be formed of a helix of the sub-units and the contraction results in an increased number of sub-units per turn. Finally the tail fibres are 2 × 130 mμ each with a molecular weight of 100,000.

Two enzymes are present in the tails of the T-even coliphage particles —lysozyme and phosphatase. The lysozyme is associated with the distal end of the tail, has a molecular weight of 15,000 and may be the baseplate, while the phosphatase is associated with the sheath and it is thought that its purpose is to split the nucleoside triphosphates, ATP and dATP which are also present. It has been suggested that there is one nucleoside triphosphate molecule and one calcium ion per sub-unit which are necessary for contraction of the sheath.

As has been already stated, the DNA of the T-even coliphages contains 5-hydroxymethyl-cytosine (HMC) and is also glucosilated depending on the strain. The DNA is about 49 ± 4 mμ long and contains approximately 200,000 nucleotide pairs. Estimates of the molecular weight of the DNA range from 110 to 160 × 10^6.

Lytic Growth Cycle

The adsorption and penetration of the T-even coliphages is fairly well understood.[3,12,13] Specific attachment of the phage to the bacterial cell is effected by the formation of electrostatic bonds between the tail fibres and the site of attachment. The base plate is then held in contact with the cell wall and the lysozyme punctures a hole at the adsorption site. The sheath then contracts, presumably forcing the core through the hole in the cell wall and also through the underlying lipid membrane. Finally, the DNA passes into the host cell through the hollow core which is just wide enough to permit the passage of the DNA double

helix. The exact mechanism of this procedure is not yet known but once inside the cell the DNA immediately becomes associated with the RNA at the cell membrane. Only the DNA and the small amount of protein associated with it enter the cell—the remainder of the phage particle remains on the outside having served its purpose of injecting the viral genome.

The theory of the sequence of events following the injection of the DNA is[13]—The double-stranded DNA first becomes associated with the cell membrane RNA to form an RNA-DNA duplex. Then the RNA is rebuilt to form new or transfigured RNA complementary to the viral DNA, whereupon the two strands of DNA separate completely. The susceptibility of a cell to a given phage is therefore dependent upon a certain degree of compatibility between the phage DNA and the cell RNA. The last step of this stage is the conversion of all the membrane RNA to transfigured RNA. This then initiates phage-specified protein synthesis. At this time 6–8 minutes after infection new viral DNA starts to be formed and replication of the double helix commences from the single-stranded phage DNA. At the same time a deoxyribonuclease also starts to break down the single-stranded DNA and phage DNA sub-units are rapidly formed. It is suggested that these sub-units are formed into complete phage DNA by means of a highly stable RNA template. By this hypothesis the RNA serves two functions, firstly to direct protein synthesis and secondly to act as a template for the formation of new phage DNA.

A theory on the formation of new phage particles has been suggested.[12] While the new DNA is being formed the protein sub-units which make up the various parts of the particle are also being synthesised in a central pool. At the commencement of particle synthesis the phage DNA or vegetative phage is coated with the L-protein, one of the internal components of the phage head. The loose DNA then condenses into the shape of the phage head by the action of a "condensation principle" which may be any or all of the internal proteins. Next the head membrane is built up from head protein sub-units and the tail is added in the same way. Finally the various appendages are attached to the tail and a new infectious virus particle has been formed.

The times of formation of the various phage components is known fairly accurately. The internal protein and the enzymes for phage DNA synthesis are formed first and can be detected 5 minutes after infection. The external phage proteins do not appear until 8 minutes after infection at which time phage DNA can also be detected. Synthesis of the early enzymes ceases about 12 minutes after infection but the formation of phage proteins continues. Formation of lysozyme commences at about the 9th minute and mature phage is first detectable intracellularly 12–15 minutes after infection. All synthesis ceases abruptly upon lysis of the bacterial cells 25 minutes after infection.

The mechanism of lysis is not yet fully understood but it appears that

the lysozyme which is synthesised from the 8th minute plays a part. The theory which has been advanced suggests that the effects of the lysozyme are initially nullified by new cell-wall synthesis which continues as long as host-cell metabolism continues. This metabolism ceases at a definite time after infection and the lysozyme then destroys the cell. When observed in a fluid culture there is a sudden and dramatic disappearance of the turbidity as all the cells lyse in a short space of time.

Lysogeny

Lysogeny has been defined as[6] "the hereditary property of certain bacteria which enables them to produce bacteriophage in the absence of external phage particles." This is a stable character and in a lysogenic culture each cell will produce another lysogenic culture. Such cultures may be found in nature or they may be produced by infecting a susceptible cell with a temperate phage. The phage is carried in the host-cell in a non-infectious form called *prophage* which is integrated with the genome of the host-cell and replicates with it. In all respects except one prophage behaves exactly like a gene.

The one exception is that under certain circumstances the prophage can be "induced" to undergo a lytic growth cycle. This induction occurs naturally and in every lysogenic culture infectious phage is continuously being produced by a small proportion of the cells undergoing lysis. With lysogenic *Bacillus megatherium* up to 19 divisions may pass before a daughter cell undergoes lysis. Thus particle production is a potential ability of a lysogenic cell and only becomes a reality when the host cell is induced and undergoes lysis.

In naturally growing cultures of lysogenic bacteria in fluid media there is a constant ratio of free phage particles to bacterial cells. The ratio is a function of the burst size (the number of particles produced by each cell undergoing lysis) and of the frequency of lysis. The rate of spontaneous lysis varies from 10^{-5} to 10^{-2} depending on the strain of bacterium and the type of prophage being propagated, but in any given lysogenic culture it is a constant fraction of the growing cells. The mechanism of spontaneous lysis is still unknown but in many cultures the rate can be artificially increased as a result of induction by U-V, gamma and X-ray irradiation and such chemicals as nitrogen mustard, organic peroxides, epoxides and ethyleneimines.

Several other factors influence induction by any of these agents. The most important is a genetic factor—some strains of a given bacterial species are inducible whereas others are not. This inducible character of a prophage appears to depend on its location in the host-cell genome and is usually observed as a property of the prophage rather than the host cell, indicating that each phage has a specific site of attachment to the host-cell genome. In most lysogenic systems the spontaneous lysis of inducible strains is higher than that of non-inducible strains. Finally

the environment must be such as to render the bacteria capable of producing phage. Environmental disturbances both before and after induction can prevent lysis. Again the mechanism of induction is not known but the inducing agent appears to act on the bacterium rather than on the prophage itself. There has been the suggestion that the prophage is normally controlled by the nuclear apparatus of the host cell and can only develop into the vegetative state when some disturbance releases it from this control. Once induced, however, the lytic process in the lysogenic bacterium is identical with that of the virulent phage except that host cell metabolism continues.

A bacterial culture rendered lysogenic by a temperate phage may acquire several new properties. Two which always result are the potential capacity to produce new phage and an immunity to re-infection with the same or related phages. This immunity is not due to an inability on the part of the phage to adsorb but the phage DNA is prevented from entering into the cell metabolism in any way, although the nature of this block is not known. In general there is no interference with the replication of unrelated phages but many specific examples of such interference are known. Occasionally a variety of other properties called lysogenic conversions may result. These are distinct from the transductions which will be discussed later and the gene producing the new property is in fact the prophage. The most interesting example of a lysogenic conversion is the production of toxin by *Corynebacterium diphtheriae*. A complete correlation has been observed between toxin production and lysogeny of the culture by one of a group of temperate phages.[16,17] Other such conversions are changes in colonial morphology and antigenic structure.

By definition a temperate phage has the ability to lysogenize a bacterial cell. When a susceptible bacterial culture is infected with such a phage some of the cells undergo a productive infection with lysis and production of new phage while others become lysogenised. Although variation in the environmental conditions alters the ratio of productive to lysogenic infections, under constant conditions this ratio is constant. At least with phage P1 of *Shigella dysenteriae* the early stages of infection are the same whether the outcome is lysis or lysogeny.[18] Finally with many bacteriophage systems the ratio of lysogenic to productive infections can be increased by increasing the multiplicity of infection.

The prophage in a lysogenic bacterium is located on a specific site on the chromosome. This site differs for different phages and the ability of a phage to be induced by U-V irradiation appears to be a function of the site of attachment rather than of the phage itself. The prophage does not however behave exactly as a normal genetic component of the host cell and the method by which it is attached to the chromosome is not yet known. The difference between prophage and vegetative phage is almost certainly due to this attachment of prophage to the

chromosome whereas vegetative phage is free within the bacterial cell. Thus prophage replicates with the host-cell chromosome where it is prevented in some way from initiating the formation of phage proteins although its presence is manifest by the addition of detectable hereditary characters to the cell. In many ways lysogeny and the cell transformations described in the previous chapter are similar. Whether they are identical or not has still to be established but most workers consider that there are some differences although the basic mechanism may well be the same.

Transduction was first demonstrated with cultures of *Salmonella typhimurium*. This phenomenon is the transference of genetic characters from one strain of bacterium, the donor, to another strain, the recipient, by a temperate phage.[19,20] The temperate phage acts as the carrier of a fragment of genetic material from the donor host to the recipient host which it lysogenises. It appears that during the maturation of the phage small segments of host-cell chromosome may be incorporated at random into a small proportion of the new phage particles. When two or more characters are closely linked on the chromosome they may be transduced simultaneously. In some cases one prophage may be transduced by another unrelated phage together with other closely linked characters. This confirms the very close association of the prophage with the host-cell chromosome. In the case of coliphage λ there is evidence that this association is so close that it persists during the phase of vegetative multiplication.[6]

When we look more closely at transduction and lysogenic conversion we observe that these could easily be gradations of a single phenomenon. At one end of the scale we have the normal gene which can be transduced but which cannot be induced. Next we have the gene which can be transduced and which can also be induced, i.e., a gene which is a prophage (coliphage λ). The third stage is reached with the prophage which can be induced but not transduced and finally there is the virulent phage which cannot give a lysogenic infection at all. The very close association of coliphage λ and the gene which controls galactose fermentation suggests that this phage is closely related to the host-cell chromosome. From this pattern it is not unreasonable to postulate that a temperate phage arises as the result of the mutation or liberation of a bacterial gene whose mutation may not become obvious until environmental conditions result in its induction. Once induced the resulting phage particles will then be capable of infecting related bacterial cells which do not normally possess that gene resulting in either a productive or a lysogenic infection. There is however some evidence that the opposite sequence may occur. Mutations of prophage occur which result in defective lysogenic bacteria in which the prophage is unable to develop into infectious particles. Some such strains can be induced by U-V irradiation when phage precursors are produced normally but no mature particles are formed, while others cannot be induced at all. In

this instance the phage genetic material can only be perpetuated indefinitely in the prophage state and can then be classed only as a gene.

Typing of Bacteria

Certain bacteria can be typed by their sensitivity to a range of phages. The stock cultures of these phages are grown on specific host strains—in the case of the salmonella phage the strain to which it was adapted and in the case of the staphylococci the strain in which they were originally isolated. The salmonella phages are specific for their host strain of salmonella species all of which contain the Vi-antigen. Therefore if a newly-isolated salmonella is sensitive to one of the stock phages it will not be sensitive to any of the others and its phage-type will then be established. With the staphylococci the position is different. Here each strain of bacterium can be infected by several phages. Therefore the phage-type in this instance is determined not as a unique relationship between one phage and its specific host but as a pattern of infection with several distinct phages. The factors determining this phage pattern are a combination of surface structure and the range of prophage carried by the bacterium. Both types of reaction are more sensitive than antigenic analysis and are useful both for the identification of a strain of salmonella or staphylococcus and for the investigation of the source of an epidemic.

