

Textbook of Medical Virology

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Preface

The great advances which have taken place in the field of virology during the last two decades have made the subject one of increasing importance to students and practitioners of medicine. Until now, most recent advances in the subject have been available only in the original literature, specialist monographs, review articles, or in advanced texts designed for the professional virologist. This book is designed to meet the needs of students and practitioners of medicine; in it, I have tried to present a simple, readable, and up-to-date account of virology in its relation to medicine. The text is, however, sufficiently comprehensive to make it useful for medical graduates reading for the Membership examination of the College of Pathologists, and for other postgraduate diplomas. Although written primarily for students, the book should prove useful to general practitioners who wish to bring themselves up to date in the subject. There is a particular need, since they are frequently concerned with the diagnosis and prevention of virus diseases.

The book is based on a course of lectures given to students at University College Hospital Medical School, and is divided into three parts. The first part is devoted to general virology, and deals with the principles of technique, structure, nomenclature, and replication, as well as with mechanisms of virus infection and the host's resistance to it. Parts 2 and 3 deal with various aspects of viral and rickettsial infections, respectively. With students in mind, and in the interests of general readability and simplicity, no detailed references have been included but credit is given to those who have made specially important contributions to the subject. Inevitably, many names which no doubt should have been included have been omitted, but I am deeply conscious of my indebtedness to all those whose long, arduous, and often brilliant research has made this book possible. Nearly all the references to original papers which may be

required can be found in the books which are listed in the guide to further reading, and others can easily be traced in the *Index Medicus*. I have not therefore considered it necessary to repeat these references again here.

My choice of arrangement and nomenclature requires a word of explanation. I have adopted, as far as possible, the nomenclature proposed by the Provisional Committee for the Nomenclature of Viruses which is likely to receive international agreement before long. This has not been allowed to prevent my using the more widely known colloquial names for the purpose of achieving greater clarity. In the arrangement of subject matter, the claims of clinical logic have been allowed to outweigh those of virus taxonomy; this is in keeping with the applied nature of clinical virology. Occasionally the superficial and dogmatic treatment of subjects which others may consider controversial has been dictated by the necessity of keeping the book within a reasonable compass.

No textbook can be written without reference to other works on the subject, and I have referred constantly to many classic works on virology. I am particularly indebted to Rivers' famous textbook *Viral and Rickettsial Diseases of Man*, now edited by Horsfall and Tamm. This may be regarded as one of the great classics of medical literature and it has served as my model in preparing this book. Most other works to which I have referred are listed in the guide to further reading, to which those requiring further information may turn.

It is a pleasure to acknowledge, with gratitude, the help and co-operation of many friends and colleagues. I am indebted to Professor G. Belyavin for his generous support and encouragement, and for reading the manuscript. Especial thanks are due to my friend and colleague Dr Peter Higgins who provided me with many tissue culture preparations and other material for illustration. I am also grateful to Professor J. F. Smith for the sections illustrating poliomyelitis, rabies, and zoster infections; and to Dr D. McSwiggan and Dr M. W. N. Nicholls who provided material infected with coxsackie virus.

I am indebted to Mr V. K. Asta for his fine and skilful drawing of Figs 3, 5-8, 15a-17a, 21, 33, 36a, 37, 38, 42, 43, 45, 46, and 49 and to University College Hospital Medical School for permission to

reproduce them. I owe particular thanks to Mr H.L. Spindler F.R.M.S., for all the photomicrography and for the photography of fresh preparations; his contribution is gratefully acknowledged. Thanks are also due to Mrs Margaret Spindler for help with some of the preparations and to Gillett and Sibert for the loan of photomicrographic apparatus. For making some of the photographic reproductions, I have to thank Mr A.C. Lees and the staff of the photographic department, University College Hospital Medical School. I also wish to express my thanks to Mr A.T. Picton and Miss S. Bevan, of University College Hospital Medical School library, who spared no effort in answering my numerous library requests.

I am especially grateful to the many authors, editors, and publishers who so readily granted me permission to reproduce figures from their various publications, and most of all to those who supplied me with actual photographs and unpublished material. Their contributions are acknowledged in the appropriate places.

My greatest thanks are due to Miss Diana Wilson who has typed and retyped the manuscript, and has been of invaluable assistance in many different ways. The unfailing patience and courtesy of Mr Per Saugman of Blackwell Scientific Publications Ltd is also gratefully acknowledged.

Finally, I wish to pay tribute to my teachers, the late Professor Wilson Smith F.R.S. with whom I was privileged to work for several years, and to Professor George Belyavin, teacher, friend, and colleague of many years standing. The following pages owe much to their influence.

PART 1

General Virology

CHAPTER 1

Introduction

The classical researches of Pasteur (1822-95) established the germ theory of infective disease beyond doubt, and before the end of the nineteenth century the causative bacteria of many diseases had been isolated and identified. There were some infective diseases, however, from which no causative bacteria could be isolated and whose aetiology remained in doubt. The first clue that another type of micro-organism might be responsible for them was provided by Ivanovsky in 1892. He observed that the mosaic disease of tobacco plants was transmitted by an agent which passed through filters whose pores were small enough to hold back ordinary bacteria. But, it was not until 1899 that Beijerinck seized upon the significance of this observation, and realized that the filterable agent of tobacco mosaic disease was something other than a bacterium, referring to it as a *contagium vivum fluidum*. Later, the term filterable virus, or virus, was applied to this type of filterable infective agent.

Infective agents similar to that of tobacco mosaic disease were soon recognized as the cause of a number of diseases of man and animals. They were characterized by the specific pathological effects produced in their hosts, their filterability, their failure to grow on ordinary bacteriological media, and by their invisibility in the light microscope. By these criteria, the viral aetiology of foot-and-mouth disease of cattle was proved by Loeffler and Frosch in 1898, that of yellow fever by Walter Reed and his colleagues in 1901, and that of poliomyelitis by Landsteiner and Popper in 1909. A few years later, the independent observations of Twort and d'Herelle showed that even bacteria were susceptible to infection with specialized viruses called bacteriophages.

The numerous unsuccessful attempts by these and other workers to grow viruses on artificial, non-living, media, using the usual bacteriological methods, led to the realization that the most

characteristic property of viruses is their dependence on living host cells for replication. Because of this, the range of viruses studied in the laboratory was for many years limited to those few which were pathogenic for experimental animals. This restriction was partially lifted by the work of Goodpasture, Woodruff, and Buddingh who, in 1931, succeeded in growing the viruses of fowlpox, vaccinia, and herpes simplex on the chorioallantoic membrane of the chick embryo. Since then, the discovery that other parts of the chick embryo are also susceptible to virus infection has led to the extensive use of chick embryos in virus studies. This advance in technique freed the virologist of some of the difficulties inherent in the use of experimental animals, which have to be fed, housed, and protected from extraneous diseases. For the first time, large scale preparation of some viruses for use in vaccines, and the investigation of some aspects of cell-virus interaction became possible.

Although the chick embryo is susceptible to a number of viruses, including those of influenza, mumps, yellow fever, and psittacosis, it is not susceptible to all. Intensive investigation of those viruses, including poliovirus, to which the chick embryo is not susceptible had therefore to wait for some further advance in technique. This was provided in 1949, when Enders, Weller, and Robbins discovered that the virus of poliomyelitis would grow in cells of human and monkey tissues maintained in artificial culture. True, others had previously used tissue cultures for growing viruses, but the difficulty of maintaining sterile conditions without the aid of antibiotics, and the necessity of subinoculation into experimental animals to demonstrate virus growth, prevented the adoption of tissue culture methods for routine use. The achievement of Enders and his colleagues was two-fold: first, they succeeded in growing poliovirus in non-nervous tissue and thereby demonstrated that the tissue tropism of the virus was not as rigidly specific as had previously been thought; secondly, they realized that the degeneration and necrosis of infected cells, which they were able to observe with the low power objective of the light microscope, was sufficient evidence of virus growth, making resort to animal inoculation unnecessary. Neutralization of this cytopathogenic effect by the appropriate antiserum left no doubt that tissue cultures could provide an effec-

tive substitute for experimental animals, with the added advantages of easy manipulation and economy.

It is no exaggeration to say that the introduction of modern tissue culture techniques has revolutionized the study of viruses. Not only have they made the production of poliovirus vaccine possible, but they have provided techniques for the selection of avirulent variants for use in live virus vaccines. Of no less importance, they have led to the isolation of a great number of new viruses, many of which were previously unsuspected. In the research laboratory, they have facilitated the study of viruses at the cellular level, and have thereby increased our understanding of the cell-virus relationship.

Concurrently with the important advances made in the biological study of viruses, exciting progress was made in the elucidation of their physical and chemical characteristics. After Beijerinck's original concept of the virus as a *contagium vivum fluidum*, it was some years before the particulate nature of viruses came to be generally accepted. Even the early visual demonstration by light microscopy of some of the larger viruses was not always accepted as evidence of the particulate nature of viruses. The issue was, however, settled in the early 1930s by Elford and his colleagues who measured the size of various viruses with collodion membrane filters, which they were able to make to any required pore size. They showed that viruses are not only particulate but that different viruses are characterized by their size, which may vary from 10 to 300 m μ .

The development of the electron microscope in the late 1930s and its application to virus studies in the following decade made the visual demonstration of even the smallest viruses possible. Thereafter, the distinctive size and morphology of various viruses was no longer in doubt. Recently, the introduction of the negative staining technique in electron microscopy has revealed a wealth of detail in the fine structure of viruses, hitherto undreamt of.

The new insight into the physical structure of viruses which has been gained in the last 20 years has been paralleled by the increase in our knowledge of virus chemical structure. A prerequisite for meaningful chemical analyses of viruses is the supply of pure preparations. This was achieved spectacularly by Stanley in 1935 when he succeeded in crystallizing the tobacco mosaic virus (TMV).

Chemically, tobacco mosaic virus proved to be relatively simple consisting of 94.4% protein and 5.6% ribonucleic acid (RNA). More recently, some of the smaller animal viruses, including the poliovirus, have been crystallized (Fig. 1). The finding that they too are nucleoproteins, consisting solely of RNA and protein, serves to show that there is a fundamentally close relationship between some of the animal and plant viruses. It is now known that all viruses con-

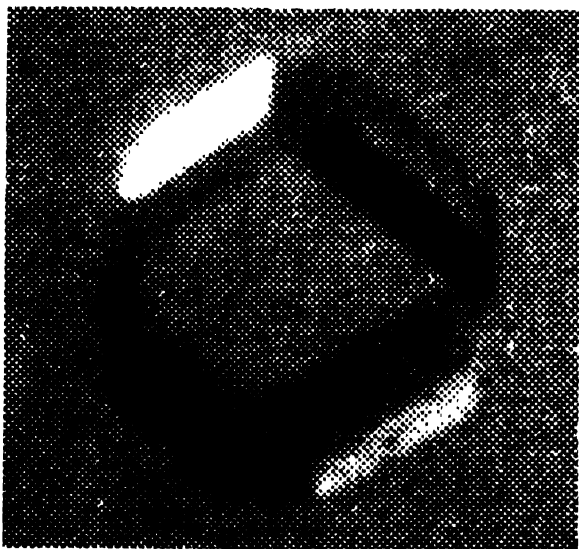


Fig. 1. Crystal of type 1 poliovirus [from Russell L. Steere and Frederick L. Schaffer (1958) *Biochim. et Biophys. Acta* 28, 241-46 (Elsevier Publishing Co, Amsterdam)].

sist essentially of protein and nucleic acid, of which the latter may be either ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), but never both. Although smaller viruses consist of protein and nucleic acid only, larger ones like influenza and vaccinia viruses are structurally more complex and possess, in addition, lipid and carbohydrate components as well as some enzymic activity.

A remarkable relationship between structure and function was established by Hershey and Chase in 1952. They demonstrated that

infection of the bacterial host cell by T_2 bacteriophage is accomplished by the intracellular penetration of the 'phage DNA only, most of the 'phage protein remaining at the cell surface. The important part which the viral nucleic acid plays in initiating virus replication was confirmed, in 1956, by Fraenkel-Conrat and by Gierer and Schramm, who showed, independently, that the RNA of tobacco mosaic virus, freed from viral protein, is infective and leads to the production of complete virus particles. Evidence that the isolated nucleic acid moiety of some animal viruses, although not all, is similarly infective has now been obtained by a number of investigators. Progeny virus resulting from infection with nucleic acid possesses all the distinctive marker characteristics of the parent virus from which the nucleic acid was extracted. It thus appears that all the information necessary for the synthesis of viral nucleic acid and protein, and for the formation of new virus particles, resides in the nucleic acid moiety. The discovery that nucleic acid is the infective principle of the virus particle and that this macromolecule is endowed with genetic functions has had a profound influence on biological thinking, far beyond the confines of virology. Indeed, at this level where chemistry, genetics and biology meet we are at the very threshold of life. For this reason, a great deal of effort is now being applied to the investigation of the chemical structure and function of nucleic acids, and the means by which they control the synthesis of proteins and exert their genetic functions. Viruses, because they are chemically the simplest organisms known, have proved to be a most important tool in these studies.

In the short span of 75 years, the rather nebulous concept of a *contagium vivum fluidum* has been replaced by a considerable corpus of knowledge about a class of micro-organisms known as viruses. Although these vary considerably in size, morphology, chemical constitution, and clinical behaviour, they possess a number of characteristics in common which differentiate them from bacteria (Table 1). They replicate only in the living host cell and are unable to grow on artificial media. Because of their small size they are filterable through bacterial filters and, with few exceptions, are invisible in the light microscope. They do not, like bacteria, replicate by binary fission but by a complex mode of replication initiated

by the viral nucleic acid. Unlike bacteria or the cells of higher organisms which contain both types of nucleic acid, RNA and DNA, viruses possess only one or other type by which they are characterized as RNA or DNA viruses. Finally, the insensitivity of viruses

Table 1. Properties of bacteria, viruses and rickettsiae

Organisms	Bacteria	Viruses	Rickettsiae
Cultivation	Artificial media	Living host cells	Living host cells
Size	> 500 mμ	< 250 mμ	500 mμ
Visibility	Light microscope	Electron microscope	Light microscope
Filterability	Non-filterable	Filterable	Non-filterable
Replication	Binary fission	Complex	Binary fission
Nucleic acids	DNA + RNA	DNA or RNA	DNA + RNA
Antibiotics	Sensitive	Not sensitive	Sensitive

to the commonly used antibiotics, effective against bacteria, is a distinctive characteristic which confronts the practising physician almost daily.

CHAPTER 2

Techniques of Virus Cultivation

I. Animal and Chick Embryo Inoculations

Animal viruses are cultivated in three types of host: susceptible laboratory animals, chick embryos, and tissue cultures. These hosts are analogous to the various types of media employed for isolation of bacteria from pathological specimens or other sources.

Animal Inoculation

Until the introduction of chick embryo and tissue culture methods, animal inoculation was the sole means of virus cultivation. Usually, virus infection produces a specific pathological effect on the animal host, which is manifested by illness or death. Although the technique of animal inoculation has now been largely superseded by other more convenient methods of isolation, there remain a few viruses for which a laboratory animal is the only susceptible host, or is the host of choice.

Age, as well as species, is important in determining the choice of an animal host. Thus for isolation of Coxsackie viruses, and some of the arboviruses, the use of suckling mice is mandatory because of their high susceptibility, which is lost after weaning. Adult mice may be used for the isolation of the viruses of herpes simplex, rabies, and some of the arboviruses, as well as for the organism responsible for psittacosis. For the isolation of Rickettsiae, guinea-pigs are the hosts of choice. Details of animal inoculation techniques used in virus diagnosis are summarized in Table 2.

Table 2. Animal hosts currently used for virus isolation

Host	Virus	Route of inoculation	Signs of infection
Suckling mouse	Coxsackie	Intracerebral or Intraperitoneal	Weakness, tremor, spastic or flaccid paralysis, and death. Generalized and local myopathy post-mortem
	Herpes simplex	Intracerebral or Intraperitoneal	Weakness, cyanosis, abdominal distension, occasional convulsions, paralysis, and death
	Arboviruses	Intracerebral	Ruffled fur, tremor, ataxia, convulsions, and death
	Lymphocytic choriomeningitis	Intracerebral	Tremor, convulsions, generalized spasticity, and death
Mouse	Rabies	Intracerebral	Tremor, paralysis, convulsions, and death. Negri bodies in brain post-mortem
	Psittacosis	Intranasal	Pneumonia and death
		Intraperitoneal	Peritonitis, necrotic lesions of liver and spleen, death
	Lymphogranuloma venereum	Intracranial	Paralysis and death
		Intranasal	Pneumonia and death
		Intracerebral	Weakness, paralysis, and death
Guinea-pig	Rickettsia tsutsugamushi	Intraperitoneal	Ruffled fur, ascites, and death. Intracellular rickettsiae post-mortem
	Rickettsiae	Intraperitoneal	Fever, rickettsaemia, and presence of antibody in animal's convalescent serum. Different types of rickettsia may be differentiated by the scrotal reaction.

Chick Embryo Techniques

Since the original discovery of Goodpasture and his colleagues, that some viruses will grow on the chorioallantoic membrane of the chick embryo, both the allantoic and amniotic membranes as well as the embryo itself have been found susceptible to at least some viruses. Viruses which grow in the chick embryo, and the particular tissues which are susceptible to them, are listed in Table 3.

Table 3. Viruses isolated in the chick embryo

Route of inoculation	Virus	Evidence of infection
Chorioallantoic membrane	Variola	Pocks
	Vaccinia	
	Herpes simplex	
	Influenza (egg adapted)	Production of haemagglutinating virus
Allantoic cavity	Mumps (egg adapted)	Production of haemagglutinating virus
	Newcastle disease	Death of the embryo and production of haemagglutinating virus
	Psittacosis (egg adapted)	Death of the embryo and production of elementary bodies
	Influenza (Primary isolation)	Production of haemagglutinating virus
Amniotic cavity	Mumps (Primary isolation)	Production of haemagglutinating virus
	Psittacosis—L.G.V.	Death of the embryo and elementary bodies in yolk sac tissue
Yolk sac	Rickettsiae	Death of the embryo and rickettsiae in yolk sac tissue
	Yellow fever	Death of the embryo and production of virus

Before inoculation by any route, fertile eggs are incubated in commercial type incubators (Fig. 2) in which they are automatically turned in an atmosphere maintained at 37.5°C , and in which the

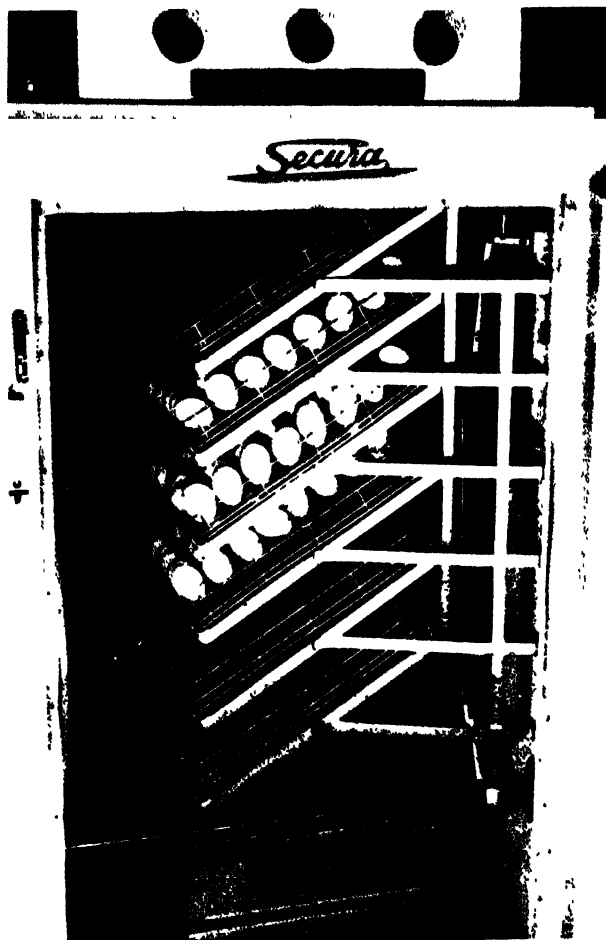


Fig. 2. Egg incubator.

relative humidity is adjusted to 40–70%. Eggs are usually inoculated after 9–12 days incubation, at which time the embryo is well formed and the amniotic and allantoic cavities are fully developed (see

Fig. 3). Immediately before inoculation, the eggs are transilluminated to confirm the viability of the embryo and its position, and to mark the position of the air sac. After inoculation, eggs are incubated for a further period at 35°C.

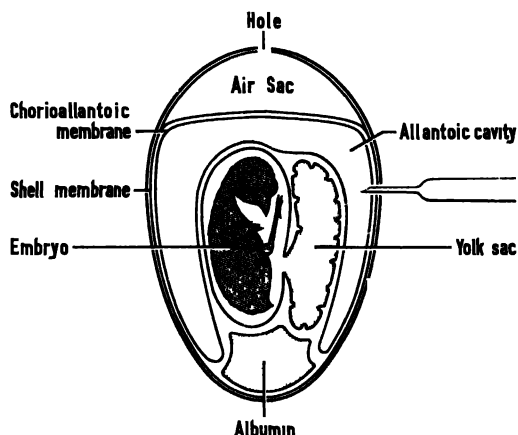


Fig. 3. Allantoic inoculation of 10-day-old chick embryo.

(a) Chorioallantoic inoculation

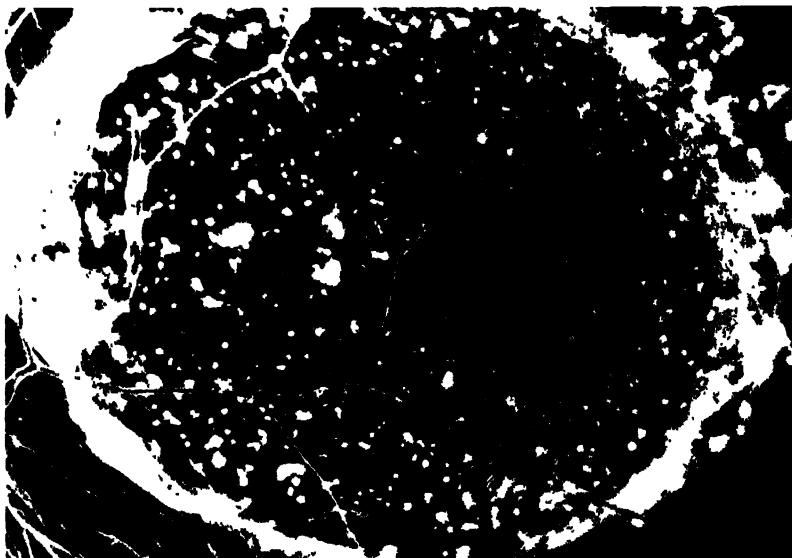
Viruses, like vaccinia, variola, or herpes, which are grown routinely on the chorioallantoic membrane do so with the formation of characteristic pock-like lesions (Fig. 4). Each pock lesion is theoretically derived from one infective unit, and is analogous to a single colony of bacteria on a bacterial culture plate. Pock-forming viruses are therefore easily quantitated by methods similar to the colony count method for counting bacteria. With some viruses, of which Newcastle Disease (NDV) and equine encephalomyelitis are examples, the criterion of infection, after chorioallantoic inoculation, is death of the embryo rather than the formation of pock lesions.

(1) *Technique of chorioallantoic inoculation*

Eggs which have been incubated for 12 days are prepared for inoculation by marking the air sac and the point of inoculation; for the latter, a site over a well developed part of the chorioallantoic membrane is chosen. A small area of shell, about 0.5 cm in diameter, is

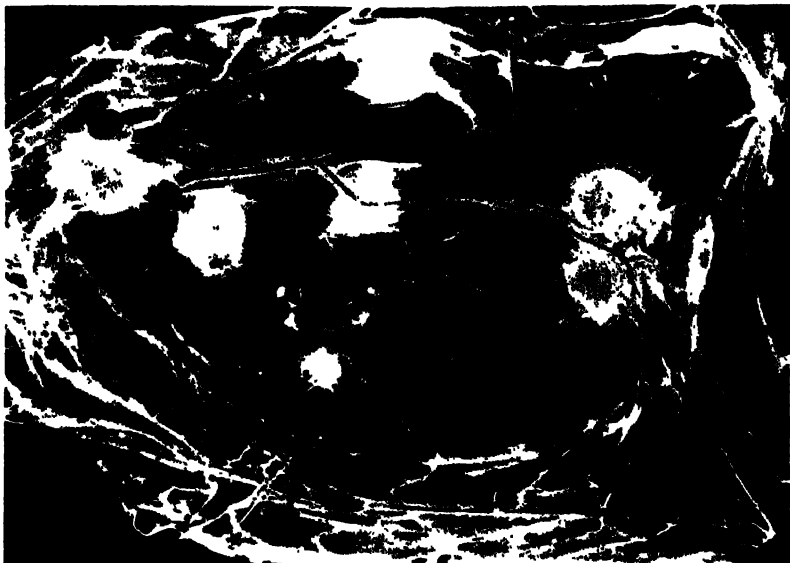


(a) Normal chorioallantoic membrane.

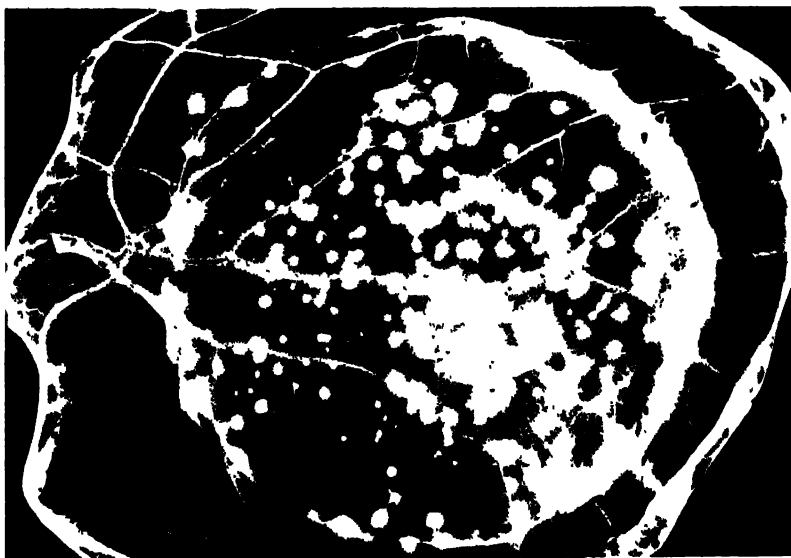


(b) Pocks produced by variola virus.

Fig. 4



(c) Pocks produced by vaccinia virus.



(d) Pocks produced by *Herpesvirus hominis* (herpes simplex).

Fig. 4

removed from the site of inoculation, with the aid of a dental drill, to expose the shell membrane; at the same time a small perforation is made in the shell over the air sac. The inoculation site is encircled by a rim of sterile paraffin wax, and the inoculum of 0.05 ml is gently deposited from a pipette onto the exposed shell membrane. The shell membrane is incised with a blunt instrument directed through the inoculum, care being taken not to damage the underlying chorioallantoic membrane, and gentle suction is then applied to the perforation over the air sac with a rubber teat. This allows the chorioallantoic membrane to fall away and become detached from the shell membrane, forming an artificial air sac. At the same time

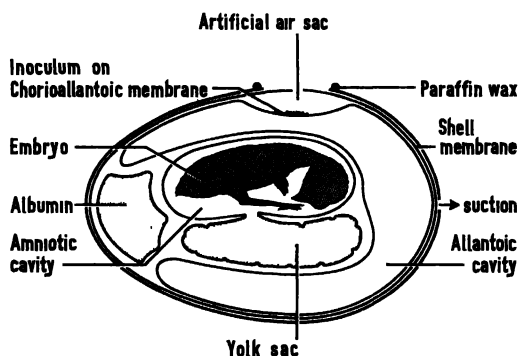


Fig. 5. Chorioallantoic membrane inoculation of 10-day-old chick embryo.

the inoculum is sucked in and distributed uniformly over the detached area of chorioallantoic membrane (Fig. 5). The inoculation site is sealed with Scotch tape, and the egg is incubated, with the inoculation site uppermost, for a period of 2-4 days, depending on the virus which has been inoculated.

After incubation, the shell over the artificial air sac is removed and the exposed chorioallantoic membrane is excised, washed in sterile saline, and examined in a Petri dish.

(b) Amniotic inoculation

Virus inoculated by the amniotic route easily reaches the embryo lung and other embryonic tissues, and this may account for the

greater efficiency of the amniotic over other routes of inoculation for primary isolation of certain viruses. Once adapted to the egg by growth in the amniotic cavity, influenza and other viruses grow equally well, if not better, in the allantoic cavity. For primary isolation of influenza viruses from human sources, amniotic inoculation is the method of choice and is extensively used in diagnosis.

(1) Technique of amniotic inoculation

Eggs which have been incubated for 9–13 days are used. The shell over the air sac is removed and the opaque shell membrane is cleared

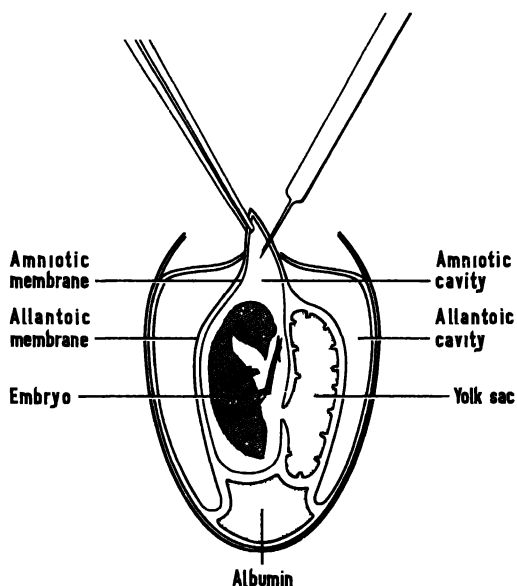


Fig. 6. Amniotic inoculation of 10-day-old chick embryo.

with a coating of sterile liquid paraffin. The underlying chorioallantoic membrane, which is now exposed, is pierced with a pair of non-toothed forceps which are used to grasp the amnion and deliver it through the chorioallantoic membrane. The inoculum, 0.05–0.2 ml is now inoculated into the amniotic cavity with a syringe or calibrated fine-tipped pipette, under direct vision (Fig. 6). The air sac is sealed with Scotch tape and the eggs are incubated for 3–5 days.

Eggs are transilluminated daily to detect the occurrence of embryonic death. Deaths occurring within 24 hours of inoculation are assumed to be non-specific in origin and the eggs are discarded. After incubation, the amniotic fluids are harvested for testing. The tape seal is removed, the underlying chorioallantoic membrane is resected and any allantoic fluid is removed, the amnion is then easily grasped with forceps and pierced with fine-tipped pipette through which the amniotic fluid is removed.

(c) Allantoic inoculation

Allantoic inoculation is the method of choice for the growth of influenza viruses. Although primary isolation of influenza virus is

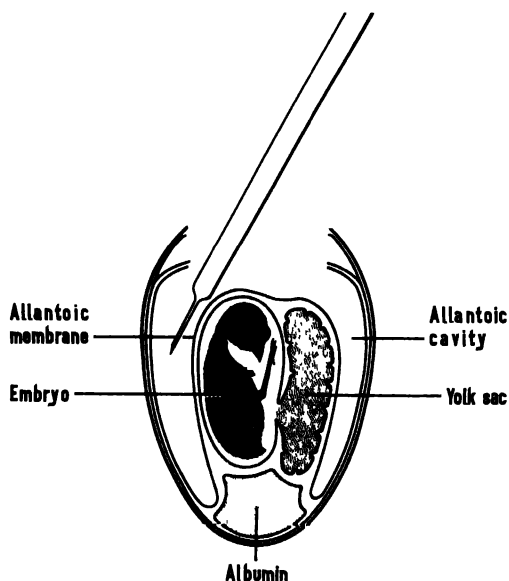


Fig. 7. Harvesting allantoic fluid from 11-day-old chick embryo.

best achieved by amniotic inoculation, the virus will grow readily in the allantoic cavity after a few amniotic passages. The advantage of using the allantoic cavity for growing egg adapted influenza virus is the relative abundance of allantoic fluid, which provides a rich source of virus for vaccine production or experimental work.

(i) Technique of allantoic inoculation

Eggs which have been incubated for 10–11 days are prepared for inoculation by marking the air sac and the point of inoculation; for the latter, a site over a non-vascular area of the chorioallantoic membrane is chosen. A small area of shell about 0.25 cm in diameter, overlying the inoculation site, is removed with the aid of a dental drill, care being taken to leave the exposed shell membrane intact. At the same time, the shell over the air sac is perforated to allow the release of any excess pressure produced by inoculation. Inoculations of 0.05–0.2 ml are made through the exposed shell membrane with a syringe or calibrated fine-tipped pipette (Fig. 3). After inoculation, the site is sealed with sterile paraffin wax.

After 48 hours incubation, the eggs are chilled at 4°C for a few hours to prevent haemorrhage during harvesting. When chilled, the shell over the air sac is removed, the underlying membranes are incised and retracted with forceps, and the allantoic fluid is easily removed with a pipette (Fig. 7).

(d) Yolk-sac inoculation

The technique of yolk-sac inoculation is used for cultivation of the

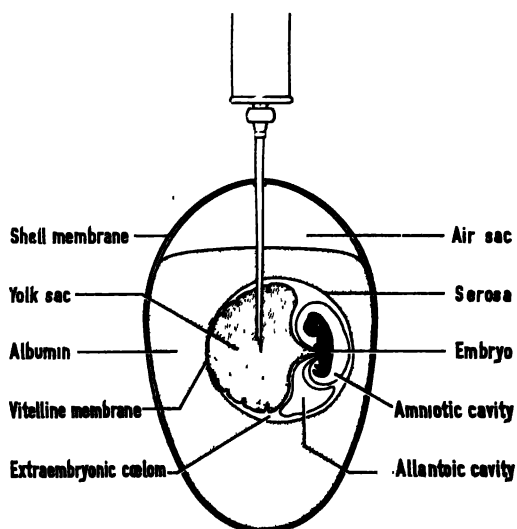


Fig. 8. Yolk sac inoculation of a 5-day-old chick embryo.

psittacosis-lymphogranuloma-trachoma group of organisms, and the Rickettsiae. The method may be used both for isolation, and the preparation of vaccines.

(i) Technique of yolk-sac inoculation

Eggs are usually used after 5-6 days incubation, at which stage the yolk sac is relatively large. The shell over the air sac is perforated and inocula of 0.5-1.0 ml are made directly into the yolk sac, using a syringe and a number 21 gauge needle. The needle is passed down the long axis of the egg for a distance of 3 cm to a point just beyond its centre, where the injection is made (Fig. 8).

After 3-8 days incubation, the shell over the air sac is removed and the underlying membranes resected. The embryo is extracted with forceps and released by cutting the membranes and the umbilical stalk. The remaining yolk sac may now be delivered into a Petri dish by traction on the distal part of the umbilical stalk.

CHAPTER 3

Techniques of Virus Cultivation

II. Tissue Culture Techniques

The survival of virus in cells maintained in artificial culture was demonstrated by Steinhardt and his colleagues, as long ago as 1913. Although they demonstrated the survival of vaccinia virus in cultures of guinea-pig and rabbit corneal tissues, they were unable to obtain unequivocal evidence of virus multiplication. This was provided by Maitland and Maitland in 1929, using hen kidney tissue suspensions, and was confirmed later by other workers. The adoption of tissue culture techniques for routine purposes was prevented at that time by the great difficulty of maintaining aseptic conditions. When antibiotics came into general use, tissue culture techniques for the growth of viruses became a possibility, and by 1949 Enders, Weller, and Robbins had succeeded in growing poliovirus in human embryonic and monkey kidney tissues.

From the growth of poliovirus in non-nervous tissue, and in tissues which in the intact animal are resistant to infection, it soon became apparent that susceptibility to a wider range of viruses is displayed by cells in culture than in their natural habitat. Furthermore, the ease with which virus infection in tissue culture could be detected provided an added advantage to a technique already preferred because of its ease of manipulation and economy.

Types of Tissue Culture

(a) Fragment cultures

Tissue cultures may be initiated from tissue fragments which are either suspended in a nutrient medium, or are fixed to the glass wall

of a tissue culture vessel or other solid base, usually with the aid of plasma or fibrin clots. When the tissue fragments are fixed, cells grow out forming a sheet on the glass or other solid surface and may be examined microscopically. This renders the cell degeneration produced by virus infection easily observable.

(b) Cell cultures

Treatment of minced tissue fragments with trypsin disperses the tissue cells so that suspensions consisting of single or small clumps of cells may be prepared. This technique, which was first described by Rous and Jones in 1916, was introduced, or more correctly re-introduced, into modern tissue culture techniques by Moscona in 1952. Inocula of trypsin dispersed cells are used to initiate suspended cell cultures in which cells are freely suspended in nutrient medium; alternatively, they may be introduced into suitable glass tubes, or other culture vessels, where they form monolayer cultures in which cells are spread out uniformly over the glass surface, in a layer one cell thick. Cell cultures derived from tissues treated with trypsin, or with chelating agents which are similarly effective in dispersing cells, have now almost entirely superseded the use of fragment cultures for virological purposes.

(i) Suspended cell cultures

Suspended cell cultures are employed in research for the study of virus-host cell interactions, as a source of cells for the initiation of monolayer cultures, and for the cultivation of viruses for use in vaccines. For best results, the cells are maintained in suspension by gentle automatic stirring devices, as in the spinner cultures described by Cherry and Hull.

(ii) Monolayer cultures

Primary cell cultures. Without doubt, monolayer cultures are the most useful for virus isolation and diagnosis. For their preparation, a number of human and animal tissues of foetal, adult or tumour origin may be used. Cultures derived directly from the appropriate tissue, as they are needed, are referred to as primary cell cultures. This type of culture, which is susceptible to a wide range of viruses,

is extensively used for virus isolation. The most commonly used tissues for primary cell cultures are monkey kidney, human foetal kidney or amniotic membrane, hamster kidney, and chick embryo tissue. The choice of tissue usually depends on its availability and the particular virus to be isolated. The use of monkey kidney or human amnion cells can be made more economical by preparing secondary subcultures from the primary monolayers, after dispersal of the cells by trypsin. Satisfactory monolayers cannot, however, be obtained after more than one subculture.

Primary cell cultures are prepared by treating minced tissue fragments, previously washed in saline, with 0.25% trypsin in phosphate buffered saline, pH 7.5, in two stages. The minced tissue is first suspended in 100 ml of trypsin solution and stirred for 10 minutes, the tissue fragments are then allowed to settle, and the supernatant which contains cytotoxic substances is discarded. The tissue fragments are now treated with a further 100 ml of trypsin solution at 4°C overnight. The dispersed cells are then deposited from suspension by light centrifugation, washed in balanced salt solution, and resuspended in sufficient nutrient medium to give concentrations of 300,000–500,000 cells per ml. The cell concentrations are adjusted by counting the cells in a haemocytometer or by using a haematocrit tube. 0.5 ml of the standard cell suspension is enough to seed a test tube. Seeded tubes are incubated at 37°C and confluent monolayers are formed after 5–10 days (Fig. 9).

Continuous cell lines. A number of mammalian cell types, usually of malignant origin, have been subcultured continuously for an indefinite period. These are referred to as established or continuous cell lines, of which the best known is the HeLa cell line. This was established by Gey and his colleagues in 1951 from a uterine cervical carcinoma excised from a patient whose initials gave the name HeLa to this type of cell. Other examples of continuous cell lines are the KB cell line derived from a carcinoma of the nasopharynx, the HEP-2 line derived from a human epidermoid carcinoma, and the FL line of human amnion.

Continuous cell lines have many advantages, and some disadvantages, for virus work. They are readily available, making it unnecessary to hold a stock of monkeys or other animals for the

initiation of primary cultures. They grow more quickly and luxuriantly than primary cell cultures, but are less susceptible for the isolation of many viruses, including echo and parainfluenza viruses. Many continuous cell lines are malignant in origin, and those which are not nevertheless display some of the characteristics of malignant cells, including abnormal morphology and heteroploid chromosomal configurations. It follows that they cannot be employed for the cultivation of viruses used in vaccines because of the theoretical



Fig. 9. Normal monkey kidney cell monolayer, stained with haematoxylin and eosin.

possibility of transferring some carcinogenic factor to the recipients of the vaccine.

All continuous cell lines display heteroploid chromosomal configurations, but recently Hayflick and Moorhead have introduced lines of human foetal cells which retain their normal morphology and normal diploid chromosomal configuration. Human diploid cells, unlike continuous cell lines, tend to die out after about 50 passages. For this reason, a cell bank is usually prepared and

ampoules of cells are stored frozen at -70°C . An ampoule is taken as required, from which monolayer cultures may be prepared and repeatedly subcultured until the culture dies out about the fiftieth passage. At this stage, another ampoule may be taken from the frozen stock and repeatedly subcultured until viability is lost. Using the bank technique a prolonged supply of diploid cells is assured.

Subcultures of continuous cell lines are prepared by methods similar to those used for primary cell cultures. The cells in culture are washed with phosphate buffered saline, free from calcium and magnesium ions, and are dispersed with a chelating agent, versene, at 37°C for 15-30 minutes. The dispersed cells are then sedimented by centrifugation, washed and resuspended in growth medium adjusted to give standard cell suspensions.

Cell Culture Media

(a) Growth medium

The development of monolayers from cell inocula requires a medium which promotes cell growth and multiplication, as well as the attachment and spread of cells on the glass surface. Such media are termed growth media and a number of different formulae are available. All consist essentially of balanced salt solutions, which provide the right conditions of osmotic pressure and pH; adequate quantities of essential inorganic ions; glucose as a source of energy; antibiotics for the maintenance of sterility; and various protein supplements to provide the various essential growth factors. Animal serum, in concentrations of 5-10%, is an essential protein supplement to which may be added in various combinations, enzymatic protein hydrolysates, yeast extract, embryo-tissue extracts, peptones, or serum fractions.

(b) Maintenance medium

Once monolayers are formed and are ready for virus inoculation a change of medium is necessary for two reasons. First, in place of cell growth and multiplication all that is required is the maintenance of cell viability and metabolism; secondly, serum must be removed

from the medium, or reduced in concentration, to exclude the presence of antibody or other virus inhibitory substances which it may contain. In vaccine manufacture, exclusion of serum from the final product is essential in order to prevent hypersensitivity reactions in the vaccine recipients. Before cells are inoculated with virus, the growth medium is therefore removed and the monolayers washed several times with balanced salt solution before a less rich maintenance medium is introduced.

Some cell cultures are more fastidious than others in their maintenance requirements. Where possible, a chemically defined medium free of protein is used. A number of complex chemically defined media are available, of which Morgan's medium 199 may be taken as an example. It contains 19 amino acids, various vitamins, several nucleic acid constituents, cholesterol, adenosine triphosphate, adenylic acid, and ferric nitrate, made up in a balanced salt solution containing glucose and antibiotics. Some cell lines, particularly continuous cell lines, require the addition of serum in concentrations of 2–5% to maintenance media, even to those as complex as Morgan's 199 medium. In these circumstances, it is necessary to test the serum before use in order to exclude the presence of antibody or virus inhibitory substances; where possible, replacement of serum with other protein supplements such as egg albumin, bovine serum albumin, or skim milk should be attempted.

Virus Inoculation

The multiplication of viruses in cell cultures is affected by a number of factors, including the temperature and the mode of incubation, as well as the composition and pH of the maintenance medium. The type of cell culture and the virus to be isolated will determine the maintenance medium used and the conditions of incubation. Thus rhinoviruses require a particularly low sodium bicarbonate concentration and their optimal temperature of growth is 33°C, unlike most other viruses whose optimal temperature of growth is 35–37°C. Measles and varicella viruses which require incubation periods as long as 7–10 days need relatively rich maintenance media. Virus

multiplication is often enhanced by rotation of the cell cultures, which facilitates the exchange of the fluid and gas phases on the monolayer. For this reason, infected cultures are usually rotated in roller drums (Fig. 10).

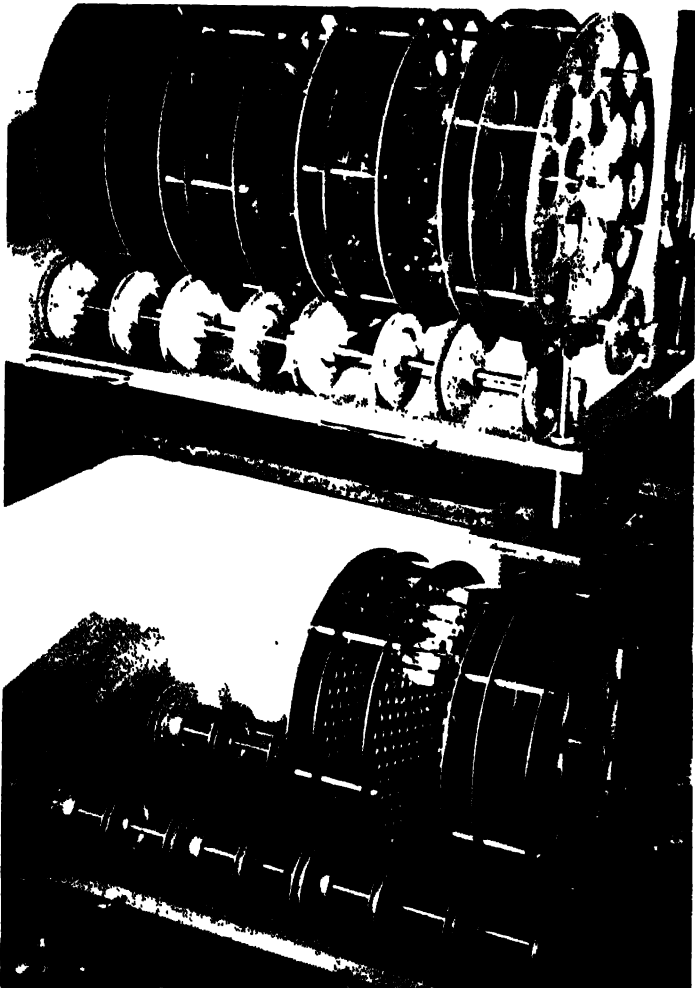


Fig. 10. Roller drum for rotation of tissue cultures.

Evidence of Viral Multiplication**(a) The cytopathic effect (CPE)**

The growth of virus in susceptible cells is recognized in one of several ways. The least complicated is the direct observation of cell degeneration and necrosis by light microscopy (Fig. 11). This cytopathic or cytopathogenic effect (CPE) is distinctive for certain types

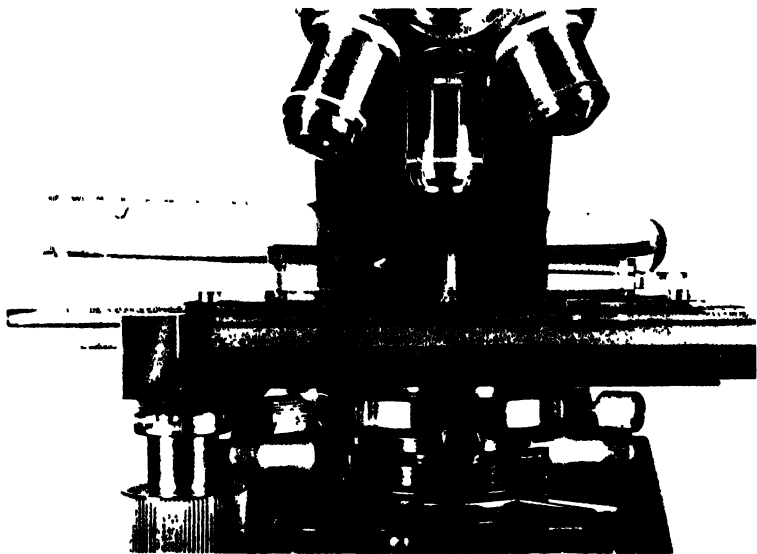


Fig. 11. Examination of tissue culture tube by low-power microscopy.

of virus. After infection with enteroviruses, cells become refractile, rounded, and smaller; their nuclei become pyknotic, and eventually the degenerated cells fall away from the glass (Fig. 12b). The formation of syncytia and multinucleate giant cells are characteristic of measles, respiratory syncytial, herpes simplex, mumps, and some parainfluenza viruses (Fig. 12c). CPE occurring within 24–48 hours of inoculation may be non-specific in origin due to the toxicity of the inoculum, particularly if this is a stool sample. The toxic effect can usually be diluted out by subculture. Sometimes, cell cultures undergo spontaneous degeneration, but this is easily recognized by

examination of uninfected cell controls which should always be incubated and examined in parallel with infected cultures. On occasion, spontaneous degeneration of monkey tissue cells may be due to contamination with endogenous simian viruses, which themselves produce CPE.

(b) Plaque formation

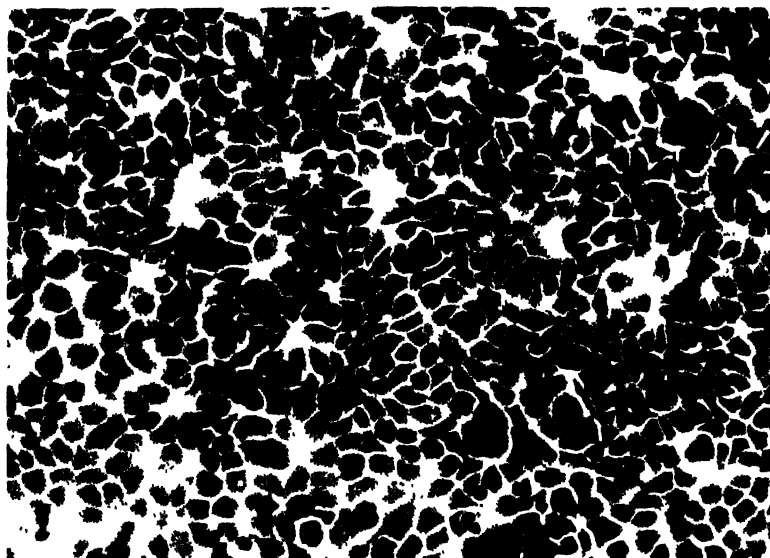
Cytopathic effects are produced by the unrestricted transport of virus from an initially infected cell, through the fluid medium, to other cells of the monolayer. The spread of virus may be restricted and localized if a thin layer of agar, incorporating the maintenance medium, is poured over the monolayer after virus has been adsorbed. As a result, localized areas of CPE (plaques) are produced which may be rendered more easily visible by staining with a vital neutral red stain. In stained preparations plaques stand out as unstained lacunae distributed in an otherwise red-stained monolayer (Fig. 13).

Theoretically, each plaque is produced from a single infective unit and is thus analogous to a pock lesion on the chorioallantoic membrane, or a colony of bacteria. The number of plaques produced by a given virus inoculum is therefore a measure of the number of infective units present, and the plaquing of viruses is a very useful method of quantitative assay. Furthermore, the size and shape of plaques produced by certain viruses is characteristic, and may be a useful criterion for virus identification. Indeed, mixtures of viruses which produce plaques of varied morphology can be purified by subculture from a single plaque, which is itself produced from a single infective unit. This is one of the methods of obtaining pure clones of avirulent variants for use in live virus vaccines.