With the number of antibiotic-resistant strains of *Staphylococcus aureus* which have arisen the importance of accurate identification of such a strain need not be stressed. In a hospital outbreak of staphylococcal septicaemia, by phage-typing the various outbreak strains and all strains isolated from nasal carriers it is possible to trace the source of the epidemic.

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CHAPTER TWENTY-TWO

THE RICKETTSIALES

EARLIER we stated that the *Rickettsiales* could not be classified as viruses and for a comprehensive classification of this group the reader is now directed to the 7th Edition of Bergey's Manual of Determinative Bacteriology. In this and the following chapter we shall very briefly review the genera of the tribe *Rickettsieae* and two genera of the family *Chlamydiaceae* respectively. The latter are more usually called the basophilic group or the Psittacosis-Lymphogranuloma-venereum-Trachoma (PLT) group of agents. Both the *Rickettsieae* and the PLT group possess DNA and RNA and contain enzyme systems similar to those of bacteria, but whereas the former multiply by binary fission the latter appear to undergo a rather more complex form of multiplication. There is little doubt that these micro-organisms are much nearer to the bacteria than the viruses and should in fact be considered and classified as bacteria. The only reasons for discussing them here are firstly that they require virological techniques for their growth and secondly the bacteriologist has no desire to accept full responsibility for them.

The *Rickettsieae* with which we shall now deal are the members of the genus *Rickettsia*, the single member of the genus *Coxiella* which are pathogenic for vertebrates, and to a lesser extent those pathogenic for insects in the genus *Rickettsiella*. All species of the genus *Rickettsia* are vectored by arthropods and most share antigens with one or more strains of *Proteus* species. On the other hand *Coxiella burnetii* is probably not arthropod-vectored and does not possess *Proteus* spp. antigens. In this chapter the evidence leading to the conclusion that the Rickettsiae are bacteria will be briefly mentioned together with the clinical syndromes with which they are associated.

Biological Properties

The Rickettsiae can be observed under the light microscope where they appear as small cocco-bacilli varying in diameter from 0·3–0·65 μ by 0·8–1·5 μ long. They are best seen in smears stained by Macchiavello or in living cells by phase contrast microscopy[1,2] where their fine structure is similar to that of bacteria and unlike that of viruses. A variety of enzyme systems has been demonstrated in the Rickettsiae and it has been claimed that one, *Rickettsia akari* is able to multiply "in vitro".[3] They multiply by binary fission like bacteria and not by replication as do all the viruses, and with the exception of *Coxiella burnetii*

the Rickettsiae will not pass through a bacteriological filter. Finally they are all susceptible to the action of broad spectrum antibiotics.

The size and shape of the Rickettsiae vary considerably and some species have two distinct forms. There is the bacillary form measuring about $0{\cdot}4 \times 1{\cdot}5\,\mu$ and the diploid form measuring $0{\cdot}25 \times 0{\cdot}45\,\mu$. These organisms stain poorly by Gram's method but Giemsa, Macchiavello and Castenada stains can be used. Cell walls similar to those of bacteria are present and a detailed analysis of the constituents of *R. mooseri* has demonstrated the presence of protein, polysaccharide and small amounts of RNA.[4] The polysaccharide consists of glucose, hexosamine, glucuronic acid and galactose while 12 amino-acids make up the protein component.

A number of enzyme systems are present in the agents of this group. Glutamic acid is oxidised readily by purified suspensions maintained in certain conditions and follows a common metabolic pathway.[5] This pathway is to pyruvic acid via α-ketoglutaric, succinic, fumaric, malic and oxalacetic acids. Synthesis of citrate from oxalacetate has also been demonstrated.[3]

The members of the genus *Rickettsia* are relatively labile and are killed by formalin, phenol, heating and desiccation. Heating at 56°C for 30 minutes kills all members of this genus but they can be preserved by snap-freezing in a mixture of alcohol and dry ice (solid CO_2), although rapid thawing is essential to prevent great loss of viability. *Coxiella burnetii* is much more stable and is relatively resistant to heating at temperatures up to 70°C, desiccation and treatment with formalin etc., and can be readily preserved at −20°C.

A variety of antigens are present in the Rickettsial cells, principally associated with the cell walls.[4,6] There are two C-F antigens of which one is a group antigen and the other is type-specific. The group antigen can be removed from the cells by repeated washing of the suspension. *Coxiella burnetii* shows "phase variation" by the complement fixation test, and only reacts when in phase 2.[7] An antigen which is present in large amounts in the cell wall is the erythrocyte sensitising substance (ESS). Sheep or human group 0 erythrocytes can be sensitised by treatment with Rickettsiae and are agglutinated by antisera against most members. This ESS antigen is common to most of the Rickettsiae and is distinct from the two C-F antigens, those involved in agglutination tests and the Weil-Felix reaction. This Weil-Felix reaction is the agglutination of certain *Proteus* species by the convalescent sera from cases of Rickettsial infections.[8] The 0 or somatic antigens of Proteus strains X2, X19 and XK are agglutinated by sera to various Rickettsiae but not by those against *Coxiella burnetii*. These reactions are called OX2, OX19 and OXK depending on the strains of Proteus which are agglutinated and are explained by the sharing of antigens between the Proteus and Rickettsial cells. Specific agglutination of suspensions of Rickettsiae also occurs and is identical with bacterial agglutination.

Fluorescent antibody staining has also been used to differentiate between the Rickettsiae and is reasonably specific.[9]

Neutralisation of infectivity can be obtained with specific sera. A toxin and a haemolysin are related to the infectivity. The erythrocytes of several animal species are lysed by certain Rickettsia. This reaction is enzymatic and requires the presence of glutamate and magnesium. The toxin is only demonstrable in suspensions of living organisms and appears to damage the capillary endothelium with death resulting from a loss of circulating blood volume. This toxin is present in all the pathogenic Rickettsiae and is neutralised, as is the haemolysin, by specific antiserum.

Growth

The Rickettsiae can be grown in a wide range of vertebrate and arthropod hosts, in eggs and in tissue cultures. Apart from their obligate intracellular growth, which is shared by other bacteria, they have the same growth cycle as bacteria and divide by transverse binary fission.[2] Although they can all be grown readily in a variety of tissue cultures the yolk sac of the fertile hen's egg is the method of choice.[10]

Yolk sac inoculation of 6–7-day-old eggs followed by several days incubation at 34–36°C is used for serial passage and for initial isolation. Infected yolk sac membranes may contain as many as 10^9 viable Rickettsiae per ml. Microscopic examination of infected cells shows that all the Rickettsiae multiply in the cytoplasm while some multiply in both the cytoplasm and the nucleus. These exceptions *R. rickettsii*, *R. conorii*, *R. akari*, *R. sibericus*, often show a preference for the nucleus. Most species occur singly, in pairs or in chains, but *Coxiella burnetii* is frequently seen in large clumps.

With the exception of *Coxiella burnetii* an arthropod vector is required to transmit the disease from one animal to another. Transovarian transmission does occur but it seems that most if not all Rickettsiae have a vertebrate as their natural host.

Classification

Not everyone is in agreement on how these organisms should be classified although there is no doubt but that they are bacteria. Subdivisions into genera and species are difficult to define and in this section we shall consider only three genera. The first genus is *Rickettsia* whose members are characterised by (1) sharing antigens with *Proteus* species, (2) being relatively labile, (3) failing to pass through bacterial filters and (4) infecting vertebrates with the production of disease. The second is *Coxiella* whose single member is filterable, more resistant to physical and chemical agents and is not arthropod vectored, while the third, *Rickettsiella* is reserved for the insect pathogens.

Within the genus *Rickettsia* the species can be grouped together on

the basis of site of multiplication and antigenic relationship. Thus, those causing typical typhus in its various epidemiological forms belong to one group, while those causing spotted fevers belong to another. Using neutralisation of toxin all members of the spotted fever group can be readily distinguished although cross-reactions occur at low levels between most strains.[11]

Manifestations of Rickettsial Infections

The diseases produced by the Rickettsiae are characterised by severe and lengthy fever, CNS involvement and a maculopapular rash. All, except Q-Fever, are arthropod vectored and most infections of man are due to transmission from infected animals—principally rodents. The louse-borne typhus fevers however, are solely diseases of man and these Rickettsiae may remain dormant in the body for years, occasionally invading the blood and giving rise to a rickettsemia—sometimes with a definite illness. Q-fever is spread primarily via the respiratory tract and the remainder by mites, fleas or ticks from their natural hosts.

Unlike the virus diseases all Rickettsial diseases respond to treatment with broad spectrum antibiotics, although prolonged administration may be necessary for complete elimination of the organisms. Successful vaccines have been produced, but for all except Q-fever the elimination of the arthropod is the ideal.

Epidemic Typhus (Louse-borne)

This is a disease associated with war and famine, it has been known in Europe since the 15th century and has probably influenced the course of history more often than any other natural occurrence. The mortality rate is usually of the order of 20% in epidemics but may be higher than 40%. In cases uncomplicated by secondary bacterial infection death occurs in the 2nd or 3rd week of illness.

Following an incubation period of 10–14 days there is a sudden onset of malaise, chills, headache, weakness and generalised aches and pains. The temperature ranges from normal to 102°F for the first few days but from the third it remains between 102°F and 105°F until the death or the recovery of the patient. The headache is severe and persistent. A non-productive cough and constipation are common features during the first week although diarrhoea and vomiting may occur. Between the 4th and 7th days a generalised eruption appears first on the trunk and then over the whole body. Only in severe cases are the face, palms and soles of the feet involved. This rash is macular or maculopapular and at first slight pressure causes it to fade After the first few days the pulse and respiration rise and the blood pressure falls. By the end of the first week of illness photophobia and conjunctival suffusion are present and the face becomes flushed.

In the second week of illness the skin lesions turn a reddish-purple

colour and no longer fade when subjected to pressure. This rash lasts for only 2 to 3 days in mild cases but in the severe case it is present to the end of the febrile period. It may become petechial or even haemorrhagic in very severe cases.

During the 2nd and 3rd weeks of illness increasing prostration and mental dullness sometimes progress to stupor or coma. The stupor is interrupted by brief periods of delirium which are most marked at night. In some cases there is a persistent cough accompanied by patches of pulmonary consolidation. Other complications which may occur at this stage are parotitis, otitis media, furunculosis, and in very severe cases, gangrene of the extremities. Death may occur between the 9th and 18th day and usually follows a deepening of the coma and extension of the pulmonary consolidation.

When the patient recovers the temperature returns rapidly to normal during the third week. Only when severe encephalitis occurs is there any delay in recovery, which is complete in 2 or 3 months. The disease is mild in children under 15 but the mortality rate rises rapidly above this age.

There is a depression of the erythrocyte and haemoglobin count during the 2nd and 3rd weeks of illness. Albumin is invariably present in the urine, often with erythrocytes.