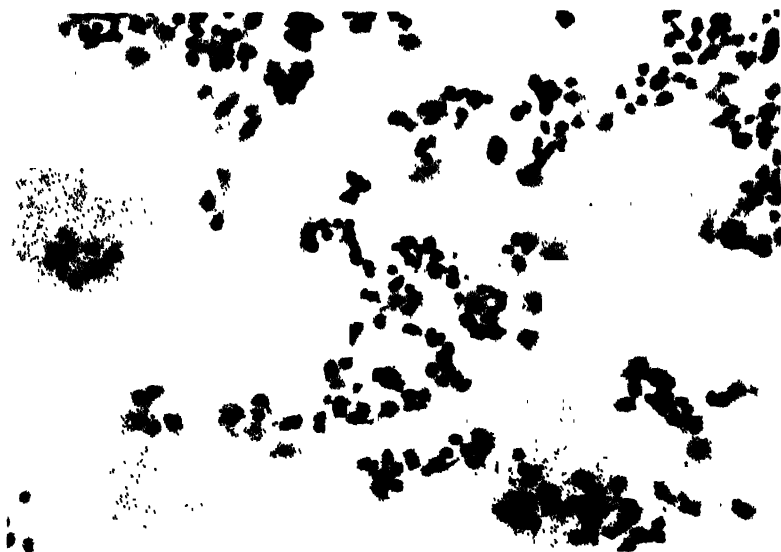
(c) Haemadsorption

Myxoviruses and paramyxoviruses which do not produce CPE in cell cultures can nevertheless be detected by the haemadsorption technique. These viruses characteristically adsorb to red blood cells and produce haemagglutination. Their presence in tissue culture may be recognized by introducing a guinea-pig red cell suspension into the culture after a few days incubation, and then examining

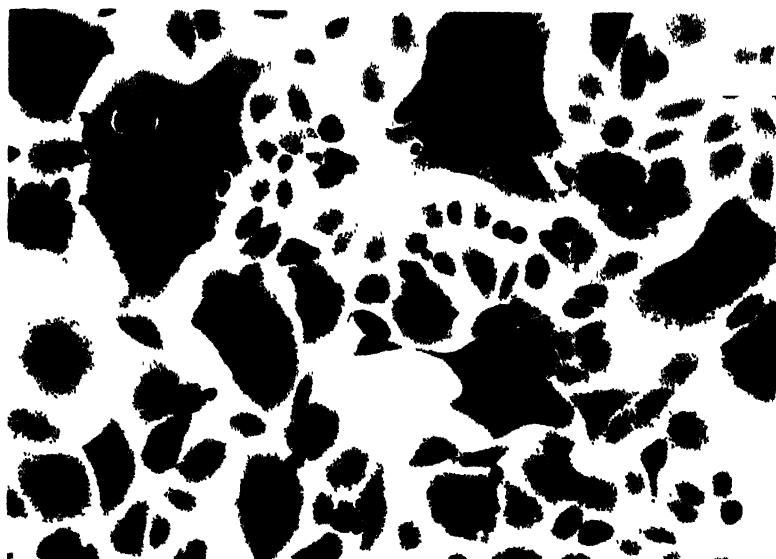
Fig. 12. Cytopathogenic effects in cell monolayers; preparations stained with haematoxylin and eosin.



(a) Normal HeLa cells.



(b) HeLa cells infected with poliovirus.



(c) HeLa cells infected with respiratory syncytial virus

infected and control cultures under the microscope. Infected cells are revealed by the clumps of erythrocytes which adhere to their surface (Fig. 14).

(d) Fluorescent antibody staining

Staining cultures with fluorescent antiviral antibody and examining them in ultraviolet light is another method of detecting the growth of viruses which do not produce CPE. The presence of viral antigen in infected cells is revealed by localized areas of marked fluorescence which are absent from uninfected control cultures.

(e) Interference

Infection of a cell with one virus sometimes prevents superinfection with another. This phenomenon of interference can be utilized for the recognition of some viruses which do not produce cytopathic effects. Cell cultures are infected with the test virus and are incubated for several days, infected and control cultures are then chal-

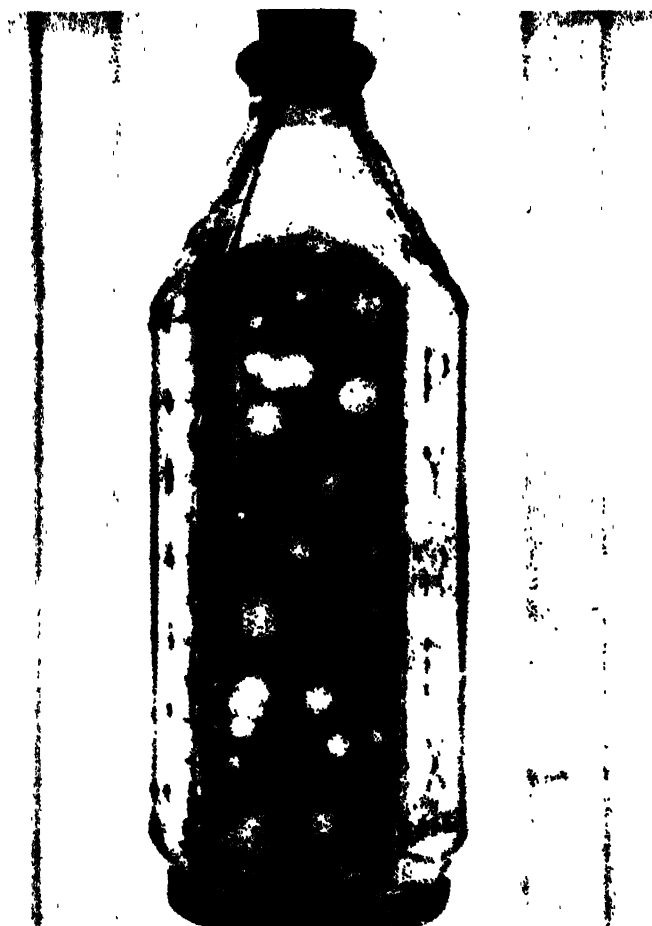


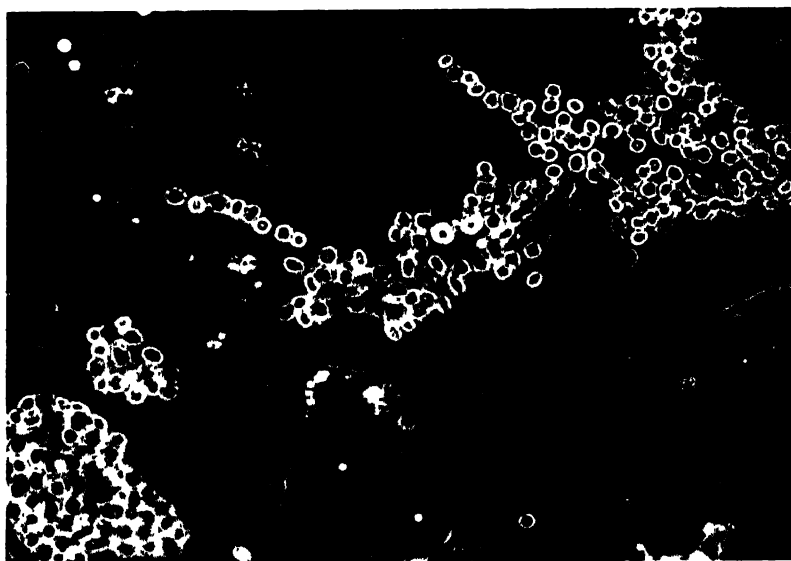
Fig. 13. Poliovirus plaques on a HeLa cell monolayer.

lenged with a cytopathogenic virus and incubated for a further 48–72 hours. Cultures in which the test virus has grown are resistant to the challenge virus and do not therefore exhibit any cytopathic effect. Control cultures and those in which the test virus has not grown remain susceptible to the challenge and undergo cytopathogenicity in the normal way. This is one of the techniques used for the isolation and identification of rubella virus.

Fig. 14. Haemadsorption viewed by phase contrast [photographs by Dr P. A. Riley].



(a) Normal monkey kidney cells.



(b) Monkey kidney cells infected with influenza virus.

CHAPTER 4

Serological Techniques in Virology

I. In vivo Neutralization Tests

Viruses are antigenic and their inoculation into patients or experimental animals, either naturally or artificially, produces an antibody response. The antibodies produced react with homologous viruses or viral antigens in vivo and in vitro, in the same way as bacteria and antibacterial antibodies. The familiar neutralization, precipitation and complement-fixation tests are therefore applicable to virological work, as are haemagglutination- and haemadsorption-inhibition tests which are widely used serological techniques in virology.

In diagnosis, serological techniques are usually employed for the identification and titration of antibodies in a patient's serum, using known viral antigens. An increase of four-fold or more in serum antibody titre against a particular virus or viral antigen, which occurs between the acute and convalescent stages of a disease, is accepted as evidence of infection with that virus. The demonstration of a rise in antibody titre is, with virus isolation, the key to diagnosis of virus infections. Of course, serological techniques may also be employed for the identification of unknown viruses or viral antigens, using known antisera.

Neutralization Tests

Virus which has reacted with its homologous antibody is rendered non-infective for susceptible hosts. Hence, antiviral antibodies are easily recognized by their ability to prevent any overt biological effects of virus infection in experimental animals, tissue cultures or chick embryos. Although in vitro tests are more convenient, the

neutralization test is still widely used because neutralizing antibodies are highly specific. Indeed, for some arboviruses and coxsackie viruses it has proved the most useful. However, non-specific neutralizing substances active against certain viruses are sometimes present in sera, and their removal by heat or chemical treatment is necessary before the neutralizing potency of sera against some viruses is tested.

(a) Experimental animals

Neutralization tests in animals are now confined, almost exclusively, to those viruses which produce overt reactions in adult or suckling white mice. The usual technique is to test serial dilutions of serum against a fixed dose of virus known to produce death, disease, or some pathological lesion in the test animals. The highest dilution of serum which protects 50% of the test animals against the effects of the inoculated virus represents the neutralization titre. Before any neutralization test, the fixed dose of virus to be used is estimated from a preliminary virus titration.

(1) Virus titration

The unit of virus activity is expressed as the highest dilution of a virus suspension which produces a required effect in the host animal, under standard conditions. To overcome any differences of susceptibility among the experimental animals and to increase the accuracy of the test, each virus dilution is tested in a group of six or more animals. The unit of activity, or titre, is then defined as the highest dilution of virus suspension which produces the required effect in 50% of the test animals. If death is the required effect, the unit of activity is termed an LD_{50} , i.e. a 50% lethal dose, which is a commonly used unit of virus activity.

The 50% end-point is calculated from experimental results by special formulae, of which the one commonly used is that proposed by Reed and Muench. For this calculation, which may be illustrated by data set out in Table 4, it is assumed that animals which die at a given dilution of virus would also die at lower dilutions (i.e. higher doses), and conversely, animals which survive at any given dilution of virus would survive at all higher dilutions (i.e. lower doses). The

accumulated number of dead at each dilution is therefore calculated by adding the figures in the third column of the table, starting the addition from each successive dilution but working from the lowest to the highest dilution. From these totals, the accumulated

Table 4. Calculation of virus infectivity titre from experimental data

Virus diln.	Log virus diln.	No dead	No. of survivors	Mortality ratio	Accumulated values			
					Dead	Survivors	Mortality ratio*	Per cent mortality
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
1:100	10^{-2}	6	0	6/6	18	0	18/18	100
1:1000	10^{-3}	6	0	6/6	12	0	12/12	100
1:10,000	10^{-4}	4 ↓	2 ↑	4/6	6	2	6/8	75
1:100,000	10^{-5}	2 ↓	4 ↑	2/6	2	6	2/8	25
1:1,000,000	10^{-6}	0	6	0/6	0	12	0/12	0

* Numerator = Figure in column 6

Denominator = Sum of figures in columns 6 and 7

↑ = Addition from highest to lowest dilution

↓ = Addition from lowest to highest dilution

mortality ratio and the percentage mortality for each dilution may be calculated. In the example, the 50% mortality end-point lies between the 10^{-4} and 10^{-5} dilutions. The exact figure may now be calculated from the formula of Reed and Muench as follows:

$$(\% \text{ mortality next above } 50) - 50$$

$$(\% \text{ mortality next above } 50) - (\% \text{ mortality next below } 50)$$

$$= \frac{75 - 50}{75 - 25} = \frac{25}{50} = 0.5$$

= calculated factor

Dilution step = 10 (fold)

Logarithm of dilution step = 1

Calculated factor \times log of dilution step

$$= 0.5 \times 1 = 0.5$$

= Figure to be added to logarithm of dilution next above 50

Logarithm of dilution next above 50 (sign ignored) = 4

Logarithm of dilution next above 50 + figure to be added

$$= 4 + 0.5 = 4.5$$

Logarithm of 50% end-point = $10^{-4.5}$ = virus titre

Reciprocal of titre

$$= 10^{4.5} = 31,620$$

= number of LD₅₀ units per inoculum volume

(ii) Neutralization titration

The titre of the virus preparation having been determined, a suitable test dose, usually 100 LD₅₀, is selected for use in the neutralization titration. In the virus titration illustrated above, 1 LD₅₀ per inoculum volume is obtained by diluting the preparation 1:10^{4.5}, 100 LD₅₀ will therefore be obtained by diluting it 1:10^{2.5}. This dose of virus is then mixed in equal volumes with serial dilutions of serum and incubated for 30 minutes at 37°C, 22°C, or 4°C, depending on the virus strain. After incubation, each virus-serum mixture is inoculated into a group of six or more animals.

Experimental results are tabulated (Table 5) and the 50%,

Table 5. Calculation of serum neutralization titre from experimental data

Serum diln.	Log serum diln.	No. dead	No. of sur- vivors	Mor- tality ratio	Accumulated values			Per cent mor- tality
					Dead	Sur- vivors	Mor- tality ratio*	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
1:200	10 ^{-2.3}	0	6	0/6	0	17	0/17	0
1:400	10 ^{-2.6}	1	5	1/6	1	11	1/12	8.3
1:800	10 ^{-2.9}	2	4	2/6	3	6	3/9	33.3
1:1,600	10 ^{-3.2}	↑ 4	↓ 2	4/6	7	2	7/9	78.0
1:3,200	10 ^{-3.5}	6	0	6/6	13	0	13/13	100
1:6,400	10 ^{-3.8}	6	0	6/6	19	0	19/19	100

* Numerator = Figure in column 6

Denominator = Sum of figures in columns 6 and 7

↓ = Addition from lowest to highest dilution

↑ = Addition from highest to lowest dilution

neutralization titre is calculated by a method similar to that used for calculating the 50% lethal dose, but the order of addition for computing the accumulated totals is reversed. The accumulated number of dead at each dilution is calculated by adding the figures in the third column of the table, starting the addition from each successive dilution and working from the highest to the lowest dilution. Similarly, the number of survivors is accumulated by adding the figures in the fourth column, starting the addition from each successive dilution and working from the lowest to the highest dilution. In the example, the 50% neutralization end-point lies between the 1:800 and 1:1600 dilutions of serum. The exact figure may be calculated from the formula of Reed and Muench as follows:

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

$$= \frac{50 - 33.3}{78 - 33.3} = \frac{16.7}{44.7} = 0.37 = \text{calculated factor}$$

Dilution step = 2 (fold)

Logarithm of dilution step = 0.3010

Calculated factor \times logarithm of dilution step

$$= 0.37 \times 0.3010 = 0.11$$

= figure to be added to logarithm of serum dilution next below 50

Logarithm of serum dilution next below 50 (sign ignored) = 2.9

Logarithm of serum dilution next below 50 + figure to be added

$$= 2.9 + 0.11 = 3.01$$

Logarithm of serum neutralization titre (50% end-point) = $10^{-3.01}$

Reciprocal of serum neutralization titre = $10^{3.01} = 1023$

Serum neutralization titre = 1:1023

(iii) Neutralization index

Another neutralization technique, in which serial dilutions of virus are mixed with equal volumes of a standard dilution of antiserum, is sometimes used. A control series in which serial dilutions of virus are mixed with equal volumes of normal serum is set up at the same time. After incubation, each serum-virus mixture is inoculated into

a group of six or more animals, and the number of deaths occurring during the observation period, usually 10 days, is recorded. The LD_{50} of virus in the presence of normal and immune serum is then calculated by the method of Reed and Muench, and the difference between the two titres, which is called the neutralization index, provides a measure of the immune serum's neutralization potency. If the virus titre in the presence of normal serum is 10^{-5} and in the presence of immune serum 10^{-3} , the neutralization index is 2 (the sign is ignored). Expressed in arithmetic terms, this signifies that the immune serum, at the dilution used, is capable of neutralizing 100 LD_{50} of virus. In comparing acute and convalescent sera a neutralization index of 2 or more is considered diagnostically significant.

(b) Tissue Culture

The majority of virus neutralization tests are now performed in tissue culture. In principle, these tests are the same, *mutatis mutandis*, as animal neutralization tests. Each monolayer tissue culture tube is analogous to an experimental animal, and any unneutralized virus in the monolayer may be recognized by some overt biological effect of infection.

(i) Inhibition of the cytopathogenic effect

The most easily observed biological effect of virus infection in monolayer tissue cultures is the cytopathogenic effect (CPE). Neutralizing antibody is therefore easily identified by its ability to prevent the CPE normally produced by a standard dose of virus.

As in animal neutralization tests, the standard dose of virus is estimated from a preliminary virus titration performed in groups of tissue culture tubes. The highest dilution of virus producing CPE in 50% of the tubes inoculated is said to contain 1 TCD_{50} (tissue culture dose₅₀). For use in the neutralization test, the dilution of virus is usually adjusted to contain 100 TCD_{50} per inoculum volume. Equal volumes of this virus dilution and serial dilutions of serum are then mixed, incubated for 30 minutes, and inoculated into groups of tissue culture tubes. The cultures are incubated under

optimal conditions, and examined at intervals for the presence of CPE. After a suitable observation period, usually 5–10 days, the number of tubes with and without CPE is scored and the results are tabulated in the style used for animal neutralization tests. The 50% neutralization end-point is then calculated by the formula of Reed and Muench.

Occasionally, sera contain cytotoxic substances, and controls must be included in which the highest concentration of serum used is inoculated alone. Other controls required are the normal cell control inoculated with maintenance medium only, and a virus control in which the virus challenge dose is titrated to confirm that it actually contained 100 TCD₅₀.

(ii) Metabolic-inhibition test

Uninfected cells actively metabolize and produce acid, converting the phenol red indicator, incorporated in the medium, from red to yellow. Cytopathogenic viruses inhibit cell metabolism and limit the amount of acid produced, so that the pH of the medium remains static, or becomes more alkaline, and the red colour of the indicator is maintained.

Using the colour changes described, neutralization tests may be performed which are similar to those based on inhibition of CPE. Neutralization of virus by antiserum allows cells to metabolize normally and behave as uninfected cells, whereas unneutralized virus inhibits cell metabolism, limits acid production, and prevents the indicator changing colour. After 6–8 days incubation under optimal conditions, colour changes may be read macroscopically, and 50% end-points calculated by the formula of Reed and Muench. Controls include titrations of challenge virus and normal cell suspensions.

(iii) Haemadsorption inhibition

Myxoviruses, which multiply in tissue culture without producing CPE, may be recognized by the haemadsorption technique. Hence, neutralization of haemadsorbing viruses by antiserum may be recognized by the absence of haemadsorption after inoculation of serum-virus mixtures and several days incubation. Haemadsorption-inhibition tests similar to those based on inhibition of CPE may

therefore be employed, in which the titre is expressed as the highest dilution of serum which prevents haemadsorption in 50% of the tubes inoculated.

Neutralization of haemadsorbing viruses *in situ* allows their rapid identification. Virus-infected cultures are incubated for a suitable period, after which the culture medium is removed and the monolayers washed with balanced salt solution. Fresh medium containing an appropriate amount of antiserum is then added and allowed to react with the cells for 1 hour at 37°C. The antiserum-containing medium is now removed and a suspension of human or guinea-pig red cells is introduced and allowed to react for 30 minutes at 4°C. Cultures treated with antiserum and untreated controls are then compared for inhibition of haemadsorption.

(iv) Plaque reduction test

The neutralizing potency of an antiserum can be measured by its ability to inhibit the formation of plaques by homologous virus. Although one of the most accurate methods for the assay of virus neutralization, the plaque reduction test is too time-consuming for routine work. In practice, a suitable virus challenge dose which produces a sufficient number of plaques under standard conditions is determined by previous titration. Equal volumes of this virus dose and serial dilutions of antiserum are then mixed and incubated for 30 minutes, before inoculation into monolayer cultures which are overlaid, after an appropriate interval to allow for virus adsorption, with an agar overlay. The number of plaques produced by serum virus mixtures, after suitable incubation of the monolayers, is compared with the number produced by virus alone. The dilution of antiserum which reduces the plaque count by 50% is the plaque neutralization titre.

(c) Chick embryos

Chick embryos provide suitable hosts for neutralization tests, in which antibody may be identified by its ability to prevent some overt biological effect of virus infection.

(i) Death of the embryo

The psittacosis-LGV group of organisms, some arboviruses, and herpesvirus are lethal to the chick embryo. The neutralizing potency of their antisera may therefore be measured by the capacity to prevent embryonic death produced by a standard dose of virus. The principles and technique of the egg neutralization test are the same as those of the animal neutralization test.

(ii) Pock reduction

The neutralizing potency of antisera against variola, vaccinia, and herpesvirus can be measured by their ability to reduce the number of pocks produced on the chorioallantoic membrane by a standard dose of the homologous virus. The principles of the test are exactly the same as those of the plaque-reduction test. The titre of the serum may be expressed as that dilution of serum which reduces the pock count by 50%.

(iii) Neutralization of infectivity

Infection of chick embryos with myxoviruses is manifested after 48 hours incubation by the production of haemagglutinating virus in the allantoic fluid. For infectivity-neutralization tests, a virus titration is a necessary preliminary. Serial dilutions of virus are inoculated into groups of six or more embryos, and after 48 hours incubation the allantoic fluids are tested for haemagglutination. From the number of eggs infected, the EID_{50} (egg infective dose₅₀) is calculated by the method of Reed and Muench.

Subsequently, serial dilutions of antiserum are mixed with equal volumes of virus, diluted to contain 100 or 1000 EID_{50} per inoculum dose. After incubation for 30 minutes at 37°C, each mixture is inoculated into groups of six or more eggs. After 48 hours, the allantoic fluids are tested for haemagglutination, and the 50% end-point, which is the dilution of serum which prevents infection in 50% of the eggs inoculated, is calculated by the formula of Reed and Muench.

CHAPTER 5

Serological Techniques in Virology

II. In vitro Methods

Complement-Fixation

The complement-fixation test is very widely applied in the serological diagnosis of virus infections. It is often preferred by experienced workers because of its relative simplicity, and because living hosts are not required. The increased popularity of the complement-fixation test has been aided by the wide range of viral antigens which have recently become available in pure and concentrated form. These antigens are prepared in tissue cultures or embryonated eggs and are concentrated and further purified by various techniques, including high-speed centrifugation, lyophilization, and treatment with fluorocarbon.

In principle, virus complement-fixation tests do not differ in any way from those employed in bacteriology. The reaction between a virus antigen and its homologous antibody is detected by the ability of the antigen-antibody complex formed to fix complement. Any unfixed complement remaining in the test is detected by the indicator, sensitized sheep red cells.

THE COMPLEMENT-FIXATION TEST

1. *The indicator—sensitized sheep red cells*

- (a) Sheep red cells + anti-sheep red cell haemolysin → sensitized sheep red cells
(b) Sensitized sheep red cells + free complement → haemolysis

2. *Positive complement-fixation reaction*

- (a) Antigen + homologous antibody → antigen-antibody complex

- (b) Antigen antibody complex + complement →
antigen-antibody-complement complex
- (c) Antigen antibody complement complex
+ sensitized sheep red cells →
antigen antibody complement complex
+ sensitized sheep red cells → no haemolysis
= positive complement-fixation reaction

3. *Negative complement-fixation reaction*

- (a) Antigen + heterologous antibody →
antigen + heterologous antibody
- (b) Antigen + heterologous antibody + complement →
antigen + heterologous antibody + complement
- (c) Antigen + heterologous antibody
+ complement + sensitized sheep red cells →
antigen + heterologous antibody
+ complement + sensitized sheep red cells → haemolysis
= negative complement-fixation reaction

Many variations in technique have been described, the details of which are available in practical handbooks. The main differences are in the volumes of reagents used, in the temperature and time of incubation, and in the use of complete or partial haemolysis as the end-point. In general, the methods used are the classical Kolmer technique, employed in the Wassermann reaction, and the now increasingly used micro-techniques performed by a drop method on smooth perspex sheets. Micro-techniques are specially suitable for virus work because the small test volumes employed are extremely economical in the use of reagents.

(a) **Titration of reagents**

In principle, both types of test are the same and both require preliminary titration of the reagents to be used. Sheep red cells and anti-sheep red cell haemolysin are available commercially and are standardized in the usual way. Complement, usually in the form of guinea-pig serum, is titrated in saline diluent, and in the presence of the maximum amount of antigen to be used in the test (Fig. 15a).

The highest dilution of complement which produces complete or partial haemolysis, either of which may be defined as the end-point, represents 1 unit of complement. In practice, the titre obtained

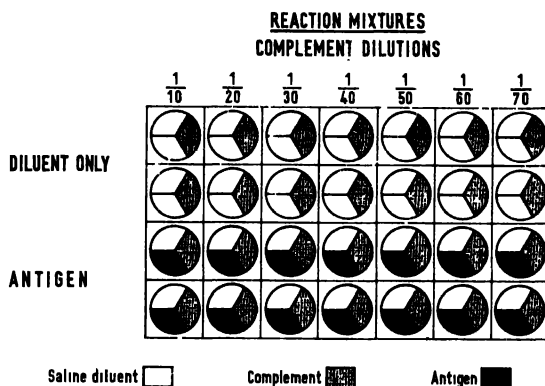
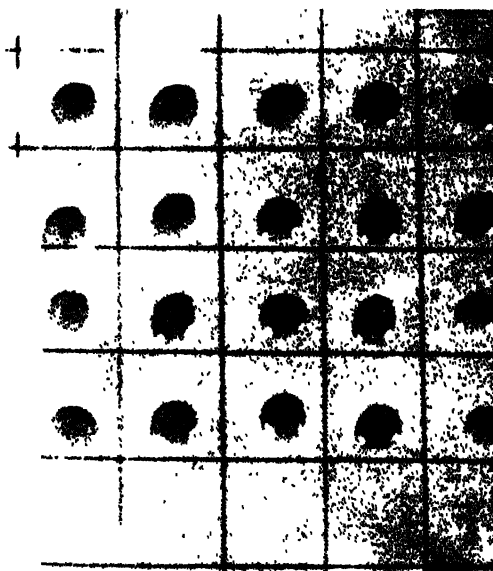


Fig. 15. Complement titration (a) Reaction mixtures.



(b) Results -- button of red cells = no haemolysis.

from replicate titrations of complement in the presence and absence of antigen is taken to represent 1 unit (Fig. 15b). Any difference between the titres obtained in the presence and absence of antigen is a measure of the pro- or anti-complementary activity of the antigen. For use in the test proper, the dilution of complement is adjusted to contain 2 units of complement per test volume

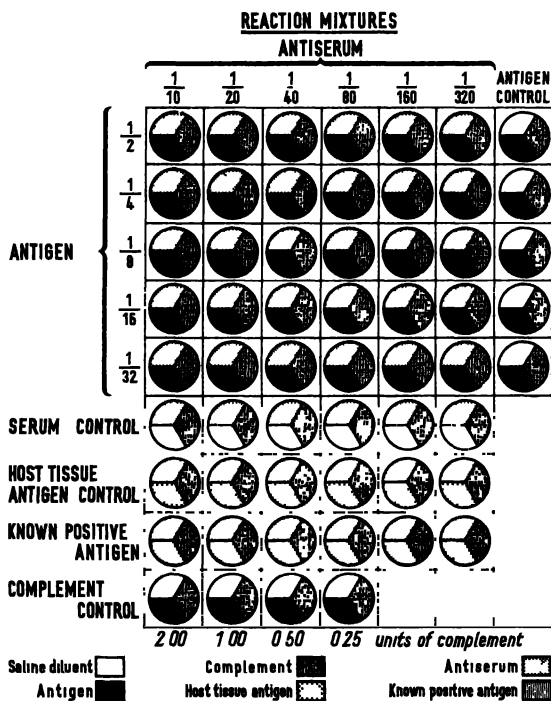
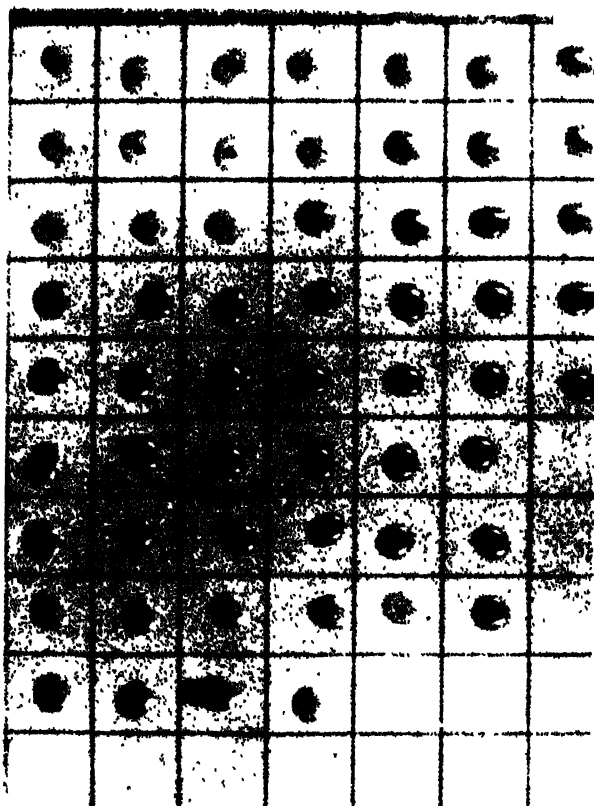


Fig. 16. Complement-fixation test—antigen titration.
(a) Reaction mixtures.

The unit of antigen is standardized by titrating serial dilutions of antigen against serial dilutions of a known potent antiserum, in 'chessboard' fashion (Fig. 16a). The highest dilution of antigen which fixes complement in the presence of the highest dilution of serum represents one unit of antigen (Fig. 16b). For use in the test, the dilution of antigen is adjusted to contain 2 units per test volume.

(b) The test

Serial dilutions of the test serum are mixed with equal volumes of complement and antigen, each diluted to contain 2 units per test volume. Depending on the technique used, the reaction mixtures are allowed to incubate at 37°C for 30–75 minutes, or overnight at 4°C,



(b) Results.

before one test volume of sensitized sheep red cells is added to each mixture. After a further period of incubation at 37°C for 30 minutes, the results are read. The end-point is the highest dilution of serum which produces complete or partial haemolysis, according to the technique used. Titrations of known positive antisera to each of the

SERUM DILUTIONS

	$\frac{1}{20}$	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{320}$	$\frac{1}{640}$
ACUTE PHASE SERUM						
CONVALESCENT PHASE SERUM						
KNOWN POSITIVE SERUM						
COMPLEMENT CONTROL						

units of complement	2 00	1 00	0 50	0 25
100	100	100	100	100
200	100	100	100	100
300	100	100	100	100
400	100	100	100	100
500	100	100	100	100
600	100	100	100	100
700	100	100	100	100
800	100	100	100	100
900	100	100	100	100
1000	100	100	100	100

**Sensitised
sheep cell control**



**Known positive
serum control**

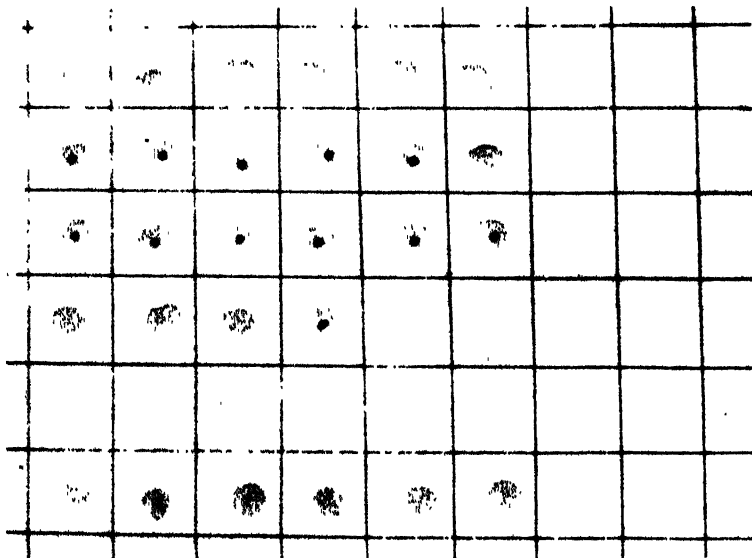
**Acute phase
serum control**

Host tissue antigen control

Acute phase serum
Host tissue antigen control
+
Convalescent phase serum

Convalescent phase
serum control

Saline diluent

Complement Serum as specified ☒Antigen Host tissue antigen 

antigens used are included in the test, and a number of other controls are required (Fig. 17a). These serve to exclude any anti-complementary activity on the part of the test serum, any complement-fixation reaction between the test serum and antigens derived from the host tissue in which the virus was grown, and autohaemolysis of the sensitized sheep red cells. A separate complement titration confirms the adequacy of the complement dose.

For diagnosis, a four-fold increase or more of antibody titre, occurring between the acute and convalescent phases of the disease, is considered significant.

Complement-fixing Antigens

Two sorts of complement-fixing antigen are found in some viruses; one, a group-specific antigen common to a particular group of viruses, and the other a type specific antigen specific for each virus type within the group. Adenoviruses, for example, are characterized by a group-specific complement-fixing antigen which is common to all the adenovirus types, each type is nevertheless distinguishable by its own type-specific antigen.

During the course of some viral and rickettsial infections, virus specific antigens which are non-infective, small in molecular size, and separable from virus particles, are manufactured in infected tissues. After sedimentation of virus particles from infected tissue suspensions by high-speed centrifugation, these antigens remain in the supernatant and are therefore termed soluble antigens.

Soluble antigens are produced in tissues infected with poxviruses, myxoviruses, respiratory syncytial virus, adenoviruses, herpesviruses, psittacosis agent, and some rickettsiae. The soluble antigen of influenza virus, the S antigen, is the one which has been most intensively studied and is the one which is best understood. It is a complement-fixing ribonucleoprotein by means of which influenza viruses are divided into two main serological species, A and B. The antigens of the viral particle, the V-antigens, are associated with the

Fig. 17. Complement-fixation test—diagnostic investigation.

(a) Reaction mixtures.

(b) Results (fifth row = serum and antigen controls).

haemagglutinin component and are strain specific as well as type specific. After disruption of influenza viruses with ether, a species specific antigen, identical with the soluble antigen, is released. It is, in fact, the internal component of the virus, which in the intact particle is known as the g-antigen (gebundenes or bound antigen); because of its internal position it is not normally revealed. The soluble antigen produced in infected tissues probably represents excess internal component not incorporated in the virus particle.

In clinical influenza infection, antibodies to the soluble antigen appear very early in the disease and disappear rapidly during convalescence. In contrast, antibodies to the V-antigens appear later and rise to a maximum during convalescence. The presence of antibodies to S-antigen is therefore evidence of recent influenza infection. Artificial immunization with killed influenza virus vaccines stimulates the production of antibodies to the V-antigens only and not at all to the S-antigen.

Haemagglutination-Inhibition

Certain viruses possess the property of attaching to red blood cells and agglutinating them. In the presence of homologous antibody, virus-red cell attachment is prevented and haemagglutination inhibited. The capacity to inhibit haemagglutination may therefore be utilized for the identification and measurement of antibodies to haemagglutinating viruses. Indeed, haemagglutination-inhibition (HI) tests are extensively used in the study of antibodies to myxoviruses, adenoviruses, arboviruses, some enteroviruses, and reoviruses.

Two virus groups, the vaccinia-variola group and the psittacosis group of organisms, elaborate soluble haemagglutinins which although separable from the virus particles provide suitable antigens for HI tests.

(a) Myxovirus haemagglutination-inhibition tests

The wide application of the haemagglutination-inhibition technique to the study of influenza viruses makes the influenza virus haemagglutination-inhibition test a model which best illustrates the

principles of the HI test. Influenza viruses agglutinate human group O or fowl red cells, but the latter are used almost exclusively because of their faster sedimentation and more easily read patterns of agglutination. One of two methods may be used, the pattern or the densitometric method.

(1) *Pattern method*

The pattern method, which is most widely used, is performed in plastic plates moulded into a design of 8 rows, each containing 10

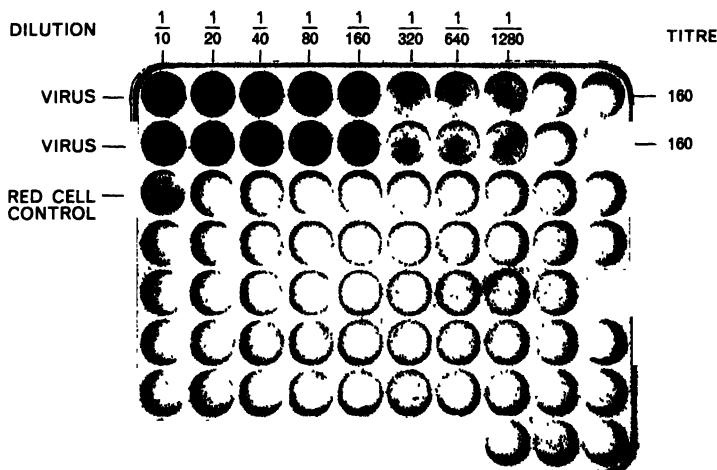


Fig. 18. Haemagglutination test—virus titration. Button of red cells = no haemagglutination.

individual cups (Fig. 18). Each reaction mixture is placed in a separate cup, to which red cells are added. Haemagglutination is easily observed by the characteristic pattern with which red cells settle in the cup. Unagglutinated cells settle in an easily recognized disc in the centre of the cup (Fig. 18).

In the test proper, serial two-fold dilutions of serum in 0.25 ml volumes of saline diluent are mixed with equal volumes of virus, diluted to contain four agglutinating doses (AD's) per 0.25 ml. The serum-virus mixtures are allowed to react for 15 minutes at room temperature before 0.25 ml of a 0.5% fowl red cell suspension is

added. Patterns of agglutination are read after 60 minutes at room temperature; the highest dilution of serum which prevents complete agglutination by four AD's of virus denotes the HI titre (Fig. 19).

The preparation of the virus challenge dose, four AD's, is based on a preliminary haemagglutination (HA) titration (Fig. 18). Serial two-fold dilutions of virus in 0.25 ml volumes of diluent are mixed with equal volumes of a 0.5% suspension of fowl red cells. The

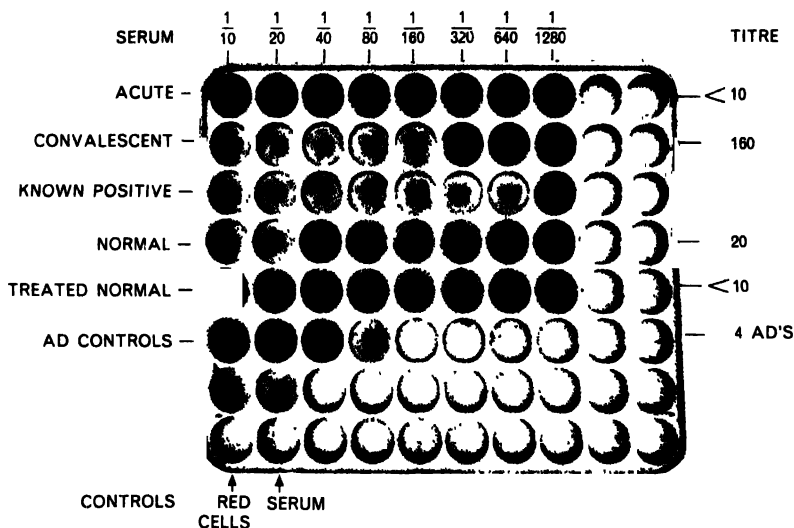


Fig. 19. Haemagglutination-inhibition test—diagnostic investigation.

highest dilution of virus which produces complete agglutination after 1 hour contains one AD per test volume. The standard dose of four AD's is prepared by adjusting the dilution of the virus preparation used. For example, if the virus HA titre is 1:160, four AD's per test volume is obtained by diluting the original virus preparation 1:40, (i.e. 160/4).

Red cell and antiserum controls, in which appropriate reagents are replaced with an equivalent volume of saline diluent, are required in each series of tests. The virus challenge dose must also be shown to contain four AD's per test volume. Test volumes containing

4, 2, 1 and 0.5 AD's are therefore prepared and checked for haemagglutination. Known positive antisera to each antigen used and normal serum from the species in which antisera were prepared, treated similarly to the test serum, are included in each series of tests (Fig. 19).

In diagnosis, a rise in HI titre of four-fold or more, occurring between the acute and convalescent phases of the disease, is considered significant.

(ii) Densitometric method

The densitometric method although more accurate than the pattern method is more time consuming and difficult to perform. It is therefore better suited to research investigations than to diagnostic routines. Titrations are carried out in slim tubes, $3 \times \frac{1}{8}$ inch, in which agglutinated cells sediment more rapidly than unagglutinated cells, leaving a clear supernatant. After a 75 minute sedimentation period, the degree of agglutination in each tube of a titration series is read by measuring the optical density at a fixed point, in a photoelectric densitometer. The end-point in both HA and HI titrations is defined as the dilution of virus or serum in which 50% of the red cells sediment in the 75 minute reaction period. This value is easily calculated by interpolation from densitometer readings.

(iii) Removal of non-specific inhibitors

The results obtained in influenza HI tests may be confused by the presence of non-specific inhibitors of haemagglutination which are present in the sera of many animal species and man. When present in high titre, they may mask inhibition of haemagglutination due to antibody and must therefore be removed, by appropriate treatment, before use.

Three types of non-specific inhibitors active against influenza viruses are recognized, and are referred to as α , β , and γ . α - and γ -inhibitors are mucoproteins which may be inactivated by oxidation with potassium periodate, and β -inhibitor is a lipoprotein which is destroyed by heat at 56°C for 30 minutes. The problem of non-specific inhibitors of influenza virus haemagglutination may also be surmounted by the use of specially selected strains, produced by

laboratory manipulation, which are insensitive to inhibitors but sensitive to antibody.

(b) Haemagglutination-inhibition tests with other viruses

The principles of the HI test described above apply to viruses, other than influenza, which haemagglutinate erythrocytes of certain species, sometimes under restricted conditions of temperature and pH only.

(i) *Arboviruses*

Arboviruses agglutinate red cells from geese or day-old chicks, each virus strain having its own optimal pH and temperature for agglutination. Lipid or lipoprotein inhibitors of arbovirus haemagglutination are found in normal sera, and pretreatment of sera with kaolin or acetone is required before use in HI tests. Naturally occurring agglutinins for goose red cells are also present in normal sera, but these may be removed by absorbing the sera with goose red cells. On the basis of HI tests, arboviruses may be divided into at least three immunological groups, A, B, and C.

(ii) *Enteroviruses*

About one-third of the currently known enteroviruses agglutinate human group O cells under varying conditions of pH and temperature (Table 6). HI tests with these viruses may be performed after

Table 6. Conditions for enterovirus HA and HI tests

Fowl red cells at 22°C	Human group O red cells at 4°C		Human group O red cells from new born infants at 37°C
Coxsackie A.7	Coxsackie A 20	Echo type 12	Coxsackie B ₁
	A.21	13	B ₃
	A.24	19	B ₅
	Echo type 3	20	
	6	21	
	7	24	
	11	29	

non-specific inhibitors present in normal sera have been removed by absorption with kaolin. These inhibitors are usually lipoprotein in nature.

(iii) Adenoviruses

With the exception of types 18 and 31, adenoviruses fall into three groups according to their capacity to produce complete or partial agglutination of rhesus or rat red cells at 37°C. Within these groups, the various types can be identified by HI tests. Pretreatment of sera with kaolin is required to remove non-specific inhibitors of haemagglutination.

Flocculation and Precipitation

(a) Macroscopic and microscopic flocculation tests

Interaction between concentrated suspensions of purified virus or rickettsial particles and their homologous antibodies at 37°C results in the visible aggregation or precipitation of the virus-antibody complexes formed. The term flocculation is usually applied to these reactions which are visible macroscopically and microscopically; flocculation reactions have now been demonstrated with vaccinia, variola, influenza, and poliomyelitis viruses, as well as with some rickettsiae. The flocculation test has not however found wide application because large amounts of virus suspension, in a highly concentrated and purified form, are necessary.

(b) Agar-gel diffusion tests

The Ouchterlony gel-diffusion technique has been extremely useful for the demonstration of precipitates formed of viruses or soluble antigens, small enough to diffuse through agar, and their homologous antibodies. Antigen and antibody are placed in wells, cut a suitable distance apart in a layer of solid agar in a Petri dish, and are allowed to diffuse towards each other over a period of about 48 hours. Visible lines of precipitate are formed where antigen and antibody meet in optimal proportions (Fig. 20). The distinct separation of precipitates formed by different antigen-antibody systems allows the separation of various antigenic components forming a

single complex. The technique is therefore particularly valuable for the antigenic analysis of virus strains, and has the advantage of economy in the use of reagents.

A micro-agar-gel technique in which gel-diffusion tests are carried out on a microscope slide coated with a thin layer of agar has

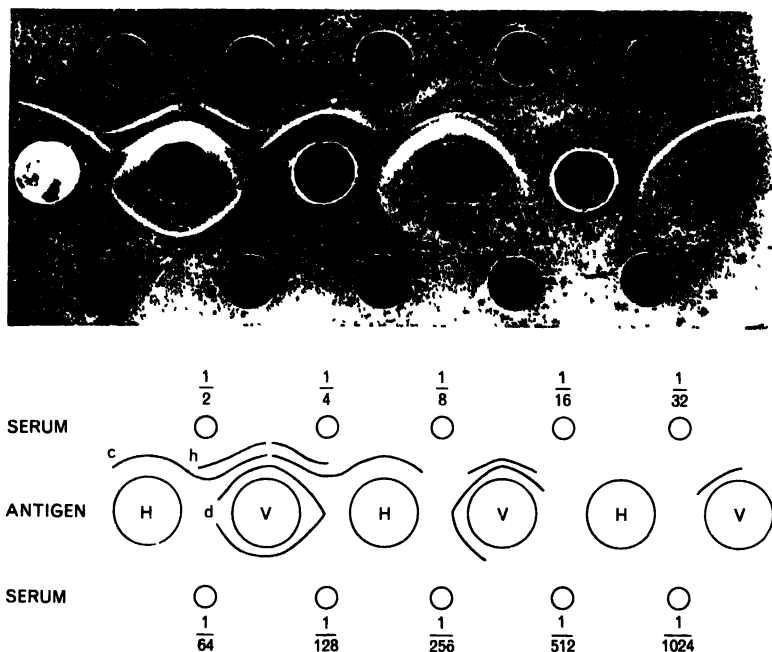


Fig. 20. Agar-gel precipitation--immunodiffusion pattern of poliovirus and homologous rabbit antiserum.

H = Heated virus V = Live virus d = D-antigen

c = C-antigen h = Non-specific host tissue antigen

recently been developed. It is particularly advantageous for certain diagnostic procedures because of the rapidity with which precipitation lines appear. Antigenic material from vesicles of patients with smallpox, for example, may be tested against the appropriate immune serum in this way, and a positive result, manifested by a line of precipitation, can be obtained in 3-5 hours.

Fluorescent Antibody Techniques

Virus antigens stained with homologous fluorescein-labelled antibodies exhibit a yellowish-green fluorescence when examined in ultraviolet light by fluorescence microscopy. In tissue smears or tissue sections, virus antigens may be located in infected cells; the technique is therefore of some diagnostic importance. In rabies, the demonstration of virus antigens in brain smears, by the fluorescent antibody technique, has proved at least as good a diagnostic procedure as the demonstration of Negri bodies, or the mouse inoculation test. In research investigations, the technique has proved extremely useful for the study of intracellular virus development and for the identification of non-cytopathogenic viruses in tissue culture.

In practice, certain precautions are required to prevent non-specific fluorescence. The antisera used must be devoid of antibodies to tissue antigens, and absorption with appropriate host tissue antigens is therefore necessary before use. To prevent interference from serum proteins, other than antibodies, which may be labelled, only the γ -globulin fraction of the antiserum is used. After antigen-antibody combination, the specimens are thoroughly washed to remove excess fluorescein-labelled material.

(a) Direct method

Two methods for staining viral antigens with fluorescent antibodies are available. The direct method, which is simple, highly specific, and the one usually used for diagnostic purposes, is based on the direct combination of antigen and its homologous fluorescein-labelled antibody.

(b) Indirect method

The indirect method is based on the combination of fluorescein-labelled antiglobulin serum with antigen-antibody complexes. In this technique, antigen is allowed to react with its unlabelled homologous antibody. After washing to remove excess antiserum, the antigen-antibody complexes are identified with fluorescein-labelled antiglobulin serum specific for the species in which the virus antiserum was prepared. For example, virus antisera prepared in rabbits

are revealed by fluorescein-labelled anti-rabbit globulin serum. The indirect method although less specific is more sensitive than the direct method, and is more suited to research investigations, and to the titration of serum antibody by the fluorescent technique.

A variation of the indirect method is one which is based on the fixation of complement by homologous antigen-antibody complexes. Virus antigen is allowed to react with its homologous antibody and guinea-pig complement, any fixation of complement by antigen-antibody complexes is then revealed by fluorescein-labelled antibody to guinea-pig complement.

CHAPTER 6

Virus Structure and Morphology

The simplest virus particles consist of protein and nucleic acid only. The nucleic acid, which may be either RNA or DNA, is enclosed in a virus protein coat which serves to protect the viral nucleic acid and to facilitate its entry into the host cell. Although it is often assumed that the nucleic acid component is situated in the centre of the virus particle, in the form of a core, it is not yet known, exactly, how the nucleic acid is arranged in relation to the protein coat. Recent evidence suggests that the relationship may be an intimate and complicated one.

About 10 years ago, Crick and Watson concluded that the amount of nucleic acid in the smaller virus particles was sufficient to code for only a very few specific proteins. From this, they predicted that the virus protein coat must be made up of a number of identical protein subunits arranged in a symmetrical array. This brilliant prediction has now been overwhelmingly substantiated by the analysis of virus structure using the modern techniques of X-ray diffraction, and electron microscopy of negatively stained preparations.

Crick, Watson, Caspar, Klug, and others have made an intensive study of the possible geometric designs which closed shells made up of identical subunits may exhibit. They conclude that shells formed of identical subunits are most efficient when the subunits are assembled in the form of a tube exhibiting helical symmetry, or in the form of a polyhedron exhibiting some form of cubical symmetry. In fact, apart from a few viruses such as the T_2 -bacteriophage and vaccinia virus, which exhibit complex structural and symmetry arrangements, all viruses so far examined exhibit either helical symmetry or a particular form of cubical symmetry known as icosahedral symmetry.

It is now clear that the basic unit contributing to the symmetry of the virus protein coat is the protein molecule. This molecular unit is beyond the range of the electron microscope, and the symmetrical arrangements to which it gives rise can only be deduced from X-ray diffraction analysis. However, the symmetrical arrangement of protein subunits leads to the formation of morphological subunits, in symmetrical array, which can be resolved by the electron microscope. It is now believed that each of these morphological subunits, which are termed capsomeres, is composed of a specific number of elementary protein subunits.

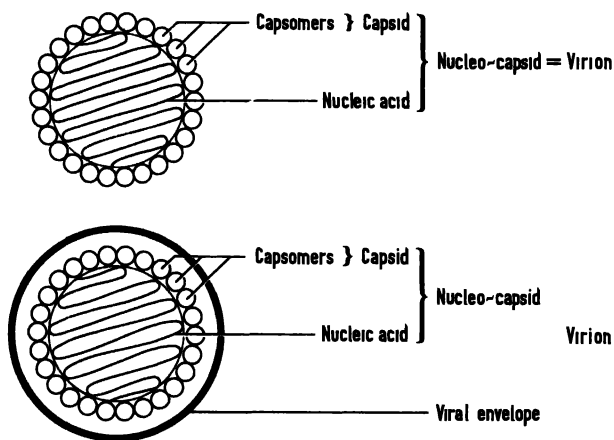


Fig. 21. Virus structure—morphological components.

The terminology, proposed by Lwoff and his colleagues, for describing the fine morphological details of virus particles has now been generally accepted. The smallest morphological unit is the capsomere, the symmetrical arrangement of which leads to the formation of the virus protein coat or capsid. Within the capsid is the viral nucleic acid, and the two components together form the nucleocapsid (Fig. 21). The nucleocapsid of some larger viruses is surrounded by a lipid or lipoprotein envelope, the whole forming the virion, or complete virus particle (Fig. 21). The virion of those viruses which do not possess an envelope consists of the nucleocapsid only.

Tobacco Mosaic Virus (TMV)

Of all viruses, tobacco mosaic virus is the one which has been most intensively studied. Its structure has been elucidated in very considerable detail by the pioneer X-ray diffraction studies of Bernal and Fankuchen, Rosalind Franklin, Caspar, Klug, and others, so

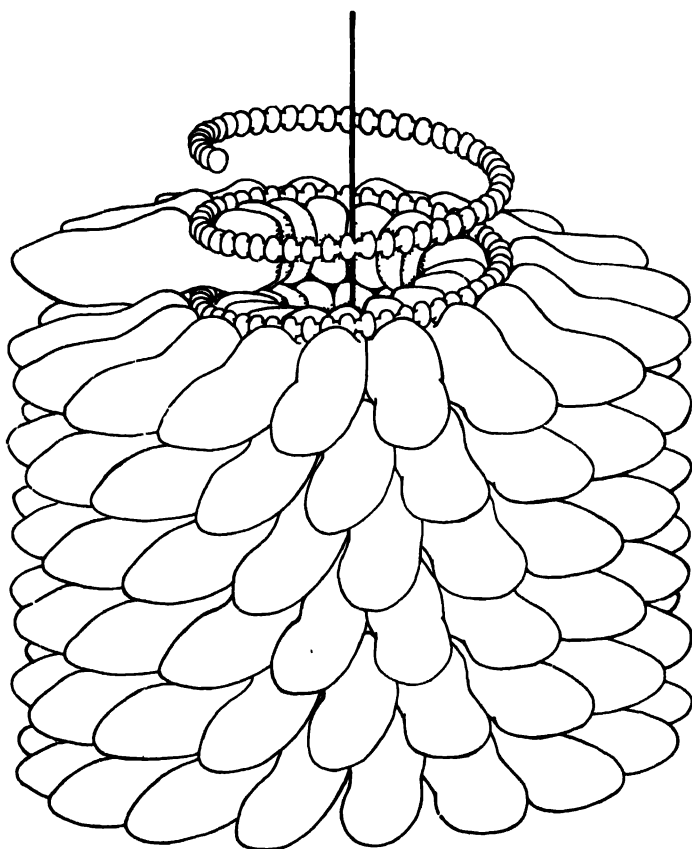


Fig. 22. Structure of tobacco mosaic virus—diagram based on X-ray diffraction studies showing axial hole, RNA helix, and protein subunits [from A. Klug and D. L. D. Caspar (1960). The structure of small viruses; in *Advances in Virus Research*, 1960, vol. 7 (Academic Press Inc., New York and London)].

that it has become the classic example of a rod shaped virus exhibiting helical symmetry. As such, it merits detailed description.

TMV is a rod-shaped particle 3000 Å in length and 180 Å in diameter. It consists of 2130 identical protein subunits wound in a helical array around an empty axial hole, 40 Å in diameter (Fig. 22). Fraenkel-Conrad, Rachamandran, and others have analysed the protein subunit even further, from the chemical point of view, and have shown that the protein subunit is made up of 164 amino acids.

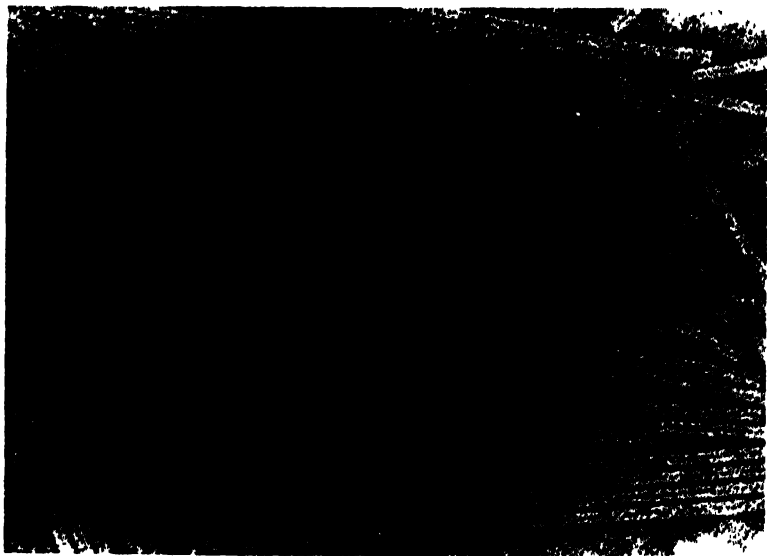


Fig. 23. Negatively stained particles of tobacco mosaic virus [from S. Brenner and R. W. Horne (1959) *Biochim. Biophys. Acta* 34, 103-10 (Elsevier Publishing Company, Amsterdam)].

The RNA of the virus is wound in helical fashion around the axial hole and is encased between the inner parts of the protein subunits (Fig. 22). Each protein subunit is in contact with three nucleotides of the RNA chain, and Gierer estimates that the total length of the chain is 6400 nucleotides. It is now beyond doubt that the length of the TMV particle is determined by the length of the RNA chain, which also contributes to the stability of the particle. This is evident

from the study of the aggregation of TMV protein subunits in the absence of RNA. In these circumstances, rod-shaped particles of variable length are formed in which a certain degree of instability is evident.

Of course, the electron microscope cannot resolve the detailed structure which X-ray diffraction has revealed. Nevertheless, electron microscopy of negatively stained preparations has confirmed the presence of the axial hole 40 Å in diameter and has revealed a helical array of morphological subunits (Fig. 23).

Animal Viruses exhibiting Icosahedral Symmetry

Crick and Watson suggested that the protein subunits of viruses which do not exhibit a rod-like structure would be arranged in some form of cubical symmetry to form a near spherical closed shell. The X-ray diffraction studies of Klug, Finch, Caspar, and others, as well as electron microscopic observations, have now established that these viruses are polyhedral in shape, and are built up from protein subunits in a particular form of cubical symmetry, called icosahedral symmetry. Viruses of this type are much more difficult to analyse by X-ray diffraction methods because of technical difficulties not encountered with rod-shaped particles. Most information has therefore come from electron microscopy, which can only display the symmetrical arrangement of the morphological subunits (capsomeres) and not that of the basic protein subunits.

Fine details of virus morphology, previously unimagined, have only become apparent in recent years following the application of the negative staining technique to viruses, by Huxley, Horne, Brenner, and others. Virus particles are coated with an electron dense material, such as potassium phosphotungstate, which produces a negative image of the virus surface contours, in the electron microscope.

(a) Adenovirus

One of the first animal viruses to be examined in this way was the adenovirus, which Horne's historic electron micrograph showed to

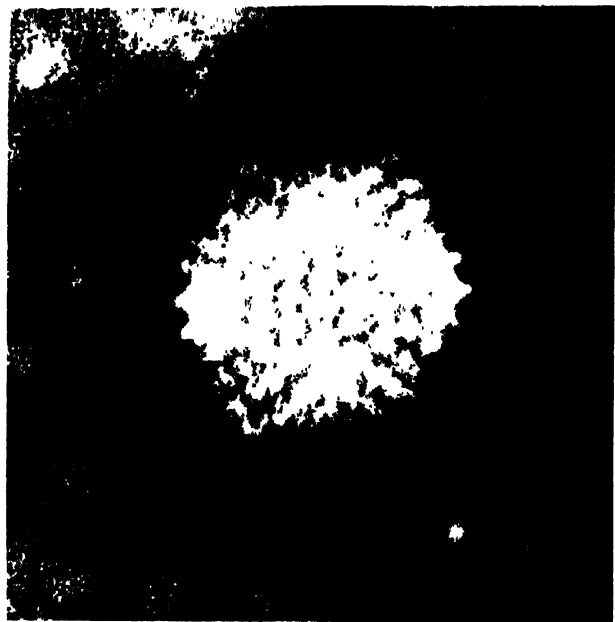
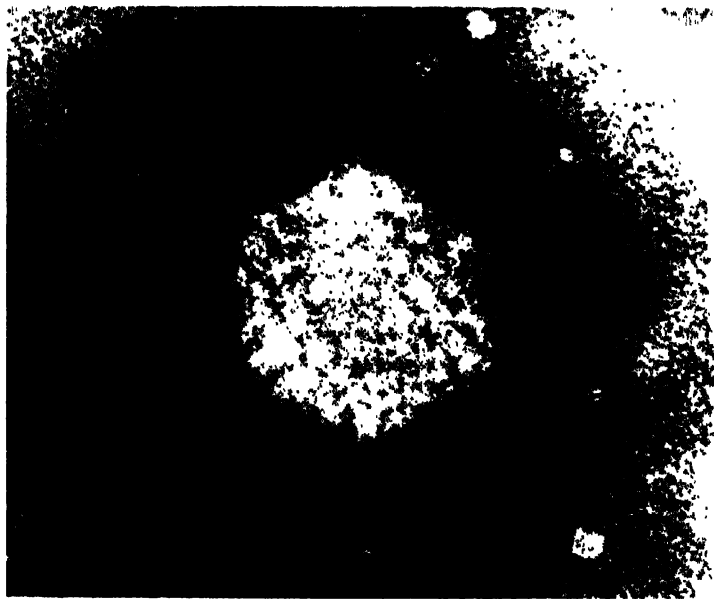
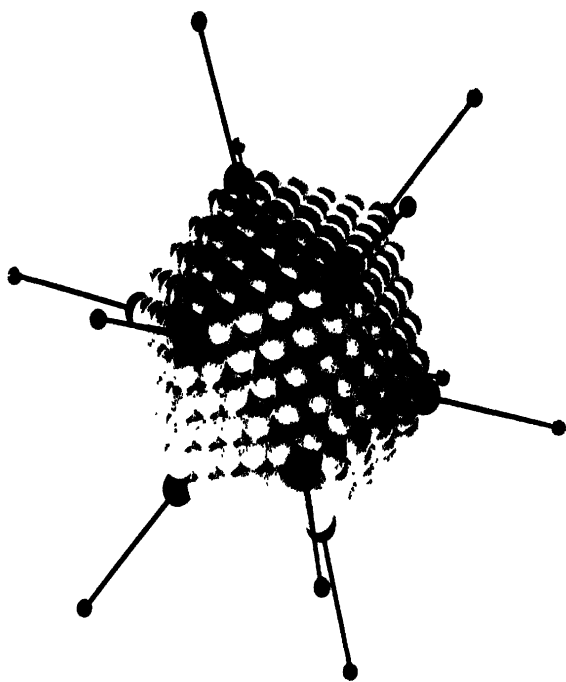


Fig. 24. (a) Negatively stained adenovirus particle showing tail structures.

(b) Negatively stained adenovirus particle showing icosahedral array of capsomeres.

[(a) and (b) From R. C. Valentine and H. G. Pereira (1965) *J. Mol. Biol.* 13, 13-20 (Academic Press Inc., London and New York).]



(c) Model of adenovirus particle [courtesy of Dr Robin Valentine, National Institute for Medical Research, Mill Hill, London].

be icosahedral in shape with 20 triangular faces. The particle is about 800 Å across and is composed of 252 capsomeres arranged in icosahedral symmetry (Fig. 24b). Recently, Valentine and Pereira have carried the analysis of the adenovirus particle further, and their electronmicrograph of adenovirus type 5 (Fig. 24a) shows tails projecting from the 12 vertical capsomeres, which possibly play some role in effecting adsorption of the virus to the host cell. Valentine and Pereira have dissected the adenovirus particle by

chemical means and have shown that the capsomeres which are situated at the vertices of the icosahedron, 12 in all, represent the 'toxin-like factor' or antigen B of the adenovirus. The tails which project from the vertical capsomeres represent the type specific antigen, and the remaining 240 capsomeres, which are arranged on the faces and edges of the icosahedron, represent the adenovirus group specific antigen (Fig. 24c).

(b) Herpesviruses

Another virus exhibiting icosahedral symmetry and a characteristic morphology is the virus of herpes simplex. This virus is one in which the nucleocapsid may be surrounded by an envelope 40–100 Å thick, from which a number of projections may be seen protruding (Fig. 25b). This envelope is probably of host cell origin and is apparently not essential for the infectivity of the virus. In the absence of the envelope the structure of the capsid becomes evident and has been described by Wildy and his colleagues (Fig. 25a). It consists of 162 capsomeres arranged in icosahedral symmetry which are not spherical, like those of the adenovirus, but are hollow elongated structures. Those situated at the vertices of the icosahedron, 12 in all, are pentagonal in outline, whereas the remaining 150, on the edges and faces of the icosahedron, are hexagonal. The necessity for two types of capsomere with different shapes has long been known to geometers, who are aware that an enclosed shell cannot be built of hexagons alone, but only from a combination of hexagons and pentagons. The virus of varicella is indistinguishable, morphologically, from the virus of herpes simplex (Fig. 26).

(c) Poliovirus

Poliovirus is one of the few animal viruses which have been studied by X-ray diffraction methods. From these studies, the construction of its capsid according to the principles of icosahedral symmetry has become evident. Particles growing in infected cells have also been examined by electron microscopy, and show well-defined capsomeres. So far the definition obtained has been insufficient for the elucidation of their symmetrical arrangement.

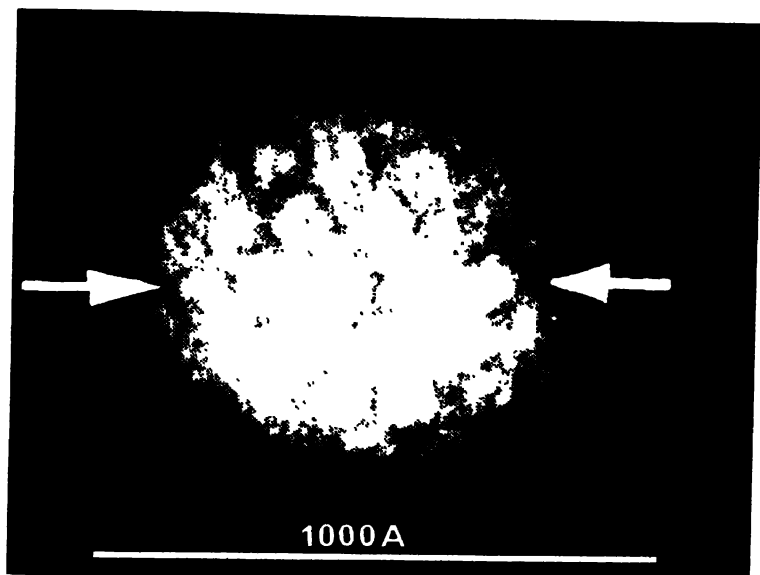


Fig. 25. Negatively stained particle of *Herpesvirus hominis* (herpes simplex).

(a) Naked capsid showing hollow capsomeres.

(b) Enveloped particle.

[From P. Wildy, W. C. Russell, and R. W. Horne (1960) *Virology* 12, 204-22 (Academic Press Inc., New York and London).]

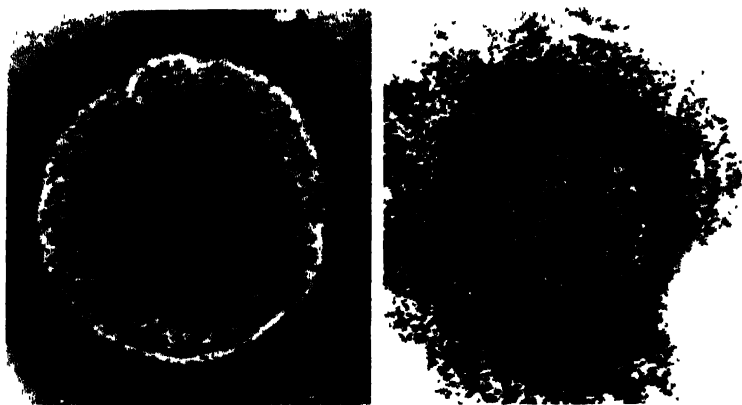


Fig. 26. Negatively stained particles of *Herpesvirus varicellae* (varicella virus).

(a) Enveloped particle

(b) Naked particle

[From June D. Almeida, A.F. Howatson, and M.G. Williams (1962) *Virology* 16, 353-55 (Academic Press Inc., New York and London)]

Animal Viruses exhibiting Helical Symmetry

(a) Myxoviruses

Myxoviruses which exhibit, at first sight, a somewhat complicated morphology do in fact exhibit helical symmetry. All myxoviruses are characterized by a lipoprotein envelope, which consists of an inner membranous layer, 60–100 Å thick, and an outer zone of evenly spaced radially orientated rods 100 Å long and 10 Å wide, which cover the whole surface (Fig. 28). Within the envelope is a coiled mass of ribonucleoprotein which becomes visible when released by rupture of the virus envelope (Fig. 29). Morphologically, negatively stained preparations of the internal ribonucleoprotein component resemble the rod-shaped particles of TMV. Thus, the inner component of influenza virus is a helical structure, 90 Å in diameter, which Hoyle and his colleagues believe to be composed of capsomeres, about 30–35 Å in diameter, surrounding an axial hole. The inner helical ribonucleoprotein component is now interpreted as the nucleo-capsid of the myxovirus particle. The exact morpho-



Fig. 27. Crystal of poliovirus particles in an infected cell. Va = vacuole [from S. Dales, H. J. Eggers, I. Tamm, and G. E. Palade (1965) *Virology* 26, 379-89 (Academic Press Inc., New York and London)].

logical relationship between the RNA and protein components of this structure remains to be determined.

From the study of myxoviruses disrupted by ether treatment, both Hoyle and Schaffer, and their colleagues, have identified the internal helical nucleoprotein component as the soluble antigen incorporated in the virus particle. In this situation the soluble antigen is known as the g-antigen (gebundenes or bound antigen). The

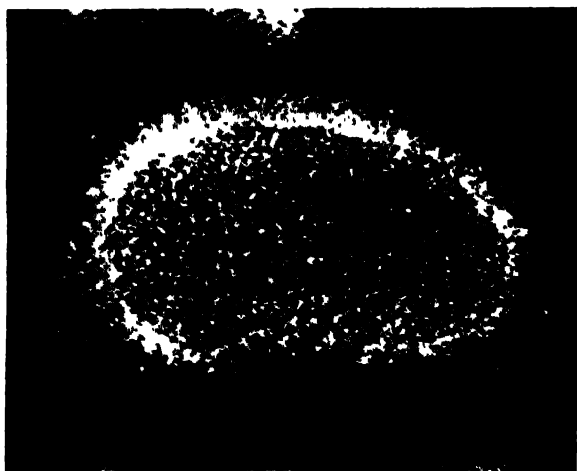


Fig. 28. Negatively stained influenza virus particle (*Myxovirus influenzae*) [electron micrograph by Dr J. P. Stevenson].

strain specific antigens have, likewise, been located in the lipoprotein virus envelope, which is also the structure responsible for haemagglutination and neuraminidase activity.

Influenza virus may also appear in a filamentous form, particularly in recently isolated strains (Fig. 30). Morphologically, the envelope of the filamentous form is the same as that of the spherical forms, but the morphology of the internal component has not yet been studied in detail.

(b) Paramyxoviruses

The morphological features of paramyxoviruses resemble those of myxoviruses, but Waterson has drawn attention to the features

which differentiate them. Myxoviruses are characterized by particles whose size varies from 800 to 1100 Å in diameter, and whose helical internal component is 90 Å wide and tightly packed inside the envelope. Paramyxoviruses, which include the viruses of mumps



Fig. 29. Negatively stained internal component of influenza virus [from L. Hoyle, R.W.Horne, and A.P.Watson (1961) *Virology* 13, 448-59 (Academic Press Inc., New York and London)].

and Newcastle disease (NDV), are characterized by larger sized particles, 1000-5000 Å in diameter, whose internal component is 180 Å wide and more loosely packed within the virus envelope. Morphological differences between myxoviruses and paramyxoviruses are associated with some differences of biological activity.



Fig. 30. Negatively stained influenza virus filaments [from P. W. Choppin, J. S. Murphy, and W. Stoeckenius (1961) *Virology* 13, 548-550 (Academic Press Inc., New York and London)].

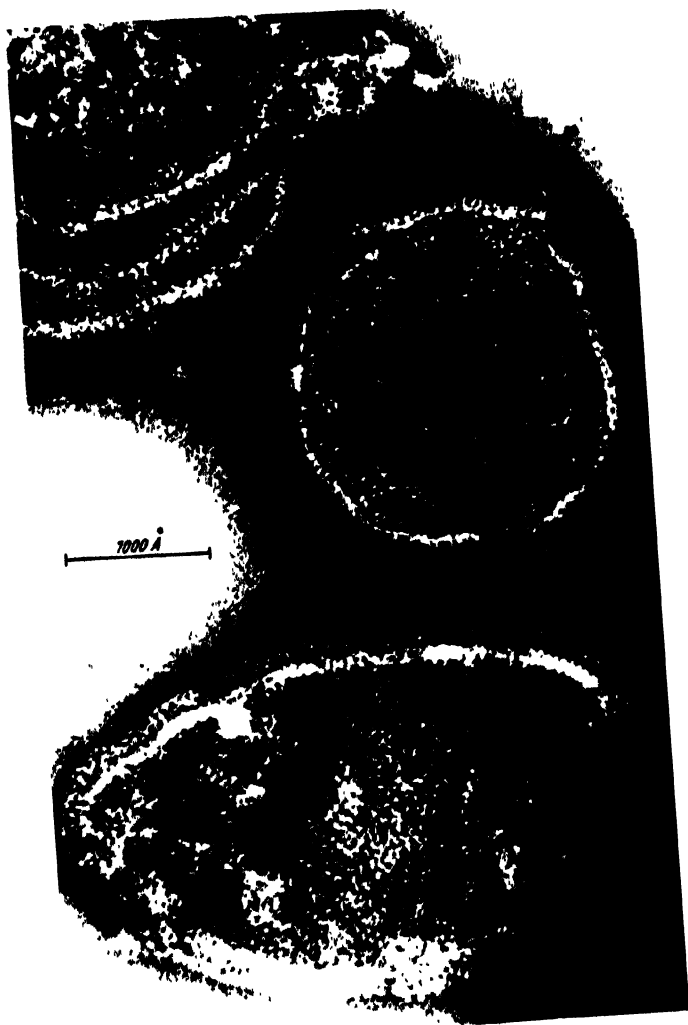
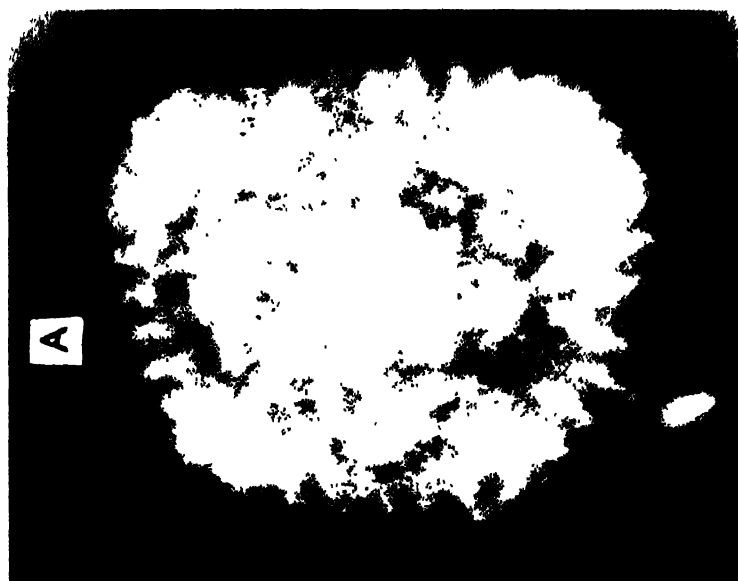
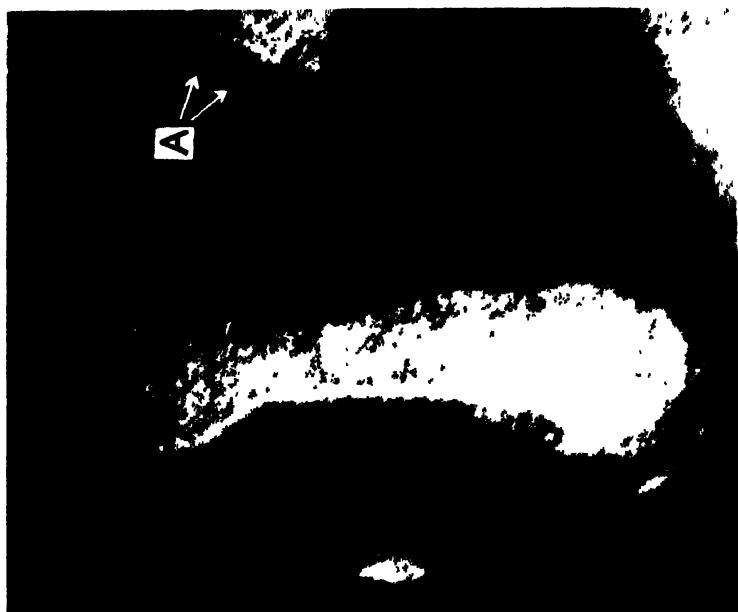


Fig. 31. Negatively stained measles virus particles; the middle particle is intact [electron micrograph by Mrs J.D. Almeida, from A.P. Waterson (1965) *Arch. f. die ges. Virusforsch.* 16, 57-80 (Springer-Verlag, Wien and New York)].



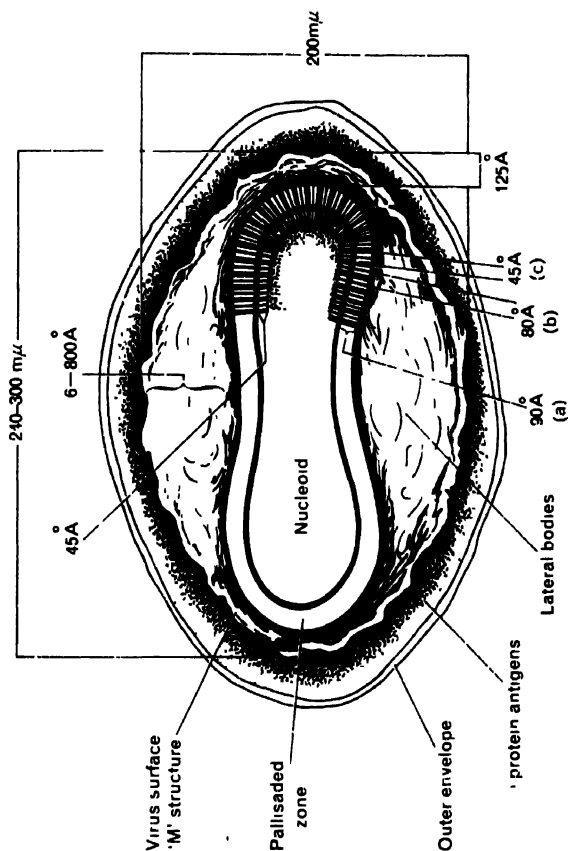


Fig. 32.

(a) Surface structure of vaccinia virus particle.

(b) Side elevation of negatively stained vaccinia virus particle.

(c) Diagram of the side elevation of a vaccinia virus particle showing structure and principal dimensions suggested by Westwood *et al.*

[(a) (b) and (c) From J.C.N. Westwood, W.J. Harris, H.T. Zwartouw, D.H.J. Titmuss, and G. Appleyard (1964) *J. gen. Microbiol.* 34, 67-78 (Cambridge University Press)].

Surprisingly, the viruses of measles and canine distemper have been found to be morphologically similar to the paramyxoviruses (Fig. 31). Similarly, the viruses of rabies and Rous sarcoma which have no biological properties in common with myxoviruses or paramyxoviruses display some of the morphological features of these groups.

Viruses exhibiting complex Symmetry

Poxviruses

The largest animal viruses, which belong to the *Poxvirus* group and include the viruses of vaccinia and variola, exhibit a very complicated structure. Vaccinia, which is the most intensively studied member of the group, is a brick shaped organism approximately $3000 \text{ \AA} \times 2500 \text{ \AA}$. Thin sections of the organism examined in the electron microscope disclose an outer double membrane enclosing a central biconcave nucleoid structure and two lateral bodies (Fig. 32b). Although the appearance of vaccinia virus particles after negative staining is dependent on the mode of preparation, the fine structure which most of the particles exhibit has been well described by Westwood and his colleagues. The surface of the virus has an irregular beaded appearance which is due to the winding of a thread-like protein structure around the virus (Fig. 32a). This thread appears to be a helical structure, 90 \AA in diameter, with a central hole, 30 \AA in diameter. In vaccinia preparations this complicated surface structure is sometimes surrounded by a thin protein membraneous envelope. The nucleoid consists of an outer palisaded zone enclosing an inner core in which the virus DNA resides (Fig. 32c).

CHAPTER 7

Virus Classification

At the time of writing no official nomenclature for viruses has been internationally agreed. As a result, colloquial and semi-official names are often used interchangeably, and the former are often more readily intelligible than the latter. For this reason, the author makes no apology for the frequent use of colloquial names.

Until quite recently, in the absence of other criteria, viruses were grouped according to their host and tissue affinities. The primary classification was therefore into viruses which infected bacteria, plants and animals, respectively. In spite of the search for more comprehensive systems of classification this division retains its general usefulness. In contrast, the subdivision of animal viruses on the basis of their tissue affinity has proved, in the light of modern knowledge, unsound. Thus poliovirus once thought to be a strictly neurotropic virus readily infects epithelial cells in culture.

A more rational classification based on biochemical and biophysical criteria, which embraces bacterial, plant and animal viruses, is now under study by the Provisional Committee for Nomenclature of Viruses. The proposals are for a binomial system of classification similar to that used in zoology and botany. In this system, viruses will be classified into Subphyla, Classes, Orders, Families, Genera, Subgenera, and Species.

In this chapter and the accompanying table (Table 7 at end of chapter), the classification of animal viruses, only, will be considered, and the discussion will be confined to those viruses which are of most interest to students of medicine. Nevertheless, the scheme and proposals put forward by the Provisional Committee will be adhered to as closely as possible.

The primary classification into two main groups or Subphyla is based on the type of nucleic acid, RNA or DNA, found in the virus particles. The subdivision of each Subphylum into Classes

is based on the type of symmetry, helical or cubical (icosahedral), which is displayed by the virus capsid. The presence or absence of a virus envelope surrounding the nucleocapsid determines the Order to which viruses of each Class belong. With the exception of the Chitovirales (see table 7), the presence of an envelope is correlated with the sensitivity of the virus to inactivation by ether. Ether sensitivity is therefore an important property in the identification of viruses. The diameter of the nucleocapsid of helical viruses, and the number of capsomeres and other geometrical criteria of viruses exhibiting cubical (icosahedral) symmetry, are the criteria by which viruses of the various Orders are divided into Families. The assignment of viruses within each Family to the various Genera is based on their different biological and antigenic properties.

Class: Ribocubica

Order: Tagovirales

Family: Arboviridae

From the table Arboviridae are seen to be ether sensitive, RNA viruses exhibiting cubical symmetry, whose virions are characterized by the presence of an envelope. All viruses belonging to this Family are transmitted from one vertebrate host to another by arthropod vectors, in which the virus undergoes replication. Hence, the term *Arbovirus* which represents an abbreviation of arthropod-borne virus.

The genus *Arbovirus* is divided into the subgenera *Arbovirus* A, B, and C, as well as some others, which are recognized on the basis of antigenic specificity. There are 17 different species or serotypes in the subgenus *Arbovirus* A, of which Western equine encephalitis (WEE) is the type species. Similarly, there are 33 different species or serotypes in the subgenus *Arbovirus* B of which yellow fever is the type species. In addition the subgenus *Arbovirus* C comprises nine different species or serotypes, and there are many others which do not fit into these three groups.

Order: Gymnovirales*Family: Napoviridae*

All viruses belonging to this Family are small in size, varying from 10-30 m μ in diameter. They are defined as small, ether stable, RNA viruses exhibiting cubical symmetry, whose virions are not characterized by an envelope. Animal viruses belonging to this Family are included in the subfamily Picornavirinae. The prefix 'Picorna' is a neologism derived from the Latin Pica, meaning small, and the abbreviation RNA, i.e. small RNA viruses. Six genera of the subfamily Picornavirinae are recognized, one of which is the genus *Picornavirus* itself. The type species of this genus is the virus of foot and mouth disease, *Picornavirus aphthae*, which is the smallest representative of the Picornavirinae.

Three genera of the Picornavirinae, *Poliovirus*, *Coxsackievirus*, and *Echovirus* are found in the alimentary tract of man and are, therefore, together known colloquially as enteroviruses. The genus *Poliovirus* comprises three species represented by the three different antigenic types of poliovirus. The genus *Coxsackievirus* is subdivided into two subgenera, *Coxsackievirus* (A) and *Coxsackievirus* (B) on the basis of their biological properties, and the nature of the lesions which they produce in experimental animals. Twenty-three different species or antigenic types of *Coxsackievirus* (A) are now known and six different species or antigenic types of *Coxsackievirus* (B). The genus *Echovirus* is divided into several subgenera according to the species from which the viruses are isolated. The subgenus *Echovirus* (*hominis*) comprises cytopathogenic viruses, originally not known to be associated with any specific disease, isolated from the human alimentary tract. Hence the term Echovirus, which is an abbreviation for enteric cytopathogenic human orphan virus. Thirty different species or antigenic types of the subgenus *Echovirus* (*hominis*) are now known.

The most recently recognized group of viruses in the subfamily Picornavirinae is the genus *Rhinovirus*. This genus comprises those viruses which cause common colds and have their natural habitat in the nose. Viruses of this type have now been isolated from a number of different animal species and strains recovered from

human sources have therefore been assigned to the subgenus *Rhinovirus (hominis)*. At least 80 different species or antigenic types belonging to this subgenus, have now been recognized.

Family: Reoviridae

Viruses belonging to the Family Reoviridae are either stable RNA viruses which are not enveloped. They differ from the Napoviridae by their larger size, the diameter of the Reoviridae measuring approximately 75 m μ , and by the presence of double-stranded RNA. A number of reoviruses have now been isolated from various animal species, not all of which are mammalian. Those from mammalian species are isolated from both the respiratory and alimentary tracts and are assigned to the subgenus *Reovirus (mammalis)*. Most of them are not associated with any specific disease, hence the term Reovirus which is an abbreviation for respiratory enteric orphan virus. There are now three different species or antigenic types of *Reovirus (mammalis)*.

Class: Ribohelica

All the ribohelical viruses of medical interest belong to the Order *Sagovirales*. These are either sensitive RNA viruses exhibiting helical symmetry and possessing a lipoprotein envelope. The two main Families of this Order, the Myxoviridae and the Paramyxoviridae were originally grouped together because of their common affinity for mucoproteins, manifested by their haemagglutinating properties. The two Families are now distinguished on the basis of their morphological characteristics and other biological properties summarized in Table 8.

Some viruses which are not characterized by affinity for mucoproteins are now included in the Myxoviridae and Paramyxoviridae Families because of their very close morphological resemblance to the original members of the group.

Family: Myxoviridae

The type genus of this family is the genus *Myxovirus* which comprises, in the subgenus *Myxovirus (influenzae)*, the viruses of

influenza. This subgenus is further subdivided into three species, A, B, and C, on the basis of the antigenic specificity of the soluble and viral antigens which are produced. *Myxovirus (influenzae)* A contains a number of subspecies according to the animal species from which the constituent viruses are recovered. In addition to the viruses isolated from man, which belong to the subspecies *hominis*, there are those which are isolated from swine (*suis*), horses (*equi*),

Table 8. Properties which distinguish Myxoviridae from Paramyxoviridae*

Property	Myxoviridae	Paramyxoviridae
Particle size	80-120 mμ	150-500 mμ
Diameter of nucleoprotein component	90 Å	180 Å
Filaments	Common	Rare
Haemolysis	Non-Haemolytic	Haemolytic
Site of soluble antigen formation	Nucleus	Cytoplasm

* After A. P. Waterson (1962) *Nature (Lond)*, 193, 1163

ducks (*anat*) and fowls (*galli*). *Myxovirus (influenzae)* A, *hominis*, is further subdivided into types on the basis of the antigenic specificity of the viral as opposed to soluble antigens. These types fall into periodic as well as antigenic groups. Viruses belonging to classical type A were prevalent in the 1934-46 period, those belonging to type A₁ (A-Prime) were prevalent in the period 1946-57, and those belonging to type A₂ (Asian) became prevalent in 1957 and remain extant to the present day. Viruses belonging to the species *Myxovirus (influenzae)* B and C are not found in animal species other than man, and they are more antigenically stable than viruses belonging to *Myxovirus (influenzae)* A.

The virus of rabies does not possess any of the biological properties of the myxoviruses but is included in the Family Myxoviridae because of its close morphological similarity to the myxoviruses. It is ascribed to the genus *Rabiesvirus*, species *canis*.

Family: Paramyxoviridae

Two genera of the Family Paramyxoviridae are recognized, the

genus *Paramyxovirus* and the genus *Bronchovirus*. The genus *Paramyxovirus* comprises the subgenus *Paramyxovirus (influenzae)*, and the species *Paramyxovirus multiforme* and *Paramyxovirus parotidis*. There are four species of *Paramyxovirus (influenzae)* which represent four different antigenic types, all of which are capable of producing respiratory infections, particularly in children. *Paramyxovirus parotidis* is the virus of mumps and *Paramyxovirus multiforme* is the virus of Newcastle disease of fowls (NDV or fowl pest).

Two genera which resemble the paramyxoviruses in morphology but have no affinity for mucoproteins are distinguished. The genus *Bronchovirus* does not have any haemagglutinating properties, and its type species *Bronchovirus syncytialis* is commonly known as respiratory syncytial virus. The other genus, which may be termed *Pseudomyxovirus*, comprises the viruses of measles, distemper and rinderpest which in spite of their different host affinities are antigenically related.

SUBPHYLUM: DEOXYVIRA

Class: Deoxyhelica

The Deoxyhelica are DNA viruses which do not exhibit icosahedral symmetry and are tentatively thought to exhibit helical symmetry. They are all characterized by the possession of an envelope and therefore belong to the Order Chitovirales. Contrary to the general rule for enveloped viruses, they may be either ether stable or ether labile. Only one Family is defined, the Poxviridae, which comprises a number of genera, only three of which are important for man.

Family: Poxviridae

Three genera belonging to the Family Poxviridae are important for man. The genus *Poxvirus* includes the two species *Poxvirus variolae*, the virus of smallpox, and *Poxvirus vaccinia*, the vaccinia virus. Other genera are the genus *Molluscovirus*, of which the species *Molluscovirus hominis* is the cause of molluscum contagiosum, and the genus *Dermovirus*, of which the species of *Dermovirus orfi* is the

cause of orf or contagious pustular dermatitis of sheep, which may be transmitted to man. A number of other genera which cause pox-like conditions in various animal species are included in the Family. Also included is the genus *Fibromavirus* which, unlike other genera included in the Family Poxviridae, is sensitive to inactivation by ether. The genus contains two species, *Fibromavirus myxomatosis*, the cause of myxomatosis in rabbits, and another species which is the cause of Shope fibroma in rabbits.

Class: Deoxycubica

The Deoxycubica, or DNA viruses exhibiting cubical symmetry, are divided into two Orders, the *Peplovirales* which are characterized by the possession of a virus envelope, and the *Haplovirales* which are not. In accordance with the general rule, the *Peplovirales* are sensitive to inactivation by ether. The Order *Peplovirales* is represented by the Family *Herpesviridae* and the Order *Haplovirales* by two Families, the *Adenoviridae* and the *Papillomaviridae*.

Family: Adenoviridae

The only genus belonging to this Family is the genus *Adenovirus*, which is divided into several subgenera according to the animal species from which the viruses are recovered. Those isolated from man belong to the subgenus *Adenovirus (hominis)*, of which there are now 31 different species identified on the basis of their antigenic specificity.

Family: Papillomaviridae

The Family *Papillomaviridae* comprises two genera, both of which include viruses which produce neoplasms in animals. The genus *Papillomavirus*, species *sylvilagi*, previously known as the Shope papilloma virus, produces a papillomatous condition in cottontail (*sylvilagus*) rabbits. The other genus *Polyomavirus* includes the important species *Polyomavirus neoformans* which is oncogenic in mice, producing a variety of malignant tumours.

Family: Herpesviridae

Viruses belonging to this Family are DNA viruses which exhibit cubical (icosahedral) symmetry, possess a viral envelope, and are sensitive to inactivation by ether. Characteristically, their morphological subunits or capsomeres are hollow units with hexagonal or pentagonal outlines arranged in icosahedral symmetry (Fig. 25). Two genera are defined, the genus *Herpesvirus* and the genus *Cytomegalovirus*. The former consists of several species, of which the ones important for man are *Herpesvirus hominis* (the virus of herpes simplex), and *Herpesvirus varicellae* (the virus of chickenpox and zoster).

The genus *Cytomegalovirus* comprises viruses which are very similar in morphology to those of the genus *Herpesvirus*, but which have a special affinity for the salivary glands of man and animals. The species *Cytomegalovirus hominis* is the one which causes the cytomegalic inclusion disease of man.

CHAPTER 8

The Bacteriophage Model

The discovery that bacteria, themselves the cause of so many diseases, are susceptible to infection by viruses was made by Twort (1915) and d'Herelle (1917). The ability of bacterial viruses to lyse and kill bacterial cells led to the hope that they would be therapeutically effective in bacterial infections. After a number of clinical trials it soon became evident that little success was to be achieved in this field, and the introduction of antibiotics made further therapeutic studies superfluous.

The real importance of bacterial viruses, to which d'Herelle gave the name bacteriophage,* is derived from the unicellular nature of their host. This makes the bacteriophage-bacterial system an ideal model for the study of host-virus relationships at the cellular level. Indeed, until the introduction of tissue culture methods for the study of animal viruses, bacterial viruses were unique in this respect. For this reason, the mechanisms of bacteriophage infection are, even now, much better understood than those of other types of virus infection.

Structure of the Bacteriophage Particle

With few exceptions, all 'phage strains which have been examined in the electron microscope possess head and tail components and exhibit a high degree of structural differentiation. Although strains differ in detail, the T-even phages of *E. coli*, which have been most intensively studied, provide the most suitable models of 'phage morphology. T₂-'phage consists of an hexagonal head 100 mμ in length and 65 mμ wide, and a tail 100 mμ long and 25 mμ wide (Fig. 33). The head is composed of an outer protein coat and an internal structure, which consists almost entirely of deoxyribose-

* The shortened name 'phage is now commonly used for the sake of convenience.

nucleic acid (DNA) but includes a small amount of protein and peptides. The tail, which is designed to effect attachment to the bacterial cell wall, is made up of an outer sheath which encloses the tail core. The tail terminates in a base plate from which project six short spikes and from which six tail fibres originate. In the unattached 'phage, the tail fibres are wound around the distal end of the tail (Fig. 33).

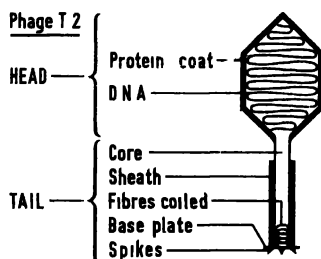


Fig. 33. T₂ bacteriophage particle.

Besides chemical and morphological differences, the head and tail components of 'phage particles differ in antigenic composition. Only the tail protein antigen is capable of producing neutralizing antibody. This antibody reacts with the tail fibres and the distal part of the tail core only, and does not react with the head protein.

Mechanisms of Bacteriophage Infection

(a) The lytic cycle of replication

(1) Adsorption

In suspension, the initial contact between 'phage particle and bacterial cell is brought about by random collision. For attachment to occur, the bacterial cell wall must be endowed with receptors specific for the particular 'phage in question. The range of bacterial strains to which a 'phage can adsorb and the range of 'phages to which a bacterial strain is susceptible is therefore limited. The specific receptors are lipoprotein or lipopolysaccharide structures incorporated in the bacterial cell wall; 'phage particles of certain

strains anchor to them through complementary receptors on the 'phage tail. From the point of view of those interested in bacterial viruses, the bacterial cell wall may be considered as a network of specific 'phage receptor sites.

The first stage of adsorption is reversible, in the absence of cations, and is therefore presumed to be of an electrostatic nature. Subsequently, after the formation of firm chemical bonds between the complementary stereo-chemical configurations on the cell and 'phage attachment sites, adsorption becomes irreversible. Electron microscopic studies show that irreversible adsorption is associated with some structural modification of the 'phage particle. Thus, on contact with the cell wall the tail fibres unwind and attach by their tips. At the same time, the tail sheath contracts exposing the tail core, which actually penetrates the cell wall. Contraction of the 'phage tail is accompanied by release of an enzyme which weakens the bacterial cell wall but does not actually destroy the specific receptors (Fig. 34).

(u) Penetration

The mechanism of 'phage penetration was elucidated by Hershey and Chase, in 1952, from experiments with radioactively labelled 'phage. After adsorption, only the DNA component actually passes into the cell, and the whole of the 'phage protein coat, of both head and tail, remains at the cell surface playing no further part in the process of infection. After discharging their DNA, 'phage 'ghosts' with empty heads, firmly attached to the cell surface, can easily be seen with the electron microscope (Fig. 35). It is now known that a very small amount of protein, no more than 3%, of the total 'phage protein, and some peptides pass into the cell along with the DNA.

In effect, the 'phage particle behaves as a 'micro-syringe'. The tail core penetrates the bacterial cell wall, already weakened by the 'phage enzyme, and afterwards is probably extruded to allow the passage of the DNA through the tail (Fig. 36). The fundamental experiments of Hershey and Chase showed beyond doubt that the nucleic acid component of bacteriophage particles is the infective principle, which contains all the information necessary for the production of new 'phage particles within the host cell.



Fig. 34. Negatively stained T₂ bacteriophage particle showing filled head, contracted sheath, exposed core, and released tail fibres [from S. Brenner, G. Streisinger, R. W. Horne, S. P. Champe, I. Barnett, S. Benzer, and M. W. Rees (1949) *J. Mol. Biol.* 1, 281-92 (Academic Press Inc., London and New York)].

(iii) *Eclipse phase and latent period*

Once inside the cell, the 'phage DNA has the function of producing new 'phage DNA and protein to be assembled into new 'phage particles. To achieve this end, 'phage DNA diverts the metabolic machinery of the bacterial host cell from making bacterial constituents to the synthesis of 'phage components.

The classical experiments of Doermann, in which bacteria were artificially lysed at intervals after infection, showed that no infective

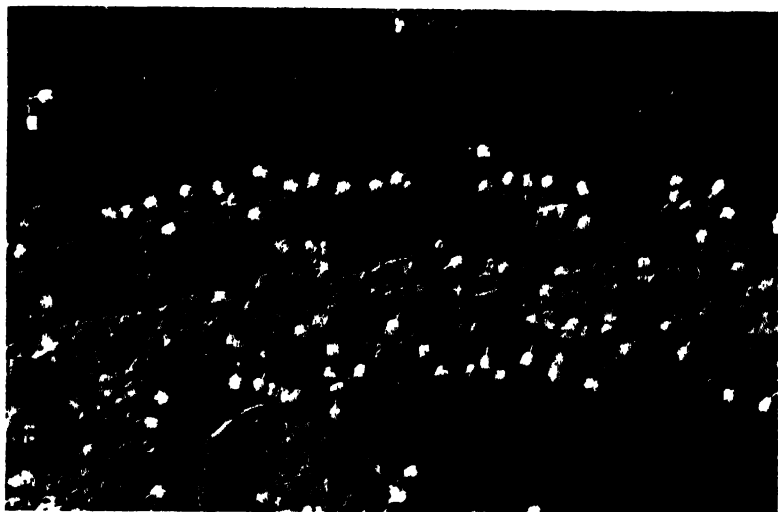


Fig. 35. T_2 'phage attached to *E. coli* cell ghost. The contracted 'phage sheath and penetrating 'phage core are easily seen [from E. Kellenberger and W. Arber (1955) *Zeitschrift f. Naturforsch.* **10B**, 698-704].

'phage was detectable for the first 12 minutes. During this period the infected cell contains only the 'phage DNA component without its protective protein coat and attachment organs, the absence of infective 'phage is not therefore surprising. At the end of this period, known as the eclipse phase, during which the 'phage remains in its non-infective or vegetative form, infective 'phage begins to make its appearance. The number of infective 'phage particles produced increases at a constant rate and reaches a maximum of about 50-100 particles per cell, approximately 25 minutes after infection. At

this stage, the cell bursts releasing progeny 'phage particles which are free to infect neighbouring cells.

Of course, in culture no infective 'phage will be detected until the cell bursts and 'phage is released. This period from the time of infection until release of infective 'phage is called the latent period.

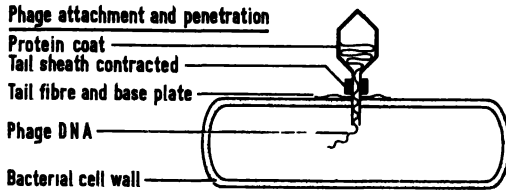


Fig. 36. Bacteriophage infection-- stage of penetration.

(a) Diagrammatic representation.

(b) Electron micrograph of Lambda 'phage; passage of DNA, through tail after incomplete osmotic shock.

[From L. G. Caro (1965) *Virology* 25, 226-36 (Academic Press Inc., New York and London).]

The length of the latent period, usually about 25 minutes, and the number of progeny 'phage released when the cell bursts, the 'burst size', vary in different 'phage-host cell systems.

(iv) Intracellular 'phage synthesis

The production of infective 'phage according to the laws of linear

and not exponential kinetics indicates that 'phage, unlike bacteria, replicates not by binary fission but by some more complicated process. Experimental evidence has revealed this process to be the independent synthesis of various 'phage components and their later assembly into mature 'phage particles.

The synthesis of 'phage DNA and protein has been studied by chemical, immunological and radioactive techniques. Synthesis of 'phage DNA commences about 6-7 minutes after infection, during the eclipse phase, and within a few minutes enough has been synthesized to pack 50-80 'phage heads. Thereafter, synthesis continues at a constant rate, so that there is always an excess of 'phage DNA available. Only about one-third of the 'phage DNA is derived from pre-existing host cell DNA, the remaining two-thirds being synthesized from nucleic acid precursors in the extracellular medium. The host cell DNA component is first broken down and then resynthesized as 'phage DNA, a process which is associated with the disappearance of the bacterial nucleus.

'Phage protein is newly synthesized from precursors in the extracellular medium, only an insignificant amount, if any, is derived from the host cell. Specific 'phage protein first appears about 10 minutes after infection, that is after 'phage DNA but before mature infective particles. Within a very short time, enough 'phage protein is synthesized to form 30-40 'phage particles and thereafter 'phage protein and DNA synthesis increase in parallel.

During the first 5 minutes after infection, some protein which is neither host cell nor 'phage-specific is formed, and probably represents the production of new enzymes required for the synthesis of 'phage DNA and protein.

The independent synthesis of 'phage DNA and protein has been confirmed by electron microscopy. Kellenberger and his colleagues found that about 8 minutes after infection, fibrils with the dimensions of DNA could be seen, and that after 10 minutes empty 'phage heads and tails made their appearance.

(v) Maturation

During the stage of maturation, by mechanisms as yet unknown, DNA becomes incorporated into the 'phage head which is joined to

the tail to produce the mature 'phage particle. Proof that the assembly of 'phage DNA and protein components takes place by random association has been obtained from experiments in which bacterial cells were doubly infected with two different 'phages. Some of the progeny obtained in these experiments contained the DNA and head protein of one 'phage and the tail protein of the other; a phenomenon known as phenotypic mixing.

(vi) Lysis

After maturation of the 'phage, the host cell lyses releasing the mature 'phage particles. Lysis is associated with the production, in the infected cell, of lytic enzymes, very closely related to lysozyme, which weaken the cell wall before the cell bursts. There is some evidence that lysis occurs when a critical concentration of the lytic enzyme has been built up.