At post-mortem the principal lesions are broncho-pneumonia, myocardial damage and petechial haemorrhages in the subcutaneous tissues and brain. Microscopically the pathology is characteristic with the Rickettsiae multiplying in the endothelial cells which line the small blood vessels.

Epidemic typhus is caused by *Rickettsia prowazekii* and is transmitted by body lice. Man is the only known host. Lice are essential for the spread of the disease because the organisms are rarely present in the secretions or excretions of the patient. The course of the disease can be shortened considerably by the administration of chloramphenicol or a tetracyclene. The temperature returns to normal within 24–48 hours and is followed by a rapid recovery. The antibiotic should be administered for three days after the temperature returns to normal otherwise there may be a recurrence of symptoms.[12]

The disease is spread by a louse which sucks the blood of a febrile patient in whom there is a rickettsemia. The louse becomes infected and excretes Rickettsiae after 3–5 days. When the louse bites an uninfected person it also defecates and subsequent scratching of the bite introduces the infected faeces through the punctured skin. Infection can also result from contact of dried louse faeces with the conjunctival and respiratory tract mucous membranes. The infected louse itself is killed by the infection usually after 7–10 days. Delousing of all persons and efficient bathing of infected persons is the best method of controlling an epidemic. Several vaccines are in use, all of which are successful in reducing the effects of the disease.[6]

Brill-Zinsser Disease

This is the name given to louse-borne typhus fever when it recurs several years after the original illness. The symptoms are similar to those of epidemic typhus but the disease is shorter lasting 7–11 days, the temperature is more irregular, the rash is often absent, complications are less frequent and the mortality rate is lower except in the aged.

Some factor which is as yet unknown stimulates the latent *R. prowazekii* and a second bout of typhus results. New epidemics are probably the result of body lice becoming infected from a case of Brill-Zinsser disease.[13] This form of the disease can be differentiated from the primary louse-borne form by the low or absent titres of OX agglutinins and by a very rapid rise in specific antibodies. There is some evidence which suggests that the Rickettsiae may remain dormant in the lymph nodes.[14]

Murine Typhus (Flea-borne)

The clinical picture of this form of typhus is similar to that of epidemic typhus but in this instance the illness is less severe. With the exception of the over-fifty age-group the mortality rate is negligible and recovery is rapid. This illness is caused by *R. typhi*, also known as *R. mooseri*, which occurs as a natural infection of rats and mice. It is transmitted to man by the rat flea and from rat to rat by either the flea or the louse. Unlike *R. prowazekii* infection of the arthropod host, infection with *R. typhi* does not result in its death. Antigenically it is possible to differentiate between *R. prowazekii* and *R. typhi* by C-F tests using washed suspensions of organisms. Nevertheless an attack of either murine typhus or epidemic typhus renders the patient immune to subsequent attacks of both. Vaccination with suspensions of inactivated Rickettsiae, on the other hand, only protects against the type incorporated in the vaccine.

Scrub Typhus

Scrub typhus, together with the various tick-borne typhus fevers to be described later are varieties of classical typhus caused by different Rickettsiae. Scrub typhus itself is caused by *R. tsutsugamushi* which is transmitted to man from small rodents by the larvae of mites. Trans-ovarian transmission of the Rickettsiae occurs among the mites who leave their larvae (chiggers) behind.

After an incubation period which may range from 6–21 days but is usually about 10–20 there is a sudden onset of illness. The principal symptoms are fever, chilliness, severe headache, conjunctivitis and lymphadenopathy. The primary lesion "eschar" caused by the chigger has a characteristic black scab within a few days of the onset of fever. This fever rises slowly during the first week of illness to 104–105°F, at which time (5th to 8th day) a macular rash appears. The rash lasts for a variable length of time and may become maculopapular, while the

remaining symptoms and subsequent course of the illness follow the pattern of classical typhus. The disease varies in severity but the mortality rate has been reduced almost to zero by the use of chloramphenicol and the tetracyclenes.

Considerable antigenic variation exists between the strains of *R. tsutsugamushi* and immunity to heterologous strains lasts for only one or two years with the result that recurrences are common. So far nothing similar to the Brill-Zinsser disease has been encountered with scrub typhus.

Rocky Mountain Spotted Fever

This is the most severe of the tick-borne typhus fevers and has been singled out as one of the most severe of infectious diseases.[15] For this reason it will be considered separately from the other tick-borne fevers. Once again this disease resembles classical typhus and we shall deal only with the differences.

The incubation period is usually 4–8 days with a sudden onset similar to typhus. The clinical picture, however, is often one of acute encephalitis and a much more variable fever with a drop of 1–3°F every morning followed by a rise during the day. Usually the rash appears between 2 and 4 days after the onset and initially resembles that of measles becoming macular, maculopapular and petechial. It is first seen on the wrists and ankles but spreads rapidly to the legs, arms and chest within 2–3 days, often involving the soles and palms and sometimes the face and scalp.

The severity of the illness and the mortality rate vary from epidemic to epidemic but death invariably occurs during the second week or not at all. In patients with prolonged fever there is a high incidence of neurological sequelae. Even in mild untreated cases convalescence is very slow while recovery from a severe infection may take up to a year.

The disease is caused by *R. rickettsii* whose natural hosts are small animals. Man is an incidental host who becomes infected as the result of a bite from an infected wood or dog tick. Infection via the respiratory tract is likely to be a rare occurrence because the Rickettsiae rapidly die in dried tick faeces.

Vaccines have proved to be successful at least in reducing the severity of the illness although complete protection is not always achieved in the adult. Finally, antibiotic therapy with or without the addition of cortisone is highly successful but in severe cases the combination appears to be more satisfactory.

Tick-borne Typhus

This is a widespread disease occurring over much of Europe, Africa, Asia and Australia. Three antigenically distinct Rickettsiae are responsible for the illness—*R. conorii* in Europe, India and Africa; *R. australis* in Australia and *R. sibericus* in Russia. These organisms

are all related to *R. rickettsii* and are all transmitted to man from their natural hosts, dogs and rodents, by the bite of a tick. All stages of the life cycle of the tick are infectious because transovarian transmission occurs. Although some differences do exist, basically they are all fairly mild illnesses with a low mortality rate.

A sudden onset with fever and chills follows an incubation period of 5–18 days. In most cases there is a local lesion (tâche noire) at the site of the bite. This lesion is 2–5 mm in diameter with a black necrotic centre and there is usually regional lymphadenopathy. Other symptoms are severe headache, muscle and joint pains, photophobia, lassitude and sometimes mental confusion. A maculopapular rash develops 3–4 days after the onset invariably appearing on the arms first and then spreading over the rest of the body including the soles, palms and face. The illness lasts 2 to 14 days and recovery is usually complete although often prolonged.

As with the other typhus fevers chloramphenicol and the tetracyclenes can be used successfully in the treatment of this disease.[16]

Rickettsialpox

Rickettsialpox is usually a mild disease with many features common to tick-borne typhus and a rash resembling that of chickenpox. The principal symptoms are those described above but the primary lesion at the site of the bite persists longer and often looks like the vesicle of a vaccinial lesion. The rash first appears maculopapular but quickly vesiculates. Black crusts are formed when the vesicles dry but there is no residual scarring. Although the rest of the body may be affected, spread to the soles and palms is unusual.

This disease is caused by *R. akari* which is antigenically related to the organisms causing Rocky Mountain spotted fever and tick-borne typhus.[17] The house mouse appears to be the main reservoir of infection and transmission to man is via the mouse mite.

Both chloramphenicol and the tetracyclenes effect a dramatic improvement within 48 hours although the latter are the most efficient.

Trench Fever

As the name implies this is a disease of war and has been recognised in epidemic form only in the two world wars. It is caused by *R. quintana* which can only be propagated in lice, man or monkeys. Transmission to man is by body lice which are not killed by the infection but whose faeces are infectious.

The incubation period is 14–30 days followed by a sudden onset with chills, headache, dizziness, nystagmus, back and leg pains, relapsing fever, tachycardia and an enlarged spleen. A rash of erythematous macules or papules appears on the trunk. Relapses occur in at least half of the cases and recovery may take up to 2 years although many

patients recover in about 6 weeks. Fatalities have never been known and the effects of antibiotics are likewise unknown.

Q-Fever

Coxiella burnetii, the causal agent of Q-fever, is widespread among such domestic animals as cattle, sheep, goats, dogs, donkeys and fowls. Man becomes infected by inhalation of contaminated dust etc. Ticks may play a part in maintaining this organism in some of the animal hosts but they do not appear to be implicated in the transmission to man. The disease itself may be mild or severe but the mortality rate is very low and although the pneumonic form is the most common some cases show no respiratory symptoms at all.[18]

Following an incubation period of about 14 days there is a sudden onset of chilliness, malaise, severe headache, weakness, muscular pains and anorexia. The fever may reach 105°F and may be diphasic. Respiratory symptoms usually develop with patchy consolidation of the lungs and severe cough. Neck stiffness is present in many cases. When the fever subsides in about 2 weeks a rapid and complete recovery is made, although relapses are known to occur.

The Rickettsiae are excreted in the sputum of men and the milk, urine and faeces of cows. They are present in the placenta and amniotic fluids of infected domestic animals and since the organism is relatively stable it remains viable in infected material for long periods.

Again treatment consists of the administration of chloramphenicol or a tetracyclene and once more the latter probably gives the best results.

The Epidemiology of most of the rickettsial diseases follows the same general pattern as that of the Arboviruses. With the exception of Q-fever an arthropod is usually required for transmission to man. Like the Arboviruses the Rickettsiae are rarely present in the secretions or excretions of the infected animal. During the rickettsemia the arthropod—tick, louse, flea or mite ingests infected blood and thereby becomes infected itself and excretes the organism in its faeces. The effects of the Rickettsiae on the arthropod host vary. *R. prowazekii* kills the human body louse whereas the organisms causing tick-borne typhus not only have no effect on the arthropod but are passed from mother to offspring.

Most of the rickettsial diseases are primarily diseases of lower animals with man as an unfortunate secondary host. Epidemic typhus however is a disease of man alone relying on the body louse for transmission. The common recurrences of typhus with a rickettsemia due to reactivation of latent Rickettsiae are probably the cause of each new outbreak. Q-fever on the other hand, although it is also a disease of lower animals requires no arthropod vector because the organisms are present in secretions and excretions and can survive for long periods outwith the body. Consequently respiratory tract infections are common following inhalation of infected dust.

A variety of vaccines have been produced against the more serious rickettsial diseases, e.g. epidemic and murine typhus, Rocky Mountain spotted fever and Q-fever. A combination of vaccination and antibiotic therapy have done much to reduce the incidence of these diseases in certain areas of the world. Control measures directed against the vector and the natural hosts are also successful but the best measure of protection is given by general cleanliness.

Laboratory Investigations are required to confirm the clinical diagnosis. With the exception of *R. quintana* all types of rickettsial organisms can be isolated from the blood. Egg and animal inoculations are used as well as serological tests which are simpler and easier to perform.