(b) The lysogenic cycle

(i) Prophage

The lytic cycle of bacteriophage replication, which results in lysis and death of the host cell, is not the only cycle of replication which the 'phage may undergo. This became evident when strains of *E. coli* were recovered from nature which, although not susceptible to lysis, produced a few free 'phage particles. These particles were easily demonstrable by their perfectly normal lytic activity against other strains. It was eventually found that the resistance of the original strain to lysis was due to infection of 99.9% of the culture with 'phage in an unusual non-infective form. This form although non-infective reproduced by replicating in synchrony with the host cell. It was thus established that bacteriophage may exist in two forms; the virulent or lytic 'phage which produces lysis of the host cell and release of infective 'phage particles, and the temperate 'phage, or prophage, which is non-infective and renders the host cell resistant to the virulent form of the same 'phage. Bacteria infected with prophage are said to be lysogenic (Fig. 37).

(ii) Induction

Very occasionally, under natural conditions, prophage may be con-

verted to virulent 'phage, producing lysis and death of the host cell and release of infective 'phage particles. This phenomenon, known as induction, may be produced spontaneously or by artificial means. Ultraviolet, X- and γ -irradiation, nitrogen mustard, hydrogen peroxide and other chemical substances are all capable of inducing prophage to produce infective virulent 'phage particles. It may be

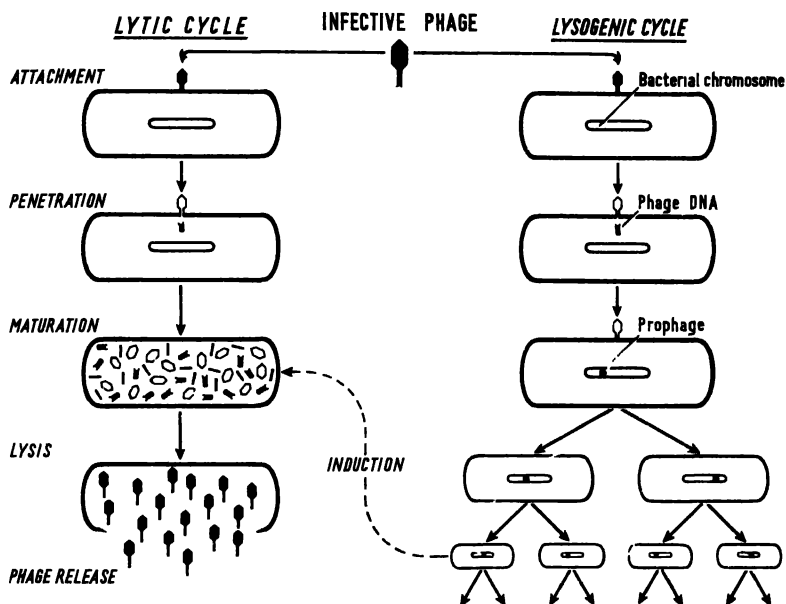


Fig. 37. Lytic and lysogenic cycles of bacteriophage replication.

noted that these substances also behave as mutagens and carcinogens, indicating that their effect is primarily on nucleic acid.

(iii) Nature of the prophage

It was suggested by Bordet as long ago as 1925 that the faculty to produce temperate bacteriophage, or prophage, was carried in the hereditary material of the bacterial cell. Since that time, much evidence has been adduced from complicated genetic experiments to indicate that prophage represents the 'phage genome, i.e. 'phage

DNA, and that it is carried on the bacterial chromosome. Apparently, prophage becomes attached to a specific location on the bacterial chromosome, without actually being incorporated into it, and is inherited in the same way as a bacterial gene. Thereafter, prophage and bacterial chromosome replicate as a whole (Fig. 37).

(iv) Transduction

The close integration of 'phage and host cell genomes as they occur in lysogenic bacteria leads to some interesting and important possi-

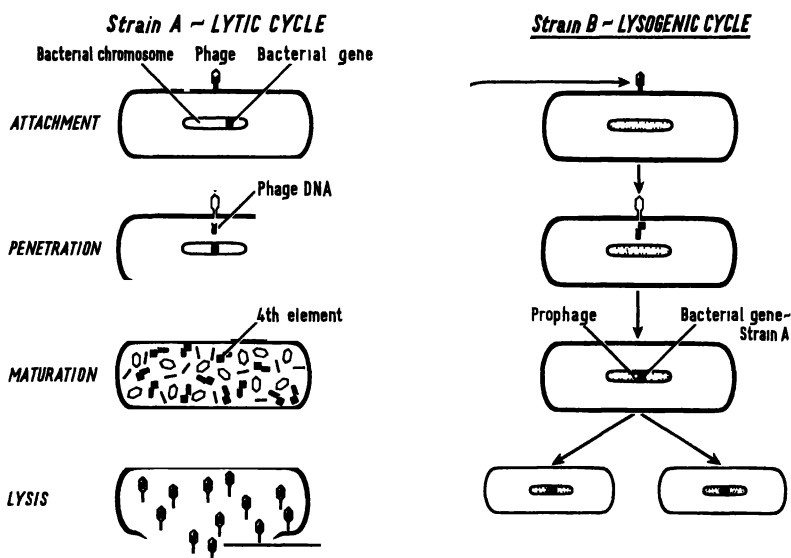


Fig. 38. Transduction; fourth element = bacterial gene

bilities for the exchange of genetic material, many of which have been demonstrated experimentally. Thus, a genetic marker may be transferred from one bacterial cell to another by a phage particle which produces lysogenic infection in the recipient cell. In this phenomenon, known as transduction, the 'phage genome plays a purely mechanical role in carrying a genetic element of the bacterial chromosome from one cell to another (Fig. 38).

(v) Lysogenic conversion

Lysogenic infection with prophage may express itself by conferring a new phenotypic character on the infected bacterial cell. Thus, some strains of *Salmonella* infected with certain prophages produce a new somatic antigen. Perhaps one of the most important examples of this phenomenon, known as lysogenic conversion, is the association of lysogenic infection of *C. diphtheriae* with the production of diphtheria toxin. Toxin is produced only by infected strains, and is released only after the prophage has been induced to produce virulent 'phage and lysis of the cell.

It is evident from the phenomenon of lysogenic conversion that prophage, attached to the bacterial chromosome, may act as a gene indistinguishable from other bacterial genes.

Biological Assay

Infection of a bacterial cell with bacteriophage is manifested by lysis of the host cell and release of progeny virus particles. Released particles infect neighbouring bacteria which, after the requisite cycle of 'phage replication, are lysed and from which the process extends. In liquid culture media, lysis of the bacterial suspension is easily recognized by loss of turbidity. On solid media, where bacteria are growing in a confluent layer and 'phage is present in low concentration, lysis is recognized by the appearance of discrete, circumscribed translucent areas, easily visible to the naked eye, which are called plaques (Fig. 39). Each plaque is produced by a single 'phage particle, and the number of plaques formed under standard conditions is therefore equivalent to the number of infective 'phage particles present in the original phage inoculum. Where plaques occur close together they may coalesce and become confluent.

For titration, serial dilutions of a bacteriophage suspension are mixed with small volumes of molten agar, cooled to 45°C, containing about 10^7 bacterial cells. Each mixture is then poured over the surface of a layer of solid nutrient agar in a Petri dish and allowed to harden before incubation at 37°C. After 24 hours incubation, the plate exhibiting 100–200 plaques is selected and the number of

plaques accurately counted. The total number of infective 'phage particles in the original suspension is then easily calculated from the dilution of the inoculum. Thus, if 0.1 ml of a 'phage suspension diluted 1 : 100,000 produces 200 plaques per plate, the total number of infective particles in the original suspension is $200 \times 10 \times 100,000$ per ml.

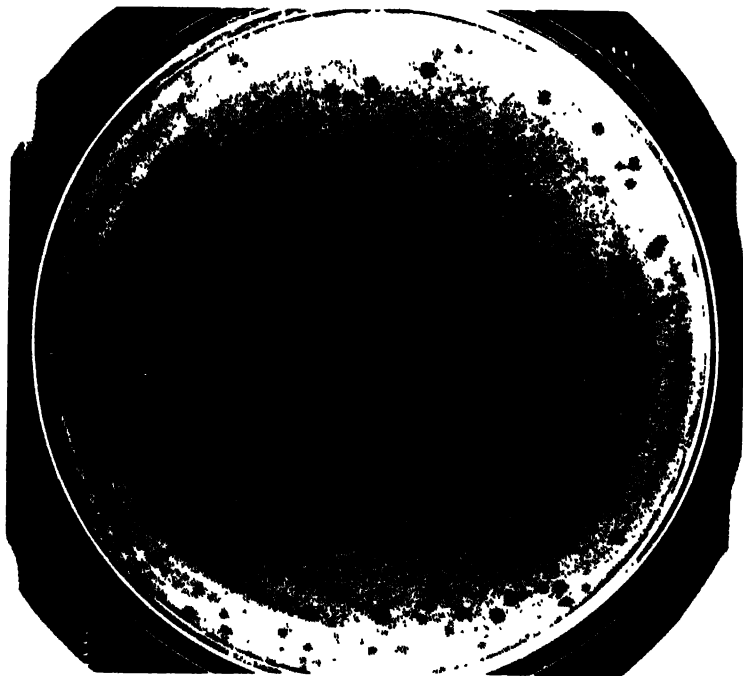


Fig. 39. *T*₂ bacteriophage plaques on a confluent layer of *E. coli*.

Plaque Size and Morphology

The size and morphology of the plaques produced may be affected by environmental variables. But where conditions are standardized and the particular plaque type breeds true, the characteristics of the plaque are determined by an intrinsic property of the particular 'phage. As such, plaque morphology may be used for identification of a 'phage and as a genetic marker.

Practical Application: Bacteriophage Typing

Bacteria which are identical by the usual serological and bacteriological tests may be distinguished on the basis of the particular 'phage or group of 'phages to which they are susceptible. This technique of 'phage typing has been of very great value in epidemiological work for tracing the source and spread of organisms through the community.

The test bacterial strain is spread on a nutrient agar plate in sufficient concentration to give a confluent growth. At the same time a number of different 'phages, in suitable dilution, are applied from fine dropping pipettes. 'Phage susceptibility is indicated by the appearance, after incubation, of clear areas of lysis in an otherwise confluent bacterial growth (Fig. 40).

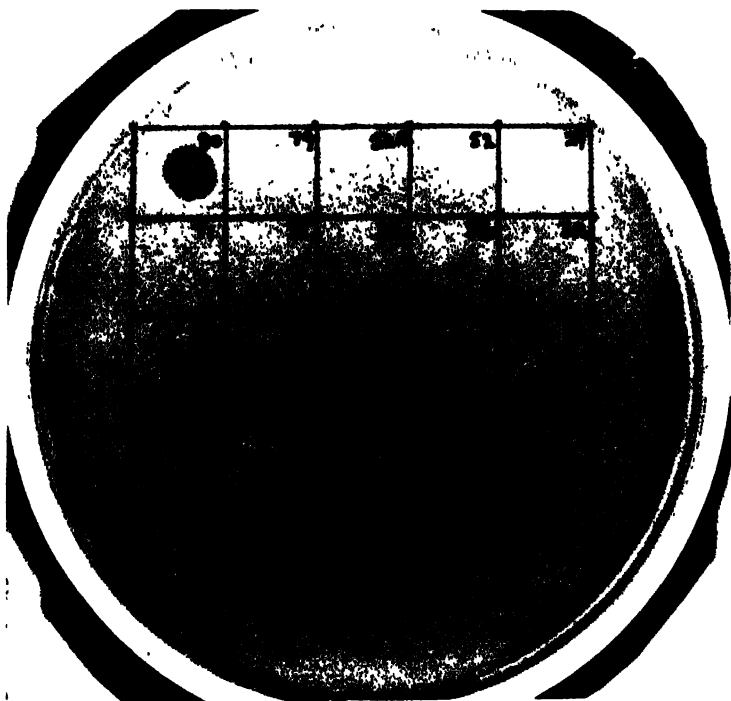


Fig. 40. Phage typing of *Staphylococcus*—Type 80/81

CHAPTER 9

Mechanisms of Cell Infection

Entry into the Host

In the metazoan, as in the bacterial host, viruses replicate exclusively in the interior of susceptible host cells. Before reaching these cells, animal viruses must first gain the interior of the animal or human host. The route of entry is usually through the skin, or mucous membranes which communicate directly with the external environment. Entry may be through a breach in continuity of the skin caused by wounding, insect bites, or artificial inoculation, or more commonly through the mucous membranes of the upper respiratory and alimentary tracts. Even generalized virus infections, like chickenpox and smallpox which do not necessarily cause signs and symptoms of respiratory infection, gain entry into the interior through the mucous membranes of the upper respiratory tract.

Virus-Host Cell Interaction

The interaction of animal viruses and their host cells are not as well understood as that between bacteriophages and bacteria. So far, only a few viruses have been studied in any detail, but myxoviruses and enteroviruses have provided excellent models for the study of cell attachment, penetration, and intracellular virus replication. Although data provided by other viruses are required before generalizations can be made with confidence, enough information is available to suggest marked similarities as well as many differences between the mechanisms of cell infection used by bacteriophages and animal viruses.

(a) Attachment

The cycle of infection commences with attachment of virus to the cell surface. The elucidation of the mechanisms of virus-cell

adsorption has been greatly facilitated by the study of the myxovirus haemagglutination reaction, in which attachment of myxoviruses to red blood cells closely simulates attachment to host-tissue cells.

(i) The myxovirus haemagglutination reaction

Myxovirus particles adsorb to red blood cells by means of specific complementary receptor sites, a number of which are distributed

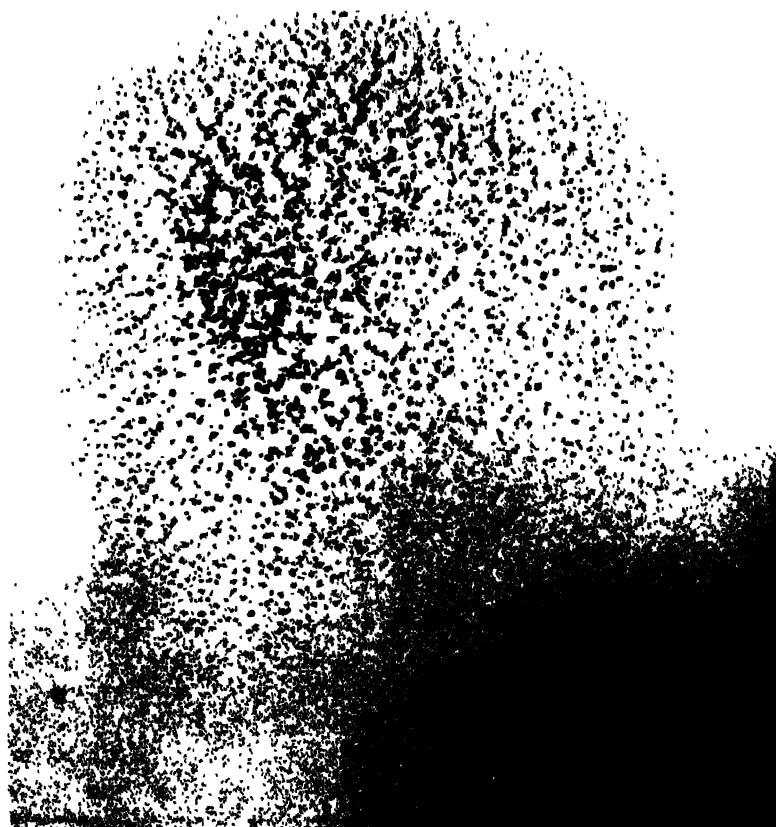


Fig. 41. Influenza virus haemagglutination—tile test.

over the surfaces of both virus and red cell. A number of virus particles become attached to each red cell and some particles attach simultaneously to more than one. In this way, a 'lattice' of red cells held together by virus particles is formed, which eventually becomes sufficiently large to be visible by the naked eye (Figs 41 and 42).

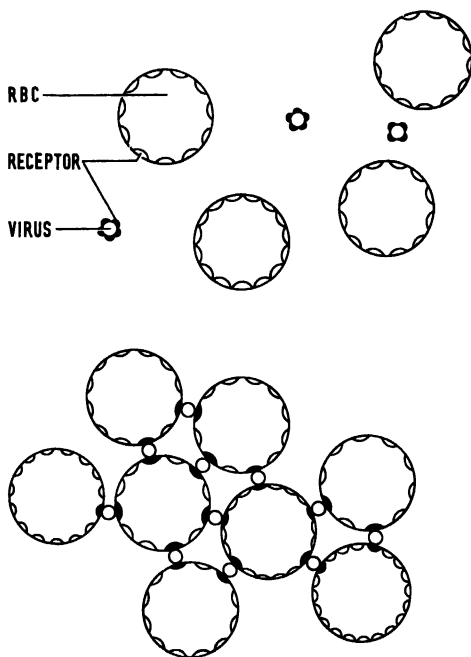
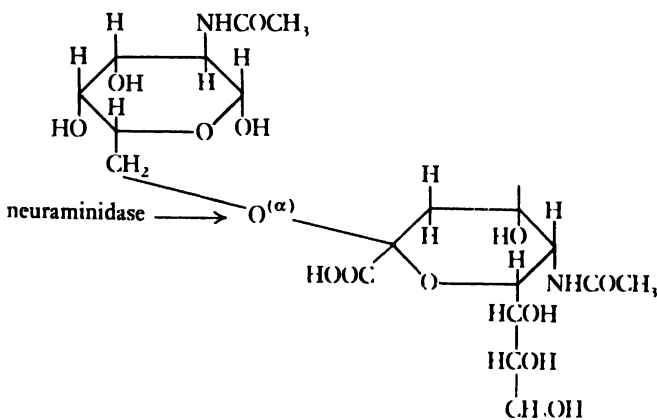


Fig. 42. Influenza virus haemagglutination

At 37°C adsorbed virus is spontaneously released from red cells with the enzymic destruction of the red cell receptors. The enzyme, now known to be neuraminidase, is incorporated in the virus surface, and is the only virus enzyme so far known. Its substrate is the carbohydrate prosthetic group of the mucoprotein red cell receptor, which Gottschalk and his colleagues have identified as 6- α -N-acetyl-D-neuraminy-N-acetylgalactosamine. After release of the split product, N-acetylneuraminic acid (sialic acid), the red cell receptor is no longer capable of adsorbing or binding virus.

Disaccharide Prosthetic Group of Receptor Mucoprotein
(after Gottschalk and Graham)



(ii) *Myxovirus host cell adsorption*

The reactions which occur between myxoviruses and red cells occur also with host cells. The close similarity, if not identity, of the receptors through which these reactions take place is evident from the destruction of host cell receptors by the purified enzyme neuraminidase. After treatment with neuraminidase, host cells no longer adsorb virus, although they are not irreparably damaged. Regeneration of the sialomucoprotein receptors begins in a few hours and is complete in a few days.

There is now no doubt that myxoviruses attach to host cells by means of specific sialomucoprotein receptors present at the cell surface. In vivo, attachment is not followed by virus release, but this is not surprising when it is appreciated that virus must penetrate the cell membrane and gain the cell interior for infection to occur.

(iii) *Attachment of other viruses to host cells*

In recent years, good evidence has been obtained that poliovirus and some other enteroviruses attach to the host cell surface by means of specific receptors. Unlike myxovirus receptors, however, these are of a lipoprotein nature. Compared with myxoviruses and enteroviruses, little is known about the mechanisms by which other viruses

attach to host cells. But, the possibility that they attach through specific receptors is suggested by the common use of this mechanism by such diverse viruses as myxoviruses and bacteriophages.

(b) Penetration

Passage of virus particles from the cell surface to the interior necessitates transmission across the cell membrane. In contrast to the bacterial cell wall, the animal cell membrane is a very much less rigid structure. The mechanism of penetration evolved by animal viruses is therefore different from that evolved by bacteriophages.

Originally, the enzymic destruction of specific receptors by myxoviruses suggested that this was a necessary step in cell penetration, but it is now beyond doubt that neuraminidase plays no part in this process. Its function remains controversial. Possibly, it acts on the mucoproteins of the respiratory secretions, reducing their viscosity and thereby facilitating contact between virus and host cell.

Much recent experimental evidence, supported by electron microscopic observations, leads to the conclusion that virus is taken into the host cell by a process of active ingestion. This process resembles pinocytosis by which fluid droplets gain entry into the cell. After adsorption, the virus particle is engulfed by the cell membrane, which eventually forms a vesicle and buds off in the interior of the cell (Figs 43 and 44). This mechanism, to which the name viropexis was given by Fazekas de St Groth, has been shown by electron microscopy to serve the needs of many different viruses. Although virus ingested in this way achieves an intracellular position, it is still surrounded by a part of the cell membrane. How the virus traverses this to enter the cytoplasm is not yet known with any certainty.

The initiation of infection by the nucleic acid moiety of some of the smaller animal viruses suggests the possibility of cell penetration by some mechanism similar to that of the T_2 -bacteriophage. So far, no unequivocal evidence that animal viruses split into nucleic acid and protein components at the cell surface, or that nucleic acid alone enters the cell, has been obtained. All the evidence indicates that the whole virus particle is incorporated into the cell and that the

subsequent breakdown into protein and nucleic acid components takes place in the interior.

(c) The eclipse phase

After penetration, most of the virus particles become non-infective and remain so for a period of some hours. This is the so-called

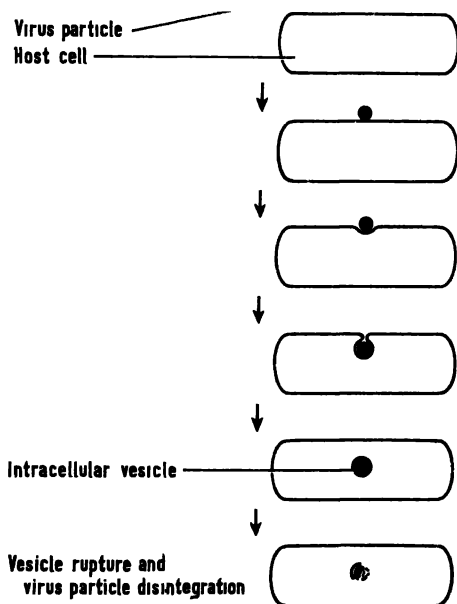


Fig. 43. Viropexis diagrammatic representation.

'eclipse phase' which, because of its regular occurrence, Lwoff considers to be one of the criteria that distinguish viruses from other micro-organisms. Radioactive labelling and electron microscopic techniques show that eclipse commences with the uncoating of the virus particle and continues while viral nucleic acid and protein are being formed. With the formation of mature virus particles, the eclipse phase comes to an end and the titre of infective virus begins to rise. The duration of the eclipse phase after infection with the

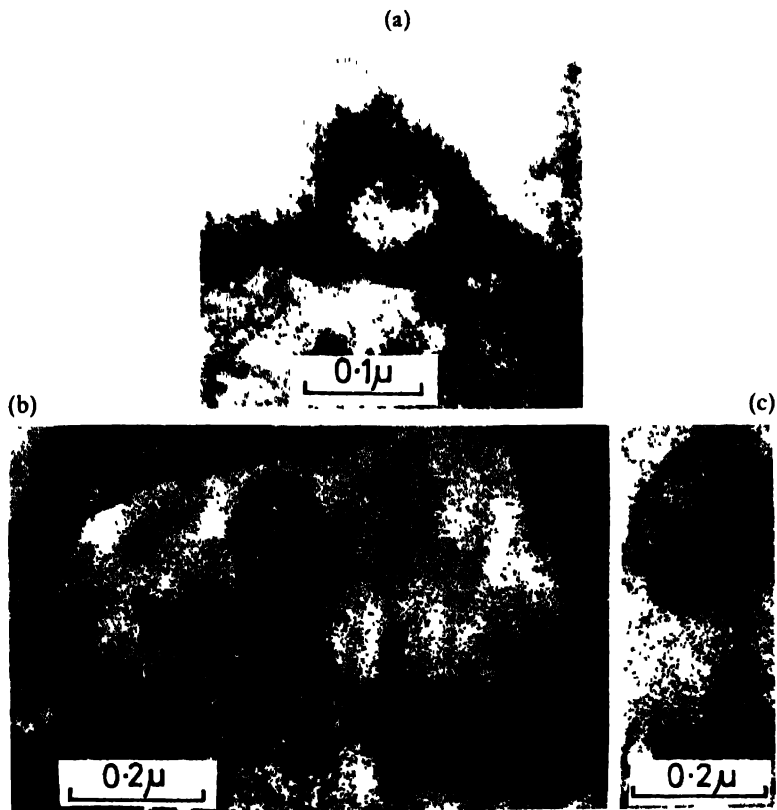


Fig. 44. Viropexis.

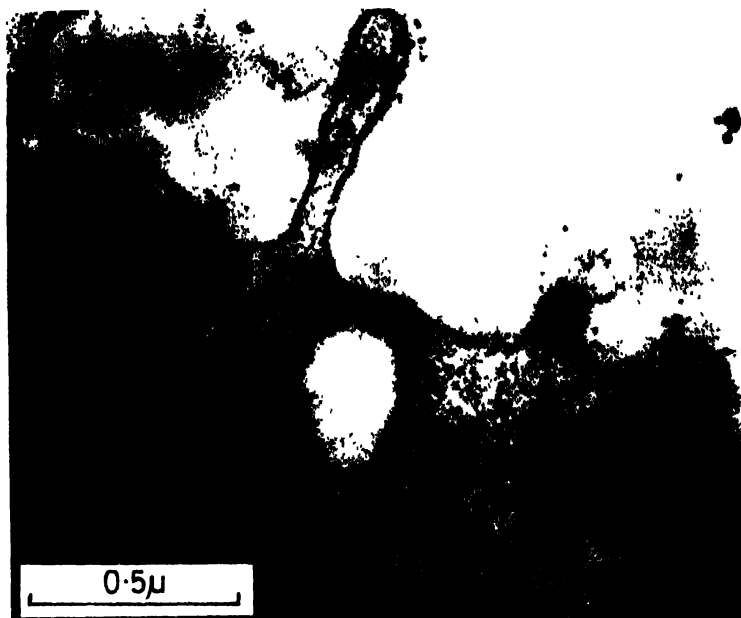
(a) Influenza virus particle in contact with surface of a monkey kidney cell.

(b) and (c) Section of chorioallantoic membrane 20 minutes after infection with influenza virus.

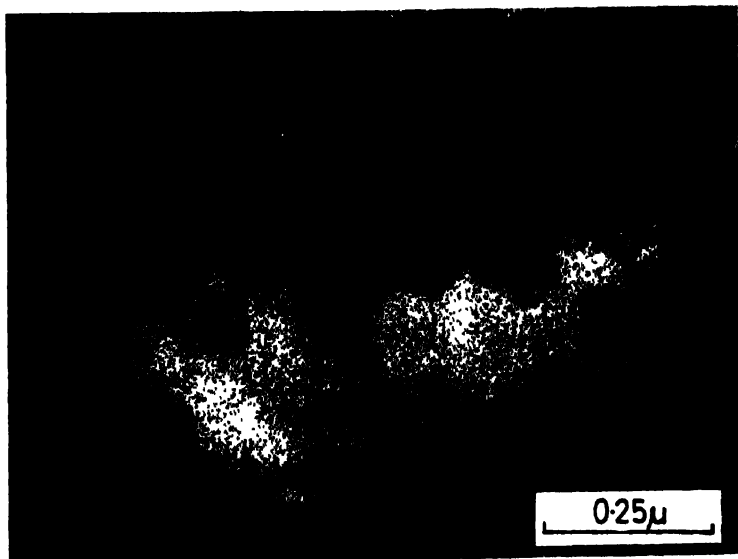
(d) Intravesicular virus particles 20 minutes after infection of chorioallantoic membrane.

(e) Intravesicular amorphous material and membranous profiles, probably representing disintegrated virus, together with recognizable virus particles in section of chorioallantoic membrane 20 minutes after influenza virus infection.

[From S. Dales and P. W. Choppin (1962) *Virology* 18, 489-93 (Academic Press Inc., New York and London).]



(d)



(e)

small RNA viruses is approximately 4–7 hours but may extend to 14 hours after infection with adenoviruses.

(d) Intracellular virus replication

Animal viruses, like bacteriophages, do not replicate by binary fission, but by the independent synthesis of viral nucleic acid and protein components and their later assembly into mature virus particles. Although some generalizations are possible, the marked differences in structure and chemical composition of various viruses make some differences in the detail of their synthesis inevitable.

After separation of the protein and nucleic acid moieties of RNA viruses, a short delay period of 1 hour or longer is apparent before viral nucleic acid and protein synthesis begins. During this period certain 'early' proteins are synthesized, possibly under the direction of the parental viral RNA, which inhibit host cell nucleic acid and protein synthesis and which are essential for the synthesis of viral RNA. One of the 'early' proteins is probably virus-specific RNA polymerase which is an essential prerequisite for the synthesis of viral RNA.

Although viral RNA acts as template both for its own replication and for the synthesis of virus-specific proteins, the synthesis of viral nucleic acid and coat protein at different times and at different sites in the cell indicates that they are synthesized independently. There is definite evidence that RNA of myxovirus and encephalomyocarditis virus is synthesized about 1–2 hours before the synthesis of viral protein. RNA and protein of poliovirus, however, are synthesized simultaneously. Although less is known about replication of DNA viruses, Cairns has shown that the synthesis of DNA and protein of vaccinia virus proceeds simultaneously.

The intracellular sites at which viral nucleic acid and protein are synthesized vary. The DNA and protein of adenoviruses are synthesized in the nucleus, whereas the DNA and protein of vaccinia virus are both synthesized in the cytoplasm. In contrast, the nucleoprotein component of myxoviruses is synthesized in the nucleus, where it appears about 3 hours after infection, and the strain specific antigens and haemagglutinin components are synthesized in the cytoplasm, where they appear about 4 hours after infection. Later,

the nucleo-protein component diffuses into the cytoplasm and combines with the newly synthesized viral proteins to form mature virus particles. This pattern is followed in the replication of encephalomyocarditis virus but has not been unequivocally proved to apply to all RNA viruses. Indeed, both the nucleoprotein and haemagglutinin components of paramyxoviruses are synthesized exclusively in the cytoplasm. Similarly, the synthesis of poliovirus RNA and protein is confined to the cytoplasm, although the possibility that poliovirus RNA is synthesized in the nucleus and rapidly transported to the cytoplasm has been suggested.

(e) Virus maturation

(1) Normal maturation

The stage of virus maturation commences with the assembly of newly synthesized viral nucleic acid and coat protein components into mature virus particles. Although the precise mechanisms by which this comes about are not yet known, the mode of maturation appears to be determined, in part, by the structure of the mature virus particle.

Many of the smaller RNA viruses, and some DNA viruses, are constructed according to the laws of cubic symmetry, and the physico-chemical configuration of the protein subunits and nucleic acid components of these viruses is believed to be such that assembly into only one final configuration is possible. The individual components may thus be considered analogous to the pieces of a jig-saw puzzle whose assembly is possible in only one particular way.

Myxoviruses and paramyxoviruses, which are more complicated in structure, mature in a more elaborate way. The nucleoprotein component, whether synthesized in nucleus or cytoplasm, becomes integrated with viral protein components just beneath the cell membrane. In the final stage of maturation, the assembled particles bulge through the cell membrane, which envelops them in the course of separation from the cell by a pinocytotic process in reverse (Fig. 45). The mature virus particles thus acquire their lipoprotein envelope from the cell membrane, which apparently acquires viral antigenic and haemagglutinating properties in the process.

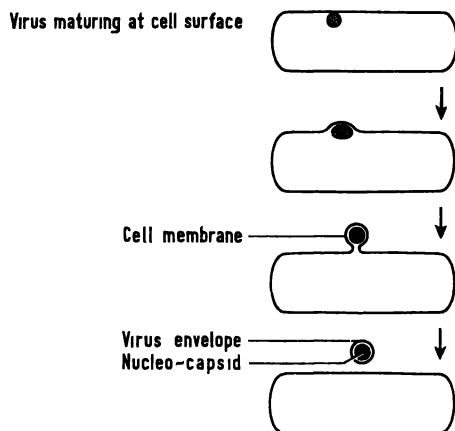


Fig. 45. Virus maturation and release — diagrammatic representation

(u) *Anomalies of maturation*

Virus maturation by the random assembly of separately synthesized viral nucleic acid and protein components is confirmed by some of the anomalies which may occur during the stage of maturation. Cells simultaneously infected with two different strains of influenza virus may produce some progeny particles which inherit a single genome but type specific antigens representative of both parent viruses in their lipoprotein envelopes (Fig. 46). This is the phenomenon of phenotypic mixing.

On occasion, both viral nucleic acid and protein are synthesized but maturation fails to occur. When high multiplicities of infection are used, maturation may be faulty and particles are produced which although normal in respect of their protein or lipoprotein coats are deficient in the nucleic acid component. Because of this deficiency such particles are non-infective and are known as incomplete virus particles.

(f) **Virus release**

Release of virus into the extracellular environment may occur with or without destruction of the host cell. Release of poxviruses and

enteroviruses, which mature in the cytoplasm, is associated with disintegration of the cell. Adenoviruses, which mature in the nucleus, are retained at the site of maturation for some time before the nuclear membrane ruptures. With extrusion of the virus particles into the cytoplasm, the cell undergoes complete disintegration and virus is released into the extracellular environment.

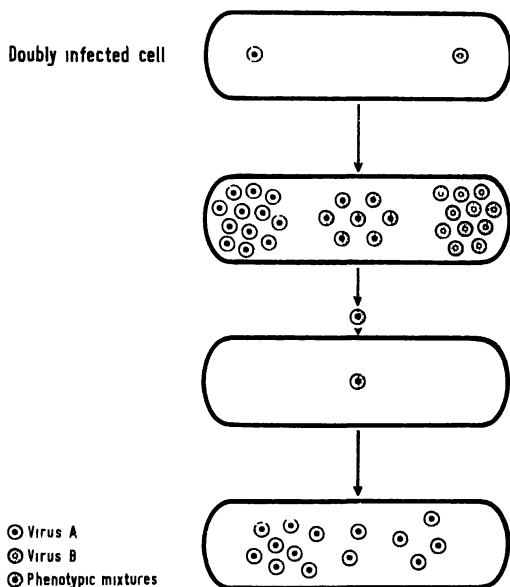


Fig. 46. Phenotypic mixing of influenza viruses diagrammatic representation

Myxoviruses, which are released by 'budding' through the cell membrane, leave the host cell intact. Eventually, however, cytopathic effects may make their appearance. The virus of herpes simplex (*H. hominis*) although it matures in the nucleus may be released without cell destruction. In the intranuclear position herpes virus particles are surrounded by a single membrane only, but passage from nucleus to cytoplasm leads to the acquisition of another membrane, which is possibly derived from the nuclear membrane. When seen in the cytoplasm the virus particles, enveloped in a double

membrane, are often found in intracytoplasmic vacuoles, from from which they are released, in some way, without cell disintegration.

Herpesviruses may spread from infected to neighbouring cells without release into the extracellular environment. The multinucleate giant cells, characteristic of infection with these viruses, indicates that intracellular transfer of virus takes place where cells are in contact, by dissolution of their contiguous membranes and fusion of their cytoplasm. The ability of viruses to spread in this way seems to be associated with the clinical manifestations of the diseases which they produce. Characteristically, herpes simplex is a recurrent localized condition which occurs in patients with a high level of circulating antibody. In these conditions virus spread is probably confined to the intracellular route, by-passing the extracellular immune environment.

CHAPTER 10

The Molecular Basis of Viral Replication

Introduction

Initiation of infection and the production of mature virus particles by the nucleic acid component of many bacterial, plant, and animal viruses, freed from viral protein, leaves no doubt that nucleic acid plays the dominant role in virus replication. The function of the viral protein coat is, essentially, protection of the nucleic acid component in the extracellular environment, and the facilitation of virus attachment for effective delivery of the infective principle into the host cell. Clearly, viral nucleic acid, whether RNA or DNA, contains all the information necessary for the synthesis of mature viral particles. It is this fact which has led to the now generally accepted view that nucleic acids serve as stores of genetic information in coded form.

The host cell has its own complement of nucleic acids in which instructions for synthesizing the many different cellular protein constituents are encoded. After virus infection, the function of host cell nucleic acid is usurped and viral nucleic acid directs the synthesis of viral proteins and nucleic acid instead of normal cellular constituents.

Nucleic Acids

Nucleic acids exist in two forms, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), both of which are found in tissue cells and bacteria but only one of which, either DNA or RNA, is present in viruses.

(a) DNA

Essentially, nucleic acids are long chain polymers of nucleotide units. In the DNA molecule, the nucleotide unit consists of one of

the purine or pyrimidine bases thymine (T), adenine (A), guanine (G), or cytosine (C) linked to a phosphate and deoxyribose sugar molecule. Chemical analyses show that the amount of adenine in DNA, from a number of sources, is almost always equal to the amount of thymine, and that the amount of cytosine is almost always equal to that of guanine. From this, it has been deduced that the DNA molecule is formed of two nucleotide chains linked to-

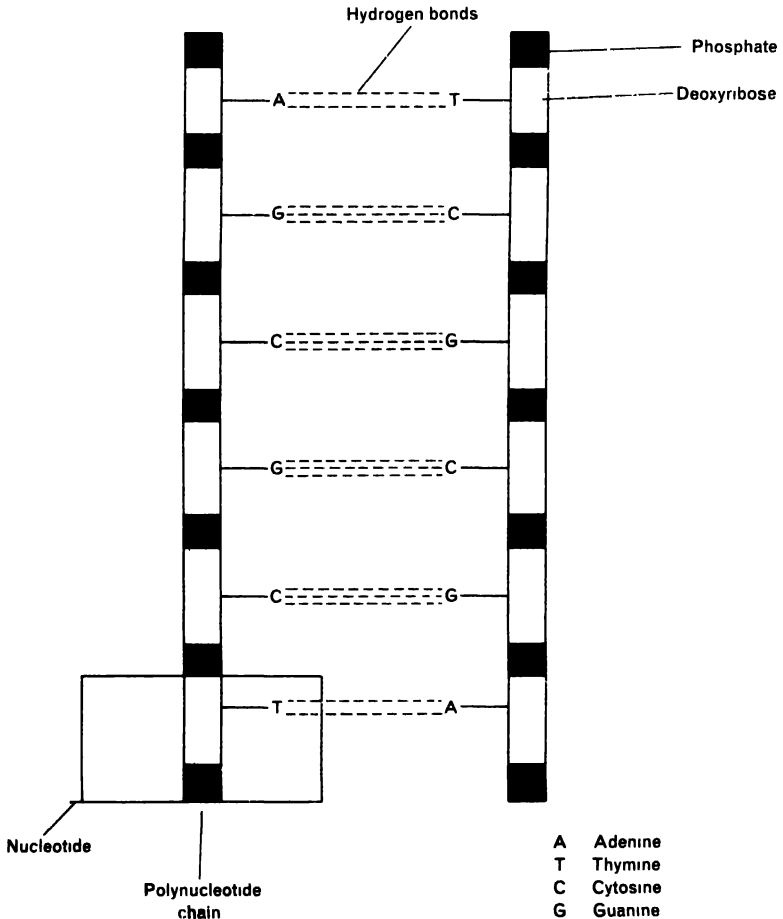


Fig. 47. Chemical Structure of DNA.

gether, through hydrogen bonds, by the complementary pairing of adenine to thymine and cytosine to guanine (Fig. 47).

From the chemical analysis of Chargaff and his colleagues, and the X-ray diffraction studies of Wilkins and others, Crick and Watson formulated the structure of the DNA molecule. They proposed the now familiar model of the double-stranded helix in which the paired polynucleotide chains are not assembled in line but are twisted spirally around each other to form the double helix (Fig. 48).

DNA Molecule

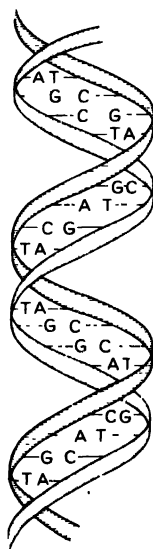


Fig. 48. DNA helix.

(b) RNA

The structure of RNA differs from that of DNA in some important details. RNA is nearly always single stranded but like DNA consists of a long chain of repeating nucleotide units. The nucleotide unit of RNA consists of one of the four purine or pyrimidine bases adenine (A), guanine (G), uracil (U), or cytosine (C), linked to a phosphate and ribose sugar molecule (Fig. 49). The nucleotide

chain therefore consists of alternating units of phosphate and ribose sugar molecules to which the bases are attached.

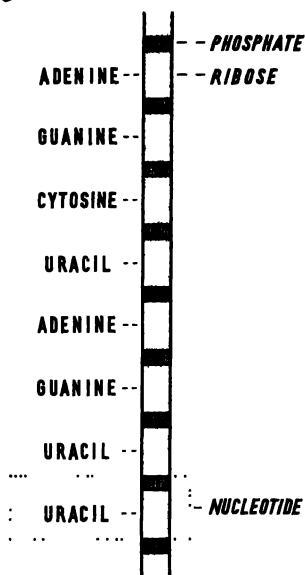


Fig. 49. Chemical structure of RNA.

DNA Replication

From the Watson-Crick model of the DNA molecule stem the current ideas of nucleic acid replication, protein synthesis and the genetic code. Briefly, the replication of DNA, whether viral or cellular in origin, is easily accomplished by the unwinding and separation of the nucleotide chains, through some mechanism yet unknown, and the formation of complementary chains, each the mirror image of its parent. In this way, two daughter molecules identical with the parent are formed (Fig. 50). In the process of replication, each polynucleotide chain serves as a template on which free nucleotides are assembled in the appropriate sequence by complementary base pairing, and are linked together with the aid of an enzyme, DNA polymerase.

The Genetic Code

Once the structure of DNA had been elucidated, it was soon realized by Gamow, Crick, and others that the code for protein synthesis

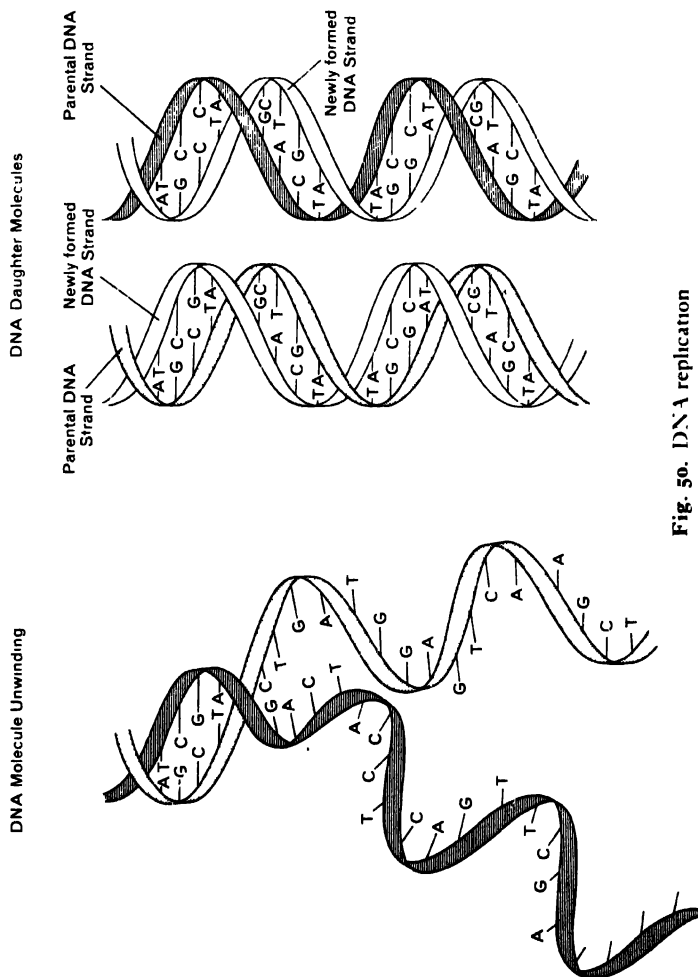


Fig. 50. DNA replication

was enshrined in the sequence of purine and pyrimidine bases along the polynucleotide chain. Although only four in number, the bases may be arranged in a variety of sequences. Originally conceived on theoretical grounds, this idea has now received experimental support from the work of Nirenberg, Ochoa, and others. Their work indicates that each of the 20 amino acids found in naturally occurring proteins is represented in the polynucleotide chain by a particular sequence of three bases, called a triplet or codon. Likewise, the amino acid sequence of any particular protein is determined by the sequence of codons in the chain.

The genetic code has not yet been universally agreed but the non-overlapping code favoured by Crick suggests that the code for a particular protein begins and ends at a fixed point on the polynucleotide chain and is read in triplets. Theoretically, 64 triplet combinations are possible by arrangement of the four bases, but only 20 are needed for the known amino acids. Excess triplet combinations are therefore probably used as alternative codes for known amino acids and, possibly, to mark the beginning and end of particular codon sequences. The amount of information that can be stored in this way may be appreciated from the fact that the DNA of T₁ bacteriophage contains some 200,000 base pairs and DNA from a mammalian cell has been estimated to possess about 500 million.

Experimental evidence of the relationship between nucleic acid base sequence and the amino acid sequence in synthesized protein has been obtained by the induction of chemical mutations. Fraenkel-Conrad, Tsugita and others have shown that treatment of TMV RNA with nitrous acid produces changes in the amino acid sequence of the TMV protein produced. This follows the conversion of cytosine to uracil and adenine to hypoxanthine, a base which resembles guanine, after nitrous acid treatment.

Protein Synthesis

In the last few years, a fascinating picture, by no means complete, has emerged of the mechanisms by which information encoded in nucleic acid molecules is translated into the synthesis of protein.

The genetic message encoded in the DNA molecule is first transcribed onto an RNA chain. For this to occur, the DNA molecule, presumably, unwinds to allow the assembly of ribonucleotides on the DNA template by complementary base pairing. The assembled ribonucleotides are then linked together with the aid of an enzyme, RNA polymerase, and the newly formed chain disengages from the DNA template which is supposed to resume its double-stranded helical form (Fig. 51).

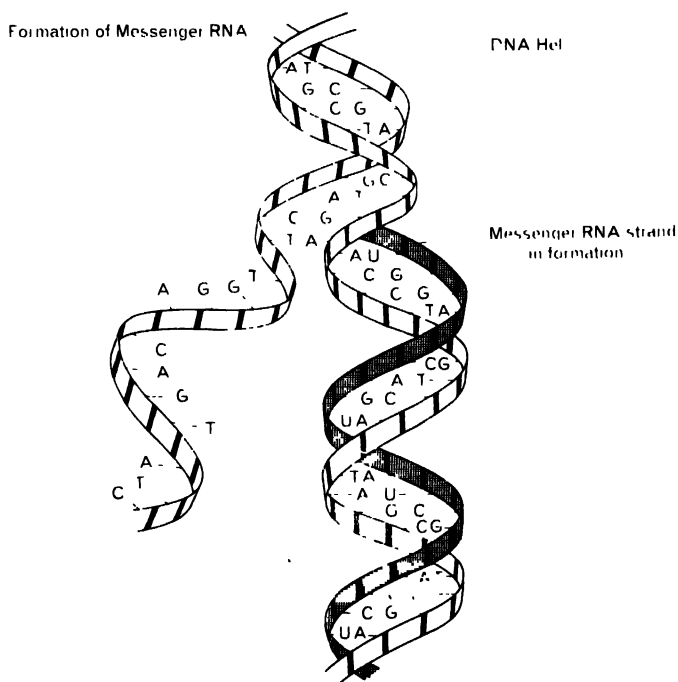


Fig. 51. DNA dependent RNA synthesis.

The newly synthesized RNA, termed messenger RNA, carries the code to the ribosomes for synthesis of the appropriate protein. Thither, the constituent amino acids are transported by yet another type of RNA, called transfer RNA, and are assembled in the sequence specified by the sequence of bases in the messenger RNA.

It appears that each of the 20 known amino acids is transported by a specific transfer RNA molecule. These molecules are short polynucleotide chains which, although single stranded, take up a U-shaped form, whose limbs are twisted over part of their length to form a double helical arrangement leaving a number of unpaired bases at each free end.

On arrival at the ribosome, it is believed that each codon triplet on the messenger RNA chain is recognized by three complementary bases, referred to as an anticodon, which are situated somewhere in the transfer RNA molecule. In this way, amino acids are assembled in their correct sequence in the polypeptide chain. Once in position the amino acids are linked by peptide bonds, effected with the aid of various enzymes, and transfer RNA molecules are released for further amino acid transportation. For synthesis of viral proteins, the cell's normal complement of transfer RNA molecules is believed to be employed.

Active protein synthesis leads to the aggregation of ribosomes and the formation of polyribosomes. Polyribosomes associated with the synthesis of viral proteins are usually larger than those found in the normal cell. Indeed, polyribosomes in poliovirus infected cells are among the largest seen by electron microscopy, and consist of about 50–70 ribosome units. The ribosomes are thought to travel along the messenger RNA chain adding amino acids in sequence to the growing peptide chain until the end of the messenger RNA chain is reached and the protein molecule is completed (Fig. 52). Probably a number of ribosomes follow each other in sequence and traverse the chain simultaneously.

Synthesis of Virus-Specific Components

After infection with a DNA virus, the viral nucleic acid takes over the cell's metabolic machinery which henceforward is employed solely in the synthesis of viral components. Soon after 'phage DNA enters its host cell and before any synthesis of 'phage DNA and protein takes place, a number of 'phage-specific enzymes are produced. These 'early protein' enzymes, apparently produced directly from the parental DNA molecule before any replication takes place,

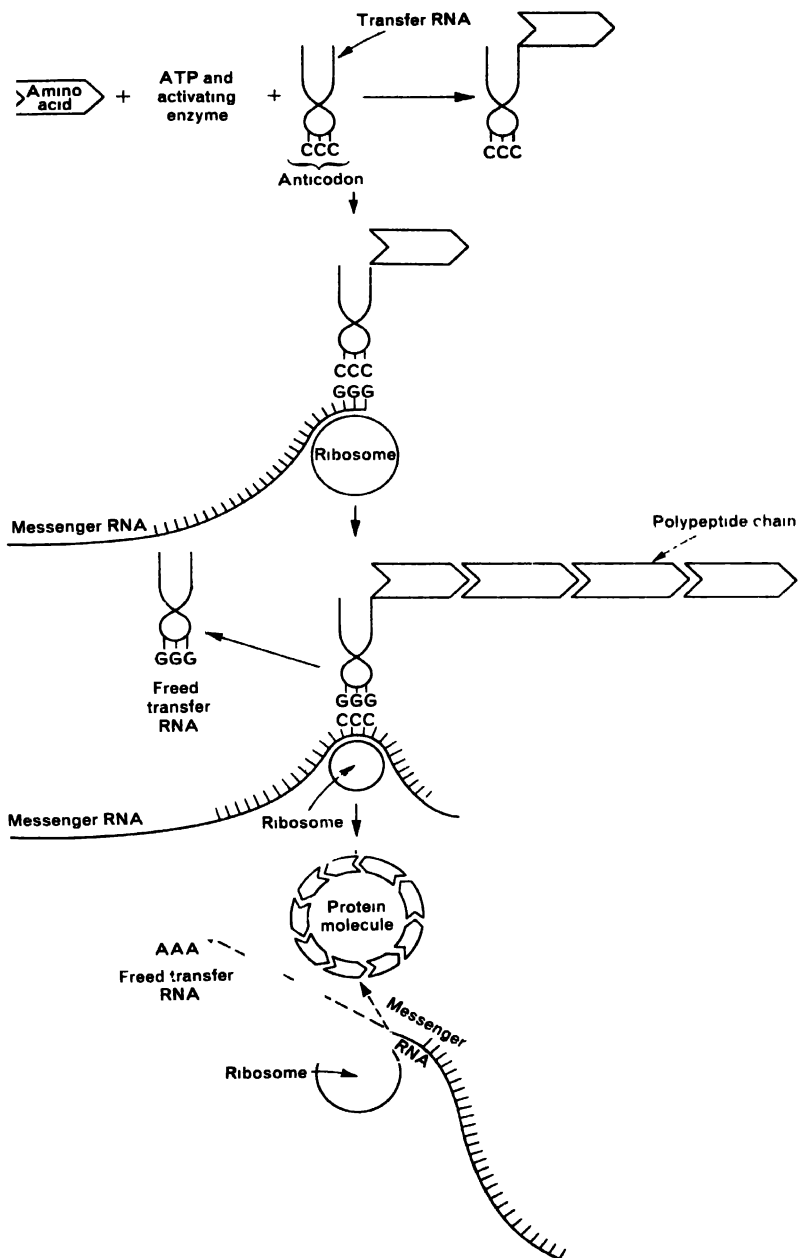


Fig. 52. Formation of protein on messenger RNA.

serve two functions. One, the suppression of host cell protein and nucleic acid synthesis; and the other, the formation of 'phage-specific polymerase which allows the replication of 'phage DNA. Subsequently 'phage-specific proteins, including the necessary 'phage-specific enzymes, are synthesized by the host cell ribosomes under the control of 'phage-specific messenger RNA (Fig. 53).