A variety of procedures are in use to detect an antibody rise—some involve suspensions of the Rickettsiae themselves and others the "X" strains of *Proteus vulgaris*. A rise in OX agglutinins is produced by infection with all the Rickettsiae except *R. akari*, *R. quintana* and *Coxiella burnetii*. This Weil-Felix response is not specific and all but *R. tsutsugamushi* give rise to OX19 agglutinins. OX2 agglutinins are less common and only occur in the tick-borne typhus fevers and Rocky Mountain spotted fever while OXK agglutinins are produced only by *R. tsutsugamushi* and *R. sibericus*. More specific results are obtained when agglutination or C-F tests are performed using suspensions of the Rickettsiae for the antigen. The agglutination of sensitised sheep red cells is a relatively non-specific test but the C-F test using a washed suspension of Rickettsiae grown in the yolk sac is the most accurate of all the serological tests.

Arthropod Pathogens

Several species of Rickettsiae, some of which are pathogenic, have been demonstrated in arthropods. Three which cause fatal diseases in members of the order *Coleoptera* have been grouped together in the genus *Rickettsiella*.[19,20] These organisms attack the beetles at the larval stage of development. There are no signs of illness—larvae cease to feed, become inactive and die. The three species differ in size and site of multiplication. Two produce crystalline inclusions and the third does not.

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CHAPTER TWENTY-THREE

THE PSITTACOSIS-LGV-TRACHOMA(PLT) GROUP

THIS group of micro-organisms known variously as the Basophilic group of viruses, the PLT group of agents, the family *Chlamydiaceae* of the *Rickettsiales* or *Bedsonia* is widespread throughout the animal kingdom and one member, Trachoma, probably causes more disease than any other micro-organism. The present position of these agents is still a subject for debate although officially they are not viruses. They have been classified in the order *Rickettsiales* which is probably correct and Linnean binomials have been allotted which is possibly premature. A ready acceptance of the names cannot be expected and after mentioning them we shall revert to their common names. The agents of the Trachoma sub-group have been placed in the genus *Chlamydia* while those of the Psittacosis-pneumonitis-lymphogranuloma sub-group have received the generic name of *Miyagawanella*. While these names are undoubtedly correct according to the rules of nomenclature there are others, such as Bedsonia, in current use which could be adopted and so provide an acceptable nomenclature for this important group of agents. As it is we shall use the term proposed by Gordon[1] and call them the PLT group.

Now the question arises—are they viruses or are they bacteria? They are definitely not "true viruses"—they possess enzyme systems, both DNA and RNA and they are sensitive to chemotherapeutic agents. On the other hand they differ from "true bacteria" in their growth cycle and in their size. They appear to be the second half of the link between the viruses and the bacteria having some virus-like properties but being essentially more like bacteria.

Biological Properties

The biological properties of several members of the PLT group have been extensively studied and all the results indicate a much closer affinity with the bacteria. The morphology of these agents is fairly uniform if one considers only the elementary bodies. At various stages in the growth cycle there are several distinct types of particle but the elementary body is the infectious particle which is adapted for extracellular existence. The agents stain readily with basophilic dyes and can then be observed in the light microscope.

By electron microscopy the elementary body has a diameter of about 300 mμ with a dense nucleoid separated from the outer membrane by an electron-lucent zone.[2,3] The outer membrane is 7 mμ thick, composed

of 3 layers and is unlike a bacterial cell wall. The nucleiod appears to be made up from twisted filaments 10 mμ in diameter while other structures are occasionally found within the elementary body and these may be analogous to the mesosomes of bacteria. The composition of the elementary body is very complex. About 60% of the total dry weight is accounted for by lipid. Of the remainder 33% is protein, 1·7% carbohydrate, 3·4% DNA and 2–7% RNA.[4,5] The cells contain muramic acid, sugar and lysine as do bacterial cells. Other constituents are a variety of enzyme systems involved in the synthesis of lysine from diaminopimelic acid; the synthesis of folic acid and at least a part of the cytochrome system.[4,6,7]

These agents are relatively heat labile and the presence of protein, sucrose and sodium ions helps to stabilise them. Folic acid also stabilises the Psittacosis sub-group but not Trachoma.[8,9] The half-life of purified feline pneumonitis virus at 37°C is 90 minutes,[10] At 4°C however infectivity is preserved for several weeks using suitable diluents. Even at −70°C the infectivity slowly decreases.

A variety of soluble and particle antigens are associated with this group. They may be detected by complement-fixation, neutralisation and haemagglutination.[10,11] There are two C-F antigens—a heat stable group antigen and a heat labile type-specific antigen. Both are present in the elementary bodies although at least some of the group antigen appears in a smaller particle form. This group antigen is enhanced in titre by boiling or treating with phenol but is destroyed by periodate (KIO_4). It is a lipo-carbohydrate while the specific antigen is probably of a protein nature. The group antigen comprises the bulk of the antigenic material of the agent. Type-specific antigens which are heat stable have been described in purified preparations of cell walls.[12]

The heat stable group antigen can also be detected by the skin sensitivity or Frei test. A reaction indicates infection with a member of the group. By treating the antigen with HCl or KIO_4 the type-specific antigen will also elicit a positive response in the immune host.

Another type-specific antigen present in the cell wall is the toxin. This material will kill mice in 18–30 hours and the effect can be neutralised by specific antiserum.

The production of a haemagglutinin is a property common to many members of the Psittacosis sub-group.[13] It is soluble, produced irregularly and in low titres, only agglutinates murine erythrocytes and is group specific. Haemagglutination occurs at 24 or 37°C but not at 4°C and the optimum pH is 7·0. Calcium and magnesium ions inhibit this reaction. The haemagglutinin contains lecithin and nucleo-protein, both of which occur in the elementary bodies. It is therefore similar to the Poxvirus haemagglutinin.

Growth

All members of the PLT group will grow in the yolk sac of the fertile

hen's egg and this is the only efficient means of isolation of the trachoma agent, although it can be adapted to tissue cultures. The Psittacosis sub-group however has a wider host range with mice, eggs, and tissue cultures all being susceptible.

The growth cycle is highly unusual and appears to fall midway between the viruses and the Rickettsiae. By electron microscopy binary fission or some form of budding or sporogenous fission can be readily observed.[3] On the other hand there seems to be an eclipse phase where no infectious virus can be found and during this time the DNA is produced in a similar manner to that of the viruses.[14] Again, however, all the agents are susceptible to chemotherapeutic agents and antibiotics suggesting that during their growth cycle they produce and utilise a variety of enzyme systems which are integral parts of the Psittacosis elementary body.[15] Altogether the members of the group cannot be classed as bacteria and even less as viruses. They occupy a mid-way position between the viruses and bacteria but there is a greater pull towards the bacteria.

The growth cycle is highly complex and although it varies slightly from one strain to another it is basically the same for all members of the group.[2,3,10,16–8] The agent adsorbs fairly rapidly to susceptible cells, is absorbed by phagocytosis and enters an eclipse phase which lasts 15–30 hours. During this non-infectious period a variety of cytological changes occur which can be observed in both the light and electron microscopes. The first change is the appearance of "initial" bodies near the nucleus which are rich in RNA and also contain DNA. These bodies enlarge with an accompanying increase in DNA and the group antigen to form "plaques" which may develop in clusters to form vesicles. The "plaques" then break up into the typical elementary bodies either by binary fission, polygonal fission, or sporogenous fission. From the morphological studies there is no doubt that the development of the PLT agents is similar to that of the bacteria and quite distinct from that of the viruses. However certain of the biochemical changes are more akin to viral than bacterial growth.[19,20] There is evidence to suggest that the DNA of psittacosis replicates like that of a "true virus" and that the elementary body is built up by the assembly of sub-units. Similarly, although these agents are sensitive to antibiotics they are only inhibited by them at certain times during the growth cycle. This again tends to suggest a growth cycle which would place them with the viruses.[20] Another group of workers[21] suggests that the members of the PLT group are obligate intracellular parasites as a result of losing the ability to form high energy compounds and thus are "energy parasites". Their properties would suggest that this is a more likely interpretation than the idea that they are unusual viruses. In fact when one compares them with the Poxviruses one can see a connecting link. The PLT group has retained some synthetic processes and both nucleic acids but requires energy from the host cell. They multiply by a system as near to binary fission as their metabolism allows

whereas the Poxviruses have lost all their own enzymatic processes but are able to form factories in the host in which they multiply as "true viruses".

Classification

It is doubtful if complete agreement will ever be reached on the precise classification of these agents. The properties which relate them to the bacteria are probably stronger than those relating them to the viruses. Yet despite suggestions to the contrary[19] their position in the order *Rickettsiales* seems to be correct and their division into two sub-groups (Trachoma and Psittacosis) would also appear to be valid. Further sub-divisions into types or species can be made on host range and antigenicity which seem to be related—each host having one or more specific antigenic types associated with it.

There are only two members in the Trachoma sub-group—trachoma and inclusion blennorrhea both of which are human pathogens. The Psittacosis sub-group contains several members which can be divided on the basis of natural host into avian, mammalian and human strains. The avian strains are psittacosis itself and ornithosis both of which also infect man. The human strains include lymphogranuloma venereum and at least two strains which cause pneumonia and which appear to be fairly specific for man. Finally the mammalian strains include meningo-pneumonitis, pneumonia virus of mice (PVM), enzootic abortion of ewes, feline pneumonitis and a variety of agents causing enteritis, encephalitis and pneumonitis in separate animal species. These agents only rarely affect man—as laboratory infections.

Manifestations of PLT Infection

In man three main types of disease follow infection by the agents of the PLT group. These are respiratory infections, usually characterised by pneumonitis, severe conjunctivitis and venereal disease. The respiratory illnesses are psittacosis and "virus" pneumonias which appear to be transmitted to man from infected birds. Lymphogranuloma venereum (LGV), as its name suggests, is the venereal disease. The psittacosis and LGV agents are closely related. Finally the eye infections are inclusion blennorrhea which also causes infections of the genital tract and of course trachoma. These last two agents are closely related and have only a comparatively distant relationship to psittacosis.

TRACHOMA as it has been called for 2,000 years is the most important disease caused by a member of this group and is probably the most important infectious disease in the world today. A recent estimate of the number of persons suffering from trachoma is more than 400,000,000.[22] It is certainly responsible for most of the blindness in the world and despite its susceptibility to sulphonamides and several antibiotics eradication programmes are doomed to failure at present due to the lack of patient co-operation in the essentially extended

treatment. Rapid re-infection is inevitable because of the general low standards of hygiene and the prevalence of flies in the endemic areas.

The incubation period has been determined by volunteer studies as 5–7 days but in nature it can probably be longer. The onset is often insidious particularly in children although it may be sudden depending on the size of the inoculum. The principal symptoms in the early stage are a follicular hypertrophy of the conjunctiva sometimes obscured in severe cases by an extensive papillary hypertrophy. The corneal lesions which develop are epithelial keratitis, sub-epithelial infiltration and extension of limbal vessels (pannus). There is a considerable exudate containing polymorphs during the acute stage which lasts for several weeks. This is followed by a chronic stage which may last for years and give rise to major conjunctival or corneal damage. The eye-lids become distorted and often complete loss of vision develops, especially when there is secondary bacterial infection. Re-infection is common because no immunity is established despite the production of antibodies.

The pathology of trachoma is made difficult due to secondary bacterial infection but there are some characteristic signs. Typical basophilic inclusion bodies (Halberstaedter-Prowazek bodies) are found in the epithelial cells of the cornea and conjunctiva. The follicle in the upper fornix is a sub-epithelial infiltration of lymphocytes and plasma cells and is a characteristic of trachoma.

The trachoma agent is susceptible to the sulphonamides and several antibiotics of which the tetracyclenes are the most efficient. Unfortunately therapy is a lengthy business carried on over weeks, even months, with the result that many patients cease to attend the clinics. Finally, the administration of cortisone is a useful test to determine the presence of dormant trachoma in a healed lesion—if the agent is present it will be reactivated by the cortisone.

INCLUSION BLENNORRHEA is a disease of the genital tract in the adult causing urethritis and cervicitis in the male and female respectively. It can also give rise to conjunctivitis, usually in new-born babies who have been infected at birth but also in older people. The diseases are not severe especially in the adult female who may show no signs of illness at all and who is probably the reservoir of infection. In the male there is a constant or intermittent discharge which is classed as a non-specific urethritis.

The conjunctivitis can be readily distinguished from that of trachoma. Here the follicular hypertrophy is predominantly of the lower fornix whereas in trachoma the upper is most usually affected, and there is only slight corneal involvement.[23] The disease is mild and self-limiting although the causal agent cannot be distinguished in the laboratory from that of trachoma.

LYMPHOGRANULOMA VENEREUM (LGV) is a chronic venereal disease which presents in a variety of forms. It has three stages and is apparently spread by females with active but mild tertiary disease.

The primary lesion appears between 3 days and 3 weeks after exposure and takes the form of a vesicle on the penis, the labia, vaginal walls, cervix, within the urethra or in the region of the anus. Occasionally non-venereal spread may result in a lesion on the hands, etc. The vesicle bursts leaving a greyish ulcer which is often painless and consequently is ignored. Sometimes the disease terminates at this stage but it usually progresses to an adenitis. In the male the affected glands are in the groin but in the female they are generally in the pelvis due to the differences in lymphatic drainage. Generalised symptoms such as fever, chills, prostration, nausea, vomiting, headache, etc., may be present at this stage with an occasional progression to pneumonitis or meningo-encephalitis. Usually, however, the disease progresses to the tertiary stage with elephantiasis of the external genitals, granulomatous lesions of the vulva and rectal strictures.

A clinical diagnosis can be confirmed by the Frei test.[24] This test is performed by injecting the heat stable antigen, called lygranum, intradermally and examining after 48 and 96 hours. The test is an allergic response and is positive if a papule 7 mm or more in diameter develops. It usually becomes positive between 7 and 40 days after the onset of adenitis. This test is a group test and is positive in cases of psittacosis as well.

The treatment of choice is a tetracylene but sulphonamides can be used.

PSITTACOSIS is a respiratory disease of man which is contracted from birds. Originally the name was specific for the disease as contracted from psittacine birds and ornithosis was the word when another species of bird was the source. Today all the infections in man are termed psittacosis and the name ornithosis is reserved as the name for the disease in extrapsittacine birds. There are many distinct strains including several which may be specific human or at least mammalian strains but the clinical picture produced by all of them is similar. The disease varies in severity from a pneumonia with a mortality rate of 20% to sub-clinical infections. Again it may be a short mild to severe illness terminating abruptly or a low-grade fever lasting over 3 months.

The incubation period is usually 1–2 weeks but may be as long as 4 and is followed by either a sudden or gradual onset of illness with chilliness, fever, anorexia, sore throat, malaise, photophobia and severe headache. A broncho-pneumonia then develops in the severe cases. Sometimes there is also a myocarditis or meningitic and encephalitic symptoms. The fever lasts from 7 days in mild cases to 3 weeks in severe cases. Early treatment with the tetracyclenes generally results in a prompt and rapid recovery but treatment must be continued for 10 to 14 days otherwise there is danger of relapse and a prolonged convalescence. In untreated cases the mortality rate is highest in the "over 40" age-group.

The Epidemiology of the PLT group falls into three separate categories, although all strains show a marked ability to give rise to latent and prolonged infections. Firstly there is LGV which is purely a venereal disease. Secondly there is trachoma which is probably spread by flies, fingers, etc., and is prevalent in surroundings of poor or non-existent hygiene. Finally, there is the large group of strains which naturally infect birds and which are passed to man in a variety of ways. Parrots, parakeets and other pet birds are the source of many cases but probably the most important source of infection is the domestic fowl. Turkeys and ducks are the most common. Some strains have been isolated which readily pass from man to man but whether they are genuine human pathogens is doubtful because they have also been isolated from extrapsittacine birds. Infection is spread by inhalation of infected material but ingestion or the bite of an infected bird can also introduce the disease.

Non-human Infections

Members of this group cause a variety of illnesses in both mammals and birds.[10] In birds psittacosis and ornithosis are widespread and epizootics are common among domestic flocks. The value of turkeys is considerably reduced when flocks are attacked although the mortality rate is only 3%. Pneumonitis occurs in a large number of mammals which includes mice, cats, opossums, sheep and goats. However, two illnesses due to this group deserve to be mentioned in greater detail.

BOVINE ENCEPHALOMYELITIS is a disease of calves characterised by fever, anorexia, inactivity and paralysis. Diarrhoea is a common feature of the illness. Most infections are sub-clinical and up to 50–100% of all adult cattle have been infected. Post mortem examination reveals a peritonitis, a fibrinous net enveloping the omentum and covering the liver and spleen, a fibrinous pleuritis, pericarditis and occasionally CNS lesions.

ENZOOTIC ABORTION OF EWES is a common disease of young ewes. The overall rate of abortion is about 5% but in clean flocks and first year ewes it may be as high as 25–30%. Abortion occurs most often during the last 2–3 weeks of the gestation period but there are many stillborn and premature lambs as well. Lesions of the foetal membranes are always present as are subcutaneous and intramuscular lesions of the foetus itself.

In non-pregnant ewes the agent produces little or no sign of illness. It is thought that in most cases the stress of pregnancy activates a latent infection acquired when the ewe was a lamb. Vaccination of the ewes before pregnancy considerably reduces (up to 85%) the rate of abortion showing that immunity is poor and disappears rapidly. This is the only instance with the PLT group where an effective vaccine is in use although it is hoped that a successful vaccine will be produced against trachoma.

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CHAPTER TWENTY-FOUR

EPIDEMIOLOGY OF VIRUS DISEASES

THE epidemiology of any virus disease depends on a combination of many distinct factors, most of which are intrinsic characters of the causal virus or viruses. These intrinsic characters are those with which we have become familiar in the course of the preceding chapters under the title "Biological Properties". The antigenic structure and stability of any given virus or group of viruses to environmental change are two of the main features which will determine the epidemiology of each disease. Host range, site of multiplication and thereby excretion are also major factors while latency and prolonged excretion go together to complete the complex called epidemiology. We shall therefore consider now the various routes by which a virus can gain entry to the body and give rise to an infection which in turn may result in a clinical illness.

A composite pattern of virus infections is illustrated in Figure 24.1, and we shall examine each step in relation to the viruses. The routes of infection and excretion are those which occur regularly and account for the vast bulk of virus diseases in man. Other routes which play a large part in some diseases of animals but only rarely involve man include milk-borne infections with foot and mouth disease and certain Arboviruses, and excretion of infectious canine hepatitis virus in the urine. In man, however, the greatest number of infections occur via the respiratory and alimentary tracts.

The Conjunctiva probably provide a means of entry for many more viruses than is commonly supposed. The classical example is furnished by the Adenoviruses which give rise to "swimming pool conjunctivitis" and epidemic keratoconjunctivitis. Members of both the Myxovirus and Enterovirus groups may also gain entry in this way while herpetic infections of the eye are not uncommon. As far as the Adenoviruses are concerned swimming is an important epidemiological factor not because the virus is present in the water, although this does occur, but because the conjunctiva become irritated and consequently are more susceptible to infection by any available virus. After infecting the conjunctival cells the virus may give rise to local symptoms or spread to the respiratory tract without ever manifesting its presence. Two types of response are seen with the Adenoviruses depending on the strain of virus and the age of the patient. In adults, the symptoms following infection with type 8 remain localised in the form of a severe keratoconjunctivitis. On the other hand, children infected with any of

the Adenoviruses have conjunctival symptoms only as a part of the syndrome pharyngo-conjunctival fever. Herpes simplex also causes

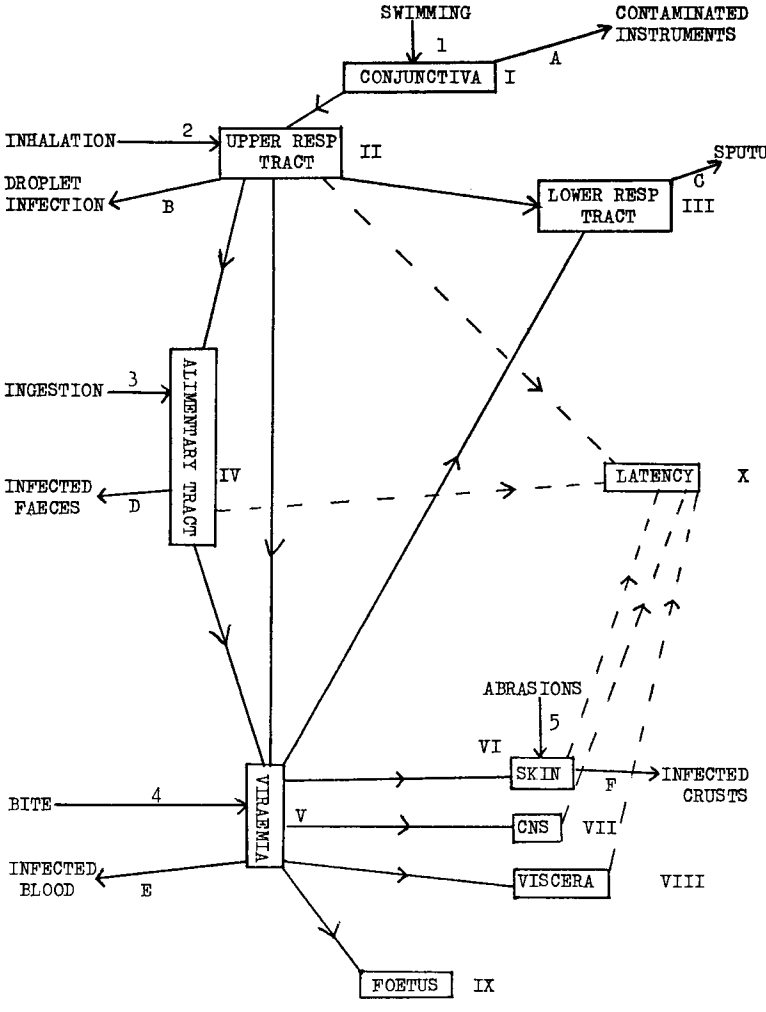

FIG. 24.1. Virus infections.

Note 1, 2, 3, 4, 5 = routes of infection of animal with a virus.
I, II, . . . X = sites of multiplication of virus in the host.
A, B, C, . . . F = secretions and excretions containing infectious virus which can cause the spread of a disease.

keratoconjunctivitis but in this instance the virus spreads to the respiratory tract, produces a viraemia, after which it passes into a latent state. The other viruses which first enter the body via the conjunctiva

probably produce little or no local reaction and spread rapidly to the respiratory tract.