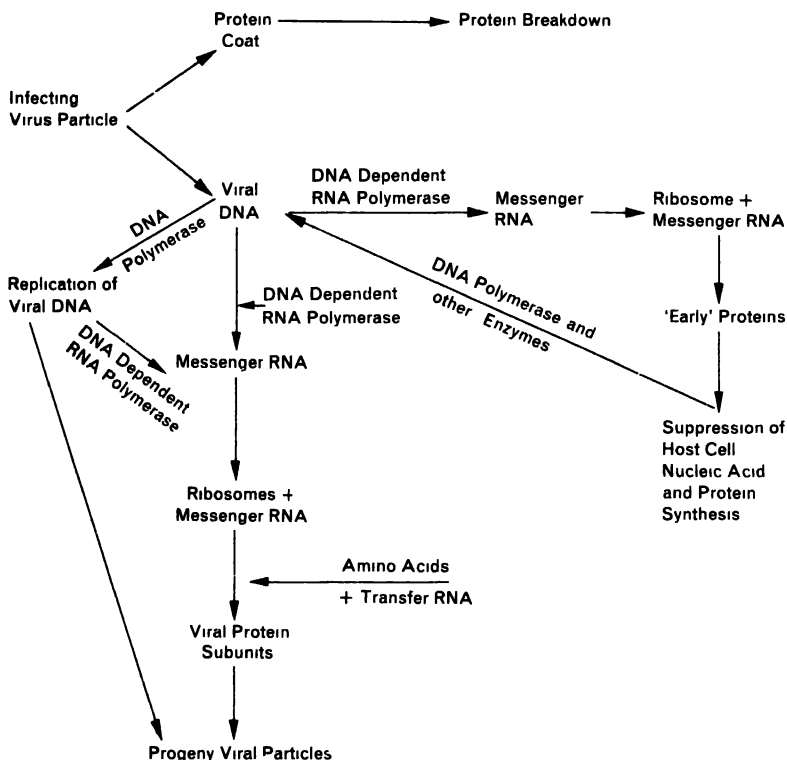


Fig. 53. Replication of DNA viruses—simplified sequence of probable steps.

More difficult to understand is how the nucleic acid of RNA viruses usurps the host cell's metabolic machinery for the synthesis of viral materials. The very existence of RNA viruses leaves no doubt that RNA itself is capable of carrying and transmitting the

genetic information of these viruses, without the intervention of DNA. This has been confirmed in experiments with actinomycin D, a substance which inhibits DNA dependent RNA synthesis but allows replication of many RNA viruses without hindrance.

The sequence of events after infection of animal cells with RNA viruses is similar to that which occurs in 'phage-infected bacterial cells. First to be produced are virus-specific 'early protein' enzymes serving two functions. One, the inhibition of host cell protein and nucleic acid synthesis; and the other, virus-specific RNA polymerase activity which allows the replication of virus RNA. Subsequently, virus-specific proteins and the necessary virus-specific enzymes are synthesized by the host cell ribosomes under the

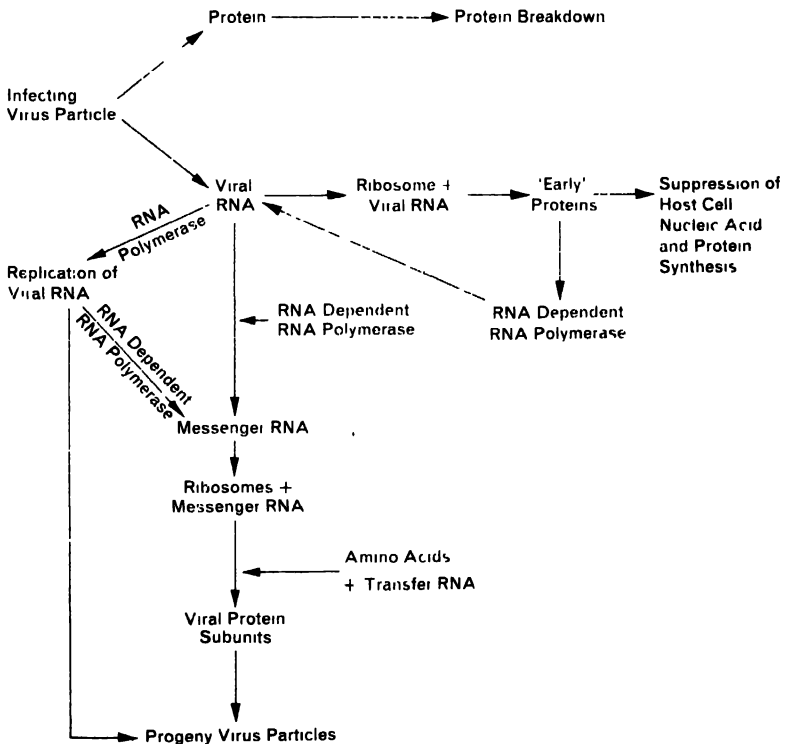


Fig. 54. Replication of RNA viruses—simplified sequence of probable steps.

control of viral RNA, presumably acting as its own 'messenger' (Fig. 54).

Recently, the sequence of events involved in viral RNA replication has become more clearly understood following Montagnier and Sanders' observation of the intermediate 'replicative' double-stranded form of RNA. This is formed of a parental and a newly formed complementary strand, of which the latter forms the template for progeny viral RNA.

CHAPTER 11

Immunity to Virus Infection

I. Non-specific Immunity

It is common knowledge that after infection with the viruses of measles, mumps, smallpox, and of many other diseases, a state of immunity is induced which persists for life. This immunity is associated with the presence of specific circulating antibodies and provides a well-recognized example of active immunity acquired in response to natural infection. As such, it does not differ in any way from specific immunity induced by bacterial infections. Although specific immunity to some virus infections is short-lived, there is no doubt that the antibody response forms a most important element in the host's defences against virus infections. Nevertheless, many individuals exhibit resistance which is unrelated to any previous infection or experience of viral antigens, and which is non-specific in character. A number of factors which contribute to this non-specific immunity are known, each of which, alone or in combination, makes an important contribution to the host's defences.

General Factors

(a) Mechanical barriers to infection

In order to reach susceptible cells viruses have to overcome a number of factors inimical to their progress. Some of these, like the skin, are purely mechanical barriers which are impenetrable except after trauma or through an insect bite. The special clearing mechanism of the respiratory tract is also mechanical in nature and is designed so that organisms trapped in the mucous secretions are swept outwards by the action of cilia to be finally discarded by coughing, sneezing, and swallowing.

(b) Chemical factors

A variety of non-specific inhibitory substances which prevent virus attachment to host cells are found in normal sera of various animal species. These include the lipid and lipoprotein substances which inhibit arboviruses, and enteroviruses, as well as the mucoprotein and lipoprotein inhibitors of influenza viruses. Many viruses are also inactivated by the properdin system. Although the part these serum inhibitors play in resistance to infection has not been precisely determined, their presence suggests a mechanism of resistance distinct from the classic immune response.

Non-specific inhibitory substances may also be present at the site of infection. Thus, non-specific inhibitors of influenza virus are normally present in the mucous secretions of the respiratory tract, and may prevent adsorption of virus to host cells. Similarly, acid gastric juice must be detrimental for those viruses which gain entry by the alimentary route. As might be expected enteroviruses are resistant to acid pH.

(c) Hormonal factors

The very severe effects of poliomyelitis in pregnant women, in whom there is a significant alteration in the balance of various hormones, suggests that hormonal factors play an important part in resistance to virus infection. The hormonal effect on resistance can also be demonstrated in experimental animals, whose susceptibility to poliomyelitis may be increased after castration. Cortisone, in particular, increases the susceptibility of experimental animals to virus infection, and especially to poliomyelitis. Although its mechanism of action is not exactly understood, it is significant that cortisone inhibits the production of interferon and depresses the antibody response.

(d) Genetic factors**(i) Species immunity**

It is well known that some species resist particular virus infections to which others are highly susceptible. Thus humans are highly susceptible to measles, although other species, with the exception of

primates, are completely resistant. Similarly, most animal species are not susceptible to human strains of influenza and poliomyelitis viruses.

(u) Individual immunity

Within each species, individuals vary in their susceptibility to infection. Although it is difficult to separate the specific from the non-specific factors which are responsible for these differences in man, animal experiments leave no doubt of the importance of genetic factors. Strains of mice resistant to specific infections can be produced by high degrees of in-breeding, and the genetic resistance to arbovirus and mouse hepatitis infections has been shown to be associated with the resistance of macrophages to virus infection.

With these experimental examples in mind, it may be safely assumed that natural selection has played a significant role in raising the natural resistance of man to those infections which have plagued him from ancient times. A remarkable contemporary example of natural selection, operating in this way, has been observed in the rabbit population of Australia, with respect to myxomatosis. When this disease was first introduced into Australia about 15 years ago, the mortality in the previously uninfected rabbit population was approximately 99%. Within a decade or so, during which the disease had become endemic, the incidence of mortality declined to about 30% or less. Admittedly, some attenuation of virus virulence had developed, but this has not been sufficient to account for the decline in mortality.

(e) Age

Newborns are particularly susceptible to virus infections, and their lack of resistance is not necessarily the result of immunological immaturity. The susceptibility of newborn and suckling mice to coxsackie and arbovirus infections, to which adults are completely non-susceptible, are striking illustrations of this phenomenon. Similarly, polyoma and Gross leukaemia viruses are actively tumorigenic only when inoculated into newborn mice. The deficient production of interferon by newborns has been suggested as the reason for their greater susceptibility to virus infection.

Non-Specific Immunity at the Cellular Level

(a) Tissue specificity

Tissue specificity is an important characteristic of viruses; indeed, tissue tropism was once the criterion by which viruses were classified. The susceptibility of tissue cells to infection depends on their capacity to allow adsorption and penetration, as well as intracellular virus replication once this has been achieved.

Much evidence has now been adduced to indicate that adsorption of virus to the cell surface is determined by the presence or absence of surface receptors specific for the virus in question. Moreover, cellular resistance to infection is often associated with the absence of specific surface receptors, and hence an inability to carry out the first stage of infection. Thus, certain cells which in the organized tissues of the intact animal are resistant to enterovirus infection, because of a deficiency in the appropriate receptor material, acquire specific surface receptors and susceptibility to infection in tissue culture. Likewise, non-primate cells which are insusceptible to infection with poliovirus fail to adsorb it to any great extent, but they are fully competent to support virus replication if intact virus or its infective RNA principle is introduced into the interior of the cell.

(b) Interference

Infection of cells with virus, live or inactivated by heat or ultraviolet irradiation, renders them resistant to superinfection with certain homologous and heterologous strains. This phenomenon, known as interference, is a manifestation of cellular immunity and is not dependent on the production of antibody. Sometimes, however, infection of a single host with two viruses does not result in the establishment of interference, but in mixed infection in which both viruses replicate side by side. The outcome of a dual infection depends, among other things, on the relative doses of the two viruses and the time interval between the two inoculations.

(c) Interferon

The mechanism of interference was not explained until 1957 when Isaacs and Lindenmann discovered that virus-infected cells pro-

duce a substance which protects normal cells against infection with some but not all viruses. This substance, which they called interferon, is a small-sized protein which protects the infected cell and may be produced in amounts large enough to protect cells at neighbouring and even distant sites.

Like the phenomenon of interference itself, interferon may be produced after infection with viruses rendered non-infective by heat or irradiation. Possibly, only the nucleic acid or nucleoprotein part of the virus is necessary to stimulate the production of interferon, and the demonstration that non-viral RNA, foreign to the host cell, may stimulate the production of interferon supports this view. It has now been established that interferon acts by inhibiting some stage in the intracellular synthesis of virus, probably the synthesis of viral nucleic acid.

Isaacs and others have attributed to interferon an important role in recovery from virus infections. In support of this view they emphasize that antibody is often not detected until the late stages of infection when recovery is already under way, and that recovery of some experimental animals from virus infections may occur in the absence of local or circulating antibody. The normal recovery from virus, but not bacterial, infections of hypo- and agammaglobulinaemic patients, who produce little or no antibody, is similarly explained. The simultaneous appearance of maximal amounts of interferon and virus in experimental animals has led some authorities to infer that interferon is responsible for the subsequent decline in virus production and recovery from infection. Some support for this view is provided by the fact that avirulent strains often produce more interferon than virulent strains. Nevertheless, the establishment of interference against heterologous infection by lymphocytic choriomeningitis virus without the production of interferon suggests that other mechanisms of cellular immunity exist.

The discovery of interferon with its antiviral properties and lack of toxicity has naturally led to the investigation of its therapeutic possibilities. Some encouragement has been obtained from experimental observations, and the protection of mice against virus infection after injection of exogenous interferon, albeit in large doses, has been demonstrated. Although some inhibitory activity against

vaccinia and conjunctival herpes after local application in man has been demonstrated, the future of interferon in the clinical field remains doubtful. Apart from technical difficulties of manufacture, its species specificity demands that interferon for human use be produced in primate cells. Moreover, the critical circumstances of timing and dosage required for its successful use in experimental animals make it unlikely that the required conditions could ever be achieved in clinical practice. To be effective interferon must be given before or very soon after virus infection, but by the time clinical signs and symptoms are evident infection is well under way. Nevertheless, its use in prophylaxis remains a possibility.

CHAPTER 12

Immunity to Virus Infection

II. Specific Immunity

A state of specific immunity, often persistent, follows recovery from virus infections. Like the specific immunity which follows bacteriological infections, it depends primarily on the presence of circulating antibody, and on the ability of the host to produce antibody in quantity, and without delay, in response to a second infection. The immune state is not always preceded by clinical infection but may follow atypical or subclinical infection.

The Antibody Response

It is well known that the immunity which follows infection with some viruses, including smallpox, measles, and mumps, persists for life, whereas immunity to others is transient and persists for no more than a few months. Those who suffer from recurrent colds and upper respiratory tract infections are only too well aware of this difference in the immune response to various viruses, which may be partially explained by the mode of infection.

Viruses responsible for the exanthemata, and some other diseases, gain entry into the host through the mucous membranes of the upper respiratory tract; from here, they are transported by macrophages, via the blood stream and lymphatics, to the spleen, liver, and other organs of the reticulo-endothelial system, where they multiply during the long incubation period characteristic of these diseases. Prior to the onset of clinical symptoms, virus is liberated from the organs of the reticulo-endothelial system into the blood stream by which it is delivered to the target organs.

Upper respiratory infections like influenza, to which immunity

is usually transient, are characterized by short incubation periods. In these, infection is usually limited to the surface epithelium of the respiratory tract, where virus, transported by the mucous secretions, spreads from cell to cell. With rare exceptions, there is little, if any, spread to the deep tissues and no viraemia.

The longer lasting immunity of those diseases which are characterized by long incubation periods and multiplication of virus in the reticulo-endothelial system is the result, according to Burnet, of the very effective and prolonged stimulus which their causative viruses provide in the actual tissue where antibodies are produced. Another important factor is the viraemia, characteristic of these diseases, which allows virus to be effectively neutralized by circulating antibody in the blood stream before it reaches the target organ. This is also one of the reasons why vaccination is a more effective prophylactic in this type of disease than it is in upper respiratory tract infections.

Although antibody is produced in response to upper respiratory tract infections and may diffuse into the respiratory secretions, preventing spread of virus from cell to cell, higher serum titres are required to provide effective antibody concentrations in this situation. Nevertheless, recent work suggests that antibodies to influenza virus may persist for a very long period indeed, so that recurrences of influenza are more likely due to the antigenic variation characteristic of this virus than to any deficiency of antibody or antibody production. Similarly, the oft-repeated recurrences of other respiratory infections are more likely due to the multiplicity of antigenic types available than to any deficiencies in the immune response.

The Action of Antibodies, *in vivo*

Antibodies are incapable of penetrating host cells and are therefore active against viruses only in the extracellular environment. In the blood or tissue fluids, antibodies combine with specific antigens on the virus surface and block the virus receptor sites, either directly or by steric hindrance. In this way, antibodies inhibit adsorption of virus to host cells and thus prevent the essential first stage of virus

infection. In the presence of small amounts of antibody some virus adsorption may take place but penetration is effectively prevented.

In the immune subject, virus may be neutralized in the early stages of infection, either at the site of infection or in the blood stream. To be effective, antibody need not prevent infection absolutely. Even if a few cells are successfully infected, the subsequent spread of virus from cell to cell might be prevented by antibody in the extracellular environment, and infection quenched before sufficient cell damage has occurred for clinical signs and symptoms to become evident.

Some viruses, like that of herpes simplex, spread to neighbouring cells by an intracellular route which by-passes the extracellular environment. This mode of spread may account for the particular clinical manifestations of herpes simplex which characteristically recurs in patients with a high level of antibody. The recurrences are believed to be due to reactivation of virus which has remained latent in the tissues, and the lesions formed probably remain localized because extracellular spread is prevented by circulating antibody.

Phagocytosis

It is probable that macrophages play an important part in clearing the body of viruses, and in this role they may be aided by the presence of antibody. Removal of virus particles from the blood stream of experimental animals by macrophages has been demonstrated, and the removal of larger particles, like vaccinia, more speedily than smaller particles has been noted. If size is an important factor, the clearance of virus from the circulation may be enhanced by agglutination of virus particles by antibody *in vivo*.

After ingestion, the initial reactions between macrophages and virus may play an important part in the outcome of infection. Non-pathogenic viruses are digested and eliminated, but more virulent viruses survive and are transported by the macrophages to the reticulo-endothelial tissues where viral multiplication ensues. If first neutralized by antibody, ingested virus fails to survive and is digested like non-pathogenic viruses. There is some evidence that

antibody promotes the uptake as well as the digestion of viruses by macrophages.

Phagocytosis of virus by polymorphonuclear leucocytes, although demonstrable in experimental systems, does not appear to play an important part in defence against virus infection. Polymorphonuclear leucocytosis is not typical of virus infections nor are accumulations of polymorphonuclear leucocytes typical of virus lesions.

Delayed Type Hypersensitivity

The classic example of delayed type hypersensitivity is the hypersensitivity to tuberculin which follows infection with tuberculosis. Unlike the humoral antibody response, it is mediated by lymphocytes and mononuclear cells at the site of antigen inoculation, where it is instrumental in localizing any infection, and may cause tissue destruction. Moreover, delayed type hypersensitivity cannot be transferred from sensitized to normal animals by means of serum but only by transfer of lymphocytes. The importance of delayed type hypersensitivity as part of the immunological response has recently been emphasized by its functional role in the homograft reaction, and in autoimmune disease, but its protective role remains controversial.

Delayed type hypersensitivity develops in response to a number of virus infections; indeed, its development in lymphogranuloma venereum is utilized for diagnostic purposes in the form of the Frei test. Its possible role in antiviral immunity, suggested by Beveridge, is supported by evidence obtained from patients with hypo- and agammaglobulinaemia. These patients, who are capable of developing hypersensitivity despite their failure to produce antibody, usually recover from virus but not from bacterial infections.

The reactions of hypo- and agammaglobulinaemic patients to smallpox vaccination provides more direct evidence of the role of hypersensitivity in antiviral immunity. It is now generally accepted that both 'accelerated' and 'immediate' reactions to smallpox vaccination, which are indicative of previous vaccination or infection with smallpox, are manifestations of delayed type hypersensitivity. It is therefore significant that some hypo- and agammaglobuli-

naemic patients respond normally to vaccination, although a few develop vaccinia gangrenosa. Kempe has suggested that the latter are those who are incapable of producing a delayed type hypersensitivity response; indeed in one of these patients, he was able to arrest the progress of vaccinia gangrenosa by passive transfer of vaccinal hypersensitivity, effected by local intradermal inoculation of leucocytes from vaccinated individuals. Systemic transfer by lymph gland grafts was, however, unsuccessful. So far, no inhibition or destruction of virus by any delayed type hypersensitivity reaction has been directly demonstrated.

Immunological Tolerance

It is possible that immunological tolerance plays some part in the clinical manifestations of disease in those infected in utero or soon after birth. Mice infected in utero with the virus of lymphocytic choriomeningitis (LCM) recover from the disease and carry high titres of LCM virus in their blood and tissues for many months without evidence of antibody production. It is noteworthy that mice exhibiting this type of persistent tolerant infection are much less susceptible to polyoma infection than normal mice.

It has been suggested by Burnet that the virus of serum hepatitis, which can only be transferred from one individual to another by blood or blood products, persists in the serum of healthy carriers as a result of immunological tolerance established after infection in utero.

Curiously, there is no evidence that intrauterine rubella infection leads to immunological tolerance. Both rubella virus and antibody are present after birth.

CHAPTER 13

Immunity to Virus Infection

III. Artificial Immunization

The relative importance of circulating antibody, delayed type hypersensitivity, interferon, and other non-specific cellular factors, in recovery from primary virus infection is still disputed, but recovery probably depends on a combination of them. In contrast, all agree on the overriding importance of circulating antibody in defence of the host against reinfection. It follows, that those who have been infected, clinically or subclinically, on one or more occasions become immune to reinfection. Those who have not been previously infected may, however, be afforded protection artificially, by active or passive immunization.

Artificial active immunity is acquired following the administration of vaccines containing live or inactivated virus; to these, the recipients respond with the production of antibodies. Artificial passive immunity is acquired after injection of specific antibodies present in human convalescent or animal immune sera.

Active Immunization

(a) Live virus vaccine

Jenner's discovery that inoculation with vaccinia or cowpox virus, which produces only mild localized lesions in man, protects against a subsequent attack of smallpox inaugurated the modern era of prophylaxis through vaccination. Although ignorant of the complexities of modern immunology, Jenner had discovered, by clinical observation, a fundamental principle of immunization. Namely, that high grades of immunity to virulent viruses are produced after infection with variants of modified virulence but similar antigenic

composition. Since that time, a number of attenuated viruses suitable for use in live vaccines have been developed, although attenuation is nowadays more usually achieved by laboratory manipulation. Today, live attenuated vaccines available include those for protection against yellow fever, poliomyelitis, rabies, and measles, as well as smallpox.

In general, vaccines consisting of live attenuated viruses are superior to those consisting of inactivated organisms. The immunity they produce is longer lasting and more nearly resembles that produced by natural infection. Where the natural route of infection is employed, e.g. the alimentary route for oral polio vaccination, tissue as well as humoral immunity is produced. In this way, alimentary infection with polio is prevented as well as viraemic spread. Beveridge suggests that the higher grade of immunity produced in response to live virus vaccines is due to the development of delayed type hypersensitivity in addition to the humoral antibody response.

Besides the high degree of immunity produced, other practical advantages accrue from the use of live vaccines. They may, like oral polio vaccine, be easier to administer, without the need for parenteral injection. Moreover, the relatively small dose of virus needed to infect makes the procedure economical and minimizes production problems. Nevertheless, in spite of their manifest advantages, certain disadvantages are also inherent in the use of live vaccines. Those, like oral polio vaccine, which are given by the natural route of infection may spread from person to person. This may be an advantage if the property of attenuated virulence remains stable but, theoretically, repeated passage by the natural route may restore the original pathogenicity and virulence of the organism. Although some increase of virulence after passage of oral polio vaccine in the human alimentary tract has been observed, its use in literally millions of people without untoward effect suggests that the danger, if any, is minimal.

Other possible dangers are inherent in the method of production. Chick embryos and primary tissue cultures of primate cells used for vaccine production may carry a number of adventitious viruses, some of which are tumorigenic. Rigid control has therefore to be

exercised to ensure the exclusion of these viruses from tissues and embryos used for vaccine production. The use of continuous cell lines, free of adventitious virus, would largely overcome this difficulty, but their resemblance to malignant cells, with the implied theoretical dangers, makes their use in vaccine production unacceptable at the present time.

(b) Inactivated vaccines

Viruses rendered non-infective by formalin or some other method of treatment produce a satisfactory antibody response when injected parenterally in sufficient quantity. Vaccines of this type are available for protection against poliomyelitis (Salk vaccine), influenza, measles, rabies, and some other infections. The immunity which they produce is usually of short duration and repeated injections are required to maintain the state of immunity at a high level.

Large amounts of virus are required for the manufacture of inactivated vaccines, which are therefore less economical to produce than live vaccines. Laboratory tests to confirm that the vaccines reach the required standards of antigenic potency are necessary, and safety tests to ensure the absence of residual live virus after inactivation are important.

The virus particles of any particular strain are composed of a number of antigens, not all of which produce the neutralizing antibodies responsible for protection against infection. For best results, purified viral antigens free from unimportant and non-antigenic components are required. Modern techniques have begun to make such preparations available and these, particularly if combined with suitable adjuvants, should provide inactivated vaccines of improved specificity and potency.

Passive Immunization

Artificial passive immunity is acquired after injection of immune serum obtained from human convalescents or from immunized animals. Usually, only the γ -globulin fraction of antiviral immune sera is used, and that from the homologous species is the most satis-

factory. For prophylaxis, passive immunization is effective if given within a few days after exposure to infection, during the early part of the incubation period. Although some therapeutic benefit may be obtained from immune serum administered within a few hours after the onset of symptoms, it is completely ineffective once intracellular viral replication is under way.

The use of γ -globulin, prepared from immune human sera, for the protection of selected susceptible individuals who have been in contact with rubella, measles, smallpox, or infective hepatitis is current practice. It is also used in the treatment of vaccinia complications. Like passive immunization against bacterial infections, that against virus infections is immediately effective but of short duration, not extending beyond 2-3 weeks.

In newborn infants, passive immunization is acquired naturally by receipt of maternal antibodies via the placenta. This is a most important mechanism of protection against virus infections during the first 6-12 months of life.

PART 2
Virus Infections

CHAPTER 14

Respiratory Infections

I. *Myxovirus influenzae*—Influenza

Clinical Features

Influenza is a much abused clinical diagnosis which, for want of a better term, is often applied to upper respiratory catarrhal infections and pyrexias of unknown origin. Uncomplicated influenza, which is characterized by distinctive clinical features and epidemiological behaviour, commences with the sudden onset of high fever accompanied by severe prostration, lassitude, generalized myalgia, anorexia, sore throat, headache, depression, and sometimes epistaxis. Nasopharyngeal congestion is common, and the decline of fever after 2–5 days is often followed by catarrhal symptoms.

Epidemiological Characteristics

Periodically, influenza outbreaks, starting from a single focus, rapidly progress in certain definite directions to achieve a world-wide spread. Between these pandemics more localized epidemics occur, usually at 2–4 year intervals; although they do not progress as extensively as the great pandemics, they may sometimes be traced along certain lines of direction. The case fatality is usually very low, but the very high incidence of infection makes the total mortality appreciable. Following the serious influenza outbreaks of 1957 and 1959 the total number of deaths from influenza in England and Wales was 6716 and 7862, respectively.

Historical Background

The periodic recurrence of rapidly spreading pandemics and the wide variations of mortality which have been noted during many

centuries of epidemiological observation are puzzling features. They were manifest in the last three great pandemics of 1889-90, 1918, and 1957, and the exceptional virulence of the 1918 pandemic, which resulted in a total mortality of 100,000-200,000 persons in England alone and some 20 million in the world at large, was unique both for its magnitude and for its unprecedented toll of patients in young adult life. Fortunately, the mortality from influenza has shown a general decline in recent years, to which the introduction of antibiotics and the more efficient therapy of pneumonic conditions have undoubtedly contributed.

Death from influenza is often the result of respiratory complications in which secondary infection with bacteria is a marked feature. This confused early investigators, and Pfeiffer, who was prominent in the investigation of the 1889-90 pandemic, actually assigned a causative role to the organism *Haemophilus influenzae*. The error of his conclusion became apparent in the 1918 pandemic, in which a viral aetiology was most favoured. It was not until 1933, however, that Smith, Andrewes, and Laidlaw were able to isolate the causative virus in ferrets.

Originally, ferrets were the only animals susceptible to the virus; later, virus grown in ferrets was adapted to laboratory mice and chick embryos. With the availability of these laboratory techniques progress in influenza virus research proceeded rapidly.

The Virus

(a) Morphology

Influenza virus particles are usually spherical in form, about 80-120 m μ in diameter. They consist of an outer lipoprotein envelope with evenly spaced, radially orientated, surface projections, 80-100 Å in length, and an internal component (Figs 28 and 55). The internal component represents the nucleocapsid, and is the ribonucleoprotein of the virus wound in helical array. The diameter of this helical component is 90-100 Å.

Sometimes, particularly in recently isolated strains, filamentous forms 1 μ or more in length make their appearance (Fig. 30). The surface of these particles is similar to that of the spherical forms but

the detailed structure of the internal component has not yet been determined.

(b) Biological properties

The study of influenza viruses has been greatly facilitated by their haemagglutinating properties and neuraminidase activity, which



Fig. 55. Negatively stained influenza virus particle showing internal helical structure [from K. Apostolov and T. H. Flewett (1965) *Virology* 26, 506-8 (Academic Press Inc., New York and London)]

have been fully described in Chapter 9. The special affinity of influenza viruses for the sialomucoprotein receptors of red blood cells and host cells is also evident in their affinity for the sialomucoprotein constituents of normal serum, which act as non-specific inhibitors of haemagglutination. The inactivation of these inhibitors, by periodate or neuraminidase treatment, is an important preliminary to haemagglutination-inhibition tests for the identification of influenza antigens and antibodies.

(c) Antigenic structure

(i) Soluble antigen (*S*-antigen)

Influenza viruses are differentiated into three species (A, B, and C), according to the specificity of the soluble antigen produced in infected tissues. The soluble antigen, because of its small particle size and its low sedimentation coefficient (66S), is readily separable from intact virus particles by differential centrifugation. It reacts only in the complement-fixation test and is serologically distinct from the antigens incorporated in the virus surface.

The soluble antigen is now known to be the internal, helical ribonucleoprotein component of the virus. In the intact virus particle, it is known as the g-(gebundenes or bound)antigen but its presence is not revealed because of its internal position. It may, however, be released after disintegration of the virus particle by treatment with ether (Fig. 29). It is the excess production of internal component which accounts for the presence of free soluble antigen in infected tissues.

Antibodies to the soluble antigen are produced only in response to infection but are not protective. They appear before the antibodies to virus surface antigens and disappear rapidly during convalescence; their identification is therefore of great value in the early serological diagnosis of influenza infection.

(ii) Viral antigens (*V*-antigens)

A viruses. Although the soluble antigens produced by all A viruses are identical, the antigenic composition of the virus surface is complex and heterogeneous, exhibiting a marked tendency for variation. Strains isolated from man in three successive periods since 1933 belong to three antigenically distinct types with only minor degrees of overlap. Between 1933 and 1946, all the strains recovered belonged to type A. Between 1946 and 1957, strains of a markedly different antigenic character were recovered; these form the type A₁ and are often referred to as A-prime strains. Since 1957, all the A viruses recovered belong to a third antigenic type, A₂, and are known as Asian strains. Within each type there is a broad antigenic relationship but, generally, strains isolated from the same epidemic

exhibit a closer degree of similarity than those isolated from different epidemics.

A number of influenza viruses which produce soluble antigen A, have now been recovered from several animal species. But their viral antigens are quite distinct from those of human strains, which are easily distinguished. So far, none of the animal influenza viruses, with the possible exception of swine influenza virus, has been found to produce infection in man.

B viruses. B Viruses exhibit a greater degree of antigenic stability, and tend to cause smaller and more localized outbreaks than A viruses. Nevertheless, two main antigenic groups may be recognized; one representing viruses recovered between 1940 and 1954 and the other, those recovered since. Recently, a new B strain of novel antigenic composition was recovered in Taiwan but has not shown any tendency to spread from its place of origin.

C viruses. These viruses are antigenically quite distinct from A and B viruses, and unlike them haemagglutinate only at 4 C. C viruses cause only sporadic cases of mild influenza-like illness, and are not an important cause of respiratory infection.

Antigenic Variation and Epidemiology of Influenza

The remarkable antigenic plasticity of A influenza viruses has given rise to the widely held hypothesis that recurrent influenza epidemics and pandemics spring from the selection of new antigenic variants, which have a survival advantage in populations immune only to the old antigens. The Asian pandemic of 1957 was indeed associated with a major antigenic change, and similar changes associated with the 1889-90 and 1918 pandemics are often inferred but are never likely to be established with certainty. It is noteworthy that the major antigenic change which occurred in 1946, although associated with widespread infection was not associated with a generalized pandemic. This suggests that a major antigenic change, although an important prerequisite for pandemic spread, is not the only factor involved.

Serological Evidence of Antigenic Variation

Investigation of sera from older people, who were alive during the 1889-90 and 1918 pandemics, has yielded some interesting retrospective information about the influenza viruses current during these periods. The discovery by Laidlaw, Andrewes, and Smith, more than 30 years ago, that antibody to swine influenza virus was present in the serum of those alive during the 1918 pandemic but not in the serum of those born sometime afterwards suggested that a virus antigenically related to the swine influenza virus was prevalent in the 1918 pandemic. The swine influenza virus was isolated by Shope from hogs in the middle-west of the United States, and is not found in other parts of the world. It has a very curious and complex life cycle with intermediate lung worm and earth worm hosts. Some hold that it represents a survival of the 1918 virus following an accidental transfer from humans to swine in this particular part of the United States.

During the 1957 pandemic, Mulder and others showed that persons of 70-80 years of age who were alive in the 1889-90 pandemic gave evidence of serum antibodies to the prototype A_2 strain before they had had any experience of the then current A_2 pandemic. This suggested an antigenic similarity between A_2 viruses and those prevalent in the 1889-90 pandemic.

The value of serological studies for the retrospective investigation of influenza has been confirmed by the surveys of Davenport and his colleagues. They found that the dominant antibody in various age groups was determined by the first influenza infection experienced. Thus in their 1953 survey, swine influenza antibodies were dominant in 30-60 age group, A influenza antibodies were dominant in the 18-30 age group, and A_1 antibodies were dominant in those under 18 years of age. Subsequent infection with heterologous viruses boosts the titre of the dominant antibody, which becomes higher than that of the homologous antibody. Davenport describes this phenomenon as 'the doctrine of original antigenic sin' through which the seasonal prevalence of various antigenic types of influenza virus is reflected in the age distribution of their corresponding antibodies in the population.

Possible Mechanisms of Antigenic Variation

Virological and serological studies indicate that a finite number of antigens form the antigenic spectrum from which A strains are composed. Jensen and Francis have detected as many as 15 different antigens in a single virus strain, and they suggest that new strains arise from quantitative or spatial rearrangement of the different antigenic components so that, periodically, one or other antigen becomes dominant and gives rise to a new virus type (Fig. 56).

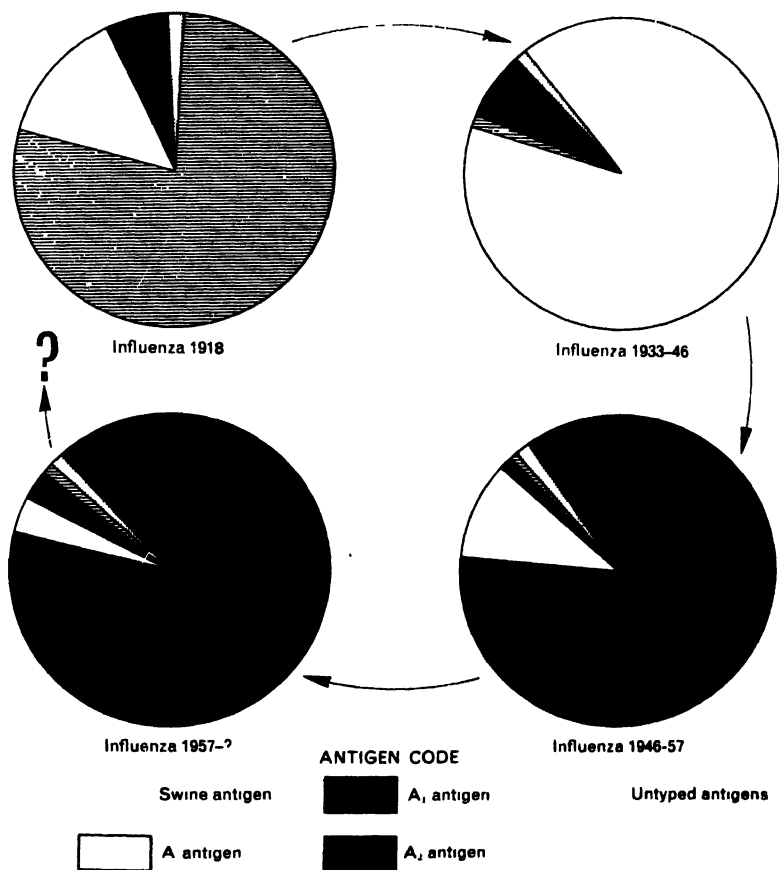


Fig. 56. Hypothetical quantitative rearrangement of influenza virus antigens.

If the virus prevalent in the 1889-90 pandemic was indeed of the A₂ variety, its reappearance in 1957 would represent the completion of the cycle of antigenic variation.

An alternative view, still held by some authorities, maintains that new types are produced by the appearance of completely new antigens which arise by mutation.

Spread of Influenza

The only great pandemic which has been studied by virological techniques is the pandemic of 1957, but the epidemiology of both the 1889-90 and the 1918 pandemics was studied in great detail in many parts of the world. The origin of both was asserted to be in some remote part of China, but in neither was there any real evidence for this. In contrast, there is definite evidence that the 1957 pandemic began in the Kwei-Chow province of China in February 1957 and spread to Hong Kong and Singapore in April. From these centres, the virus was distributed by land, sea, and air to other parts of the world, within a few weeks.

Pathology of Influenza

Much of our knowledge of the pathology of influenza comes from the study of influenza infection in ferrets, which is similar to that which occurs in man. Infection is mainly localized in the nasal turbinates, where the ciliated epithelium is destroyed. During the healing stage, stratified epithelium develops from the congested basal layers to replace the necrosed ciliated epithelium, but after 14-21 days the ciliated epithelium is restored to normal. When ferrets are inoculated intranasally with adapted strains, under anaesthesia, haemorrhagic pneumonia, consolidation, and irregular necrosis of the tracheobronchial mucosa is produced.

Similar lesions are produced in man. Infection is usually localized in the ciliated epithelium of the nasal mucosa, but in fatal cases of influenzal pneumonia, without secondary bacterial infection, irregular necrosis of the tracheobronchial mucosa, accompanied by vascular congestion and exudate, occurs. Some necrosis of alveolar cells,

focal haemorrhagic exudate, as well as the development of a hyaline membrane, is also seen in the lungs of fatal cases.

Complications of Influenza

The most common complication of influenza is lower respiratory tract infection due, more often than not, to secondary bacterial infection. This may lead to tracheitis, bronchitis, or bronchiolitis, and in children to obstructive laryngotracheobronchitis or croup.

More serious, is influenzal pneumonia which begins soon after the onset of influenza. Although usually due to secondary bacterial infection, influenza virus alone may produce pneumonia which is sometimes fatal, particularly in patients with pre-existing heart or pulmonary disease. Indeed, Hers and his colleagues estimated that 20% of their fatal cases of pneumonia, in the 1957 pandemic, were due to uncomplicated virus pneumonia.

Nevertheless, the organism recovered from a high percentage of cases of influenzal pneumonia is *Staphylococcus pyogenes*, which seems to have a peculiar affinity for the influenza-infected lung. The syndrome of influenzal staphylococcal pneumonia, in which the patient may develop grave circulatory collapse and death within a few hours, is a characteristic and greatly feared complication of influenza. Other organisms, including *Strept. pneumoniae* and *Haemophilus influenzae*, are also responsible for some cases of pneumonia following influenza. Sometimes, the patient may recover from the acute phase of the influenzal attack, and a few days or weeks may go by before the complicating pneumonia makes its appearance. This syndrome of post-influenzal pneumonia is usually due to *Strept. pneumoniae*.

Another serious, sometimes fatal, but comparatively rare complication of influenza is influenzal encephalopathy or post-influenzal encephalitis. In the former, the brain shows intense congestion but few other pathological changes; in the latter, which usually occurs a few days after the acute attack, perivascular demyelination with haemorrhagic encephalitis is characteristic. So far, the presence of influenza virus in the brains of fatal cases has not been unequivocally proved.

Laboratory Diagnosis

Laboratory confirmation of the clinical diagnosis is not often called for but laboratory investigations may be important for public health purposes during an epidemic. Laboratory diagnosis depends on virus isolation, and the demonstration of a four-fold rise in antibody titre occurring between the acute and convalescent phases of the disease.

(a) Virus isolation

Throat washings or nasal swabs, taken during the acute phase of the illness, are collected in 50% broth-saline, containing penicillin and streptomycin. Specimens are held at 4°C for not more than 24 hours; any specimens stored for longer periods are held at -70°C. 0.2 ml is inoculated by the amniotic route into six, 10-13-day-old chick embryos, which are then incubated for 2-4 days at 35°C. After incubation, the amniotic fluids are harvested and tested for haemagglutination with human group O cells. Ideally, three consecutive amniotic passages should be attempted before a negative result is accepted. After isolation in the amniotic cavity, the virus is usually easily adapted to the allantoic cavity from which sufficient virus may be obtained for serological identification, using antisera against known types of influenza viruses.

Nowadays, isolation in primary monkey kidney tissue cultures provides another routine method of isolation, and is especially useful for the isolation of B viruses. Although not cytopathogenic, the presence of virus is easily demonstrable by the haemadsorption test, and typing is likewise easily accomplished by the haemadsorption-inhibition test.

Of course, failure to isolate the causative virus does not necessarily rule out the clinical diagnosis of influenza.

(b) Serological diagnosis

Serological diagnosis is retrospective and requires the demonstration of a four-fold rise in antibody titre occurring between the acute and convalescent phases of the disease.

(i) Haemagglutination-inhibition (HI) tests

Sera used in HI tests must be treated to remove non-specific haemagglutination inhibitors. Heating at 56°C for 30-60 minutes effectively removes the heat labile β -inhibitor, and treatment with M_{190} potassium periodate for 15 minutes followed by 1% glycerol saline inactivates the α - and γ -mucoprotein inhibitors. Ideally, the antigens used in these tests should be strains selected for their high sensitivity to antibody and their low sensitivity to non-specific haemagglutination inhibitors.

(ii) Complement-fixation (CF) tests

The complement fixation test, which may be performed with viral (V) or soluble (S) antigen, has the great advantage that it is not complicated by the intervention of non-specific inhibitors. Antibody to S antigen is not produced after vaccination with inactivated vaccines nor does it persist beyond convalescence, its appearance is therefore proof of infection. It is, however, only species and not strain specific. In contrast, antibodies to the V antigens are strain specific and persist beyond convalescence. The CF test using V antigens is now considered by many to be the serological technique of choice.

(iii) Neutralization tests

Neutralization tests in eggs, mice and tissue culture may be employed, but are not used routinely.

Prophylaxis

(a) Inactivated vaccines

The short incubation period of 1-3 days, the high infectivity, and the rapid spread of influenza, make isolation and quarantine of no avail in prophylaxis. The only effective measure of control is vaccination with formalin-inactivated virus. To be effective, vaccination must be performed before the onset of any epidemic, but even under the very best conditions it is only about 70% effective, and more usually its effectiveness is nearer 50%.

The antigenic composition of the vaccine presents a problem of some difficulty, because it is impossible to predict with certainty which antigenic type of virus will become prevalent in any future season. On two occasions, in 1946 and 1957, the available vaccines were found to be useless after a major antigenic change had occurred in the epidemic strain. Generally, representative strains of A and B viruses, of recent prevalence, are incorporated in the vaccine but some authorities include classical A and A₁ strains to broaden the antigenic spectrum and embrace as many antigens as possible.

Not only is it difficult to predict which antigenic type of virus is to be expected; it is not even possible to predict, with any certainty, whether an epidemic will materialize at all. Many inoculations are therefore given in vain, particularly as the immunity produced is short-lived. The occasional allergy to egg proteins, contained in egg-produced virus vaccines, and the occurrence of other febrile reactions which lead to a significant loss of working time, are further shortcomings in a vaccine already less than ideal.

The many disadvantages of influenza vaccination make the immunization of the general population unnecessary; nevertheless, vaccination is recommended for those at special risk. The mortality of influenza falls most heavily on those over 65 years of age, on patients with chronic chest and heart conditions, and on patients with diabetes and other debilitating diseases; all these should therefore receive vaccination between September and December, before the influenza season starts.

One dose of aqueous vaccine, administered subcutaneously, is sufficient to produce an antibody response in adults who have been previously vaccinated. Two doses, separated by an interval of 6-8 weeks, are recommended for adults not previously vaccinated, and for children over the age of 6 years. The dosage of vaccine for children is half that for adults.

Recently, adjuvanted vaccines, in which the antigen is combined with a mineral oil base and an emulsifying agent, have been introduced. These produce a better and longer lasting antibody response, and their use may eliminate the need for annual vaccination. Although some adjuvanted vaccines produce unpleasant local reactions after intramuscular injection, the more recently introduced

adjuvants have caused few local reactions and it is probable that adjuvanted vaccine will soon become the vaccine of choice.

Vaccines composed of purified viral antigens, extracted from virus particles, have recently been on trial in the United States. These vaccines are potent antigens and produce few reactions, because all extraneous unwanted protein is removed.

The main danger of influenza comes from the great pandemics due to new antigenic variants. The preparation of suitable vaccines against these variants, in time to combat the pandemic, is therefore of considerable public health importance. For this reason, World Health Organization reference laboratories have been established in various parts of the world so that new antigenic variants may be immediately identified, wherever they originate. Prompt distribution of any new isolate to laboratories in other parts of the world allows production and distribution of a new vaccine before the pandemic arrives by natural epidemiological routes. In the short time available, production of sufficient vaccine to immunize the whole population is clearly not possible and attention has to be confined to certain priority groups whose services are essential to the community. These include personnel employed in the health, security, and transport services. In addition, older age groups, patients with chronic chest and heart conditions, and others at special risk should also be immunized.

(b) Live vaccine

Live attenuated virus vaccine, administered intranasally, has been used extensively in Eastern Europe, where a substantial degree of success has been claimed. In this country, trials of live vaccine have not, so far, been very successful, and antibody responses were lower than those obtained with inactivated vaccine. Moreover, febrile reactions although not common in adults were common in children, for whom the vaccine proved unsuitable.

Before live influenza vaccines can be adopted with confidence, several difficulties inherent in their production remain to be resolved. A satisfactory avirulent strain which is sufficiently infective for man and whose stability on passage is assured has yet to be found.

Also, the chick embryos used for vaccine production must be free of avian oncogenic viruses.

Treatment

In the absence of any specific antiviral treatment for influenza, general symptomatic treatment is sufficient for uncomplicated cases. Antibiotics are not advised for routine prophylaxis against secondary bacterial infection, except in special risk cases, i.e. patients with chronic chest and heart conditions or other debilitating diseases. Antibiotic treatment is, of course, mandatory once bacterial complications have been diagnosed, and staphylococcal lesions in both patients and contacts require antibiotic treatment.

CHAPTER 15

Respiratory Infections

II. *Paramyxovirus influenzae* Parainfluenza Infections

In 1953, a virus antigenically distinct but otherwise resembling influenza virus was isolated, post mortem, from the pneumonic lung of a child in Sendai, Japan. Since then, similar viruses have been isolated from mild upper respiratory infections in patients of all ages, and from severe and even fatal lower respiratory infections in infants and young children. Originally called Sendai viruses, their basic similarity to influenza virus in morphology, haemagglutination characteristics, and ether sensitivity, led to their classification as *Paramyxovirus influenzae*, and to the name parainfluenza viruses by which they are commonly referred to. Four different antigenic types have now been recognized in strains isolated from humans, and several strains, antigenically distinct from the human ones, have been isolated from animal species.

The Virus

(a) Morphology

Morphologically, parainfluenza virus particles resemble those of influenza; they are spherical particles consisting of an internal helical ribonucleoprotein component, surrounded by a lipoprotein envelope from which protrude radially orientated projections, 10-15 m μ in length. The larger overall size of parainfluenza viruses distinguishes them from influenza virus, as does the increased diameter of their internal helical component. The average particle diameter varies from 100 to 250 m μ and may reach 800 m μ , likewise

the diameter of the internal component is approximately twice that of influenza viruses and measures 180 Å.

(b) Haemagglutination and haemolytic properties

Parainfluenza viruses agglutinate red blood cells from a number of species by attachment to sialomucoprotein receptors present on the erythrocyte surface. Virus elution with destruction of receptors and release of sialic acid is brought about by the action of viral neuraminidase, but parainfluenza type II, like mumps and Newcastle disease viruses, binds irreversibly to erythrocytes at 37°C. When guinea-pig or human red cells are used, optimal haemagglutination titres are obtained at 37°C, but with fowl red cells optimal titres are obtained at 4°C.

(c) Antigenic composition

Four different antigenic types of human parainfluenza viruses have been recognized by the usual serological procedures of complement fixation, haemagglutination or haemadsorption-inhibition, and neutralization. In complement-fixation tests, both the virus surface and the internal g-(gebundenen)antigens are type specific and only slight heterologous cross reactions have been observed. So far, each type of parainfluenza virus appears to be antigenically stable and does not exhibit the variation characteristic of influenza viruses.

Although parainfluenza viruses isolated from various animal species are antigenically distinct from those of man, some antigenic relationships are demonstrable. Such relationships have been demonstrated between murine strains and parainfluenza type I; between two simian strains, SV5 and SV41, and parainfluenza type II; and between some bovine strains, isolated from shipping fever in cattle, and parainfluenza type III.

(d) Cultivation

(i) Chick embryos

With few exceptions, parainfluenza viruses grow poorly in chick embryos which are not sensitive enough hosts for primary isolation. Some tissue culture adapted strains of types I, II, and III grow to

low titre in the amniotic cavity, and some strains of types I and II have been adapted to the allantoic cavity.

(u) Tissue culture

Primary tissue cultures of monkey kidney cells are the ones of choice for isolation and cultivation of parainfluenza viruses, but tissues from a variety of species, particularly those from human sources, are susceptible. On primary isolation, cytopathic effects are minimal but become more marked on repeated passage, and in stained preparations eosinophilic intracytoplasmic inclusion bodies may be observed. Parainfluenza type II, particularly, produces a cytopathic effect characterized by the formation of syncytial giant cells and focal cell destruction.

More usually, the growth of parainfluenza viruses in tissue culture is recognized by the haemadsorption technique; positive results are obtained after 5-7 days incubation, but on primary isolation 2-3 weeks incubation may be required. Sometimes, the results of haemadsorption are invalidated by the presence of simian parainfluenza viruses SV5 or SV41 which are common contaminants of monkey kidney cells. Although the intervention of these viruses may be controlled by incorporation of the appropriate antiserum into the cell culture medium, the antigenic cross reaction between simian viruses and parainfluenza virus type II makes this practice undesirable.

(ui) Animal inoculation

Hamsters and guinea-pigs are susceptible to parainfluenza virus infection and produce antibody responses, but they do not suffer from any clinical signs of infection.

Diseases caused by Parainfluenza Viruses

(a) Adults

Parainfluenza viruses are not a major cause of disease in adults, but they have been isolated from some cases of mild upper respiratory infection, clinically manifested as colds or 'flu-like illnesses. In

various surveys, parainfluenza viruses have been found responsible for 29% of the mild upper respiratory infections investigated.

(b) Children

In children, parainfluenza viruses are responsible for a significant number of severe and even fatal cases of respiratory infection. In the District of Columbia, Parrot and Chanock with their colleagues, isolated parainfluenza viruses from 2-4% of children admitted to hospital with severe bronchitis, pharyngitis, bronchiolitis, or bronchopneumonia. Most of these belonged to parainfluenza type III, although types I and II were also found. Of children attending as out-patients with mild upper respiratory infections, described as rhinitis, pharyngitis, or bronchitis, 6% yielded parainfluenza viruses. A much closer aetiological relationship was found in children admitted with croup, otherwise known as acute obstructive laryngotracheobronchitis, from 29% of whom parainfluenza viruses, mostly belonging to type I, were isolated. When serological evidence of infection was taken into account, the incidence of parainfluenza infection was approximately twice that revealed by virus isolation.

Similar patterns of parainfluenza infection have now been observed in England and other countries. Banatvala and his colleagues in a survey of non-hospitalized patients in Cambridge isolated parainfluenza viruses from as many as 75% of children with croup, and most of these belonged to parainfluenza type I. In their survey, parainfluenza infection accounted for 18% of respiratory infections in patients of all ages investigated during the course of one year, but in periods when parainfluenza viruses were prevalent they accounted for as many as 40% of the respiratory infections.

Epidemiology

Parainfluenza viruses have been isolated in all parts of the world and in every season, although they tend to be most prevalent in autumn and winter. Exceptionally, outbreaks may reach epidemic proportions.

Primary infection with parainfluenza viruses is acquired during

the early years of life, and by 4 years of age most children have been infected with the type III virus. Primary infection with parainfluenza viruses types I and II, which spread less rapidly than type III, tends to be more delayed, but most children have been infected by 8 years of age. Primary infection may be clinical or subclinical, but reinfections, which are common, are generally less severe because of the protection afforded by the presence of antibody. Boosting of the antibody level by multiple reinfection is probably responsible for the high state of immunity to parainfluenza viruses found in adults; antibody to the type III virus is found in 90–100% of adult sera and antibody to type I virus in 60–100% of sera.

Laboratory Diagnosis

(a) Virus isolation

Posterior pharyngeal swabs or throat washings, collected in balanced salt solution containing antibiotics and 0.5% bovine albumin or gelatin as a stabilizing medium, should be stored for not more than a few hours at 4°C before inoculation. Any further delay requires storage at -70°C although freezing and thawing affects parainfluenza viruses adversely.

Primary monkey kidney cells in culture provide the most susceptible host tissue; human cells may be used but are less sensitive, particularly if they are continuous lines. After 5–7 days incubation, any virus isolated is recognized by haemadsorption techniques; some strains require more prolonged incubation, and cultures failing to yield virus at 5 days should be retested for haemadsorption at 5-day intervals for as long as 3 weeks. Uninoculated control cultures are always included in haemadsorption tests because of the not uncommon presence of simian viruses SV5 and SV41 in primary monkey kidney cell cultures.

Any haemadsorbing virus isolated is subcultured, and typed by haemadsorption-inhibition with appropriate antisera, inactivated at 56°C for 30 minutes and freed from non-specific mucoprotein inhibitors by neuraminidase or periodate treatment.

(b) Serological tests

In the absence of successful virus isolation, resort to serological

methods of diagnosis may be necessary. Complement-fixation, haemagglutination-inhibition, or neutralization tests, may be employed; a four-fold rise, or more, in antibody titre occurring between the acute and convalescent phases of the disease is considered diagnostic. The serological tests are not always specific because minor antigenic relationships between various types of parainfluenza viruses may produce heterotypic antibody responses. Thus infection with a type I or type II virus may produce a type III antibody response, particularly if a type III virus was responsible for the first parainfluenza virus infection. Heterotypic antibody responses have also been noted between parainfluenza and mumps viruses.

Prophylaxis

The recognition that parainfluenza viruses are a cause of severe lower respiratory infections in early infancy and childhood has stimulated efforts to produce an effective vaccine. Some success has already been obtained in experimental trials with formalinized inactivated vaccines; their incorporation in a multivalent vaccine, with other constituents necessary for immunization in early childhood, would be a desirable development for the production of maximum protection against respiratory infection with the minimum number of injections.

Treatment

No specific antiviral treatment for parainfluenza virus infections is available, symptomatic treatment has therefore to be resorted to.

CHAPTER 16

Respiratory Infections

III. *Bronchovirus syncytialis*

Respiratory Syncytial Infection

A hitherto unrecognized virus was isolated in 1956 by Morris, Blount and Savage from a chimpanzee with upper respiratory infection. Originally called chimpanzee coryza agent (CCA), its tendency to form syncytia in tissue cultures led to its description as respiratory syncytial (RS) virus. Because of morphological and certain other similarities to paramyxoviruses, it has been assigned to the Family Paramyxoviridae, but in the genus *Bronchovirus*, to distinguish it from the haemagglutinating members of the genus *Paramyxovirus*.

Since its original isolation, *Bronchovirus syncytialis*, commonly referred to as RS virus, has been recovered from laboratory staff in contact with infected chimpanzees, and, by Chanock and his colleagues, from children with severe lower respiratory infections.

The Virus

(a) Morphology

RS virus particles are roughly spherical in shape, although filamentous forms have also been described. The spherical particles consist of an inner helical ribonucleoprotein component, surrounded by an outer lipoprotein envelope from which protrude radially orientated projections (Fig. 57). The diameter of these particles has been variously estimated to be 90-300 m μ .

After treatment with ether or detergent, the inner helical component is released and the outer envelope fractures into fragments



Fig. 57. Negatively stained respiratory syncytial virus particle and two filaments showing regularly arranged peripheral projections [from B. Bloth, A. Espmark, E. Norrby, and S. Gard (1963) *Arch. f. die ges Virusforsch.* 13, 582-86 (Springer-Verlag, Wien and New York)].

which take on a rosette-like appearance, and resemble the structures seen after ether disintegration of myxo- and paramyxoviruses. The diameter of the inner component is not accurately known but has been variously estimated to be 100-120 Å and 130-180 Å.

(b) Biological properties

RS virus, which resembles the paramyxoviruses morphologically, shares some biological properties with them; it is ether-sensitive, acid-labile at pH 3.0, and forms syncytia with intracytoplasmic inclusions in tissue cultures. However, its failure to grow in chick embryos, to haemagglutinate or to haemadsorb, and its deficiency in the enzyme neuraminidase distinguish it from the paramyxoviruses and account for its inclusion in a separate genus, *Bronchovirus* (Table 7).

(c) Antigenic composition

(1) Viral antigens

RS viral antigens may be identified by complement-fixation and by neutralization tests, but only the latter have revealed minor antigenic differences between some recently isolated strains and the original prototype Long strain, isolated in 1956. The significance of these antigenic variations is not yet known but the recovery of different antigenic types from the same epidemic is noteworthy.

(2) Soluble antigens

In infected tissue culture fluids the major part of the RS complement-fixing antigen is associated with non-infective components separable from the virus particles, i.e. with soluble antigens. Three distinct soluble antigenic components have been recognized by Coates, Forsyth, and Chanock, of which one probably represents the internal ribonucleoprotein component in excess, one probably represents a lipoprotein antigen not incorporated in the virus particle, and the third, which is capable of stimulating the production of neutralizing antibody, is thought to represent a protein component of the viral envelope in excess.

(d) Cultivation

RS virus grows in a number of continuous human cell lines and in human diploid cells; of these, HEP-2 cells are the most sensitive. Primary monkey kidney cell cultures are very much less sensitive and not suitable for virus isolation.

The cytopathogenic effect of RS virus is very characteristic and is manifested by foci of large syncytial cells which contain large numbers of nuclei (Fig. 12c). Usually occurring within 14 days, these changes, which may be delayed for 21 days, are followed by cellular degeneration. Eosinophilic intracytoplasmic inclusions may be seen in stained preparations, but these do not apparently consist of virus particles. Like myxo- and paramyxoviruses, replication of RS virus, as far as is known, is confined to the cytoplasm and maturation takes place at the cell surface.

RS Virus and Respiratory Infection

It is now well established that infection with RS virus is one of the most important causes of severe respiratory illness in infants and young children, particularly in those under the age of 1 year. In adults, it is an occasional cause of mild upper respiratory illness.

RS infection in infants usually commences as an upper respiratory tract infection, after an incubation period of 3–7 days; spread to the lower respiratory tract occurs in about half the cases, in whom bronchitis, bronchiolitis, or bronchopneumonia becomes manifest. In some surveys of children admitted to hospital with lower respiratory tract infection, RS virus has been found to be the responsible organism in 25–30% of cases. The severity of a first infection with RS virus accounts for the severe nature of the illness in young infants, subsequent reinfections are modified in the face of a degree of partial immunity.

Pathology

Inflammation of the nose and throat occurs at the onset of the disease and, in infants, spreads to the lower respiratory tract where necrosis and desquamation of the bronchial epithelium, round cell infiltra-

tion of the bronchial wall, and interstitial pneumonia, occur; evidence of virus infection is provided by the intracytoplasmic inclusions which may be present in the bronchial epithelium. The production of exudate may lead to bronchiolar obstruction and atelectasis; secondary bacterial infection, often by *Haemophilus influenzae*, may supervene.

Epidemiology

Yearly outbreaks of RS infection, particularly in the winter months, occur in most parts of the world. The virus is spread by droplet infection and dissemination is rapid in closed communities. Infection in early life seems to be a universal experience and most children have been infected by the age of 2-4 years. Reinfection is not uncommon and produces a milder illness as well as reinforcement of the state of immunity.

Immunity

Natural passive immunization of newborn infants by maternal antibody disappears within a few months of birth. Subsequently, about 25% of infants under the age of 1 year acquire antibodies to RS virus, and the proportion increases to 100% by the age of 7.

Laboratory Diagnosis

(a) Virus isolation

Sputum or pharyngeal swabs, collected in stabilized medium similar to that used for parainfluenza viruses, are inoculated into cell cultures as soon as possible. Specimens should not be stored for more than a few hours at 4°C; for longer periods specimens must be stored at -70°C.

Several continuous human cell lines and human diploid cells are satisfactory for isolation of RS virus. Primary monkey kidney cells are much less sensitive and not suitable for isolation. Successful isolation is most likely if specimens are taken as soon after the onset of illness as possible, although virus has been recovered as long as

7–10 days after onset. The typical cytopathogenic effect of RS virus usually appears within 14–21 days and the identification of the isolate may be confirmed by complement-fixation or neutralization tests. Sometimes, three or four serial passages may be necessary to provide sufficient amounts of virus for serological tests.

(b) Serological tests

A four-fold rise, or more, in the titre of complement-fixing or neutralizing antibodies to RS virus, occurring between the acute and convalescent phases of the illness, is considered diagnostic. In infants under the age of 6 months the antibody rise may be delayed for 4–6 weeks, whereas high titres of antibody may be found in the acute phase sera of patients over 4 years of age who have been previously infected.

Prophylaxis

In the absence of any general measures of control, the development of an effective vaccine holds out the best prospect of preventing RS infection in infants and young children. Formalin-killed RS virus vaccines have been used in limited experimental trials and have been shown to be antigenic, but so far no vaccine is generally available. The development of a multivalent vaccine consisting of RS and the three main types of parainfluenza virus antigens would fulfil an important requirement for the prevention of severe respiratory infection in infancy.

Treatment

No specific treatment is available. Antibiotic therapy for any secondary bacterial infection, and general symptomatic treatment, is prescribed.

CHAPTER 17

Respiratory Infections

IV. Rhinoviruses The Common Cold

The common cold is the most familiar of all infections, and there must be few, if any, of the general population who have not suffered from this condition at one time or another. Although not usually serious for the individual, its widespread dissemination makes the total economic loss from mild ill health and short absences from work very appreciable.

The viral aetiology of the common cold syndrome was first demonstrated in 1914, by Kruse, who succeeded in transmitting colds to volunteers by inoculating them with bacteria-free filtrates of nasal secretions obtained from patients with the cold syndrome. Since that time, Kruse's results have been abundantly confirmed, particularly in recent years, by Andrewes and his colleagues at the Common Cold Research Unit, Salisbury, England.

First attempts to recover common cold viruses in tissue culture provided, at best, equivocal results. Later, in 1956, the recovery of a cytopathogenic virus, JA virus, from patients with common colds was reported by Price from the U.S.A., and this was followed in 1957 by the recovery of virus 2060 by Pelon and his colleagues. However, it was not until 1960 that the technique for recovering cytopathogenic viruses from common colds with any degree of regularity was discovered by Tyrrell and his colleagues. They used monolayer cultures of human embryonic kidney cells, constantly rotated at a temperature of 33°C, in a medium whose pH range was rigidly held between 6.8 and 7.3. In this way, a new group of cytopathogenic viruses responsible for the common cold syndrome was

discovered, to which the name rhinoviruses was applied. More than 80 different serotypes are already known.

The Virus

(a) Morphology

So far, little is known about the detailed morphology of rhinoviruses. They are about 30 m μ in diameter, and negatively stained preparations show them to be made up of capsomeres arranged in cubical symmetry, similar in structure to other members of the subfamily Picornavirinae.

(b) Biological properties

Like other members of the subfamily Picornavirinae, rhinoviruses are small RNA viruses, which are resistant to ether. They are distinguished from the other genera of this subfamily, collectively referred to as enteroviruses, by their lability in acid media at pH 3.0, by their relative stability to heat at 50°C, and by their failure to multiply in the mucous membrane of the alimentary tract.

(c) Antigenic composition

More than 80 different serotypes of rhinovirus have been recognized by means of neutralization tests; possibly, more remain to be discovered. So far, there is no evidence that different serotypes vary in pathogenicity; indeed, more than one serotype has sometimes been isolated from the same epidemic.

(d) Cultivation

If the critical conditions of temperature and pH (33°C and pH 6.8-7.3) described by Tyrrell and his colleagues are adhered to, rhinoviruses will grow and produce typical cytopathogenic effects in human embryonic kidney or human diploid cells. Some strains, particularly after first isolation, will grow in continuous lines of human cells such as the KB or HeLa cell lines. Although all strains recovered will grow in human cells, about 20% of them will grow and produce cytopathogenic effects in monkey kidney cells under the same conditions. Two distinct types of rhinovirus are thus

distinguished; H strains, which grow in human cells only, and M strains, which grow in both human and monkey kidney cells.

The cytopathogenic effect resembles that produced by enteroviruses but may take longer to develop. Within 2–3 days of inoculation, foci of cytopathogenicity appear in which the cells become refractile and irregular in outline, later the nuclei become pyknotic and the cells round up and fall away from the glass (Fig. 58). After several days the cytopathogenic effect spreads throughout the monolayer.

Using the tissue culture techniques described, rhinoviruses are recovered from only 25–30% of patients exhibiting the common cold syndrome. Recently, some additional strains of rhinovirus, and other viruses which do not grow in human or monkey kidney cell monolayers, have been isolated in organ cultures of the human embryonic trachea, by Tyrrell and his colleagues.

Clinical Features

The symptomatology of the common cold is familiar to all; primarily an acute catarrhal inflammation of the nasal mucous membrane, it leads to profuse nasal discharge and sometimes nasal obstruction; mild inflammation of the conjunctivae and other parts of the upper respiratory tract may supervene. In adults, malaise may be prominent but pyrexia is usually absent, or of minor degree, and recovery occurs within a few days or a week. Complications due to secondary bacterial infection may ensue, of which the most common are sinusitis, otitis media, and bronchitis.

Pathology

In Hilding's studies of nasal mucous membranes from patients with colds, swelling and hyperaemia were accompanied by leucocytic infiltration and by necrosis and shedding of the epithelium. Epithelial regeneration was complete by the fourteenth day.



Fig. 58. Cytopathogenic effects of rhinoviruses in human embryonic kidney (HEK) cells, stained with haematoxylin and eosin.

(a) Normal HEK.

(b) HEK infected with rhinovirus.

Epidemiology

The common cold is world-wide in its distribution, and in temperate climates the incidence of infection rises during the winter months, sometimes reaching epidemic proportions. Man is the only source of infection, which is spread by droplets, and, possibly, by fomites contaminated with infected secretions. The patient may be infectious during the short incubation period of 1–3 days, but is most infectious during the first day of illness and may continue to be so for about 4 days. Volunteer experiments, serological studies, and isolation of rhinoviruses from asymptomatic cases, indicate that only about one-third of those infected actually develop clinical symptoms.

Colds are most frequent in infants and children, and the incidence of infection declines significantly after middle age; consequently, the risk to adults is greater in homes where there are children. Overcrowding at home or at work also increases the risk of infection, as does the greater population density of urban compared with rural communities.

Contrary to popular belief, there is no unequivocal evidence that exposure to cold and damp conditions increases susceptibility to infection. The maximum incidence during the winter period may well be due to the seasonal change in living habits which promotes crowding in confined spaces, with little or no ventilation, for the sake of warmth.

Immunity

Recovery from infection is usually associated with the production of antibody and resistance to infection with the homologous serotype. The antibody response may, however, depend on the infecting strain. Taylor-Robinson and his colleagues have shown that the response to M strains is greater and longer lasting than that of H strains; indeed, only about half of those infected with H strains responded with a four-fold rise of antibody titre.

The short-lived immunity to the common cold, under natural conditions, is undoubtedly related to the multiplicity of rhinovirus

serotypes; obviously, antibody to one serotype will not provide immunity to another, although heterotypic antibody responses have occasionally been demonstrated in man. The distribution of antibodies to a few serotypes has been studied in detail; they have been found in about 70% of the adult population but only infrequently in infants and children. This suggests that the lower incidence of colds in older adults is the result of their wider immunological experience.

Laboratory Diagnosis

Virological and serological tests are rarely necessary for routine diagnosis but are valuable research tools.

(a) Virus isolation

Nasal or throat washings collected within 4 days of the onset of illness, and preferably on the first day, are inoculated immediately, or stored at -70°C . Human embryonic kidney or human diploid cells are used for primary isolation, any isolates are later passaged in monkey kidney cells for the detection of M strains. After incubation at 33°C and at pH 6.8-7.3, the cytopathogenic effect usually becomes evident within a week but may take longer, so that cultures should be observed for 10-14 days.

Isolates are distinguished from other Picornavirinae by their acid lability at pH 3.0, and the serotype is subsequently identified by neutralization tests. Because of the large number of different serotypes, typing is a technique beyond the scope of all but special research laboratories.

(b) Serological tests

The multiplicity of antigenic types makes routine serological diagnosis impracticable, but a patient's serum can be tested against his own virus isolate and a four-fold rise of antibody titre, occurring between the acute and convalescent phases of the illness, may often be demonstrated.

Prophylaxis

(a) General measures

Avoidance of overcrowding and care to observe hygienic precautions when coughing and sneezing are useful measures.

(b) Vaccination

Contrary to what might have been expected, the isolation of rhinoviruses has not brought a successful solution to the problem of the common cold by prophylactic vaccination. Although formalin-killed vaccines of a few specific serotypes have been shown to be effective against infection with the homologous virus, the multiplicity of antigenic types makes it unlikely that a vaccine effective against all the 80, or more, serotypes will become available in the near future, if at all.

It seems superfluous to add that antibacterial vaccines, often recommended, cannot possibly protect against rhinovirus infections. True, they may offer some protection against bacterial complications but controlled trials have produced only equivocal results.

Treatment

Therapy is confined to non-specific remedies for the relief of symptoms.

CHAPTER 18

Respiratory Infections

V. Adenoviruses

Adenovirus Infections

Adenoviruses were first isolated in 1953, by Rowe and his colleagues, from human adenoid tissues excised at operation. Shortly afterwards, Hilleman and Werner isolated them from patients with acute respiratory infections and atypical pneumonia. So far, 31 different antigenic types of adenovirus have been recovered from human sources, but only a few of these are actually pathogenic.

The Virus

(a) Morphology

The very characteristic morphology of adenovirus particles, revealed by negative staining methods, was first demonstrated by Horne and his colleagues. Each particle is made up of 252 capsomeres arranged in icosahedral symmetry to form an icosahedron, approximately 80 m μ in diameter, some of whose 20 equilateral triangular faces are easily visible (Fig. 24b). Recently, Valentine and Pereira have shown that the capsomeres situated at each of the 12 vertices give rise to tails, about 200 Å in length, which terminate in small knob-like structures, about 40 Å in diameter, which may well serve as attachment organs (Fig. 24a). Inside the virus capsid is the DNA core, whose structure and arrangement have yet to be elucidated.

(b) Biological properties

All adenoviruses are resistant to ether and acid pH and, with the exception of types 18 and 31, agglutinate erythrocytes from one

or more mammalian species. Four groups of adenoviruses, based on their ability to agglutinate rhesus monkey or rat red cells, have been defined by Rosen (Table 9). Those belonging to Group I agglutinate rhesus monkey but not rat erythrocytes, whereas those belonging to Group II agglutinate rat erythrocytes to full titre and rhesus

Table 9. Rosen's grouping of adenoviruses based on haemagglutinating properties

Group	Adenovirus types	Red cells haemagglutinated	
		Rhesus monkey	Rat
I	3, 7, 11, 14, 16, 20, 21, 25, 28	+	—
II	8, 9, 10, 13, 15, 17, 19, 22, 23, 24, 26	±	+
III	1, 2, 4, 5, 6, 12	—	+ in presence of heterotypic immune serum
IV	18, 31	—	—

monkey erythrocytes to lower titre or not at all. Group III adenoviruses fail to agglutinate rhesus monkey erythrocytes but agglutinate rat erythrocytes in the presence of heterotypic antiserum to any other Group III virus. Non-haemagglutinating adenoviruses, types 18 and 31, are assigned to Group IV. Type 12 adenovirus, until recently included in Group IV, has now been shown to behave as a Group III virus by Schmidt, King, and Lennette.

The adenovirus haemagglutinins are separable from the virus particles, and are identical with the soluble antigens B and C (see below). Pereira and his colleagues have shown that the factor producing complete haemagglutination is antigen B, and the factor which requires the presence of heterotypic immune serum for haemagglutination is antigen C, itself a breakdown product of antigen B. Although no elution of adenovirus haemagglutinins occurs at 37°C, which is the optimal temperature for adenovirus haemag-

glutination, the adenovirus receptors on human group O cells are partially susceptible to neuraminidase.

(c) Antigenic composition

The antigenic composition of adenoviruses has been studied by complement-fixation, haemagglutination-inhibition, neutralization and gel-diffusion reactions. Although only a few types of adenoviruses have been studied in any detail, a complex antigenic composition has already been revealed. In addition to the infective virus particle, infected cells produce three soluble antigens, each of which represents a virus morphological unit produced in excess and therefore not incorporated in completed virus particles.

(i) Soluble antigen A

Recently termed the hexon antigen, this is a group complement-fixing antigen common to all types of adenovirus. Examination of soluble antigen A in the electron microscope has shown it to consist of the individual capsomeres which compose the virus capsid. It thus represents capsomeres produced in excess.

(ii) Soluble antigen B

In adenovirus type 5, Valentine and Pereira have identified this antigen, recently termed the penton antigen, with the morphological unit situated at each of the 12 vertices of the viral icosahedron. This consists of a capsomere from which extends a spike terminating in a small knob-like structure. It is believed that the capsomeric component of antigen B represents the toxin-like 'cell-detaching' factor produced by adenoviruses, and that it is responsible for direct haemagglutination.

(iii) Soluble antigen C

Tryptic digestion of the capsomeric component of antigen B leaves the spike and knob constituent which constitutes the type-specific antigen C. This is the structure which is responsible for indirect haemagglutination which occurs only in the presence of heterotypic immune serum. Valentine and Pereira suggest that the type-specific component of antigen C is located in the terminal knob, the

blocking of which by antiserum results in neutralization of infectivity.

Cultivation

Adenoviruses are easily cultivated in a number of continuous lines of human cells, of which the HeLa and KB cell lines are the most susceptible, as well as in human diploid cells. Primary cultures of monkey kidney cells are much less sensitive.

Cytopathogenicity usually becomes evident within a few days, but up to 10–21 days may be necessary for its maximum development. Foci of cells round up, and form irregular clusters of enlarged dark granular cells which degenerate and fall away from the glass (Fig. 59).

Two separate phases of the adenovirus cytopathogenic effect have been identified. One is an early cytopathic effect occurring within a few hours after infection, in which the cells round up, aggregate into clusters, and eventually fall away from the glass. These changes are due to the 'cell-detaching' factor of antigen B, and take place in the absence of viral multiplication and its associated intranuclear changes. The second phase of the adenovirus cytopathogenic effect is due to actual viral multiplication and formation of infective virus. This is characterized by nuclear changes, the detailed morphology of which depends on the virus serotype.

In stained preparations, two distinct types of nuclear changes have been described, one produced by adenovirus types 1, 2, 5, and 6, and the other produced by types 3, 4, and 7. In the former, intranuclear eosinophilic inclusions which form within about 14–16 hours are often seen. Later, the inclusions become granular, basophilic and Fielgen-positive, and break down to form basophilic granular clusters in the now enlarged nucleus. Eventually, the nucleus takes on a glassy appearance and the basophilic granular clusters aggregate to form dense intranuclear basophilic masses. Some adenovirus type 5 strains produce intranuclear protein crystals which are eosinophilic, Fielgen-negative and do not contain virus particles. In contrast, adenovirus types 3, 4, and 7 do not often produce intranuclear eosinophilic inclusions, although type 7

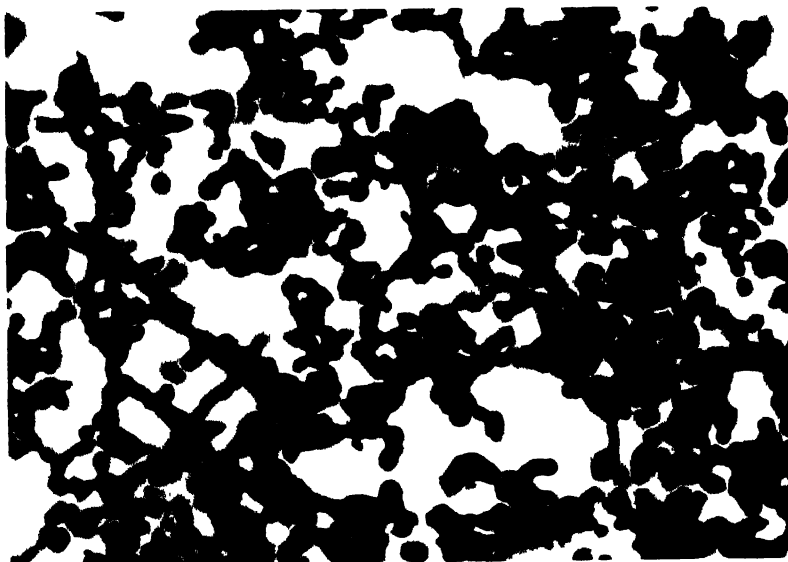
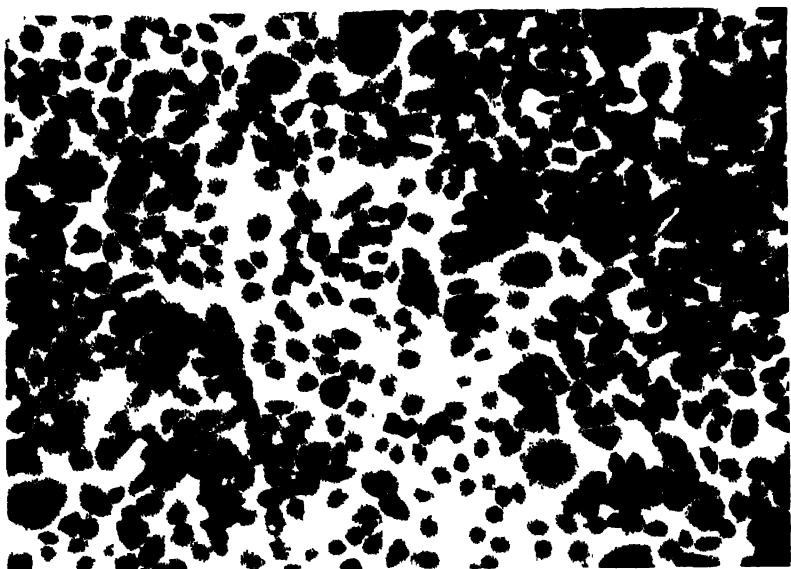


Fig. 59. Cytopathogenic effects of adenoviruses in HeLa cells, stained with haematoxylin and eosin.

(a) Normal HeLa cells.

(b) HeLa cells infected with adenovirus.

may do. Instead, about 14 hours after infection the chromatin becomes rearranged into basophilic lattice and network patterns, and draws away from the nuclear membrane. Later, crystal-like inclusions develop around a central basophilic mass, which when mature are Fuelgen-positive, basophilic, and composed of virus particles arranged in crystalline array. In the final stage the nucleus takes on a rosette-like appearance with a dense central basophilic, crystalline mass.

Because of their intranuclear position, and possibly because of their crystalline arrangement, adenovirus particles are not readily extruded into the extracellular environment; indeed, only about 2–6% of the virus produced is found in the culture fluid. Unlike cells infected with enteroviruses, adenovirus infected cells continue to metabolize after the onset of cytopathogenic changes and their culture media are therefore more acid.

Clinical Syndromes and Pathology

Adenoviruses have a pronounced affinity for the mucous membranes of the respiratory and alimentary tracts, the conjunctiva, and for lymphoid tissue. After primary infection, there is a tendency for some adenoviruses to persist in lymphatic tissue, in a latent form. Latent asymptomatic infection is commonest in childhood, and up to 90% of the adenoids removed at operation may be found to be latently infected, especially with types 1, 2, and 5. Evidence of latent adenovirus infection in mesenteric lymph nodes, removed at laparotomy, has also been obtained.

Certain adenovirus types are now well-recognized causes of specific clinical syndromes which have been classified by Huebner and others as follows:

(a) Acute febrile pharyngitis

An illness of a few days duration, acute febrile pharyngitis is characterized by cough, mild sore throat, pyrexia, pharyngitis, and sometimes by mild cervical lymphadenitis. Usually occurring in epidemics, the condition is most common in children but occasional

cases occur in young adults, particularly in military recruits. Types 1, 2, 5, and 6 are the adenoviruses most commonly involved.

(b) Pharyngoconjunctival fever

Adenoviruses most commonly responsible for pharyngoconjunctival fever belong to types 3 and 7. The illness is characterized by fever, pharyngitis, conjunctivitis which is often unilateral, and sometimes by cervical lymphadenitis. Occasionally, gastro-intestinal symptoms become evident, particularly in children, and virus may be isolated from the faeces.

(c) Acute respiratory disease (ARD)

Sometimes known as febrile catarrh, this influenza-like illness is characterized by pyrexia, malaise, rhinitis, and mild pharyngitis which sometimes develops into tracheobronchitis or laryngitis. The disease, which is uncommon in civilian populations, appears almost exclusively in epidemic form among young adults herded together in military recruit camps. The adenoviruses most commonly responsible for outbreaks of ARD are types 3, 4, and 7, although types 14 and 21 have been recovered from outbreaks in Holland.

(d) Pneumonia

The clinical and X-ray findings in pneumonia due to adenoviruses are similar to those of primary atypical pneumonia, but without the development of cold haemagglutinins or agglutinins for *Streptococcus* MG. The condition may be fatal, particularly in children under 4 years of age, and typical cytopathic effects of adenovirus infection have been found, post-mortem, in the epithelium of the bronchi and lung alveoli. Types 3 and 7 are the adenoviruses most commonly found in cases of pneumonia.

(e) Epidemic keratoconjunctivitis

Although conjunctivitis, alone or in conjunction with respiratory infection, may be due to other types of adenovirus, epidemic keratoconjunctivitis is always due to type 8. It is characterized by follicular conjunctivitis which is often unilateral, and by the later development of subepithelial corneal opacities which usually disappear

within a few weeks but may take much longer. Outbreaks tend to attack those engaged in occupations like welding, in whom occupational irritation of the conjunctiva is conducive to infection.

(f) Gastro-intestinal infections

Some cases of respiratory infection due to adenoviruses are accompanied by symptoms of vomiting, diarrhoea and abdominal pain. Although adenovirus infections in which gastro-intestinal symptoms only were present have been reported, and adenoviruses may frequently be recovered from the faeces, further investigation is required to establish the role of adenoviruses in outbreaks of gastro-enteritis.

Some evidence that adenoviruses are one of the agents responsible for mesenteric adenitis and that adenovirus infection may play a role in the production of intussusception has been reported.

Epidemiology

Adenovirus serotypes most commonly associated with respiratory infection are world-wide in their distribution, but the distribution of other serotypes is not known in detail. Characteristically, adenovirus infections occur in the winter and spring seasons, and they are most frequent in the closed communities of children and young adults found in schools, institutions, and recruit camps. Among recruits, infections fall most heavily on new entrants at the beginning of their training whereas permanent staff, whose immunity has been built up to high level by previous infections, are usually spared.

Man is the only source of human adenovirus infections and the virus is spread by droplets; however, the frequent recovery of adenoviruses from faecal specimens makes the faecal-oral route of spread possible. The occurrence of conjunctivitis suggests the conjunctiva as another possible route of entry, to which conjunctival irritation is a predisposing factor. Hence the association of outbreaks of pharyngoconjunctival fever, and conjunctivitis, with swimming and with those working conditions which provoke conjunctival irritation.

Immunity

Immunity to infection with specific types of adenovirus is correlated with the presence of homologous neutralizing antibody in the serum. Serological surveys show that neutralizing antibodies to types 1 and 2, and to a lesser extent to types 5 and 6, are acquired early in childhood. By the age of 4–5 years, some 60–70% of children have acquired antibodies to types 1 and 2. In contrast, antibodies to types 4 and 7 are not found in a high proportion of children and the susceptibility of young adult recruits to these types is thereby explained.

Laboratory Diagnosis

(a) Virus isolation

Adenoviruses may be isolated from the throat, nasopharyngeal and conjunctival secretions, and from the stools. In a recent survey of children, virus was recovered two or three times more frequently from nasal than from throat swabs. It may be noted that virus from latently infected adenoid tissue, removed at operation, is not usually recoverable by the orthodox technique of inoculating cell homogenates into tissue cultures. The excised tissue must be maintained in culture until cytopathic degeneration becomes evident, before virus can be transmitted to HeLa cells in the usual way. Probably, this is due to the very small amount of virus in the original tissue.

Swabs and specimens, collected in balanced salt solution or broth with added antibiotics, are inoculated as soon as possible. Refrigeration at 4°C is required if inoculation is delayed for a few days, specimens stored for longer periods must be frozen at –20°C or better at –70°C. Specimens taken within the first few days of illness are the most likely to yield virus.

Adenovirus isolations are best made in HeLa cells but other continuous lines of human cells, such as KB cells, or human diploid cells may be used. The lower numbered adenovirus types produce typical cytopathogenic effects within about a week of inoculation, but the higher numbered types usually take 2 weeks or longer. Sometimes, incubation must be prolonged for as long as 30 days before evidence of virus growth is obtained. In the absence of any

cytopathogenic effect, cells from the culture should be used as the inoculum for further subculture.

Identification of any isolate is confirmed by complement-fixation tests, using standard reference sera containing the group specific complement-fixing antibody. Allocation to one of the four groups of adenoviruses, described by Rosen, is then effected by haemagglutination tests with rat and rhesus monkey erythrocytes. The differentiation of individual serotypes within each group is established by haemagglutination-inhibition tests with type-specific immune rabbit sera, but types 18 and 31 are identified by neutralization tests. Some cross reaction between members of the same group may occur in haemagglutination-inhibition tests.

(b) Serological Tests

A four-fold rise, or more, in the titre of antibody against adenoviruses, occurring between the acute and convalescent phases of the disease, is diagnostic. The complement-fixation test using the group specific complement-fixing antigen is the method of choice, although it will not reveal the specific serotype responsible for infection. Other serological techniques are not usually employed in routine diagnosis. It may be noted that rises of complement-fixing antibody are less readily detected in children than in adults.

Prophylaxis

(a) General

In closed communities, avoidance of overcrowding and attendance to matters of personal hygiene are important although of limited usefulness.

(b) Vaccination

The multiplicity of adenovirus serotypes and their relatively minor contribution to the number of respiratory infections which occur in adult civilian populations makes vaccination of the general population both difficult and unnecessary. In contrast, outbreaks of adenovirus infection in recruit camps are of some military and

economic importance and make vaccination of recruits a necessary military requirement. For this purpose, formalin-killed polyvalent adenovirus vaccines, administered in one dose subcutaneously, have been used with some success. Types 3, 4, and 7, the serotypes most commonly encountered in these outbreaks, are the ones incorporated in the vaccine; other types may be added if and when they are found to be important.

Adenovirus for vaccines is usually grown in monkey kidney cells because of the potential carcinogenic hazard inherent in the use of human heteroploid cell lines. The poor growth of adenovirus in these cells, and the possibility of contamination of monkey kidney cells with the simian oncogenic virus SV40, led Couch and his colleagues of the National Institutes of Health in the U.S.A. to experiment with live virus vaccines produced in human diploid cells. Small scale trials, in which live adenovirus types 4 and 7 were administered in enteric-coated capsules to provide an attenuated primary infection of the mucous membranes of the alimentary tract, have produced sufficiently promising results to encourage further development.

Adenoviruses and Tumour Formation

In 1962, Trentin and his colleagues discovered that adenovirus type 12 possessed the remarkable property of producing sarcomatous tumours in newborn hamsters. This was the first demonstration of oncogenic properties in a virus of human origin. Later, adenoviruses of types 18, 3, 7, and 31 were also found to be oncogenic in newborn hamsters, and sometimes in newborn mice as well. The significance of these observations and their bearing on the problem of cancer in man is, as yet, uncertain and will be discussed in Chapter 50.

After tumour induction, infective virus is no longer recoverable from tumour tissue. Evidence of its viral origin is nevertheless revealed by the production of a virus specific antigen in tumour cells and by the production of antibody to this antigen in tumour bearing animals; moreover, incomplete virus particles have now been detected in tumour tissue by Smith and Melnick.

Animal Adenoviruses

A number of viruses which resemble human adenoviruses in their morphological and biological properties but which form distinct serotypes have been recovered from various animal species. None of these viruses cause disease in man, and most are non-pathogenic in their host species. The following animal adenoviruses have been described:

(a) Simian

One serotype has been isolated from a chimpanzee and 12 from monkeys. They share the group complement-fixing antigen with human strains and grow best in monkey kidney cells.

(b) Bovine

Two serotypes have been isolated from calves. They share the group complement-fixing antigen with human strains and grow best in calf cells.

(c) Canine

Canine hepatitis virus is an adenovirus. Antisera to the human strains react with the complement-fixing antigen of canine hepatitis but antisera to canine hepatitis do not react with the complement-fixing antigen of the human strains. This virus grows best in canine tissues.

(d) Murine

One strain has been isolated from mice; it shares the adenovirus group complement-fixing antigen and grows best in murine tissues.

(e) Avian

A strain isolated from chicken tissues, which is known as GAL (Gallus-adenovirus like) virus, resembles adenoviruses morphologically and produces a similar cytopathogenic effect. It does not, however, possess the adenovirus group complement-fixing antigen and its classification as an adenovirus remains in doubt. It grows best in embryonic chick tissues.

CHAPTER 19

Acute Infectious Fevers caused by Paramyxoviridae

I. *Paramyxovirus parotidis* Mumps

Mumps, which is one of the common acute infections of childhood, is characterized by painful enlargement of the parotid glands. The causative virus is closely related to the parainfluenza viruses which it resembles in both morphology and general properties; it is therefore assigned to the same genus, in which it is specified as *Paramyxovirus parotidis*.

The Virus

(a) Morphology

In conformity with other Paramyxoviruses, the virus of mumps is composed of an internal helical ribonucleoprotein component, surrounded by a lipoprotein envelope from which protrude radially orientated rod-like projections (Fig. 60). The diameter of the virus particles varies from 100–250 m μ and the diameter of the internal helical component is approximately 150–170 Å.

(b) Biological properties

(i) *Haemagglutination*

The virus of mumps agglutinates red cells of various species by attaching to the sialomucoprotein receptors utilized by both myxo- and paramyxoviruses. After adsorption, elution may occur with destruction of the erythrocyte receptors by the viral neuraminidase enzyme.

(ii) Haemolysis

The activity of mumps virus neuraminidase is comparatively weak, and at 37°C some mumps virus particles bind irreversibly to red cells which undergo haemolysis. The haemolytic reaction, which is mediated through the sialomucoprotein erythrocyte receptors, is possibly enzymic in nature.



Fig. 60. Negatively stained partially disrupted mumps virus particle revealing the helical internal component, part of which has been released [from R.W.Horne and A.P.Watson (1960) *J. Mol. Biol.* 2, 75-77 (Academic Press, London and New York)]

(c) Antigenic composition

The antigenic composition of mumps virus has been studied by neutralization, haemagglutination-inhibition and complement-fixation tests. Two distinct complement fixing antigens are produced in mumps infected tissues, the soluble (S) antigen and the viral (V) antigen.

(i) The soluble (S) antigen

The small-sized soluble (S) antigen is liberated from infected tissues

and is distinct from the virus particle. It represents the viral internal ribonucleoprotein component produced in excess. In its bound form (the g- or gebundenes antigen), it is the internal ribonucleoprotein component, which is non-reactive in the complement-fixation test unless released from the virus particle by ether treatment.

(ii) The viral (V) antigen

The larger sized viral (V) antigen, which represents the viral particle itself, is extremely stable and shows no evidence of antigenic variation. A minor antigenic relationship between the V-antigen of mumps and the antigens of NDV and parainfluenza viruses has been demonstrated.

(d) Cultivation

(i) Chick embryos

Amniotic, allantoic, and yolk sac routes of inoculation have been used for the cultivation of mumps virus in the chick embryo; of these, the amniotic route of inoculation is the route of choice. Seven to eight-day-old chick embryos are inoculated amniotically and incubated for 5–7 days at 37°C. Any virus isolated is detected in harvested amniotic fluids or amniotic membrane suspensions by haemagglutination or complement-fixation tests. Sometimes, insufficient virus is present after the first amniotic passage to be revealed by these tests and two or three ‘blind’ amniotic passages may then be necessary before virus is detected. After a variable number of amniotic passages most strains can be adapted to the allantoic cavity.

(ii) Tissue culture

Isolation of mumps virus is readily achieved in monkey kidney or HeLa cells. A cytopathogenic effect is produced which is usually characterized by the formation of syncytia and multinucleate giant cells (Fig. 61). After infection, a cytolytic effect with formation of syncytia and giant cells may occur in the absence of viral multiplication. This effect is thought to be related to the haemolytic properties of the virus.

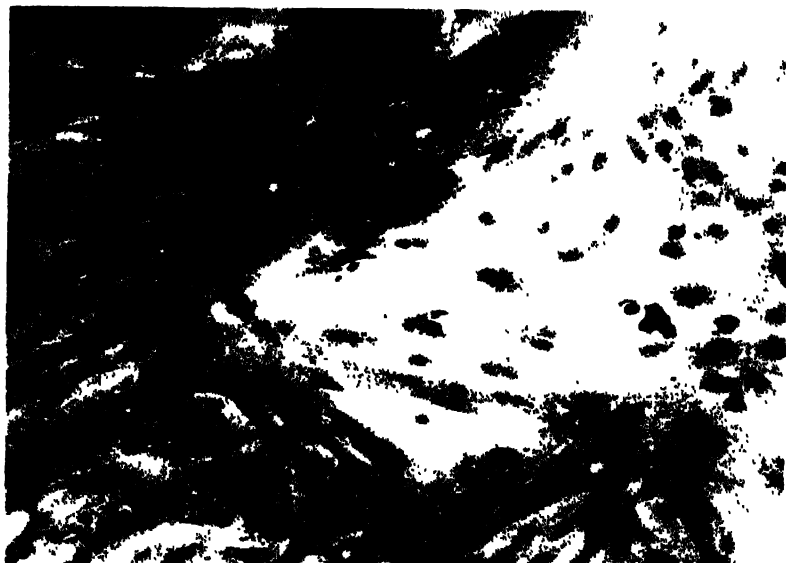


Fig. 61. Cytopathogenic effects of mumps virus in monkey kidney cells, stained with haematoxylin and eosin.

(a) Normal monkey kidney cells.

(b) Monkey kidney cells infected with mumps virus.

After adaptation to the chick embryo, mumps virus loses the ability to grow in mammalian cells but grows well in chick embryo fibroblasts, which are resistant to unadapted virus.

Clinical Features

Mumps is a disease of sudden onset characterized by an initial modest pyrexia and painful enlargement of the parotid glands. In more than 90% of cases, the parotid glands are bilaterally involved but not always simultaneously, sometimes infection spreads from one side to the other after an interval of a few days. Occasionally, the submaxillary or sublingual glands may be involved. The course of the disease in children is benign and the glandular swellings usually subside within a week or 10 days; death is extremely rare.

In patients beyond the age of puberty, the disease is often more severe and the ovaries, testes, central nervous system, pancreas, or other organs may be affected. Sometimes, orchitis, meningo-encephalitis, and other complications may occur without any parotid or other salivary gland enlargement. The incidence of severe and complicated cases in adults may be as high as 35%.

Pathology and Pathogenesis

Examination of parotids excised from experimentally infected monkeys shows the lesion to be one of inflammation, degeneration, and necrosis of the parenchyma cells. The mode of viral spread within the infected patient is, however, much less certain. Some hold that virus reaches the parotid gland from the oral cavity via the parotid duct, afterwards spreading through the blood stream to other organs. This sequence of events cannot account for those cases in which symptoms and signs of disease occur in other organs before infection becomes manifest in the parotid gland itself. Primary multiplication in the epithelium of the upper respiratory tract, and possibly in the draining lymphoid tissue, followed by viraemia and localization of virus in various glands, especially the parotids which are usually the first and most often affected, is a

more likely course of events. Passage of virus through the blood-brain barrier leads to infection of central nervous system.

Epidemiology

Man is the only source and reservoir of infection, from whom the virus is spread by droplets, direct contact, or, rarely, by fomites contaminated with infected saliva. Epidemiological investigations suggest that a patient is most infectious from 2-3 days before the onset of illness until 2-3 days after. Virus has, however, been isolated from patients as long as 7 days before the onset of illness and for 9 days afterwards, so that these may be considered the maximum limits during which the patient is infectious. Urinary excretion of virus has also been observed for 14 days after the onset of illness. Clearly, the patient may be infectious during part of the incubation period, which averages 18 days but may extend from 12 to 26 days.

Mumps is endemic in all parts of the world and epidemics, which are not uncommon in closed communities of children and young adults, tend to occur in winter and spring. Although large epidemics tend to occur every 7-8 years, the disease appears to be less widespread than some other acute infectious fevers. Thus, only 60% of the 1-20 age group have a clinical history of mumps although 90% have a history of measles. This discrepancy is, however, more apparent than real because it is now well established that only 30-40% of those infected with mumps actually display any clinical manifestations. Children in the age group 5-9 are the most prone to clinical attack and are the main spreaders of the disease, transmitting infection to their family or school contacts.

Immunity

For the first few months of life, infants are protected by passive immunity, derived from the passage of maternal antibody through the placenta before birth. Active immunity acquired after mumps infection is usually life-long, although second attacks do occur on occasion.

The delayed hypersensitivity reaction which occurs after intra-

dermal inoculation of inactivated mumps virus, in individuals who have had previous clinical or subclinical mumps infection, suggests that tissue factors may play some role in immunity to the disease. Significantly, agammaglobulinaemics do not show any predisposition to repeated attacks of mumps.

Laboratory Diagnosis

Isolated or complicated cases in which the diagnosis is in doubt may require laboratory confirmation.

(i) *Virus isolation*

A swab taken from the area around the parotid duct, or specimens of saliva, urine or, where appropriate, cerebrospinal fluid are collected in broth-saline solution to which antibiotics have been added. Specimens are inoculated into an appropriate host as soon as possible, but virus remains viable after storage for short periods at 4°C, or for longer periods at -70°C in a protein containing medium.

Amniotic inoculation of chick embryos, although still an important method of isolation, has been largely superseded by tissue culture methods. Isolation in monkey kidney cells is the method of choice, although HeLa cells may also be used. Any virus isolated may be identified by haemadsorption, or by the occurrence of cytopathogenicity after 6-7 days. Identification is confirmed by haemadsorption-inhibition or by neutralization tests. At least two consecutive passages are required before a negative result is accepted.

(ii) *Serological tests*

A rise of four-fold, or more, in the titre of mumps antibody, occurring between the acute and convalescent phases of the disease, is diagnostic. Although neutralization tests are the most sensitive for the detection of antibody, haemagglutination-inhibition or complement-fixation tests are more often used for routine purposes because of their greater simplicity.

Complement-fixation is the method of choice, and the antibodies to both the S and the V antigens may be investigated. Antibody to the S antigen appears before that to the V antigen, and may even be

present in the acute phase serum; it disappears within a few months. In contrast, antibody to the V-antigen persists for several years.

Prophylaxis

(a) General measures

The patient should be isolated until the parotid gland swelling has subsided; during this period his crockery, and other articles contaminated with infected sputum, should be disinfected. Because of the high incidence of subclinical infection, the efficiency of isolation procedures for the control of epidemics is limited, and quarantine is not called for.

(b) Vaccination

Both formalin-inactivated and live attenuated mumps virus vaccines are available. In general, vaccine is not recommended for children, in whom the disease is mild and provides life-long immunity. Susceptible adults may, however, benefit from vaccination, and vaccines have been used with some success in protecting young personnel in army recruit centres where epidemics of mumps are sometimes a serious problem. Vaccination has also been used, with equivocal results, to protect susceptible adults after exposure to infection, on the assumption that immunity will develop during the relatively long incubation period. In practice, few adults are susceptible because even those without a clinical history of mumps are, in fact, immune as a result of subclinical infection. Susceptible individuals can, however, be identified by the absence of neutralizing antibody in their serum, or by skin hypersensitivity tests. A positive skin test is strong but not absolute evidence of immunity.

(i) *Inactivated vaccine*

Formalin-killed vaccine, prepared in chick embryos, is given subcutaneously in three injections; an interval of 4 weeks separates the first two injections and 6–12 months the second and third, after which immunity is provided for 1–2 years.

(ii) Live vaccine

A live attenuated virus vaccine which, it is claimed, provides immunity in children for at least 5 years has been used extensively in the U.S.S.R. More recently, an American live attenuated strain, the Jerryl Lynn strain level B, grown in chick embryo tissue culture, has been shown by Wiebel and his colleagues to be a safe, highly effective vaccine which is not communicable after subcutaneous administration.

(c) Passive immunization

Passive immunization of susceptible adults, with human convalescent serum, after exposure to infection has been practised with variable results.

Treatment

There is no specific antiviral treatment for mumps and therapy is symptomatic. γ -Globulin prepared from human convalescent serum may be effective in preventing orchitis in adults, if administered within 24 hours of mumps parotitis.

CHAPTER 20

Acute Infectious Fevers caused by Paramyxoviridae

II. *Pseudomyxovirus morbilli* Measles

Measles is one of the common exanthemata of childhood, characterized initially by catarrhal symptoms which are followed by the development of a generalized maculopapular rash. Although long known to be of viral aetiology, the causative virus did not become available for investigation until 1954 when Enders and Peebles successfully isolated the virus in tissue culture.

The Virus

(a) Morphology

The virus of measles is a roughly spherical particle, 120–250 m μ in diameter, which consists of an internal helical ribonucleoprotein component, surrounded by a lipoprotein envelope from which protrude radially orientated rod-like projections (Fig. 31). In common with other Paramyxoviridae the diameter of the internal helical component is approximately 170 Å.

(b) Biological properties

Like other Paramyxoviridae, measles virus is ether sensitive; ether treatment disrupts the virus particle releasing the internal component and small pieces of the disrupted envelope, the latter because of their surface projections take on the form of rosettes 300–600 Å in diameter. In contrast to viruses belonging to the genus *Paramyxovirus*, measles virus agglutinates and haemolyses monkey erythro-

cytes, only, and does not elute from them; indeed, agglutination is optimal at 37°C. Moreover, measles virus does not utilize the specific sialomucoprotein haemagglutination receptors.

Although the measles virus particle itself haemagglutinates, the haemagglutinating activity is mostly mediated by a small particle, non-infective haemagglutinin. This small particle haemagglutinin, which is most sensitive for the detection of haemagglutination-inhibiting antibody, may also be released from the intact virus particle by ether or heat treatment. In contrast, only the intact virus particle is concerned in the production of haemolysis.