Although virus will be present in conjunctival secretions these are not usually disseminated, and spread from conjunctival infections is purely mechanical. Again Adenovirus type 8 with the outbreaks known as "shipyard eye" is the best, in fact the only important example of this kind of spread. Isolations of Adenoviruses have been made from lake water and ophthalmic solutions. For a virus to be spread in these ways it must be relatively stable to withstand the adverse conditions to which it is exposed, and for this the Adenoviruses are admirably equipped being among the more robust of the viruses. Consequently the conjunctiva are not of much significance in the dissemination of viruses since the majority of viruses which enter the body via the conjunctiva are spread from the respiratory tract.

The Upper Respiratory Tract is probably the most common mode of entry of a virus into the body. It is also without doubt the most important means of dissemination of the viruses since droplet infection is a feature of most virus diseases. Virtually all the known viruses with the exception of the Arboviruses cause respiratory tract infections even although clinical illness is not recorded. Many viruses never progress beyond the respiratory tract, the most common examples being the Rhinoviruses and Myxoviruses. The Enteroviruses and Adenoviruses always invade the alimentary tract, usually without producing symptoms. This difference is due to the stability of the viruses to acid pH. Both the Adenoviruses and the Enteroviruses are very stable whereas the Rhino- and Myxoviruses are relatively labile and accordingly pass through the stomach to the intestinal mucosa only on rare occasions. Other viruses give rise to viraemia direct from the respiratory tract. Of these the Enteroviruses, smallpox, and some Myxoviruses are the most common while other Myxoviruses and the Adenoviruses may reach the lower respiratory tract producing a wide range of respiratory illnesses, many of which are complicated by conjunctivitis, pneumonia and alimentary tract symptoms.

Inhalation of infected material may also assist in the spread of disease. Smallpox is probably the most striking example of this means of entry where inhalation of powdered smallpox crusts is sufficient for infection to occur. This virus is very stable and smallpox will remain viable for months, if not years, in dried crusts.

Virus is present in the nasal and pharyngeal secretions and is readily transmitted in droplets of moisture from the respiratory tract. The stability of the virus determines the type of spread. If the virus is labile as are most of the Myxoviruses then overcrowded, warm, humid atmospheres provide the ideal conditions for the spread of infection. The Adenoviruses and Picornaviruses which are more stable are less dependent upon such rapid dissemination although they do spread rapidly in closed communities.

The Lower Respiratory Tract is usually invaded from the naso-pharynx but occasionally infection of the lungs may follow a viraemia. Bronchitis, bronchiolitis and pneumonia are the common clinical entities and they can be described as "dead end" infections—the disease has spread as far as possible when the lungs are reached. The commonest invaders are the Myxoviruses although the Adenoviruses not infrequently reach the lower respiratory tract, while members of most of the other groups find their way there on occasion. Direct invasion, while not impossible, is highly improbable and dissemination of virus present in the sputum is probably slight. Therefore when a virus reaches the lower respiratory tract is has come to a "dead end".

The Alimentary Tract is the main site of multiplication of the small ether resistant viruses—the Entero-, Adeno-, and Reoviruses. Infection can occur by direct ingestion of virus-contaminated material or by the downward spread of virus from the respiratory tract. Apart from occasional cases of diarrhoea or abdominal discomfort there is generally little or no manifestation of such an infection. Some Enteroviruses and Adenoviruses cause specific diarrhoea but intestinal infection is usually the precursor of a viraemia or the end of an abortive or inapparent infection. Multiplication of the virus occurs in the Peyer's patches and occasionally mesenteric adenitis or intussusception may occur. Prolonged excretion of the virus in the faeces follows invasion of the intestines and specimens of faeces from infected persons provide the commonest source of the ether-resistant viruses. Faecal contamination is undoubtedly one of the major factors in the spread of these viruses and the method by which the lake water, mentioned earlier, became infected. Probably most Enterovirus epidemics are started by faecal spread of the virus from a symptomless chronic excreter. How great a part faecal contamination plays in the subsequent spread of the disease depends on the age-group involved and of course the standard of hygiene. The lower either of these are, the greater is the spread. Nevertheless these viruses must be stable to survive in sewage long enough to infect a new host.

A Viraemia may develop in three ways in the course of a virus infection. Smallpox, measles, mumps and other viruses sensitive to acid pH enter the blood stream from the respiratory tract. The Enteroviruses which are able to colonise the alimentary tract as well may enter from either although it is assumed that they do so from the intestines. Thirdly the Arboviruses by virtue of their arthropod vector are injected directly into the blood stream. The viraemic stage of most virus infections is devoid of symptoms and acts purely as a dispersal mechanism which distributes the virus to the parts of the body which cannot be infected directly.

Only certain viruses regularly give rise to a viraemia whereas this is rarely, if ever, the case with others. In the first category one finds the Enteroviruses, measles, mumps, rubella, smallpox and varicella while

the Adenoviruses, most Myxoviruses and the Rhinoviruses, in descending order of incidence of viraemia, comprise the second. Systemic symptoms are very common with Influenza virus infections but are due almost certainly to a toxic effect which results from the large amounts of complete and incomplete virus produced. The rarity of viraemias can be attributed to several factors. The Influenza and Rhinoviruses are not equipped for growth in the fevered body as their optimum growth temperature is 33–35°C. Therefore although the virus is shed into the blood stream it usually fails to multiply. The Adenoviruses rarely gain access to the blood stream because they tend to be retained within the infected cells. They do, however, occasionally succeed and successfully multiply, producing a detectable viraemia.

The position of the Arboviruses is entirely different. Infection in nature is the result of the injection of virus by an infected arthropod. The arthropods themselves become infected after feeding from an animal during the viraemic stage of illness. Since these viruses are rarely excreted and are in any case very labile the arthropod is the sole means of dissemination. The occurrence of laboratory infections in the absence of a vector indicates that normal spread would occur if suitably infected secretions were available and it is possible that a reservoir species of animal does exist where this occurs.

Before leaving viraemias two other diseases are spread in a similar manner. The first is rabies which is disseminated by the bite of a rabid animal. The localisation of the virus in the salivary glands makes this type of infection possible and the very marked lability of the virus suggests that any other means of spread would be difficult. The second is serum hepatitis which is an artificial disease of modern medicine and can only arise from the occurrence of chronic viraemias.

The Skin becomes involved in many virus infections usually following a viraemia. The only exceptions are the local lesions produced by various animal Poxviruses which gain entry through skin abrasions and, of course, the herpes simplex infections. This manifestation of skin involvement may be a mild, fleeting rash as is produced by the Adenoviruses and Enteroviruses; the more severe rashes of rubella and measles and finally the disfiguring rashes of chickenpox and smallpox. While the former are of little consequence when compared with smallpox, severe chickenpox is difficult to distinguish from a mild smallpox. A skin rash is only infectious when vesiculation and crusting are involved. In the case of smallpox this is a serious problem due to the potency of the virus in dried crusts for months. Further spread of the virus does not occur although latent skin infections are common with herpes simplex.

The Central Nervous System is attacked frequently by many Enteroviruses, Arboviruses and by certain members of the Myxovirus group, while most of the other viruses gain access at one time or another. The limiting factor is the low ratio of viraemic to non-viraemic infec-

tions. As one would expect the manifestations vary with the sites of invasion but the most common are encephalitis, often with permanent residual physiological and psychological damage and an aseptic meningitis sometimes accompanied by paralysis. Again this is a "dead end" for the virus and is only an "overspill" infection which can occur with any virus but no further dissemination can follow.

The Viscera are attacked by a wide variety of viruses. The heart is not often singled out for special attention during a viraemia but certain Coxsackie B viruses produce myocarditis and pericarditis with little evidence or any other symptoms. The liver on the other hand fairs relatively badly and may be severely damaged by several viruses. Mumps virus is undoubtedly the least selective of the viruses and is able to multiply quite happily in virtually any tissue. Once again this is a "dead end" for the virus and no further dissemination can occur.

The Foetus can be attacked by several viruses in the course of a viraemic illness in the mother. Rubella and the Coxsackie B viruses are the two best known examples of this type of spread. The latter usually occur as *in utero* infections of the foetus shortly before birth and present as fatal myocarditis in the neonatal period, while the former is of importance in the first trimester when foetal damage results in many congenital abnormalities. There is not sufficient known of rubella for any explanation of this but it does seem possible that other viruses cross the placental barrier but do not cause such noticeable damage. This suggests that rubella and for that matter the Coxsackie B viruses have some unusual and as yet unidentified property.

Latency is a property of many of the viruses, in particular the Herpesviruses and Adenoviruses. Rabies virus is also a good example of a latent virus in certain vertebrates. This ability to lie dormant in the tissues is not yet understood but it may represent a stable symbiotic state between the virus and the host cell. Changes in environmental conditions may cause a breakdown in this state of equilibrium allowing the virus to multiply and produce cold sores in the case of herpes simplex and shingles in the case of varicella virus. Hormonal stimulation may also occur and this suggests that both physiological and psychological stress may be instrumental in any such reactivation. In view of what is known of cell transformation latency may yet prove to be a important factor in the epidemiology of cancer.

Before discussing the other factors which play their part in the incidence of viral diseases it is worth while to make some comments on the pattern outlined above. Under the right conditions any virus can give rise to any type of symptom. All viruses infect the respiratory tract, but only some regularly produce symptoms—the remainder give inapparent infections. The viraemic stage of illness is the dividing line: once across this barrier any virus can localise itself anywhere. It is true that an Adenovirus or Enterovirus rash is unlike smallpox but it is nevertheless a rash and there are many occasions on record where a

syndrome with a specific aetiology such as mumps or pleurodynia has been related to a virus entirely different in its normal pathogenicity. The basic properties of each virus therefore determine its normal method of attack but conditions change and the normal procedure may then alter accordingly.

Immunity and Environment

The immunological properties of the virus itself and those of the host in relation to the virus are two of the main factors which determine viral epidemiology. Under certain circumstances the environment is also a deciding factor in the natural history of virus diseases. First we shall consider how the apparent immunological response of the host is determined by the site of multiplication of the virus.

The host produces circulating neutralising antibody in response to all infections irrespective of where the virus is localised. If the virus requires to pass through a viraemic stage before the infection can become an illness then the presence of even small amounts of circulating antibody will prevent the illness from developing. Thus an infection with measles, mumps, smallpox, yellow fever, etc., which are single immunological types give complete protection for life. In a community where these diseases are endemic the adult population rarely, if ever, contracts the disease because large epidemics occur in each new population of children. Thus every few years there is an outbreak of mumps or measles when the number of susceptible children is large enough. Smallpox is different today because of vaccination. The Arboviruses are complicated by the essential arthropod vector but we shall deal with this separately. However, exactly the same pattern is seen with the Enteroviruses but since there is a multiplicity of antigenic types causing each syndrome, immunity to one virus does not mean immunity to the relevant syndrome. Therefore every year there is an outbreak of central nervous system disease but the same virus usually recurs only about every four years. Again the majority of patients are children although we shall see how changing patterns of illness have followed improvements in hygiene.

When the virus remains localised in the naso-pharynx as do most Myxoviruses and the Rhinoviruses specific antibody must be present in the secretions for it to prevent infection. This requires high levels of circulating antibody. It is possible to detect antibody to the Rhinoviruses in nasal secretions immediately upon recovery from an infection but as time passes this antibody disappears and reinfection with the same virus is possible. This occurs with the Rhinoviruses and the Myxoviruses. In the case of the latter, second infections are usually milder than the first and appear only as common colds. In general the respiratory viruses tend to recur with relative frequency although the Adenoviruses are exceptional because only low levels of circulating antibody are required for complete protection. This difference may be

accounted for by the long incubation period (at least 7 days) of members of this group which allows the antibody levels to rise, compared with the 1 or 2 days of the others.

The presence of antibody does not, however, prevent a recurrence of latent infections but the symptoms are usually localised and systemic illness is rare. Thus the rash in shingles is strictly limited in extent, whereas the primary chickenpox is a systemic illness with a widespread rash.

Antigenic heterogeneity also determines the epidemiology of a virus. We need not consider further those viruses which are antigenically stable but which occur in a great multiplicity of types. Immunity to each of these is the same, long or short, depending on the virus group but cross-immunity does not occur. The Influenza viruses are the most interesting of viruses which show a great lability of antigenicity. This is most marked with the A types which undergo a major antigenic change every decade or so. When this happens the entire population of the world is at the mercy of the new virus which completely replaces its predecessors in that sub-group. Consequently large localised outbreaks occur and sometimes a pandemic. In the period 1956–1960 new antigenic types of both A and B completely replaced those previously known in the world. There has been a suggestion that there may be a limited number of distinct antigenic types of each sub-group, but even if this is so at least half a century will separate consecutive appearances of the same type.

The last of the general epidemiological determinants is the environment wherein change may lead to large outbreaks of disease. This is particularly true of the Adenoviruses and acute respiratory disease (ARD) of military recruits. The Adenoviruses are widespread among children and most persons have been infected by types 1, 2, 3, 5 and possibly 6 by the time they leave school. Types 3 and 7 cause large outbreaks of pharyngoconjunctival fever during the summer months and may occur as confined epidemics in boarding schools, orphanages, etc. Type 7 is less common than type 3 and type 4 is rarely found in the civilian population. When a large number of military recruits are gathered together a variety of factors come into play to produce serious outbreaks of ARD. Adenovirus carriers will be present in the group so that types 4, 7 and to a lesser extent 3 will have an almost virgin population to infect. Furthermore the men are in relatively close contact and are undergoing training which will be of a more tiring and demanding nature than the average recruit will have experienced previously. Thus the virus has a non-immune population under physical stress with ideal conditions, especially in winter, and types 3, 4 and 7 all give rise to explosive outbreaks during the winter months. However, vaccination has successfully reduced the loss of man-hours due to this illness.

Finally, the epidemiology of the Enteroviruses has also been altered due to the changing environment but in this instance the change is a

long term problem rather than a localised acute problem. In communities with poor or non-existent sanitation, a low standard of hygiene and generally poor living standards, the infant population meets the assault of the multitude of Enteroviruses. No doubt some of the infants succumb as a result of these infections but when living conditions are poor there is always a high infant mortality rate. Again, a few children will be crippled but in the younger age-groups the Polioviruses are quite mild and the bulk of such a population is then immune. As sanitation, general hygiene and living conditions improve faecal spread of the Enteroviruses is reduced until the time is reached when very few infant infections occur. As the age of infection rises and with the reduced infant mortality rate due to these improved conditions the increasing mortality rate in the older children and adults becomes most marked, so infantile paralysis becomes paralytic poliomyelitis. This has almost certainly occurred with other Enteroviruses which cause outbreaks in the summer and early autumn and which infect school children principally but can cause serious illness in the adult.

Arthropod Vectors

Insects can transmit viral infections in two ways. In the first they act solely as mechanical carriers but in the second they become infected themselves and transmit the virus by "biting". Ordinary house flies are the chief culprits of mechanical transmission. It is possible to isolate every known virus present in a community from house flies caught in the area. Although mosquitoes and other blood-sucking insects may act mechanically on occasion, they are usually infected themselves when transmitting a virus. These arthropod-borne viruses belong to two groups, the EMC sub-group of the Picornaviruses and the Arboviruses themselves. There are also two epidemiological cycles involved, one is the man-arthropod-man while the other is the vertebrate-arthropod-man cycle with man as an incidental "dead end" host. This is the difference which sets the epidemiology of the Arboviruses apart from the other diseases of man. When the cycle is man to man with or without a vector most cases during an outbreak occur in the centres of population. However, when the cycle is vertebrate to man the majority of the cases occur in rural areas and are often accompanied by an epizootic among some wild or domesticated bird or animal.

Epidemic Spread

Finally, there is the rate of spread of an epidemic. Although most viruses can be passed readily to close contacts, for an outbreak to spread it must be conveyed by man. Thus two factors must be taken into account, namely the incubation period of the disease and the length of time required for an infected person to reach his or her destination. There is, of course, the random nature of the selection of the carrier since before a virus will spread from one population centre to another a

sufficient number must be infected for at least one traveller to become infected and carry the virus. In recent years the most important change has been in the ease with which intercontinental travel can be undertaken, and the corresponding dangers inherent when possible smallpox contacts can move from country to country in a relatively short space of time. Prior to long-distance aviation everyone who travelled from Africa or the East, where smallpox is endemic travelled by sea. Consequently anyone who was incubating smallpox on embarkation had developed the disease before the ship reached Europe. Nowadays a person may become infected one day and be in Europe the same or the following day with another 10 days or so before any symptoms appear. Even the Arboviruses which can only spread to the geographical limits of their vector appear to be assisted in their spread by means of modern transport.

In conclusion it is possible to make one important correlative observation. Our knowledge of the properties of viruses and their specific epidemiology is now such that the epidemiology of any given virus can be predicted with a fair degree of accuracy from its biological properties or vice-versa, the general characters of a virus can be predicted from its epidemiology.

CHAPTER TWENTY-FIVE

VACCINATION

IN the previous chapter we discussed the spread of diseases, the various invasion routes of the body and the multitude of viruses which cause respiratory symptoms and which cannot easily be conquered. In the appropriate chapters mention has already been made of the production and use of the available vaccines. We now turn to the international standards governing these vaccines, the methods of production of inactivated and live-attenuated vaccines and the alterations in epidemiology brought about by vaccination against smallpox, poliomyelitis, the Adenoviruses and yellow fever.

Two distinct types of vaccine are in use—killed and live virus suspensions. The former type are still used for poliomyelitis and Adenovirus infections while the latter are used for yellow fever, smallpox and poliomyelitis. Live vaccines by their very nature give better protection because viral multiplication takes place and simulates a natural infection whereas killed vaccines produce only antibody. This advantage is to some extent balanced by the ever present possibility that live-attenuated strains may revert to virulence and thereby cause epidemics. On the other hand inefficient safety precautions have already led to the disastrous "Cutter incident" in which many people were vaccinated with inadequately formalinised poliomyelitis vaccine which contained live and *virulent* virus.[1] We shall consider first the inactivated and then the live-attenuated vaccines.

Adenovirus Vaccines

Although these vaccines have been tried in the civilian population they are necessary only in the Services. Protection is essential against types 3, 4 and 7 which cause most of the acute respiratory disease (ARD) in military recruits and the vaccines in use contain either types 4 and 7 or types 3, 4 and 7. Most persons who are vaccinated with the bivalent vaccine produce antibody to all three types. No international standards have been prescribed for this vaccine but some mention should be made of those in use in the U.S.A. Acute respiratory disease was known for many years before the discovery of the Adenoviruses and the success of the first vaccines was demonstrated within two years of the establishment of the association between the Adenoviruses and ARD.

The virus strains which are used were all isolated in human or simian primary cell cultures and then adapted to monkey-kidney cells

maintained in a serum-free medium.[2] Each antigenic type is processed as a separate pool and the requisite vaccine is prepared by mixing equal volumes of each pool. Completely degenerated cell cultures are homogenised and filtered through a bacteriological filter and inactivation is achieved by treating the suspension with a 1:4000 dilution of formalin at 36°C for 6 days, and shaking occasionally. The formalin can be removed by dialysis or by chemical neutralisation and the vaccine can then be tested for the presence of live virus, in particular an Adenovirus, Poliovirus or SV_{40}. This latter virus is often present in monkey-kidney cell cultures as a latent infection. Bacteriological tests are also carried out before the vaccines are prepared.

Many trials of these vaccines have been run in the U.S.A. and in Europe[2–6] and most have proved to be highly satisfactory providing that the vaccine is given within the first few days of the arrival of new recruits at a Service station. A mild local reaction may appear at the site of inoculation but there are usually no major ill effects with these vaccines. The reduction in total respiratory disease varies with the outbreak as there is often an outbreak of influenza as well. However, when only Adenovirus is involved the reduction varies from 70 to 90% of the expected cases. The most marked reduction is in the pneumonic cases while the least is in the non-hospitalised cases. It is certain that even in those cases where protection is not complete there is still a reduction in the severity of the illness. Only a very low level of antibody is necessary to give complete protection and only a single inoculation is required.

The effect of the Adenovirus vaccines has been seen only in military camps where their introduction has saved many man-hours and reduced the number of discharges due to pneumonia. Their possible value in the civilian population is negligible on account of the vast amount of respiratory illness caused by viruses other than the Adenoviruses.

Poliovirus Vaccine

There are two types of Poliovirus vaccine in use today—the Salk-type formalin-inactivated and the Sabin-type oral live-attenuated vaccines. With the exception of the "Cutter incident" when 149 cases of paralytic poliomyelitis were caused by the direct inoculation of vaccine containing live, virulent type 1 Poliovirus, no untoward effects have been observed with either vaccine. However, many batches of oral vaccine have since been shown to contain SV_{40} virus. Infections of the alimentary tract have resulted but so far no illnesses or tumours have developed. The elimination of this and other simian viruses from the live vaccines is an essential part of vaccine production.

The requirements for inactivated poliomyelitis vaccines have been laid down[7] and will now be reviewed. The choice of strain of each of the 3 types of Poliovirus depends on the manufacturer, the only requirement being that it will produce an adequate vaccine. Several

strains have been used and most have proved to be satisfactory. The virus for the vaccine must be grown in cultures of monkey-kidney cells maintained in a protein-free medium so that any animal serum used at any stage will be present in a maximum concentration of 1 part per million of the final vaccine. Antibiotics may be added but penicillin must not exceed 200 units per ml. Pools of the individual types are grown and processed separately. It is also recommended that each pool should be filtered through a Seitz S1 pad before inactivation but any equivalent filter may be used. Finally, the virus suspension must have a titre of at least 10^6 TCD_{50} and be free from *Mycobacterium tuberculosis* —any pools containing this organism must be discarded.

Inactivation should commence as soon as possible and certainly not later than 72 hours after filtration. Formaldehyde is the most common inactivating agent and it has been recommended that the material should be filtered during the inactivation period.[7] This has now been discarded in many countries without any obvious deleterious effects.[8] The standard by which an inactivation procedure is measured is the consistency with which the virus pools are successfully inactivated.