(c) Antigenic composition

Neutralization, haemagglutination-inhibition, and complement-fixation tests have been employed for the antigenic analysis of measles virus. So far, only one antigenic type of measles virus has been recovered in all parts of the world, and no evidence of antigenic variation has been obtained. In spite of morphological and other similarities to paramyxoviruses, no antigenic relationship between them and measles virus has been demonstrated. But, an antigenic relationship has been demonstrated between the viruses of measles, canine distemper and rinderpest. These three viruses, which resemble one another morphologically and which produce similar disease patterns in monkeys, are now considered to form a closely related group referred to by Waterson and Almeida as pseudomyxoviruses.

In support of these antigenic relationships, many sera from individuals with a history of measles have been shown to contain antibodies to distemper virus, and some sera from dogs recovering from distemper have been shown to contain antibodies to measles virus. Although children inoculated with canine distemper vaccines do not apparently produce measles antibody, immunization of ferrets and dogs with live measles vaccine has produced partial protection against canine distemper. Similar cross reactivities between the viruses of canine distemper and rinderpest have been noted, and sera from cattle infected with rinderpest have some neutralizing activity against measles virus.

(d) Cultivation

(i) *Tissue culture*

Although transmission of measles to human volunteers, using bacteria-free blood obtained from patients in the acute phase of the disease, was achieved as far back as 1905, and its transmission to monkeys as early as 1911, it was not until 1954 that Enders and Peebles succeeded in isolating measles virus in human kidney cells. It is now known that measles virus will grow in a number of primate cells, of which human and monkey kidney cells are the ones of choice, although human amnion is also suitable in spite of its lower sensitivity. Because of the occasional presence of adventitious viruses in monkey kidney cells, which produce cytopathogenic effects not unlike measles, human cells are preferable for virus isolation. After adaptation to primary cultures of susceptible cells, measles virus will also grow in many continuous lines of human cells.

In tissue culture, measles virus usually produces small cytoplasmic vacuoles, syncytia, and multinucleate giant cells; and, eventually, destruction of the cell sheet ensues (Fig. 62). In stained preparations, eosinophilic inclusion bodies are present in both nuclei and cytoplasm. Another type of cytopathogenicity, characterized by the formation of spindle shaped fibroblast-like cells, is commonly seen in human amnion cell cultures, particularly after passage of the virus; sometimes both types of cytopathogenicity may be seen in the same culture. The attenuated Edmonston vaccine strain produces predominantly the spindle-cell type of cytopathogenicity, but it is not known if this property is directly correlated with attenuation.

Propagation of measles virus in suspensions of human and monkey mononuclear leucocytes lends support to the view that these cells play an important part in the transport of virus to various internal organs during the incubation period of the disease.

(ii) *Chick embryos*

Although some evidence for the growth of measles in chick embryos and chick embryo tissue cultures was obtained before the first isolation in primate kidney cultures, the difficulties of demonstrating the

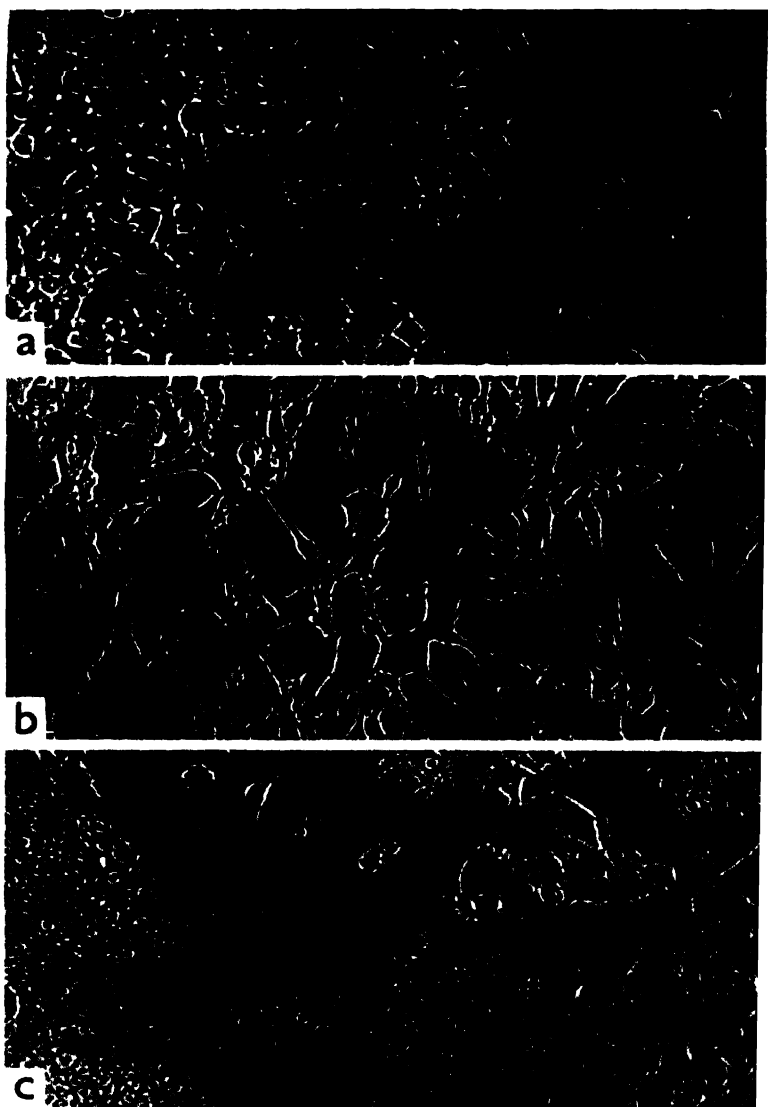


Fig. 62. Cytopathogenic effects of measles virus in HeLa cells.

(a) Normal HeLa cells.

(b) Strand forming cytopathic effect produced after low dilution passage.

(c) Giant cell cytopathic effect produced after high dilution passage.

[From F.G.Oddo, R.Flaccomio, and A.Sinatra (1961) *Virology* 13, 550-52 (Academic Press Inc., New York and London).]

progeny virus, in monkeys, made the results difficult to evaluate. Since that time, strains adapted to human amnion cells have been shown to grow in the amniotic cavity of the chick embryo, as well as in chick embryo cell cultures.

Clinical Features

Measles is characterized by a prodromal illness, lasting 2–4 days, in which high fever, malaise, conjunctivitis, rhinitis, profuse nasal discharge, and tracheobronchitis are prominent symptoms. Towards the end of the prodromal period, Koplik spots, which are pathognomonic of measles, make their appearance on the buccal mucous membrane; these are simple macular lesions with a central white spot. Three or four days after the onset of symptoms, a macular or maculopapular rash develops on the face and neck, extending to cover the whole body within 24–48 hours. With the full development of the rash, the fever and catarrhal symptoms subside and recovery begins. Two to four days after its eruption, the rash begins to fade and the skin takes on a brownish discoloration which is followed by desquamation.

Death from uncomplicated measles is rare, but complicated cases account for a death rate of 2 per 10,000 notifications in the U.K. In parts of the world where malnutrition, lack of medical care, and poor social conditions, prevail, the mortality from measles may reach 5–10%.

Complications

Complications are most frequent in adults, and in children under 1 year of age.

(a) Respiratory complications

Respiratory complications may take the form of croup (laryngo-tracheobronchitis), bronchitis, otitis media, and pneumonia; although usually due to secondary bacterial infection, these complications may be due to the measles virus itself. Thus bronchopneumonia due to uncomplicated measles infection, in which

multinucleate giant cell formation is characteristic, has been described. Usually, this type of pneumonia occurs in patients suffering from some other chronic disease, and may occur in the absence of a rash. Significantly, it has occurred, on occasion, in patients who have failed to develop antibody and in whom proliferation of virus has persisted for some time.

(b) Post-measles encephalitis

This condition, which usually follows the rash after about 4–5 days, is characterized by perivascular demyelination, round cell infiltration, congestion, and petechial haemorrhages in the brain. Evidence for viral replication in the brain is controversial, and the lesions are widely held to arise from an immunological reaction between brain tissue and an auto-antibody formed as the result of infection.

In a recent survey which included the mildest cases, post-measles encephalitis was estimated to occur in approximately 1 per 1000 cases of measles, and 11 deaths from the condition were recorded in 1964.

Pathology

Reticulo-endothelial giant cells, accompanied by mononuclear cell infiltration, have been found in the tonsils, lymph nodes, thymus, spleen, intestinal lymphoid tissue, and kidneys during the incubation period. At the same time, epithelial giant cells containing intranuclear and intracytoplasmic inclusions have been found in the trachea and bronchi. With the onset of the prodromal illness, multinucleate giant cells may be found in the nasal secretions and sputum, but these disappear soon after the appearance of the rash; significantly, giant cells are not typical of lesions found in patients who succumb during the clinical course of the disease.

Little is known about the pathology of the rash, but inclusion bodies which may be due to the presence of virus have been described in epithelial and vascular endothelial cells. Vascular congestion, oedema, epithelial necrosis, and round cell infiltration, characterize the skin lesions but giant cells are significantly absent. This supports the view of some authorities who suggest that the rash

is the result of vascular endothelial damage arising from a hypersensitivity reaction.

Pathogenesis

Observations in human cases, experimental results obtained in monkeys who are highly susceptible to infection, and the model of exanthematous diseases which Fenner's experiments on Ectromelia in mice have provided, allow us to form a provisional but not yet substantiated concept of the pathogenesis of measles. It is believed that the virus gains entry through the epithelium of the respiratory tract, and possibly through the conjunctiva. At these primary sites of infection and in the draining lymph glands, the virus proliferates for the next 24–48 hours before distribution by the blood stream, possibly in reticulo-endothelial cells, to the various internal organs. Occasionally, this stage of primary viraemia is associated with an 'illness of infection' in which pyrexia, catarrhal symptoms, and sometimes a transient morbilliform rash, appear a short time after exposure, to be followed after the appropriate incubation period by a typical attack of measles. Following the primary viraemia, and during the rest of the incubation period, the virus probably multiplies in the organs of the reticulo-endothelial system and in the mucous membranes of the respiratory tract.

About the fifth day after exposure, virus produced in the reticulo-endothelial system and in the respiratory tract overflows into the blood stream, producing a secondary viraemia which allows distribution of virus to the target organs, namely skin, mucous membranes, and probably the central nervous system. At the end of the incubation period and at the onset of the prodromal illness, i.e. the tenth or eleventh day after exposure, virus is abundant in the nasopharyngeal secretions. Four days later the rash appears, and within 24 hours of its appearance virus is no longer recoverable, or is present only in very low titres. The disappearance of infective virus is associated with the appearance of neutralizing antibody in the circulation; it is assumed that proliferation of virus during the incubation period leads to the production of antibody which, after reaching a sufficient level, is responsible for the elimination of virus and recovery from the disease.

Epidemiology

Man is the only natural source and reservoir of infection, from whom the infection is transmitted, during the prodromal phase, mainly by droplets, but sometimes by direct contact or by fomites contaminated with infected secretions. Virus may also be excreted in the urine but this is probably not an important source of transmission. Although not infective during the incubation period, which is usually 10–11 days, patients become infective from the onset of catarrhal symptoms until 2–3 days after the rash appears.

Measles is endemic in most parts of the world and epidemics occur in urban communities approximately every 2 years, usually in winter or spring. The highest incidence of the disease is in children 2–4 years of age, but in non-urban communities, where the population density is lower, the age of maximum incidence rises to 5–7 years. Over the age of 15 years, 97% of the population have experienced a clinical attack of measles.

Although the death-rate from measles has fallen considerably since the beginning of the century, no decline in the incidence of the disease has been observed. Indeed, the highest number of cases in this country since the disease became notifiable in 1940 was recorded in 1961, when there were 763,511 cases. In the same period, the case-fatality ratio declined from 0·21% in 1940 to an average of 0·02% in the years 1958–65. Of course, antibiotics have contributed to the decline in mortality from bacterial complications, but better standards of nutrition, higher standards of medical care, and improvement of social and economic conditions, have also been effective. In some underdeveloped parts of the world where these standards have not yet been attained, the mortality may reach 5–10%. A high mortality in both young and old age groups is also characteristic of isolated communities which have not previously experienced outbreaks of measles. Thus, in the Greenland outbreak of 1951 there was a 100% incidence of infection and a mortality of 18 per 1000.

Immunity

During the first few months of life, infants are protected by passive immunity, derived from the passage of maternal antibody through

the placenta before birth. With the decline of this passive immunity towards the end of the first year, infants become susceptible to the disease. Following an attack of measles, antibody is acquired and usually appears within 24–96 hours of the onset of the rash, reaching its maximum titre in 1–8 weeks; thereafter, the titre declines slowly but antibody remains detectable for many years, and in most cases throughout life. After an attack, immunity is life-long and second attacks are extremely rare; indeed, some second attacks described in the past may have been exanthemata due to rubella or echovirus infections.

Laboratory Diagnosis

Laboratory diagnosis, although rarely required for the clinically obvious case, may be necessary for the diagnosis of atypical cases, particularly those which occur without a rash; laboratory investigation may also be required for the diagnosis of post-measles encephalitis, and for confirming a diagnosis from post-mortem material.

(a) Virus isolation

During the prodromal period, virus isolation may be attempted from the blood, throat, conjunctival secretions, or urine. Throat washings or swabs are collected in culture media adjusted to pH 7·2, to which antibiotics have been added. Urinary specimens, likewise, are adjusted to pH 7·2 after addition of antibiotics, centrifuged at 4°C, and the resuspended sediment used for inoculation. Successful isolation from blood specimens is most likely if leucocytes, prepared after erythrocyte sedimentation and centrifugation of the plasma supernatants, are used as the inoculum.

Primary cultures of human or monkey kidney cells, or human amnion cells, are used for isolation, and cytopathogenicity together with the formation of multinucleate giant cells usually occurs within 5–10 days, but may be delayed for as long as 21 days. Identification of the isolate is confirmed by neutralization tests with known measles antiserum, and immunofluorescent techniques may also be used.

(b) Serological tests

A rise of four-fold, or more, in the titre of measles antibody, occurring between the acute and convalescent phases of the disease, is

diagnostic. Neutralization, haemagglutination-inhibition and complement-fixation tests may be used, of which the neutralization test is the most sensitive and the complement-fixation test the least, although quite suitable for routine purposes.

(c) Cytology

In a small proportion of cases, multinucleate giant cells are demonstrable in nasal secretions collected from the upper turbinates, but the test is not of much diagnostic importance.

Prophylaxis

(a) General measures

The patient is isolated from the onset of the disease until 1 week after the rash has appeared, and his crockery and other articles contaminated by infected secretions should be sterilized. Quarantine of contacts is not usually called for.

(b) Vaccination

The cultivation of measles virus in tissue culture has made possible the production of live attenuated and formalin-killed measles vaccines.

(1) *Live attenuated vaccine*

Several attenuated measles virus strains, whose capacity to produce disease has been reduced, have been derived from the Edmonston strain isolated and developed by Enders and his colleagues. This strain was originally attenuated after 24 passages in human kidney cells, 28 passages in human amnion and 6 passages in the chick embryo; subsequently, it has received several passages in chick embryo cell cultures in which it has been maintained. After attenuation, the Edmonston strain revealed a marked reduction of its virulence for monkeys although its antigenic potency remained unaffected. The subcutaneous or intramuscular administration of this strain to children has proved highly effective in producing an antibody response and protection against measles, but clinical reactions, although not unusually severe, have been too frequent for

its general acceptance as a vaccine strain. In various vaccine trials about 80% of the vaccinees responded with pyrexia and about 50% with a mild erythematous rash.

In order to lessen the reactions after vaccination, injections of human γ -globulin have been given with, or a few days after, vaccination. Simultaneous administration of γ -globulin, in a separate syringe and at a different site, does not unduly affect the serological response or the immunity produced, but the necessity of an accurate balance between the doses of vaccine and γ -globulin, as well as the greater expense and complexity of administration, does not commend the technique for large scale routine immunization. Administration of live vaccine one month after an injection of killed vaccine has also been tried, with some success, but the same criticisms apply.

To achieve successful immunization with one injection of live vaccine, derivatives of the Edmonston strain have been further attenuated, by both Schwartz and Goffe, through prolonged passage in chick embryo tissue cultures. Vaccines made of these strains give rise to fewer clinical reactions than the original Edmonston strain although they are just as potent antigenically. But even with the most attenuated Schwartz strain some 8% of the vaccinees, in a recent Medical Research Council Trial, developed a mild rash, and 36% a pyrexia of more than 101°F. In some trials the incidence of reactions has been even higher.

(ii) Inactivated vaccine

Virus for the formalin-killed vaccine is prepared in monkey kidney cells from the original Edmonston strain, and the final product contains an alum adjuvant. Although administration of killed vaccines is safe and free from clinical reactions, at least three injections, spaced at 1 month intervals, are necessary to produce a satisfactory antibody response. Even after this regimen, the level of antibody and degree of protection soon decline. The multiple injections, short-lived immunity, and the large amount of virus required to ensure antigenic potency, do not herald a bright future for this vaccine, but its use in conjunction with live vaccine may be of some benefit.

(iii) The problem of measles vaccination

The acceptance of an effective vaccine against measles requires that it should be harmless, easy to administer, and effect long lasting immunity in the individual and the herd; at the same time it should fulfil a definite need and be economical to apply. The live attenuated vaccine available is economical, easy to administer, and apparently harmless, although it gives rise to mild clinical reactions in a small proportion of vaccinees. These reactions are nevertheless sufficient to make the vaccine less than generally acceptable to parents, if not to the medical profession. It does however produce effective protection against measles and, although the few years since trials commenced are insufficient to judge how long the immunity will last, the indications are that it may be lasting.

Because measles in this country is generally a mild disease, only a vaccine which satisfies the highest criteria will be acceptable for mass vaccination. It must give rise to few reactions and produce immunity equivalent to that produced by natural infection. Artificial immunity falling short of this requirement may simply postpone infection from childhood to young adult life, when the results are much more severe. If further and more extensive trials show the present live measles vaccine to be effective in producing long lasting immunity without untoward side effects, its use on a mass scale to prevent the considerable morbidity of the disease and to reduce the number of deaths, which in 1964 amounted to 73, would be acceptable. In the meantime, vaccination of children suffering from chronic or debilitating diseases which may be exacerbated by measles should be seriously considered. Vaccination is however contraindicated in leukaemia, in patients undergoing steroid therapy, and in those allergic to egg protein.

In geographical areas where measles is a severe disease with a relatively high mortality, the need for a measles vaccine is much more pressing. The introduction of measles vaccine in India, Africa, and South America is an important development for these areas where its use is both necessary and desirable in spite of its shortcomings.

(c) Passive immunization

Children under 2 years of age, in whom the risk is greatest, and those suffering from chronic or debilitating diseases which may be exacerbated by measles, may be protected by administration of human γ -globulin after exposure. If administered within 3 days of exposure complete protection may be expected, but administration on the fourth to sixth day after exposure leads only to modification of the attack; administration after the sixth day is usually ineffective. Serological studies suggest that subclinical infections leading to prolonged immunity are frequent after administration of γ -globulin, in spite of the absence of overt disease.

Treatment

In the absence of any antiviral drug active against measles virus, treatment remains symptomatic. Antibiotics are employed for the treatment of bacterial complications but are not recommended for routine prophylaxis.

CHAPTER 21

Acute Infectious Fevers due to Paramyxoviridae

III. Rubella Virus German Measles

Rubella, or German measles, is one of the acute epidemic exanthematous diseases of childhood and young adult life, characterized by mild pyrexia, lymphadenopathy, and a rash. In spite of its usual clinical mildness, the disease has assumed a position of major importance following Gregg's original observation, in the Australian epidemic of 1940, that infection in early pregnancy is associated with the development of congenital defects in the foetus.

At the time of writing, knowledge of the rubella virus is still too scanty to allow its complete characterization and classification, but its morphological and biochemical similarities to those viruses belonging to the Family Paramyxoviridae make its provisional inclusion in this group convenient until its classification has received international approval. No official generic name has yet been proposed.

The Virus

(a) Morphology

Rubella virus particles are extremely pleomorphic, and their diameter has been estimated by filtration and electron microscopic techniques to range from 100 to 300 m μ . Recently Phillips and his colleagues of Baylor University, Houston, Texas have described the morphology of negatively stained particles; these consist of an internal helical component surrounded by an envelope from which

protrude rod-like projections, about 10–12 m μ in length, suggesting a morphological resemblance to the paramyxoviruses.

(b) Chemical and physical properties

The replication of rubella virus in the presence of iododeoxyuridine indicates that it is an RNA virus. This, and its inactivation by ether and sodium deoxycholate, substantiate its relationship to other viruses belonging to the Family Paramyxoviridae. Rubella is also sensitive to chloroform and formalin; it is thermolabile, destroyed by heating at 60°C, but stable at –60°C for more than 1 year.

(c) Biological properties

Although originally thought to be non-haemagglutinating, this year Stewart and his colleagues described rubella haemagglutination of erythrocytes from day-old chicks at 4°C. Rubella haemagglutination tests and haemagglutination-inhibition tests are now therefore available.

(d) Antigenic composition

Preliminary studies indicate that rubella viruses isolated from different epidemics are antigenically indistinguishable, and no evidence of antigenic variation has been forthcoming.

(e) Cultivation

Techniques for the isolation and propagation of rubella virus in tissue culture were not developed until 1962. In that year Parkman, Buescher, and Artenstein described cultivation of rubella in primary cultures of African Green (*Cercopithecus*) monkey kidney cells, and Weller and Neva succeeded in growing rubella in primary cultures of human amnion cells. No cytopathogenic effects are produced in African Green monkey kidney cells, but the growth of virus is indirectly demonstrable by the resistance of infected cultures to challenge with a cytopathogenic virus, such as echovirus 11. This interference effect produced by rubella virus develops about 5–10 days after infection, but may take longer if the rubella inoculum is small.

Although cytopathogenic effects are produced in human amnion

cells, they are delayed for 20–60 days and are not prominent. Characteristically, only individual cells are affected and they take on a rounded, refractile, and amoeboid appearance; eventually the affected cells undergo dissolution and disappear. The cytopathic effect may be enhanced by repeated passage, but the yields of virus remain low. In stained preparations, eosinophilic intracytoplasmic inclusions and irregularities of the nuclear chromatin may be observed.

More recently McCarthy and his colleagues have described a cytopathogenic effect in a transformed rabbit kidney cell line (RK-13) after rubella infection. In these cultures, for which strict pH control is required for healthy maintenance, infected cells become pleomorphic before they are destroyed and replaced by surviving cells, which become enlarged and elongated. Less easily recognized cytopathogenic effects are produced by rubella in primary rabbit kidney cell cultures.

Cytopathogenic effects are produced by rubella in some other cell lines, and that produced in a continuous line of rabbit corneal cells, described by Leerhy and confirmed by Phillips, Melnick, and Burkhardt, has many advantages. Cytopathogenicity is produced in 5–7 days, and the cells are no less sensitive to rubella than African Green monkey kidney cells, which until now have proved the most satisfactory for isolation from clinical specimens.

Clinical Features

(a) German measles

A maculopapular rash, which appears after an incubation period of 18 days, and sometimes 14–21 days, is usually the first sign of illness. Appearing first on the face and neck, it spreads rapidly to cover the trunk and limbs, subsiding after 2–3 days. Mild general symptoms may precede or accompany the rash; these include mild pyrexia, headache, malaise, sore throat, and suffusion of the eyes. Lymphadenopathy, affecting particularly the post-auricular, occipital, and cervical lymph glands, is typical and may precede the rash by a day or two, or sometimes longer. Lymphadenopathy without a typical rash is a well recognized manifestation of the disease. Leucopenia

and the presence of Turk cells in the blood are characteristic but not pathognomonic of rubella.

Very rarely, an attack of rubella may be complicated by the occurrence of transient arthralgia, thrombocytopaenic purpura, or encephalitis, from which recovery is the rule. Encephalitis, which is a much less common complication of rubella than of measles, is not of the demyelinating variety.

From virological studies, it is now well known that subclinical attacks of rubella occur during the course of an outbreak. The ratio of subclinical to overt cases has been reported to be as high as 6·5 : 1 in adult military populations, although a ratio nearer 1 : 1 has been observed in civilian populations of children and young adults.

The recognition, in recent years, that rubelliform rashes may be produced by infection with echo, coxsackie, and other viruses make it certain that some previous outbreaks of rubella have been mistakenly diagnosed. This is a factor of some statistical importance in assessing the relationship between rubella and foetal defects.

(b) Congenital rubella and the rubella syndrome

The most important complication of rubella is the teratogenic effect of intrauterine infection acquired from an infected mother. It is now well established that 15–20% of infants born of mothers infected in the first or early in the second trimester develop some major congenital malformation affecting usually the eyes, heart, ears, or brain. Recently, other manifestations of intrauterine infection have been recognized, each of which may occur alone or in all possible combinations to form the rubella syndrome. These include thrombocytopaenic purpura, hepatosplenomegaly, encephalitis, jaundice, interstitial pneumonitis, myocarditis, glaucoma, X-ray abnormalities of the long bones, low birth weight, and retardation of growth in the post-natal period. Months or years may pass before visual and auditory defects or mental retardation become evident, long-term follow-up of infants at risk from maternal rubella is therefore very important. No less important than the effects of congenital rubella on the infant is the increased risk of abortion, stillbirth, and premature delivery after maternal rubella.

When maternal infection is acquired during the first month of

pregnancy, the overall risk to the foetus approaches 50%, but this drops by half if infection is acquired in the second month, and to about 7% if infection is acquired in the third month.

Pathogenesis

The susceptibility of rhesus monkeys to experimental rubella infection has allowed Parkman, Phillips, and Meyer to follow the course of infection in these animals, which serve as suitable models for the investigation of the disease. Although no overt illness is produced in monkeys, virus is recoverable from the blood for 11 days after infection and disappears with the appearance of antibody. Virus may be recovered from the nasopharynx and from rectal swabs between the fourth and seventeenth days after infection, and evidence of infection in the placenta and amnion of pregnant monkeys, infected early in the gestation period, has been demonstrated but no congenital malformations in the foetuses have been observed.

The sequence of events in humans after natural and experimental infection is similar. Virus may be recovered from the pharynx as early as the fourth day after infection and reaches its highest concentration there during the period of the rash, sometimes persisting for 8 days or longer after the rash has disappeared. In the blood, and sometimes in the urine, virus is present about 1 week before the onset of the rash and disappears with its eruption, when circulating antibody also becomes manifest. Virus is also demonstrable in the stools for several days before and after the rash.

It is now generally accepted that rubella virus gains entry via the upper respiratory tract. The virological studies indicate that the virus multiplies at the site of infection, and possibly in other organs, during the incubation period. From these sites, virus is released into the circulation where it is found for about 1 week before the onset of the rash. The disappearance of virus from the blood stream when the rash appears, and the simultaneous appearance of circulating antibody, suggests a relationship between these events; the possibility that an antigen-antibody reaction of the delayed hypersensitivity type may be responsible for the rash, as von Pirquet first postulated to explain the rash of measles, suggests itself.

With the elucidation of the pathogenesis of rubella infection, the pathogenesis of congenital rubella has become clearer. Selzer, in 1963, was the first to recover rubella virus from a foetus delivered of a mother infected in the first trimester of pregnancy. Since then, two patterns of foetal infection have been described by Alford and his colleagues. In one, occurring in about 70% of infected foetuses, infection is limited to the placenta; in the other, occurring in the remaining 30%, infection is disseminated in all the foetal organs and persists as a chronic intrauterine and post-natal infection. Thus infants who reveal evidence of congenital rubella infection at birth may yield virus in the throat, urine, faeces, conjunctiva, or cerebrospinal fluid, for several months after birth, and in a few cases for as long as 1 year. Prolonged excretion of virus is usually associated with those who are most seriously affected, among whom there is a high perinatal mortality. Surprisingly, in spite of intrauterine infection and prolonged excretion of virus in the post-natal period, no state of immunological tolerance is produced. Indeed, these infants develop high titres of antibody which persist for some years, and so far no satisfactory explanation has been offered for this paradoxical situation.

Recently, mothers exposed to rubella in early pregnancy but who do not develop clinical rubella have been observed to give birth to infants who appear normal at birth but who shed virus and develop high titres of antibody. These infants are a particular hazard as sources of infection capable of transmitting rubella.

Boué, Plotkin, and Boué have shown that rubella infection of certain types of human embryonic cells, in culture, inhibits metabolic activity and cell division, and leads to chromosomal abnormalities. This kind of effect occurring in a foetus infected in the early weeks of pregnancy, when the organs are being formed, would explain the high risk of congenital malformations experienced at this period. Infection of the foetus after the organs have been formed is believed by Naeye and Blanc to retard intrauterine growth. They showed that infants with congenital rubella were underdeveloped at birth and that deficient numbers of morphologically normal cells were present in their organs. Moreover, they observed retardation of growth in the post-natal period which may have been due to continued viral proliferation.

Epidemiology

Rubella is endemic in all parts of the world and large epidemics occur at intervals of approximately 5–10 years. Outbreaks tend to occur in winter and spring, and are particularly marked in closed communities such as boarding schools and recruit camps. Older children and young adults are affected rather more frequently than in the other exanthemata of childhood.

Man is the only naturally susceptible host, and sources of infection are provided by clinical and subclinical cases of the disease, including infants with congenital rubella. It is generally assumed that rubella infection is spread by droplets, and the ease with which virus may be recovered from the nasopharynx supports this assumption. However, the now recognized excretion of virus in the urine and stools makes transmission by the intestinal–oral route or by indirect contact also possible. Contrary to the previously held assumption that rubella is infectious only during the period of the rash, virological studies have revealed that virus is shed in the nasopharynx for at least one week before and after the rash. Indeed, virus has been recovered from an infected volunteer as long as 21 days after the rash.

Immunity

For the first few months of life, infants are protected from infection by passive immunity, transmitted by passage of maternal antibody through the placenta before birth. Thereafter, persistent immunity is acquired after an attack of rubella. Serological evidence of past infection is present in about 20% of pre-school age children, about 80% of the 17–20 year age group, and in 80–90% of women of child-bearing age. It is thus only in 10–20% of women of child-bearing age that the dangers of congenital infection are to be feared.

Infants with congenital rubella infection not only shed virus at birth and for some months thereafter but develop high titres of neutralizing antibody which persist for some years. Alford's investigations show the 7S type of antibody, probably of maternal origin, to be dominant soon after birth, the 19S type becoming significantly apparent during the first 7 months of life. This suggests

production of antibody by the foetus after birth, in response to persistent infection. Sometimes, retrospective proof of intrauterine infection is provided by the high titres of neutralizing antibody which have been found in clinically normal infants born of mothers infected with rubella in early pregnancy.

Laboratory Diagnosis

As a general rule, there is no difficulty in making the clinical diagnosis of the disease in large outbreaks, but laboratory diagnosis of subclinical or atypical cases may be of considerable importance in assessing any risk to contacts who are in the early stages of pregnancy. Similarly, laboratory evidence that the causative agent of a rubelliform rash is a virus other than rubella may radically alter the prognosis given to those infected in early pregnancy. Laboratory tests are also sometimes necessary for the diagnosis of congenital rubella in infants who appear clinically normal, and the results are important both for prognosis and for the identification of sources of infection which would otherwise be unrecognized.

(a) Virus isolation

Most of the techniques of virus isolation now available are either slow or technically difficult to perform, but the recent introduction of a continuous line of rabbit corneal cells for rubella isolation indicates that the development of rapid routine methods may be possible. Meanwhile, isolation in primary human amnion cells or in transformed RK-13 rabbit kidney cells has the advantage of easily observed cytopathogenic effects. Primary African Green monkey kidney cell cultures although more sensitive for rubella isolation have the disadvantage that indirect interference methods are necessary for the recognition of infection.

For best results, specimens should be taken at or near the onset of the rash, although virus may be isolated for at least 1 week afterwards. Nose and throat swabs are required because one may be positive and the other negative, and specimens which are not immediately inoculated should be refrigerated at 4°C for short periods or -70°C for longer periods.

For the laboratory diagnosis of congenital rubella in infants, virus may be isolated from the throat, urine, stools, cerebrospinal fluid, and from the conjunctiva when eye lesions are present.

Identification of new isolates is confirmed by neutralization tests using rubella antisera prepared in rabbits.

(b) Serological tests

Confirmation of the diagnosis or, in the absence of virus isolation, retrospective diagnosis may be made by demonstrating a rise of four-fold, or more, in the titre of rubella neutralizing antibody, occurring between the acute and convalescent phases of the disease. In congenital rubella, high titres of neutralizing antibody may be present at birth and persist for more than 6 months.

The recent introduction of an ether extracted complement-fixation antigen, by Schmidt and Lennette, has enhanced the usefulness of the complement-fixation test, which is very adequate for routine diagnostic purposes. But the more rapid decline, over a period of years, in the level of complement-fixing antibody compared with that of neutralizing antibody makes the complement-fixation test unsuitable for the detection of remote past infections.

A haemagglutination-inhibition test has now been introduced by Stewart and his colleagues based on rubella haemagglutination of 1-day-old chick cells at 4°C. The titres of HI antibodies are higher and more persistent than those of CF antibody, but sera must be absorbed with kaolin and 1-day-old chick red cells, before use, to remove non-specific inhibitors and natural agglutinins.

Prophylaxis

(a) General measures

The prolonged excretion of virus for at least 1 week before and after the rash, and sometimes longer, together with the now evident frequency of subclinical infections makes the control of rubella by isolation and quarantine procedures quite ineffective. Indeed, deliberate exposure to, rather than isolation from, infection is advised for healthy female children and young adults. However,

pregnant women should be kept away from known sources of infection until all danger of transmission is passed.

(b) Vaccination

(1) Inactivated vaccine

Formalin-killed vaccines although possessing the advantage of safety, particularly in women of the child-bearing age, do not usually produce long-lasting immunity. Attention has therefore been concentrated on the development of a live attenuated rubella vaccine.

(2) Live vaccine

A suitable live attenuated rubella vaccine must be one which provides long-lasting immunity and which is not transmissible to susceptible contacts, i.e. one which provides no risk to pregnant women. Modification of the disease syndrome is less important, since rubella is itself a mild disease. The development of a live attenuated rubella vaccine with these properties has now been reported by Parkman and Meyer of the National Institutes of Health in the U.S.A. Trials with this vaccine, although so far only of a preliminary nature, reveal it to be both satisfactory and safe. Although vaccine virus was recovered from the throats of some vaccinated children, there was no evidence of spread to intimate contacts. Further development will, doubtless, make a satisfactory safe live attenuated vaccine generally available in the not too distant future.

(c) Passive immunization

Until such time as rubella is controlled and finally eliminated from the population by the use of vaccines, only pregnant women need be protected from the disease. Those previously infected and who possess neutralizing antibody are in no danger of infection and do not transmit virus to the foetus, attempts have therefore been made to protect those mothers who are susceptible and have been exposed to infection, by administration of pooled human γ -globulin. Although no protection against artificial infection has been demonstrated, some protection against naturally acquired clinical rubella has been observed when γ -globulin is given before or soon after

exposure, and some reduction in the degree and duration of viraemia has been demonstrated in γ -globulin treated patients. The efficiency of γ -globulin in preventing congenital rubella defects has not however been proved, and further data are required. Nevertheless, in the absence of therapeutic abortion, γ -globulin should be administered as soon as possible after exposure as the only possible, albeit uncertain, means of protection.

Treatment

There is no specific antiviral treatment of rubella.

CHAPTER 22

Enterovirus Infections

I. Polioviruses—Poliomyelitis

Poliomyelitis, sometimes known as infantile paralysis, is one of the most dreaded of all infectious diseases because of its well-known paralytic manifestations. In fact, it occurs most commonly in an inapparent or non-paralytic form. Although the disease has probably existed since ancient times, it was not until the latter half of the nineteenth century that it was recognized, by Heine and Medin, as a clinical entity. The increasing prevalence of poliomyelitis in Europe and North America, since that time, stimulated clinical, epidemiological, and pathological studies of the disease, which culminated in the establishment of its viral aetiology by Landsteiner and Popper in 1908-9, and by Flexner and Lewis in the following year. This advance marked the beginning of a long series of difficult laboratory investigations which have resulted, in the last decade, in the effective control of the disease by prophylactic vaccination.

The Virus

(a) Morphology

Poliovirus is a spherical particle approximately 30 m μ in diameter. X-ray diffraction and electron microscopic studies suggest that the capsid is made up of a number of capsomeres arranged in icosahedral symmetry. The actual number of capsomeres is not yet agreed but estimates of 32, 42, and 60 have been made.

Because of their particle symmetry, poliovirus preparations are readily crystallized; indeed, poliovirus was the first animal virus to be crystallized, by Schaffer and Schwerdt, in 1955. Crystalline arrays of poliovirus are sometimes seen in the cytoplasm of infected cells (Fig. 27).

(b) Chemical and physical properties

Essentially, poliovirus is a ribonucleoprotein in which RNA accounts for 25-30% of the virus particle. It is resistant to ether and other detergents and is acid stable at pH 3.0, but infective virus is readily inactivated by formalin and chlorine in the absence of organic material. Likewise the virus is inactivated by heat and ultra-violet light although its antigenic potency is preserved.

(c) Antigenic composition

Three distinct antigenic types of poliovirus have been defined by neutralization and complement-fixation tests. Each type is stable and shows no tendency for antigenic variation, although very minor antigenic differences among strains within a single type have been demonstrated by refined neutralization techniques.

Two distinct complement-fixing antigens can be separated by density gradient centrifugation. The D antigen, which is type specific, stimulates the production of neutralizing antibodies and is mainly associated with infective virus particles. The C antigen, which is less specific and reacts with heterotypic antisera in *in vitro* tests, is mainly associated with less dense non-infective particles which are deficient in RNA. The D type-specific antigen is converted into the less specific C antigen by heat at 56°C or by ultra-violet irradiation (Fig. 20).

(d) Cultivation**(i) Experimental animals**

Originally, intracerebral inoculation into rhesus monkeys was the sole method of poliovirus isolation. Later, the susceptibility of cynomolgous monkeys and chimpanzees to infection by the alimentary route led to the modern conception of poliomyelitis as primarily an alimentary infection. Armstrong's adaptation of type II poliovirus to cotton rats and mice, in 1939, greatly simplified the work on poliomyelitis by making available small, inexpensive, and easily handled laboratory animals. Since the advent of tissue culture techniques, the use of laboratory animals for cultivation of poliovirus has become mainly a matter of historical interest, although monkeys are used for testing the pathogenicity of vaccine.

(ii) Tissue culture

The discovery that poliovirus grows in human and monkey epithelial cells, with the production of cytopathogenic effects, was made by Enders, Weller, and Robbins in 1949. Since that time, the study of poliovirus, and indeed virology as a whole, has been revolutionized, and the production of polio and other virus vaccines has been made possible.

Primary cultures of monkey kidney cells are the most sensitive to poliovirus and are universally used, but various primary and continuous cultures of human cells are also susceptible, especially HeLa and human amnion cells. In general, cells from non-primate species are insusceptible to infection with poliovirus; their insusceptibility appears to be due to the absence on their surface of lipoprotein receptor sites specific for poliovirus. Significantly, the development of susceptibility to poliovirus infection by cells in tissue culture, which in the organized tissues of the intact animal are resistant, is associated with the production of receptor material and the capacity to adsorb virus.

Tissue culture cells infected with poliovirus produce characteristic cytopathogenic effects. The cells round up, become refractile, and exhibit marked nuclear pyknosis, before degenerating and falling away from the glass surface (Fig. 12b). Usually, the whole of the cell sheet is destroyed within a week.

In stained preparations, eosinophilic intranuclear inclusions may be seen before the nucleus shrinks, undergoes pyknosis, fragments, and becomes displaced by a central eosinophilic mass which develops in the cytoplasm. Ultimately, the cytoplasm disintegrates and the newly formed virus is released.

Clinical Features

(a) Inapparent infection

About 90–95% of all infections with poliovirus are inapparent and produce no signs or symptoms. In these, infection is limited to the alimentary tract and can only be identified by virus isolation from the stools or by serological methods.

(b) The minor illness

The minor illness is a mild febrile condition which lasts 24–48 hours and precedes the major illness by about 2–5 days (Fig. 63). It is accompanied by symptoms of headache, anorexia, vomiting, naso-

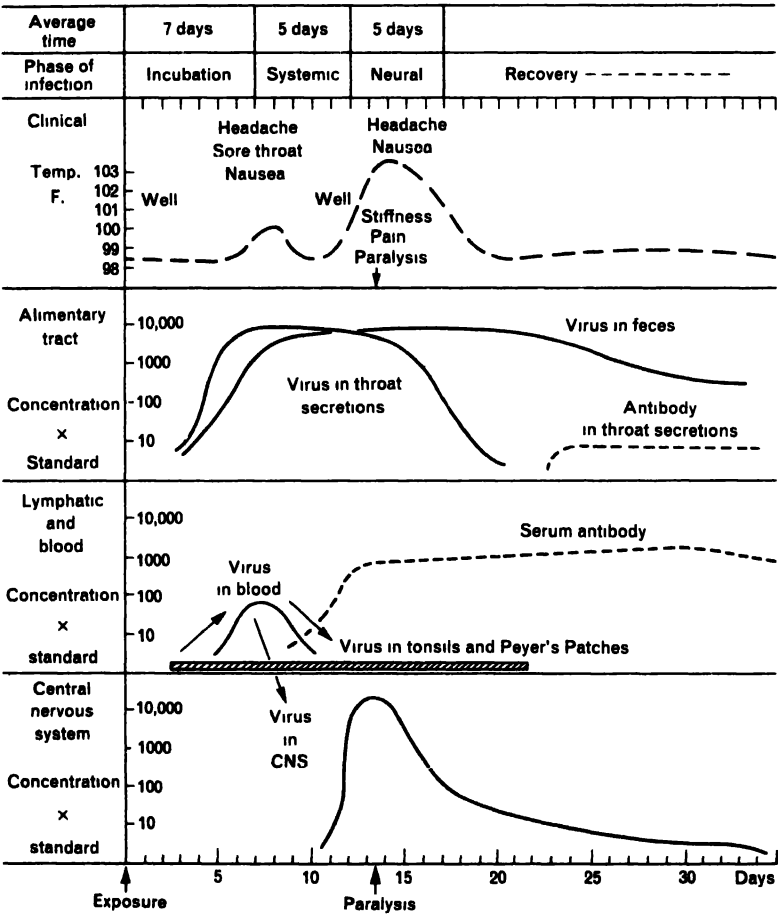


Fig. 63. Course of poliomyelitis infection showing time relations of clinical and pathogenic events [from D. Bodian (1957) Mechanisms of infection with polio-viruses; in *Special Publications of the New York Academy of Sciences*, vol. 5, pp. 57–72 (by permission of the author and The New York Academy of Sciences)].

pharyngeal congestion, sore throat, and occasionally by some gastrointestinal disturbance, but not by any localizing signs of infection in the central nervous system. Except in epidemics, the minor illness is often unrecognized clinically or is diagnosed as a 'cold' or 'flu', and its significance is usually grasped only in the light of the later developing major illness. Contrary to a widely held belief, the minor illness is not a regular feature of poliomyelitis and occurs only in about one-third of children, and fewer adults, in whom the diagnosis of poliomyelitis is made.

The minor illness is now seen as the clinical manifestation of viraemia, which may resolve and lead to uninterrupted recovery. When this occurs, the clinical syndrome is labelled abortive poliomyelitis, and such cases account for about 4–8% of all poliovirus infections. Less fortunate cases, after an interval of a few days in which a sense of particular well-being may be experienced, pass on to the major illness, the onset of which is heralded by a recurrence of pyrexia.

(c) The major illness

(i) Preparalytic stage

The major illness, which reflects involvement of the central nervous system, represents the first clinical signs of infection in the majority of cases (Fig. 63). It is characterized by pyrexia, headache, nausea and vomiting, and by signs of mild meningeal irritation which produce pain and stiffness of the neck and back. Pain and tenderness of the limbs due to generalized hypersensitivity may be present at this stage, and cerebrospinal fluid analysis reveals an increased protein and cell content.

In some patients the disease is halted at this stage and recovery ensues uneventfully without the development of paralysis. When this happens, the clinical syndrome is labelled non-paralytic poliomyelitis or aseptic meningitis due to poliovirus.

(ii) Paralytic stage

One to five days after the onset of the major illness, and sometimes longer, patients passing into the paralytic stage develop flaccid

paralysis of particular muscle groups. Paralysis develops over the next 3 days, and its extent depends on the site and extent of the lesions in the central nervous system. After a short stationary period recovery begins, and the function of paralysed muscles may improve over the next 4–6 weeks; any improvement which takes place is usually complete after 6 months. Rarely, a second paralytic phase follows 3–4 days after the first.

It is estimated that only 1–2% of all individuals infected with poliovirus develop the major illness.

(d) Bulbar poliomyelitis

Paralysis is usually limited to those muscles supplied by the spinal nerves, but in about 10% of paralytic cases paralysis of muscles innervated by the cranial nerves predominates. Sometimes a combination of these two forms, bulbospinal poliomyelitis, occurs. Bulbar poliomyelitis is especially serious because involvement of the cranial nerve nuclei leads to paralysis of the pharyngeal, laryngeal, and palatal muscles, and inability to swallow and respiratory obstruction quickly follow in the absence of appropriate treatment. Involvement of the respiratory and vasomotor centres in the medulla may further complicate the condition and lead to respiratory failure and circulatory collapse; the very high mortality of the bulbar forms of poliomyelitis is therefore easily understood.

Respiratory failure may also occur in the spinal form of poliomyelitis when the cervical and dorsal segments of the cord, innervating the intercostal muscles and the diaphragm, are affected

Pathogenesis

A significant contribution to our knowledge of poliomyelitis has been made by experiments on chimpanzees, in whom the pattern of infection with poliomyelitis resembles that in humans to a remarkable degree. The results of these experiments have now been confirmed in studies on humans fed with attenuated vaccine strains and by virological investigation of patients with poliomyelitis. From these studies, Bodian and Sabin, both of whom are pioneers in this field of research, have deduced possible sequences of infection

which, although they differ in detail, provide current concepts of poliomyelitis infection (Figs 64 and 65).

(a) Infection of the alimentary tract

Virus gains entry into the pharynx or alimentary tract after ingestion or inhalation and lodges, initially, in the tonsils and surrounding lymphoid tissue of the oropharynx, or in the Peyer's patches of the small intestine. Later, virus is found in the draining lymph glands

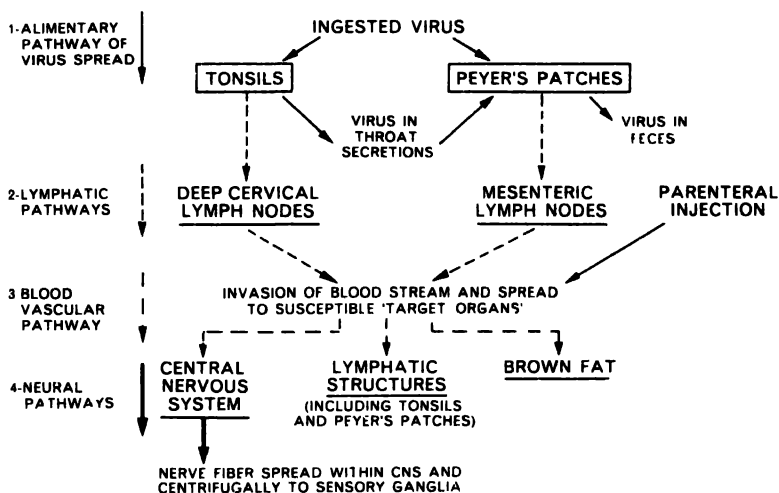


Fig. 64. Pathogenesis of poliomyelitis proposed by Bodian [from D. Bodian (1956) *Amer. J. Hyg.* 64, 181-97].

of the cervical and mesenteric regions. According to Bodian, the virus multiplies in the lymphoid tissue, but Sabin concludes that virus reaches the draining lymph glands after initial multiplication in the superficial cells of the alimentary mucosa.

One to three days after ingestion of virulent strains by chimpanzees, or avirulent strains by humans, virus is recovered in the throat secretions and faeces. Similarly in patients with poliomyelitis, virus is recovered from the throat secretions and faeces for a period of about 1 week before the onset of the major illness. Afterwards, virus continues to be present in the throat secretions for

about 1 week but may be recovered from the faeces for several weeks after the onset of illness, and sometimes for months.

In those who experience natural inapparent infection or are fed with attenuated vaccine strains, infection remains localized at the

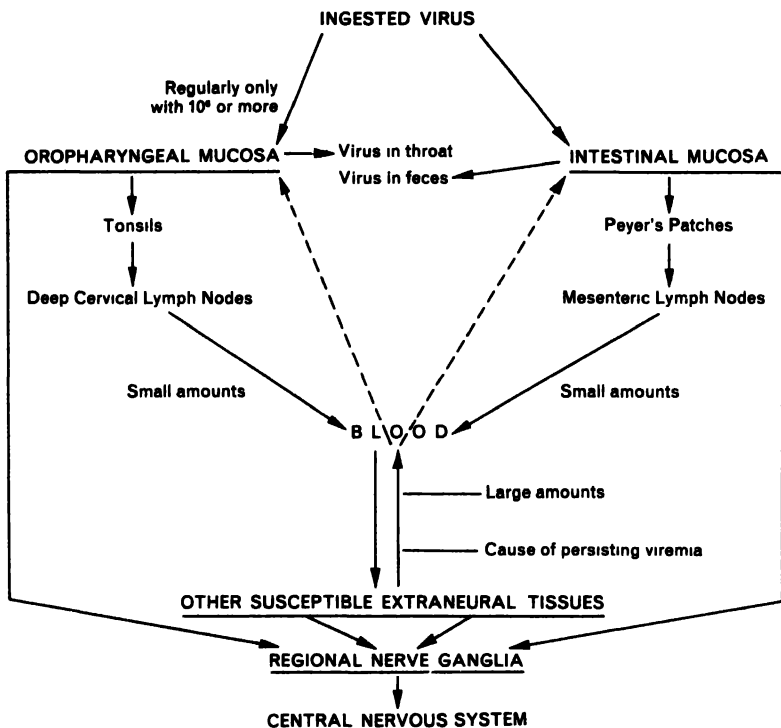


Fig. 65. Pathogenesis of poliomyelitis proposed by Sabin [from A.B. Sabin (1956) *Science* 123, No. 3209, 1151-57 (American Association for the Advancement of Science)].

primary sites of multiplication and does not spread beyond the draining lymph glands. Fortunately, this is sufficient to stimulate an effective antibody response which provides long term immunity to the homologous virus type.

(b) The stage of viraemia

After primary virus multiplication, virus spreads to the lymphatics and the blood stream. It is now generally accepted that the presence of virus in the blood stream is responsible for the minor illness. In chimpanzees, the virus spreads from the blood stream and lymphatics to the interscapular brown fat and regional lymph nodes, where further multiplication takes place and from where more virus may be shed into the blood stream, maintaining the viraemia.

If infection is arrested at the viraemic stage, the clinical course is one of abortive poliomyelitis or inapparent infection.

(c) Spread to the central nervous system

Bodian's view that the central nervous system is infected by way of the blood stream is widely accepted, but evidence that poliovirus may spread along peripheral nerve fibres, in experimental animals, has led to some controversy. Faber suggests that virus spreads along the nerve fibres and multiplies in the peripheral ganglia, from where some virus may spill over into the blood stream as the rest continues on its course along the nerve fibres to reach the central nervous system. Sabin also favours the view of nerve fibre spread to the central nervous system, and suggests that only virulent strains, which have the capacity to multiply in lymphoid tissue and various viscera, give rise to viraemia; avirulent strains, which do not multiply in extraneural tissues, other than the epithelium of the alimentary tract, fail to spread along the nerve fibres or give rise to viraemia.

Probably, both viraemic and neural routes of spread to the central nervous system are employed. Indeed, if viraemic spread was not of prime importance it is unlikely that circulating antibody, produced in response to vaccination with Salk's inactivated vaccine, would be so effective in preventing the paralytic form of the disease, in spite of its failure to prevent infection of the alimentary tract.