Testing of each monovalent pool for the presence of residual infectious virus is carried out on two samples of not less than 500 ml each, one taken at the expected time required to reduce the initial virus titre by a factor of 10^6 and the other at the end of the process. The formaldehyde should be neutralised with bisulphite and then dialysed before inoculation of tissue cultures. These cultures should be taken from two batches of cells, and at least 3 cm^2 of cell sheet should be allowed per ml of vaccine. Sub-cultures must be made after one week and two week's incubation when the primary cultures are discarded. The sub-cultures are then observed for one week. If live virus is found to be present, the pool may be processed again or it may be discarded depending on the national controls. If after re-processing satisfactory inactivation is not achieved the pool must be discarded. When suitably inactivated pools have been produced they are combined to form the trivalent vaccine.

An aliquot of not less than 1,500 ml of this trivalent vaccine is tested in tissue cultures as described above before dispensing into the final containers. Monkey safety tests must be performed on either the bulk product or the bulk product and the final containers, which are tested at the same time for bacteriological sterility. The monkey safety test is more sensitive than the tissue culture test for detecting viable Poliovirus in formaldehyde treated suspensions, and the sensitivity may be improved by prior concentration of the vaccine.[9,10]

The monkey test should be performed with not less than 20 healthy *Macaca* monkeys who do not possess Poliovirus antibody. They should be inoculated via the intraspinal, intrathalamic and intramuscular routes and 150 to 250 mgm of cortisone should administered at the same time. These animals are observed for 17 to 19 days, sacrificed and then examined histologically.

The final requirements are tests to ensure that the vaccine is not toxic, is antigenically potent and contains not more than 0·35 mgm of nitrogen per ml. Vaccines should be stored at 0–10°C and should not be used more than 12 months after the potency test.

Before considering the effect of this Salk-type vaccine we shall look at the requirements for live Poliovirus vaccine. Three sets of attenuated strains of the three Poliovirus types have been developed—by Sabin, Koprowski and Lederle. These satisfy to a greater or lesser extent the following criteria:

(1) Low neurovirulence for monkeys, as determined by the cerebral route of inoculation.

(2) Good immunising effectiveness, as determined by evidence of infection and of antibody response when administered as a monovalent vaccine.

(3) Genetic stability on human passage, as determined by tests of the viruses isolated from vaccinated persons and their contacts.

(4) Lack of paralytogenic properties in man, as determined by field trials which demonstrate both the safety and effectiveness of the procedure.

(5) Restricted capacity to spread, as determined by family, institutional and community studies.

(6) Reduced capacity to invade tissues remote from the alimentary tract, as determined by the absence or low incidence of viraemia in the course of infection.

The preparation of virus for the live vaccines follows the same procedure as that for inactivated vaccines up to the filtration stage. At this point the presence of simian viruses, in particular B virus and SV_{40} is eliminated by keeping 15–25% of the cell cultures as controls and discarding all batches with these viruses present. Probably most of the simian viruses are harmless but every effort should be made to eliminate them.

Many trials and general mass vaccination schemes have been conducted, using both the Salk and Sabin-type vaccines.[8] In most cases both vaccines have proved successful. In the U.S.A. the efficiency in reducing poliomyelitis has been 82–90% after 3 doses of Salk vaccine and 86–96% after 4 doses. Similar results have been obtained in Canada, Australia, Western Europe and South Africa, but in the U.S.S.R. and Eastern Europe the protection by the inactivated vaccines was relatively poor.

Experience with the live-attenuated vaccines has been more consistent and we shall consider the results with the different strains. The Sabin strains have been administered in South Africa, Mexico, Europe and the U.S.S.R. and very few adverse reactions (about 3 per 100,000) have been noted. These disorders took the form of transient fever, nausea, vomiting and intestinal symptoms. In all areas where administration of three doses of the vaccine over a period of two months was completed

before the expected seasonal epidemic the protection was over 90%. Administration of the vaccine during or just prior to the epidemic was also effective in reducing and shortening the outbreak although the protection was not as good. The Koprowski strains have been used only in Poland and the Congo (Leopoldville) Republic. The protection rates have been somewhat lower (70–90%) than those achieved with the Sabin strains. Finally, the Lederle strains have been administered in the Americas with success comparable to that of the Sabin strains.

The optimal age for administration of oral vaccine has not yet been established but available evidence suggests that this is immediately after birth.[11] From the point of view of administration this time is most convenient and there is less chance of interference from other Enteroviruses, while the presence of maternal antibodies appears to be less serious than might be expected.

The final problem is still hypothetical although most workers are agreed on its ultimate appearance. The attenuated vaccine strains do not spread well in the community themselves but when a sufficiently large number of persons in any area have been immunised the wild strains are eliminated. Other Enteroviruses, however, are present and it seems more than likely that in time they will cause major outbreaks of paralytic disease. It is significant to note that the first outbreak of meningitis associated with ECHO 7 virus occurred in Aberdeen, Scotland when the other three major centres of population in the country were suffering an outbreak of Poliovirus type 1 which, although introduced to Aberdeen was unable to spread owing to the high proportion of vaccinated persons.[12]

Yellow Fever Vaccine

Although two types of live vaccine are in use today the cutaneous scarrification method is not yet covered by WHO requirements.[13,14] The Dakar vaccine for cutaneous scarrification carries a risk of severe post-vaccination reactions, especially in children. On the other hand the similar vaccine prepared from the 17D strain is antigenically inadequate. The recommended vaccine is therefore the 17D strain as prepared for subcutaneous injection. We shall look at the recommendations advanced for the Dakar-type vaccine after detailing the requirements for production of the 17D subcutaneous injection vaccine.

The strain of virus in use must be the modified yellow fever virus of a type not transmissable from man to man by the bite of an insect and it should induce protection for a period of at least 6 years after vaccination. The only strains in use which meet these requirements are the 17D strains and sub-strains. The seed and vaccine lots must be grown in chick embryos and should not contain any human protein or serum. Primary and secondary seed lots are prepared, freeze/dried and stored at −50°C (or at −70°C if not dried). The flocks used for vaccine production must be free from disease especially the lymphomatosis viruses.

The primary and secondary seed lots must pass a test in monkeys for viscerotropism and neurotropism. Ten monkeys must be inoculated with 0·25 ml of vaccine in the frontal lobe and observed for 30 days.

To pass the viscerotropism test blood taken from the monkeys 2, 4 and 6 days after inoculation must be positive but cannot contain more than 500 mouse LD_{50} per 0·03 ml. At least 90% of the monkeys must become immune within the 30 days but not more than 20% of the monkeys may develop encephalitis for the seed lot to pass the neurotropism test. Finally, sterility and guinea pig safety tests are carried out on the seed lots which can then be used to grow vaccine virus.

The infected embryos are harvested when they are 12 days old, pulped, clarified and freeze/dried in ampules. These final containers are then reconstituted and tested for bacteriological sterility and other safety factors. The guinea pig safety test is performed by inoculating 8–10 human doses of vaccine intraperitoneally into each of two guinea pigs. The animals must remain healthy for 21 days. Three final containers must also be titrated in mice and most contain not less than 1000 LD_{50} in the volume used as a single human dose. Finally, there cannot be more than 0·25 mg of protein nitrogen in a single human dose.

The vaccine must be kept at $-5°C$ after preparation and preferably below $-25°C$ until it is distributed. It must not be used more than 18 months after the potency tests or 12 months from the date of issue by the manufacturer, whichever is the sooner and must be administered only by subcutaneous injection.

Similar requirements have been proposed for a vaccine to be administered by cutaneous scarrification but the following modifications may be made: The seed lots and vaccine lots may be grown in tissues and animals other than the chick embryo; the constituted vaccine should contain more than 500,000 LD_{50} per ml; there is no need for a requirement concerning the quantity of protein nitrogen in the vaccine. At present the French neutrotropic strain is used in the Dakar vaccine despite the relatively high incidence of complications. Even the 17D strain can cause an encephalitis although the incidence is very low. Nevertheless the efficiency of the two types of vaccine in preventing yellow fever is very high and man to man spread has been virtually eliminated. Unfortunately alternative hosts are present in most endemic areas with the result that revaccination is always necessary.

The development of a vaccine which would be more potent than the 17D strain without the complications of the Dakar strain when administered by scarrification would greatly assist in the maintenance of a high level of immunity in those areas with jungle yellow fever.

Smallpox Vaccine

The history of smallpox vaccine was mentioned earlier so that we now come to the regulations governing production of such vaccines from the great variety of strains of vaccinia virus.[15]

Any known strain of vaccinia other than those known as "neurovaccine" may be used providing it infects man, rabbits and chick embryos. All primary seed lots of vaccine are prepared by growing the virus in the skin of an animal but the secondary seed lots may also be prepared in chick embryos or tissue cultures. All seed lots should be maintained either in dried frozen or glycerinated form and should be free from bacterial contaminants. When virus for the preparation of vaccine is grown on the skin of an animal very strict precautions must be taken to ensure that the animal is healthy and the skin as near as possible to surgical asepsis for inoculation and harvesting. The most widely used animals are sheep, calves and buffalo. Recently, however, vaccine has been produced in eggs and tissue cultures. The flocks supplying the eggs must be free from salmonellosis, tuberculosis, Newcastle disease, fowl pest and avian lymphomatosis, while the tissue cultures cannot be of human origin.

The subsequent processing of material prepared from the skin of animals, lymph, is directed towards the elimination of bacteria. Glycerol and/or antibacterial substances may be added but antibiotics should be avoided. In some cases bacteria may be removed by centrifugation. Virus prepared from eggs or tissue cultures may again have the addition of glycerol and/or antibacterial substances as a precaution against later contamination. Glycerol is only added when the vaccine is to be kept in the liquid form. The material prepared in this way must then pass potency and sterility tests. Lymph vaccines must also be tested for *Escherichia coli*, haemolytic streptococci, coagulase-positive staphylococci, *Bacillus anthracis* and *Clostridium* species. If either of the latter two are present the vaccine must be discarded but with the former steps can be taken to eliminate these organisms. Vaccines prepared in eggs or tissue cultures must be bacteriologically sterile.

The bulk vaccine and final product must pass one or other of the following potency tests:

(1) Scarrification of 10 cm^2 of the skin of two rabbits and inoculation with 0·2 ml of a 1:10,000 dilution of vaccine must produce more than one characteristic discrete vesicle.

(2) There shall be more than 5×10^7 pock-forming units per ml. of vaccine.

(3) The vaccine shall titre at least 7×10^7 LD_{50} in eggs.

The use of tissue cultures for potency assay has been suggested and initial studies show that they will be satisfactory when a standard titre has been defined.[16]

After the potency tests the final product must again pass the sterility tests and a final potency test after 4 weeks incubation at 37°C. It must contain no human protein. All liquid vaccines must be kept below −10°C and all dried vaccines at less than +10°C. The vaccine must not be used more than 12 months after the potency test and 3 or 6 months after issue for liquid and dried vaccine respectively.

Smallpox vaccination is very efficient in reducing the incidence of smallpox and providing effective revaccination is carried out the disease can be prevented completely. Unfortunately, complications are not infrequent especially in adults receiving a primary vaccination. Young children up to about 2 years show a very low incidence of such complications so that vaccination should always be carried out in infancy.

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