(d) Infection of the central nervous system

Failure to check poliovirus infection at the primary site of multiplication or at the stage of viraemia leads to invasion of the main target organ, the central nervous system. In experimental animals, there is

a sharp rise in concentration of virus in the central nervous system a day before the onset of the major illness, and high concentrations of virus are found at this site in both animals and human patients for a few days after its onset. It is at this stage of virus multiplication in the central nervous system that the outcome of the infection is decided, depending on whether the damage sustained by the motor neurones is temporary or permanent. Within 7 days, the concentration of virus falls to minimal or non-detectable levels, although occasionally virus may persist for longer periods.

(e) The outcome of central nervous system infection

The damage sustained by motor neurones depends on the amount of virus reaching the central nervous system, the virulence of the infecting virus, and the resistance of the host cells. Many of the factors which determine these are unknown but some observations of significance have been made.

(1) *Virus virulence*

It is well known that poliovirus type I is more virulent than types II and III, and that virus strains within each antigenic type may vary in virulence.

(2) *Host resistance*

Age. Paralysis is usually more severe in adults than in young children indicating that age may affect the vulnerability of the host cell neurone.

Hormones. Certain hormonal disturbances in the host predispose to severe paralysis, in particular the hormonal changes which occur in the physiological state of pregnancy. Significantly, administration of cortisone also increases the severity of the disease.

Trauma. The enhancing effect of trauma and the increased susceptibility to the severe bulbar form of poliomyelitis after recent tonsillectomy is recognized. This may be due to the increased facility with which virus can gain entry into nerve fibres exposed at the site of trauma. Alternatively, increased permeability of the blood-brain barrier, after reflex stimulation of blood vessels in the motor area supplying the affected part, may account for the en-

hancing effect of trauma. Similar explanations are invoked to explain the increased incidence of paralysis in limbs which have recently received injections of various prophylactics.

Physical exertion. Severe physical exertion immediately before the onset of the major illness has a pronounced effect on the subsequent development of paralysis, particularly in the muscle groups which have been overexerted. It is thus not uncommon for the right upper limb to be affected after a strenuous game of tennis, or for the lower limbs to be affected after a strenuous walk, and continuation of any form of physical activity after the onset of the major illness may have a disastrous effect on the outcome of infection.

Pathology

No histological lesions due to poliovirus replication in the extra-neural tissues of the alimentary tract and lymphatics have been observed, but infection of the target cells, namely the motor neurones of the anterior horn of the spinal cord and of the motor centres of the brain stem, produces typical pathological changes. Infected neurones undergo chromatolysis with dissolution of the Nissl substance (Fig. 66); the nucleus becomes eccentric and its chromatin aggregates to form, eventually, an eosinophilic intranuclear inclusion body. Up to this stage the lesion is reversible and the cell may recover. When this occurs, the Nissl substance regenerates, first at the periphery of the cell cytoplasm, and then in the central portion of the cell which returns to its normal morphology within 6–8 weeks, although the intranuclear inclusion may remain. In the absence of recovery, the cell undergoes complete necrosis within a few days and lyses or is phagocytosed by polymorph and microglial cells. Degeneration of the axon follows as a natural consequence.

The cellular changes may be accompanied by a greater or lesser degree of inflammatory reaction, consisting of inflammatory exudation and infiltration of the tissue substance, first by polymorphs, and then by lymphocytes, mononuclear and microglial cells, which tend to form perivascular accumulations (Fig. 67). Some congestion of the meninges is also seen.



Fig. 66. Chromatolytic effect of poliovirus infection.
(a) Normal monkey neurone.
(b) Monkey neurone infected with poliovirus.

The site and extent of motor neurone destruction determines the paralytic manifestations of poliovirus infection. When only a few neurones are destroyed, or their destruction is widely disseminated rather than specifically localized, little or no paralysis ensues because of the wide margin of compensation available. For permanent

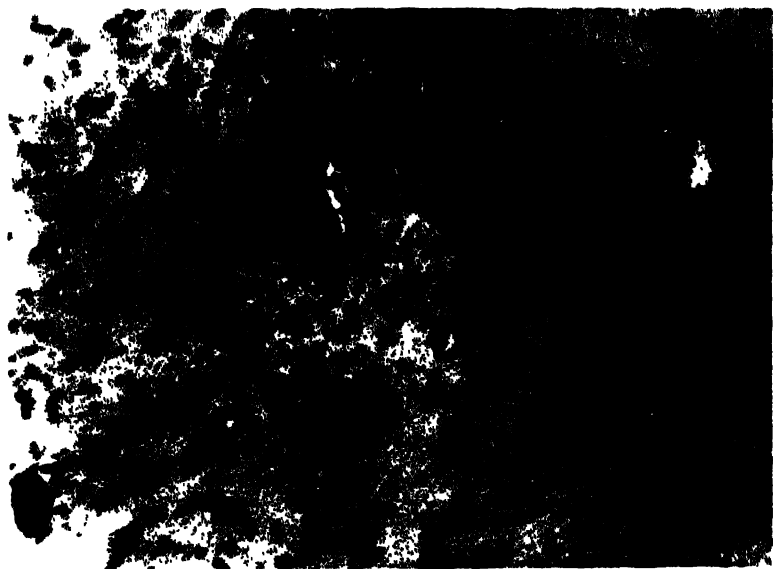


Fig. 67. Inflammatory reaction in the lumbar cord of a monkey infected with poliovirus.

Note chromatolysis of infected neurones.

paralysis to occur, a high proportion of the neurones in the affected segment must be destroyed.

Contrary to a commonly held view, lesions may sometimes be found in the posterior columns and in the posterior root ganglia, and these may be responsible for pain and tenderness which are often experienced during the course of the major illness.

Epidemiology

Poliomyelitis is endemic in all parts of the world, and epidemics tend to occur in summer and early autumn in temperate zones but

at any time of the year in the tropics. Man is the only naturally susceptible host, in whom clinical, subclinical, and inapparent cases serve as sources of infection. Of these, subclinical and inapparent infections account for more than 90% of the cases and paralytic cases for only 1–2%.

The incubation period of the disease, from the time of infection to the onset of the major illness, is usually 7–12 days but may extend from 3 to 21 days. Virus is demonstrable in throat secretions within 36 hours of infection, and in the faeces within 72 hours, it is therefore evident that virus is excreted during the incubation period as well as after the onset of illness. In fact, virus is usually demonstrable in the oropharynx about 1 week before and after the onset of illness, and in the faeces about 1 week before and 3–6 weeks afterwards. Occasionally, virus is demonstrable in the faeces for some months after illness.

The oral and faecal excretion of virus makes two routes of epidemiological transmission possible, namely oral–oral transmission by droplets of pharyngeal secretion and indirect faecal–oral transmission. The observation that poliomyelitis is most infectious during the period when virus is found in the oropharynx suggests that oral–oral spread by droplets is of major importance. However, much epidemiological information emphasizes the importance of the faecal–oral route. Thus poliovirus is much more widely distributed in communities of low socio-economic levels, in which standards of hygiene are low, than in better-off communities which enjoy a higher standard of hygiene. If spread by the faecal–oral route is important, it is perhaps surprising that outbreaks of poliomyelitis are rarely, if ever, traced to infected water, milk or food. Probably, the very large number of inapparent infections and the absence of any viral multiplication in those vehicles important for the spread of enteric infection obscure the true mode of spread.

The presence of poliovirus in sewage makes the domestic fly a possible intermediate vector in the transfer of virus along the faecal–oral route. Although there is no evidence that poliovirus multiplies in the fly, there is no doubt that flies may act as mechanical vectors and contaminate food. However, there is no unequivocal evidence that flies play a major role in the spread of poliomyelitis.

Immunity

It is well known that patients who have recovered from an attack of poliomyelitis are rarely subject to a second attack, and never to a second attack with the same virus type. There is no doubt that this immunity is associated with the presence of neutralizing antibody in the circulation, and to the resistance of the alimentary tract to reinfection.

Serological surveys show that the state of humoral immunity to poliomyelitis in the population determines, to a large extent, the epidemiological behaviour of the disease. Thus the profound change in epidemiological behaviour of poliomyelitis in Europe and North America, which has occurred in this century, is related to the lowered state of herd immunity resulting from the general rise in hygienic standards. Previously, early infection made paralytic cases most usual in infants, hence the name infantile paralysis; more recently, later infection has rendered the higher age groups susceptible and paralytic cases occur in them with increasing frequency.

Most members of isolated communities which have had no recent experience of poliomyelitis are found to lack protective antibodies. Peart has described the importation of poliomyelitis in such a community in the Canadian Arctic; he found the incidence of paralytic poliomyelitis to be extremely high, amounting to 60% of those infected, and, contrary to the usual pattern of infection, the disease attacked all age groups, affecting infants least of all.

In contrast, urban populations over the age of 4–5 years, particularly in tropical or semitropical areas where there is much overcrowding and low standards of hygiene, possess a high level of antibody. The infrequent occurrence of paralytic poliomyelitis in the higher age groups of these populations indicates that infection occurs at a very young age, perhaps even under the cover of natural passive immunity of maternal origin.

The different epidemiological behaviour of poliomyelitis in immune and non-immune populations became very evident in World War II, when serious epidemics of poliomyelitis occurred in American and Allied troops in the Middle East but spared the local populations.

Laboratory Diagnosis

(a) Virus isolation

For diagnosis, virus is isolated from the throat or faeces. Virus is most often isolated from the faeces but more than one specimen should be tested because virus excretion may be intermittent. In contrast to other enteroviruses, poliovirus is only rarely isolated from cerebrospinal fluid.

Throat swabs and faecal extracts are inoculated into primary cultures of monkey kidney or human amnion cells, or into cultures of HeLa cells or other continuous lines of human epithelial cells. Cytopathogenic effects are produced in a few days, and any virus isolated is identified and typed by neutralization or complement-fixation tests using antisera to the three types of poliovirus.

(b) Serological tests

A four-fold rise, or more, in the titre of neutralizing or complement-fixing antibodies, occurring between the acute and convalescent phases of the disease, is diagnostic. Both neutralizing and complement-fixing antibodies appear soon after the onset of the major illness, so that a high titre in the first serum specimen may obscure the antibody rise which occurs during the course of infection.

Neutralizing antibodies usually appear before complement-fixing ones and persist for long periods of time, usually for life. In contrast, type specific complement-fixing antibodies appear a few days later than neutralizing antibodies and persist for only a few years. A high titre of complement-fixing antibody in the acute phase serum is therefore indicative of recent infection, and is of diagnostic importance even in the absence of a four-fold rise in titre. Only the type-specific D antigen (i.e. unheated virus) should be used in complement-fixation tests because antibodies to the C antigen, although they appear earlier, react with heterotypic strains. Exceptionally, no rise of complement-fixing antibodies is demonstrable, and the occasional rise of heterotypic antibody titres may complicate serological diagnosis.

Prophylaxis

(a) General measures

During an outbreak of poliomyelitis certain public health measures are called for. Since the ratio of inapparent to clinical infections may be of the order of 100 or 1000 to 1, isolation of the patient cannot have any effect on the spread of the virus in the community, or indeed in the home. Nevertheless, isolation precautions are observed in hospital until virus ceases to be excreted, because spread of infection to medical attendants and other patients has been recognized as a hazard. Similarly, although universal quarantine of contacts is of no avail in checking the spread of infection, quarantine of intrafamilial child contacts for 21 days is prudent because children are the most effective spreaders of infection.

The public should be advised to be particularly careful in matters of personal hygiene, food should be protected from flies, and contact with affected families should be avoided, as should contact between affected and unaffected communities. Avoidance of overexertion at these times is particularly important, and elective nose and throat operations should be postponed until after the epidemic is over. The once popular routine closure of swimming baths is probably unnecessary, but the water should be chlorinated and overcrowding avoided. With modern immunization techniques for halting epidemics (see below), routine closure of schools is unnecessary.

(b) Vaccination

Ever since the discovery of poliovirus one of the primary aims of poliovirus research has been the production of a safe, effective vaccine against poliomyelitis. Early experimental work soon showed that immunization of monkeys with living and inactivated poliovirus produced some degree of immunity to experimental infection, and an antibody response. Immunization of humans with infected monkey cord suspensions, inactivated with sodium ricinoleate or formalin, was attempted as early as 1935, but the occurrence of several cases of paralysis brought these heroic trials to an abrupt end. It was not until the introduction of tissue culture techniques, in 1949, that the large scale production of virus, and the develop-

ment of a poliovirus vaccine suitable for use in human populations, became a practical possibility.

(i) Inactivated virus vaccine

The problems involved in the large scale production of virus in tissue culture, and its inactivation by formalin with the preservation of antigenic potency, were eventually solved by Salk and his colleagues. By 1953 they had succeeded in showing that a formalin-killed trivalent vaccine was effective in producing a substantial antibody response, and the Francis field trial of 1954, in the U.S.A., established its protective efficacy in poliomyelitis. Some cases of poliomyelitis were produced in the U.S.A. following the use of certain early batches of Salk vaccine which were later shown to contain residual amounts of live virus. The introduction of filtration to remove particulate matter, in addition to formalinization, has now ensured complete inactivation of the virus and the safety of the vaccine is no longer in doubt.

The trivalent formalin-killed Salk vaccine is given by subcutaneous injection and the dosage schedule recommended consists of two primary doses, with an interval of 6 weeks between each, followed by a third dose 7–12 months later, and a fourth dose given after an interval of 12 months or longer. Immunization should be initiated at 3 months of age, and children vaccinated in infancy may be given the fourth dose at the age of school entry. With this regimen a very satisfactory degree of protection is achieved, and in some areas the incidence of poliomyelitis has declined more than 90% after mass Salk vaccination; in England and Wales, the fatality rate has been reported to be more than 10 times as high in unimmunized as in immunized children.

Antibodies to types I and III polioviruses, produced after a complete course of Salk vaccine, tend to fade away within a few years but antibody to type II poliovirus is more persistent. Of course, individuals vary in their antibody responses, but those who have not previously been infected with polioviruses, and are devoid of antibody to any of the three types, generally respond less well than those who have. The level of antibody induced by vaccination may be maintained, in some individuals, by natural inapparent infection

which acts as a 'booster'. Indeed Salk has suggested that the effectiveness of inactivated vaccine may depend not only on the level of circulating antibody at the time of infection but also on priming of the immunological apparatus, so that antibody responses to natural infection will be of the secondary type in which high titres are rapidly achieved.

Vaccination with Salk vaccine, although effective in reducing the incidence of paralytic poliomyelitis, does not prevent infection of the alimentary tract. Nevertheless, there is some evidence that poliovirus, particularly type II, has disappeared from well-vaccinated communities. One explanation, offered by Marine and his colleagues, for this phenomenon is based on the observation that less antibody is required to clear poliovirus from the throat than from the intestine. They suggest that antibody in the throat after vaccination may be sufficient to prevent oral-oral spread by droplets, the route believed to be of major importance in communities with high standards of hygiene. If so, Salk vaccine should prove less effective in communities with low standards of hygiene, in which virus is spread mainly by the faecal-oral route, unless very high antibody titres are achieved.

The duration of protection produced by Salk vaccine is still uncertain, and large doses of virus administered by repeated injections are required to produce effective immunity. Moreover, the production of vaccine of standard antigenic potency is difficult. For these reasons, and the occasional outbreak of poliomyelitis in well-vaccinated communities, the use of Salk vaccine has been largely replaced, in recent years, by live attenuated virus vaccines.

(ii) Live attenuated vaccine

The superiority of live attenuated vaccines, over the killed variety, in producing long lasting immunity has long been recognized. It is not therefore surprising that the search for attenuated poliovirus strains began as soon as appropriate techniques became available. Koprowski and his colleagues were the first to develop an attenuated vaccine strain by mouse adaptation of a type II poliovirus, and showed that it produced a harmless alimentary infection and a substantial antibody response in humans. Since that time three differ-

ent groups led by Koprowski, Cox, and Sabin, respectively, have developed attenuated strains in tissue culture.

Originally, attenuated strains for use in vaccines were selected for their low neurovirulence in monkeys. This property is now known to be frequently correlated with two other stable genetic characters demonstrable *in vitro*; namely, inability to replicate at 40°C and delay in appearance of plaques in media containing low concentrations of bicarbonate. The assumption that low neurovirulence in monkeys is correlated with low neurovirulence in man has been amply confirmed in the large-scale use of live poliovirus vaccine. The very high efficacy of the live vaccine has now been demonstrated in a large number of field trials, in which oral administration of live attenuated strains has been shown to produce a pattern of infection which resembles that of natural inapparent infection.

After oral vaccination, immunity develops within 7–10 days and is similar to that produced after natural infection, and may be as long lasting. The great advantage of orally administered live vaccine, over the Salk type, is its ability to render the alimentary tract immune to reinfection with natural wild strains of poliovirus, which have therefore been eliminated from highly vaccinated populations. How far this immunity of the alimentary tract is due to humoral or cellular factors is not yet certain.

As in naturally occurring infections, vaccinated individuals may excrete virus in the faeces for several weeks. The possible spread of virus to unvaccinated contacts may be considered an added advantage of live vaccines, extending coverage in vaccination programmes; but some authorities have raised the possibility of genetic instability in vaccine strains after passage through the human alimentary tract. Limited laboratory evidence that vaccine strains may acquire some of the characteristics of neurovirulent strains has been obtained, but complete reversion has not been demonstrated. In practice, about 350 million individuals in all parts of the world have received live vaccine in the last few years without significant harmful effects; this suggests that the danger of reversion is not one of practical importance.

The *in vitro* acquisition of neurovirulence by attenuated vaccine

strains is also a possible hazard, and continued vigilance by vaccine producers is necessary to ensure that the characteristics of attenuation are maintained. In 1964, the Surgeon-General's Committee on Oral Poliomyelitis Vaccination in the United States called attention to a few cases of poliomyelitis which may possibly have been induced by oral vaccination. The incidence of these cases was particularly high in males over 30 years of age inoculated with the type III strain. The risk, however, proved to be small and was estimated to be 0·4 per million with type III, 0·16 per million with type I, and 0·02 per million for type II. Sabin has challenged these figures as overestimates, and no increased risk to adults has been found in the United Kingdom.

The resistance of the alimentary tract, after oral vaccination, to reinfection with wild strains of poliovirus makes possible a break in the chain of transmission and the eventual elimination of naturally occurring polioviruses from the community. Ideally, this could be achieved by immunizing 100% of the susceptible population within a short period of time. In Czechoslovakia, where the vaccination programme has come nearest to this ideal, poliovirus is reported to have been eradicated. Once achieved, the very absence of poliomyelitis may lead to a false sense of security, and this makes the vaccination of new generations of children, in the first year of life, imperative if the development of a new susceptible population, in which poliovirus can reappear and flourish, is to be prevented.

The rapid induction of alimentary tract resistance to reinfection, after oral vaccination, makes possible the arrest of naturally occurring epidemics by mass oral vaccination of the exposed populations, using a heterologous type of attenuated poliovirus. This technique, which interrupts the spread of the naturally occurring epidemic strain, has now been used in several epidemics with apparent success.

Recently, the presence of simian virus 40 in some of the monkey kidney cultures used for vaccine production has given cause for concern. This virus is oncogenic in hamsters and although the risk of oncogenic activity in humans is probably extremely small, if present at all, monkey tissues for vaccine production are now screened for SV40 before use.

(iii) Oral vaccination schedules

Various schedules based on the use of monovalent, bivalent, and trivalent vaccines have been used. Monovalent vaccines are favoured in the U.S.A., and the sequence recommended is type II, followed by type I and then type III. The three doses are given at 6 week intervals and, in infants, may be given at the same time as diphtheria-per-tussis-tetanus injections. To complete the course a dose of trivalent vaccine is recommended at 1 year of age. A further dose of trivalent vaccine is recommended for those at school age, and for those adults who have been exposed to special risk in epidemic areas or have entered into military service. Ideally, the course of immunization should begin in infancy, between the ages of 6 weeks and 3 months.

Trivalent vaccine, in which the concentration of each type of virus is adjusted to overcome any interference effects between the different types, is the one currently used and favoured in the United Kingdom, where vaccination is offered to all up to the age of 40 years. Vaccination is begun between the ages of 3-6 months when the depressive effect of maternal antibody has receded. Three doses of trivalent vaccine are given at 4-6 week intervals and a fourth dose at 2 years of age is recommended for those in whom the course is completed before the age of 6 months. A further dose of trivalent vaccine at the age of school entry is recommended for all children immunized in infancy, and one for adults exposed to special risks in epidemic areas is also recommended.

Oral vaccine, which is easily administered on a cube of sugar or in syrup, is best given during the winter months when other enteroviruses, which may interfere with the implantation of vaccine virus in the alimentary tract, are less prevalent. Trivalent vaccine may also be used to boost the immunity of persons previously vaccinated with Salk vaccine.

Although there are few contraindications to oral poliomyelitis vaccination it is prudent to postpone vaccination in patients who are suffering from an acute illness, or who are in the first four months of pregnancy. Routine vaccination of patients on steroid therapy is contraindicated, and tonsillectomy should be delayed until 2 weeks after completion of a course of oral vaccination.

(c) Passive Immunization

Although human γ -globulin is effective in preventing paralytic poliomyelitis when given soon after exposure, the large doses required, and the current use of oral vaccine in the face of epidemics, make its use superfluous, except in special cases. It may be of use if administered to a very close contact within 2 days of exposure.

Treatment

There is no specific antiviral treatment for poliomyelitis.

Table 10. Inactivated and live-attenuated poliovirus vaccines compared

Character	Inactivated vaccine	Live-attenuated vaccine
Infection of alimentary tract with wild virus	Not prevented	Prevented
Individual immunity	Effective	Effective
Herd immunity	Not effective	Effective
Eradication of poliovirus from community	Not effective	Effective
Onset of immunity	A few weeks	A few days
Duration of immunity	A few years. Probably requires repeated injections to maintain suitable antibody levels	As in natural infection. Theoretically life-long but further serological surveillance required
Control of epidemics	Not effective	Effective
Safety	Safe	Safe in practice but requires continued vigilance

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Table 10. cont.

Character	Inactivated vaccine	Live-attenuated vaccine
Administration	By injection	Oral administration (easier and more pleasant)
Administration	None	Interval of a few weeks necessary between each type in monovalent vaccines. Multiple doses of trivalent vaccine, in which concentration of each type is adjusted, overcomes interference effects
Spread to unvaccinated subjects	None	Spreads but no danger in practice from reversion to virulence. Extends vaccination programme
Economy	Large amounts of virus per dose required	Small amount of virus per dose, only, required
Storage	Twelve months at refrigerator temperature	Must be frozen for long-term storage. Six months at refrigerator temperature and 1 week at room temperature

CHAPTER 23

Enterovirus Infections

II. Coxsackieviruses Coxsackie Virus Infections

Coxsackieviruses were first isolated by Dalldorf and Sickles, in 1948, from two patients who lived in the village of Coxsackie, New York State. Although both patients were suffering from proven poliomyelitis, the associated coxsackieviruses were revealed by isolation in suckling mice. Since that time, two groups of coxsackieviruses, A and B, have been recognized by the lesions which they produce in suckling mice. Twenty-three antigenic types of Group A and six of Group B are now known, some of which are pathogenic.

Properties of Coxsackieviruses

(a) Morphology

Coxsackievirus particles, which are about 28 m μ in diameter, resemble poliovirus particles in size and density. Preliminary studies also indicate a morphological resemblance to poliovirus, and the ready crystallization of coxsackieviruses is evidence of a symmetrical capsid structure.

(b) Chemical and physical properties

Like other enteroviruses, coxsackieviruses are resistant to ether, are stable at pH 3.0, and are thermostable at 50°C in the presence of 1 M magnesium chloride (MgCl₂).

(c) Biological properties

(i) *Haemagglutination*

Coxsackievirus A, types 20, 21, and 24, and coxsackievirus B,

types 1, 3, and 5, haemagglutinate human group O red cells. The red cell receptors used, which are probably of a protein or lipoprotein nature, are quite distinct from the sialomucoprotein receptors of myxo- and paramyxoviruses, and the haemagglutinin is the infective virus particle itself. Non-haemagglutinating strains of haemagglutinating types sometimes occur, and coxsackievirus A7 will agglutinate fowl red cells of the type susceptible to agglutination by vaccinia virus (Table 6).

(ii) Interference

Infection with Group B viruses interferes with infection by polioviruses, but Group A viruses may infect concurrently with poliovirus.

(d) Antigenic composition

Two antigenic components have been demonstrated in the few types which have been studied in detail by Schmidt and her colleagues. One, is a group complement-fixing antigen which reacts with human immune serum, only, and is found in the light non-infective fraction separated by density gradient centrifugation. The other, is a type-specific antigen which reacts with human immune serum in neutralization and precipitation tests and with monkey and mouse immune sera in complement-fixation tests. This antigen may be converted to the group-specific antigen by heat at 56°C for 30 minutes, a reaction which resembles the D → C conversion observed with polioviruses.

So far, coxsackieviruses A1-24 and B1-6 have been distinguished by type-specific neutralization and complement-fixation tests. One virus, previously classified as coxsackievirus A23, has now been reclassified as echovirus type 9. Antigenic cross reactions between coxsackieviruses A3 and 8, A11 and 15, and A13 and 18, and between various B strains have been noted, and four antigenic variants of coxsackievirus A20 are known.

(e) Cultivation

(i) Suckling mice

Although Group B and some Group A coxsackieviruses are now known to grow in tissue culture, the original isolations were only

possible in suckling mice and these animals remain the only susceptible host for most of the Group A viruses. It is the pattern of infection produced in suckling mice, inoculated by any route, which serves to distinguish Group A and Group B viruses. Viruses belonging to Group A produce acute generalized necrosis of striated muscle which leads to generalized flaccid paralysis and death (Fig. 68). In contrast, Group B viruses infect the brain of the suckling mouse and produce acute cellular necrosis and encephalomalacia. This results in the production of spastic paralysis, tremors, and death. Other lesions produced by Group B viruses are acute necrosis of the interscapular brown fat, acute myocarditis, hepatitis, and pancreatitis. Necrosis of striated muscle, if it occurs at all, is not prominent and remains localized.

(u) Tissue culture

In general, all Group B viruses grow in primary cultures of monkey kidney cells, as well as in human amnion, HeLa and other continuous human cell lines. Of the Group A viruses, only A₉ grows readily in monkey kidney cells but A₁₁, 13, 15, 18, 20, and 21 may be primarily isolated in human cell lines, and some of the other Group A viruses can be adapted to grow in human amnion cells.

Clinical Syndromes produced by Coxsackieviruses

Much virological and epidemiological evidence has now been adduced to confirm the aetiological role of some coxsackieviruses in various clinical syndromes. But even those which are pathogenic produce inapparent or subclinical infections in approximately the same ratio as polioviruses. The mere isolation of virus from the stools is not therefore proof of a causative role in disease, for which further evidence is required. A concomitant rise in the titre of homologous antibody, and the recognition of a characteristic epidemiological behaviour of the disease, are important additional observations. The wide spectrum of clinical syndromes produced by a single coxsackievirus type, and the number of different virus types which

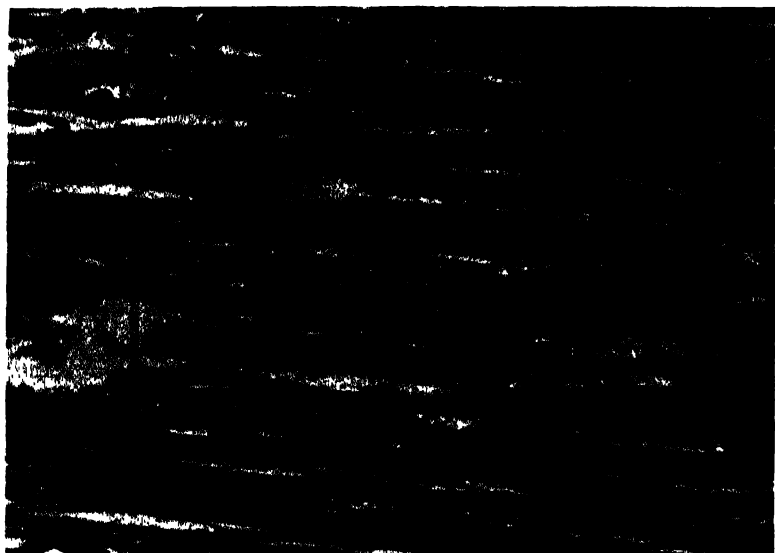


Fig. 68. Necrosis of striated muscle produced by coxsackievirus A5.

(a) Normal striated muscle of mouse hind leg.

(b) Striated muscle of mouse hind leg showing degeneration after infection with coxsackievirus A5.

may produce a single clinical syndrome, make the aetiological role of any coxsackievirus difficult to establish.

(a) Aseptic meningitis

Aseptic meningitis, which is one of the commonest conditions caused by coxsackieviruses, is characterized by fever, headache, and the clinical signs of meningeal irritation. Mild pharyngitis may be present in the initial stages, and the cerebrospinal fluid manifests a slightly elevated protein level, and a moderately increased cell count with relative lymphocytosis. Occasionally, encephalitis or lower motor neurone paralysis, indistinguishable from poliomyelitis, occurs but complete recovery is the rule.

There is no doubt that many illnesses previously diagnosed as non-paralytic poliomyelitis were, in fact, cases of aseptic meningitis due to coxsackie or other enterovirus infection. The coxsackieviruses usually involved are B1-6, A7, and A9, and sometimes other A types. These viruses tend to be prevalent in the summer months in temperate climates, and epidemics are likely to occur.

(b) Herpangina

Herpangina is an acute infectious disease of childhood and young adult life which is caused by a number of coxsackie A viruses, particularly A2, 5, and 6. The condition, which often occurs in epidemics, is characterized by an acute febrile onset, sore throat, and vesicular lesions in the faucial area of the oropharynx. The vesicular lesions, which are usually 5-14 in number, soon ulcerate and leave small, punched-out, grey-coloured ulcers. Occasionally, signs of systemic infection such as headache, abdominal pain, vomiting, and myalgia may occur, and, in adults, involvement of the meninges has been reported. The condition of herpangina has to be distinguished from herpetic stomatitis in which the lesions are characteristically present in the anterior part of the mouth and on the buccal mucosa.

(c) Epidemic myalgia (Bornholm disease)

Epidemic myalgia, which usually occurs in localized epidemics, is characterized by pyrexia and acute stabbing pains in the chest and abdomen, often worse on inspiration. Orchitis is a recognized

complication of the condition, and during epidemics aseptic meningitis, alone or in association with myalgia, may appear. Coxsackievirus B, types 1-5 are the viruses which may produce epidemic myalgia.

(d) Pericarditis

Several cases of acute benign pericarditis due to infection with coxsackie B viruses have now been recognized. The condition may be fatal in the newborn.

(e) Myocarditis

Neonatal or intrauterine infection with coxsackie B viruses may lead to the development of myocarditis in the newborn. The condition, usually occurring a few days after birth, carries a high mortality rate, and is sometimes associated with encephalitis, and, less commonly, with hepatitis. Outbreaks of the condition have been reported from nurseries and maternity units, and often a history of aseptic meningitis, myalgia, or mild respiratory infection occurring in the mother or close contact a week or so before delivery may be obtained.

Some evidence of myocarditis due to infection with coxsackie B viruses in older children and adults has also been obtained.

(f) Exanthemata

Both coxsackie A and B viruses have been incriminated in a number of outbreaks of febrile illness in which maculopapular rashes, alone or in combination with aseptic meningitis, are characteristic. Sometimes, the rash is accompanied by pharyngitis, lymphadenopathy, or conjunctivitis.

(g) Hand, foot, and mouth disease

Hand, foot, and mouth disease is a specific febrile condition caused by coxsackievirus A16. It is characterized by the eruption of vesicles or ulcers in the oropharynx or fauces, and by a maculopapular rash, later becoming vesicular, on the hands and feet.

(h) Respiratory infections

Mild upper respiratory infection due to coxsackievirus A21, previously known as Coe virus, occurs in military recruit camps but is not common in the general population. Pneumonitis due to coxsackie A9 infection has also been reported.

Pathogenesis

The pathogenesis of disease due to coxsackieviruses is believed to resemble that of poliomyelitis. After initial virus multiplication in the pharynx and gastro-intestinal tract, haematogenous spread distributes the virus to target organs, infection of which leads to the various clinical manifestations of infection. In the majority of infected individuals, spread of infection is checked at the sites of initial multiplication, and infection is inapparent.

Epidemiology

The epidemiology of coxsackieviruses resembles that of polio- and other enteroviruses. Like them, coxsackieviruses are world-wide in their distribution and become prevalent in the summer and autumn seasons in temperate climates.

Man is the only naturally susceptible host, in whom infection is usually inapparent. Virus is probably transmitted by the faecal-oral route and by the oral transmission of droplets of pharyngeal secretion. The higher incidence of infection in children and in communities with low standards of hygiene emphasizes the importance of the faecal-oral route of transmission. Mechanical transmission of coxsackievirus by flies has also been demonstrated.

Some neonatal infections are thought to be acquired from maternal sources by intrauterine infection. This is a hazard now well recognized in maternity units.

Laboratory Diagnosis**(a) Virus isolation**

Virus may be isolated from the faeces and, depending on the clinical syndrome, from the cerebrospinal fluid, pharyngeal secretions,

vesicle fluid or blood. Unless isolated from normally sterile sites, such as cerebrospinal fluid, blood, or vesicle fluid, virus isolation provides only circumstantial evidence of an aetiological role. A concurrent rise in titre of homologous antibody lends support to the causative role of any isolate.

(i) Suckling mice

Suckling mice provide the only susceptible host for most coxsackie A viruses, and for a few exceptional coxsackie B strains. Groups of mice less than 48 hours old, and preferably less than 24 hours old, are inoculated by the intraperitoneal, intracerebral, and subcutaneous routes, and are examined for typical signs of coxsackie infection every day for 14 days.

(ii) Tissue culture

At the same time as mouse inoculation, specimens are inoculated into primary cultures of monkey kidney cells which are observed for at least 7 days. If no cytopathogenic effect develops within this time, a further passage is made before the negative result is accepted. Although monkey kidney cells are the cells of choice for the isolation of coxsackieviruses B1-6, A9, and A16, HeLa cells may be used for isolating coxsackieviruses B1-6, A11, 13, 15, 18, 20, and 21.

Any tissue culture isolates are identified as enteroviruses by their resistance to ether, and by their stability at both pH 3.0 and at 50°C in the presence of 1 M Magnesium chloride (MgCl_2). Identification as coxsackievirus depends on the pathological changes produced in suckling mice, but serological typing is a matter of some difficulty. In practice, pools of antisera to various virus types, blended to allow the identification of virus with the minimum number of neutralization tests, are used. Virus may also be typed by complement-fixation, and haemagglutination-inhibition tests may be used for strains which haemagglutinate.

(b) Serological tests

A rise in antibody titre, of four-fold or more, occurring between the acute and convalescent phases of the disease, is diagnostically significant, particularly if demonstrated against the actual virus

isolated. Heterologous antibody rises sometimes obscure the correct identification of the serotype causing infection.

Control

(a) General measures

As in other enterovirus infections, the effective inactivation and disposal of sewage, high standards of personal hygiene, and control of flies are necessary prophylactic requirements.

(b) Vaccination

The multiplicity of coxsackievirus types and their relatively low pathogenicity makes the development of vaccines impracticable and hardly necessary at the present time.

Treatment

There is no specific antiviral therapy for coxsackievirus infections.

CHAPTER 24

Enterovirus Infections

III. Echoviruses Echovirus Infections

Using tissue culture techniques, a number of viruses have been isolated from the human alimentary tract which are non-pathogenic to laboratory animals and are usually unassociated with disease in the human host. Because of their habitat, the nature of their isolation, and their lack of association with any particular clinical syndrome, they became known as enteric cytopathogenic human orphan viruses or, in abbreviation, echoviruses. Although some of these viruses are now known to be associated with various clinical syndromes, the name persists. So far, 30 different antigenic types of echovirus have been recognized.

Properties of Echoviruses

(a) Morphology

Echoviruses are about 28 m μ in diameter, and those types which have been examined after negative staining are seen to consist of capsomeres arranged in cubical symmetry. According to Mayor, the number of capsomeres is 32.

(b) Chemical and physical properties

Like other enteroviruses, echoviruses are RNA viruses which are resistant to ether, stable at pH 3.0, and survive at 50°C in the presence of 1 M magnesium chloride (MgCl₂).

(c) Biological properties

Some types of echovirus agglutinate human group O red cells, and the haemagglutinin is the infective virus particle. Red cells from the

'cord' blood of newborn infants are the most sensitive to haemagglutination, but adult red cells are usually suitable for routine purposes. The optimal conditions for haemagglutination are different for various echovirus types; for this reason, echovirus haemagglutination tests are performed at 4°C and 37°C as well as at pH 5·8 and 7·3. The erythrocyte receptors used are distinct from the sialomucoprotein receptors specific for myxo- and paramyxoviruses, and are believed to be lipoprotein in nature.

Table 6 lists the haemagglutinating types of echoviruses, but it should be noted that some strains belonging to these types do not haemagglutinate.

(d) Antigenic composition

Thirty different antigenic types of echoviruses have been identified by neutralization, complement-fixation and, where appropriate, by haemagglutination-inhibition techniques. Some strains which react poorly with homologous prototype antisera can be identified by their own antisera, and are called 'prime' strains. In contrast, infection with some other strains of echovirus may produce a heterologous antibody response specific for another echovirus type.

(e) Cultivation

Echoviruses grow readily in monkey kidney cells producing characteristic enterovirus cytopathogenic effects. Human diploid cells and human amnion cells may also be used, and the latter are the cells of choice for echovirus type 21. HeLa cells are not suitable for initial isolation of echoviruses but some types become adapted after laboratory manipulation.

Different morphological types of plaque produced by the various types of echovirus on different types of cell have been used as a criterion of identification.

Clinical Syndromes produced by Echoviruses

Sufficient virological and epidemiological evidence has now been adduced to establish the association of most types of echovirus with certain clinical syndromes. Even so, inapparent and subclinical

infections are common, and the ratio of clinical to subclinical infections is similar to that which occurs with polioviruses. This, together with the production of a variety of clinical syndromes by a single virus type and the production of a single clinical syndrome by a number of different virus types, makes the aetiological role of any particular type of echovirus in any specific clinical syndrome difficult to prove.

Isolation of virus alone is not therefore proof of a causative role in disease unless isolated from the blood or cerebrospinal fluid, which sites are normally sterile. Concomitant rises of homologous antibody in infected patients and a characteristic epidemiological behaviour of the disease provide important corroborative evidence of a causative role.

(a) Aseptic meningitis

Aseptic meningitis is a common clinical manifestation of echovirus infection, and sometimes occurs in epidemics. Twenty-four of the 30 known antigenic types have been observed to be associated with this syndrome.

Some types have also been reported to be associated with lower motor neurone paralysis, indistinguishable from poliomyelitis, but usually mild and leading to uninterrupted recovery.

(b) Exanthemata

Mild febrile conditions accompanied by maculopapular and other rashes, occasionally occurring in conjunction with aseptic meningitis, have been observed. Sometimes, a mild degree of lymphadenopathy accompanies the condition which may then be confused with rubella.

(c) Gastro-enteritis

In some outbreaks, echoviruses have been isolated more frequently from patients with gastro-enteritis than from healthy controls, but definite proof that echoviruses are a common cause of gastro-enteritis has not yet been obtained. Nevertheless, echoviruses types 11, 14, and 18 have been incriminated, with some degree of confidence, in

outbreaks of gastro-enteritis occurring in newborn and premature infants housed in maternity units.

(d) Respiratory infection

Although one of the first viruses isolated from the 'cold' syndrome was originally labelled echovirus type 28, it has now been recognized as a rhinovirus and has been excluded from the echovirus group. Another virus associated with upper respiratory infections and originally labelled echovirus type 10 has also been excluded and assigned to the genus *Reovirus*. Of the remainder, a few types of echovirus have been shown to be associated with mild upper respiratory infections, usually accompanied by mild pharyngitis and sometimes by gastro-intestinal disturbances.

Pathogenesis

The recovery of echoviruses from the oropharynx, intestinal tract, and occasionally from the blood stream itself, suggests that the pathogenesis of echovirus infection resembles that of poliomyelitis and coxsackievirus infection. This assumes initial multiplication in the pharynx and intestinal tract, with subsequent viraemia and spread to the target organs. Inapparent infections, in which spread of virus is limited to the primary sites of multiplication, probably occur with the same frequency as in poliomyelitis.

Epidemiology

In temperate climates, echoviruses are mainly prevalent in the summer and autumn months and are recovered most frequently from children, particularly in those communities where poverty is responsible for low standards of hygiene. It is noteworthy that more than one type of echovirus may be prevalent in a single outbreak of infection.

Man is the only naturally occurring host and virus is believed to be transmitted mainly by the faecal-oral route. Transmission by the oral-oral route probably also occurs, and flies could conceivably act as mechanical vectors.

Laboratory Diagnosis

Because virus isolation alone is not proof of an aetiological role, except when isolated from the blood or cerebrospinal fluid, a significant rise of antibody titre against the actual virus isolated is required for the acceptance of an echovirus aetiology.

(a) Virus isolation

Echoviruses may be isolated from throat swabs, stools, cerebrospinal fluid where appropriate, and occasionally but not as a routine from blood. Isolation is most successful if attempted during the first week of the disease.

Primary cultures of monkey kidney cells represent the host of choice, but human amnion and human embryo kidney may also be used. Typical enterovirus cytopathogenicity is produced within 7 days, and any virus isolated is identified as an enterovirus by its resistance to ether and to media at pH 3.0, as well as by its heat stability at 50°C in the presence of 1 M magnesium chloride (MgCl_2). After serological and animal pathogenicity tests have excluded polio and coxsackie viruses, typing of the isolate is a difficult task usually confined to specialized laboratories. Neutralization tests using pools of different echovirus antisera, blended so as to allow identification of the virus with the minimum number of tests, are employed. Complement-fixation and, where appropriate, haemagglutination-inhibition tests may also be used for typing purposes.

(b) Serological tests

The multiplicity of echovirus types, and the not infrequent antibody responses to heterologous types, makes diagnosis solely by serological means impracticable. But a significant rise in antibody to the actual virus isolated is important corroborative evidence in assigning to it an aetiological role.

Control

(a) General measures

High standards of hygiene, inactivation and effective disposal of

sewage, together with the control of flies, aid in checking the spread of echoviruses.

(b) Vaccination

The multiplicity of antigenic types makes vaccination an impractical approach to control at present.

Treatment

No specific antiviral therapy is available.

Animal 'Echo' viruses

A number of enteric cytopathogenic viruses have been isolated from various animal species. These have been given appropriate initials such as ECSO (Swine), ECBO (bovine), and ECMO (monkey). None of these animal strains is pathogenic for man, and they are not related antigenically to the human echoviruses.

CHAPTER 25

Reovirus Infections

I. Reoviruses

Reovirus Infections

A virus originally classified as *Echovirus* type 10 now forms the type species of a separate genus to which the name *Reovirus* has been applied by Sabin. Reoviruses are distinguished from echoviruses by their larger size, the characteristic cytopathogenic effect which they produce, their wide host range, and their association with both the respiratory and alimentary tracts. The name *Reovirus* is derived from the latter property and is an abbreviation for respiratory enteric orphan virus. Three serological types of *Reovirus* are now known.

Properties of Reoviruses

(a) Morphology

Reoviruses are approximately 60–75 m μ in diameter, and are therefore about three times as large as echoviruses. The reovirus capsid is composed of 92 capsomeres arranged in icosahedral symmetry but its detailed structure is not yet known with certainty (Fig. 69).

(b) Chemical and physical properties

Reoviruses are RNA viruses, but their RNA is present in an unusual double stranded form which is unique among animal viruses. Two plant viruses whose RNA is present in the double-stranded form, the wound tumour virus and the rice dwarf virus, are of the same size and shape as reoviruses and a relationship has been suggested. Indeed, these plant viruses are now classified in a separate genus belonging to the same Family, Reoviridae.

Reoviruses are resistant to ether, and are relatively stable to heat, particularly in the presence of magnesium ions. Although resistant to chemical disinfectants such as formalin, lysol, and phenol, reoviruses are rapidly inactivated by 70% ethanol.

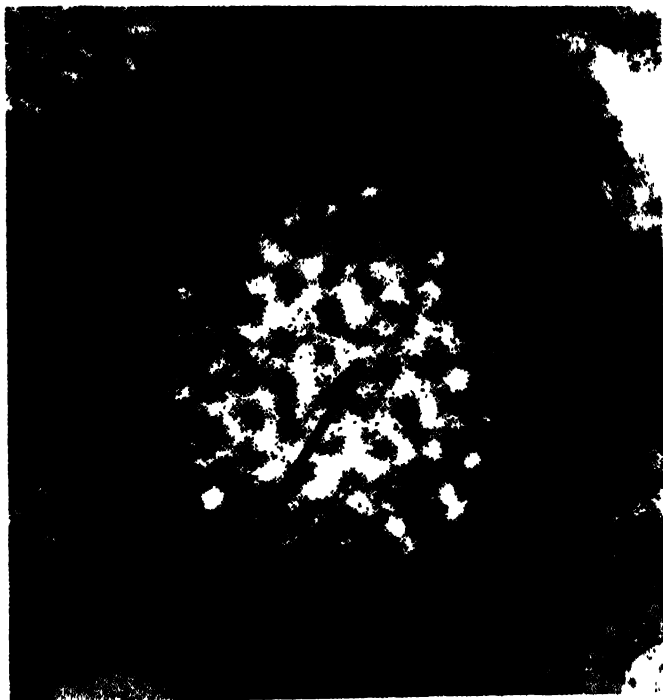


Fig. 69. Negatively stained *Reovirus* type I particle showing hollow capsomeres [from Liane E. Jordan and Heather Donald Mayor (1962) *Virology* 17, 597-99 (Academic Press Inc., New York and London)].

(c) Biological properties

Human group O erythrocytes are agglutinated by reoviruses, utilizing a periodate sensitive receptor distinct from the sialomucoprotein receptor of myxo- and paramyxoviruses. Type 3 reoviruses, only, agglutinate bovine erythrocytes through a neuraminidase sensitive sialomucoprotein receptor, although neuraminidase itself is absent from type 3 reoviruses.

(d) Antigenic composition

Three serological types of reovirus have been distinguished by haemagglutination-inhibition tests, and type 2 has now been subdivided in four subtypes. Neutralization tests are also suitable for typing, but complement-fixation tests are not because of a common complement-fixing antigen which is present in all three serological types.

(e) Cultivation**(i) Tissue culture**

A wide variety of cells in tissue culture are susceptible to reovirus infection, including monkey kidney cells, human amnion, and HeLa cells. Virus multiplies only in the cytoplasm which undergoes granular degeneration, and infected cells, in which the nucleus remains intact, do not disintegrate as readily as those rendered necrotic by enteroviruses. In stained preparations, intracytoplasmic, perinuclear inclusions may be seen.

In contrast to echoviruses, reoviruses grow in non-primate as well as primate cells.

(ii) Chick embryos

Some strains of reovirus have been adapted to the chorioallantoic membrane or amniotic cavity of the chick embryo.

(iii) Mice

Newborn mice may be used for reovirus isolation, but prior natural infection may be a source of confusion which prevents their use for routine isolation. Nevertheless, the effects of reovirus type 3 infection in newborn mice, which have been studied by Stanley and his colleagues in Australia, are of interest.

After infection, newborn mice develop an acute illness and lesions in the liver, pancreas and central nervous system. A few mice, which survive, develop a chronic disease in which infective virus is no longer present but in which lesions occur in the liver, spleen, pancreas, central nervous system, and skeletal muscles. Stanley suggests an autoimmune origin for this type of infection, and, significantly,

the administration of spleen cells from chronically infected mice to normal, newborn, genetically compatible mice may produce virus-free lesions in the spleen, liver, and pancreas, as well as retardation of growth or the 'runting' syndrome. These mice may either recover or develop malignant lymphoma, and thus provide added significance to the isolation of reovirus type 3 from Burkitt's African lymphoma in humans.

Clinical Manifestations of Reovirus Infection

Reovirus infection is extremely common and generally asymptomatic, but reoviruses have been isolated from children with respiratory, intestinal, hepatic, and central nervous system disease, and from some with undifferentiated febrile illnesses and exanthemata. In few of these syndromes has the aetiological role of reoviruses been unequivocally proved, but Joske and his colleagues isolated a reovirus from the brain of a fatal case of hepato-encephalitis occurring in an infant, and Tillotson and Lerner isolated a reovirus from the lung of a child aged 5 years who died of pneumonia following a pyrexial rash. Reovirus infections may not always therefore be without danger.

Epidemiology

Serological evidence indicates that reovirus infection is widespread in both humans and animal populations. Animal species showing evidence of infection include birds, cattle, mice, guinea-pigs, monkeys, and domestic animals. So far, no differences have been found in reovirus strains recovered from human and animal sources, and the transfer of infection from one host species to another is possible. Direct evidence has not yet, however, been obtained.

Widespread infection in the human population is probably acquired in childhood, and reoviruses are most commonly isolated from children. The more ready isolation of reoviruses from the faeces than from the upper respiratory tract, as well as their presence in sewage, suggests that the faecal-oral route of spread is the most probable.

Laboratory Diagnosis**(a) Virus isolation**

Faecal specimens or rectal swabs are more likely to yield reoviruses than swabs taken from the oropharynx. Monkey kidney cells are most suitable for isolation but HeLa cells may also be used; typical cytopathogenic effects are usually produced, but if the amount of virus in the inoculum is small a second passage may be required before cytopathogenic effects become apparent.

Identification and typing of any isolate is performed by haemagglutination-inhibition tests using appropriate antisera.

(b) Serological tests

After infection, a four-fold rise, or more, in the titre of haemagglutination-inhibiting or neutralizing antibody against the homologous serotype, occurring between the acute and convalescent phases of the illness, is diagnostic. A similar rise in complement-fixing antibody is evidence of reovirus infection without definition of type.

Treatment

There is no specific antiviral treatment.

CHAPTER 26

Poxvirus Infections

I. Poxviruses

General Description

A group of enveloped DNA viruses which are thought to exhibit helical symmetry, and which belong to the Family Poxviridae, are the cause of a number of pox diseases in man and animals. Three genera belonging to this Family cause disease in man, and of these the most important is the genus *Poxvirus*. Three species of the genus *Poxvirus* are of medical importance; *P. variolae*, the causative organism of smallpox; *P. vaccinae*, the organism used for vaccination against smallpox; and the virus of cowpox. Two viruses belonging to other genera are of lesser medical importance; these are *Molluscovirus hominis*, the cause of molluscum contagiosum; and *Dermovirus orfi*, the cause of orf or contagious pustular dermatitis of sheep which may be transmitted to man.

Poxvirus vaccinae, commonly known as vaccinia virus, is the most thoroughly studied member of the group. Because of its non-pathogenic nature and easy availability, vaccinia virus serves as an ideal model for the group as a whole and particularly for the viruses of vaccinia, variola, and cowpox which are very similar in all respects except pathogenicity.

Properties of Poxviruses

(a) Morphology

The virus of vaccinia, from which those of variola and cowpox do not differ, is a brick-shaped particle $200-250 \text{ m}\mu \times 250-320 \text{ m}\mu$ whose complex morphology has been revealed by thin section and negative staining techniques.

In section (Fig. 32b) the virus has been shown to consist, essentially, of a double-layered membrane surrounding a biconcave nucleoid structure, in which is enclosed the DNA core. Beneath the membrane, on either side of the biconcave nucleoid structure, are two lens-shaped bodies whose significance is unknown.

The morphology after negative staining depends, according to Westwood and his colleagues, on the state of the virus particle. When impenetrable by stain, the surface structure is revealed in some detail (Fig. 32a). A complex pattern of interlacing threads wound around the virus particle gives the surface a beaded appearance, which is accentuated by the double helical structure of the threads themselves. In unpurified preparations, a smooth outer envelope which covers the usually seen beaded surface structure is often present.

When the state of the virus particle allows the stain to penetrate, details of the internal structure are revealed, although the complex surface structure is masked and appears as a simple outer layered membrane (Fig. 32c). The exposure of the internal nucleoid structure reveals an outer pallisaded zone enclosing an inner zone of filamentous material, probably DNA.

(b) Chemical properties

Essentially, the viruses of the vaccinia variola-cowpox group consist of DNA, protein, and lipid. The virus of vaccinia has been analysed in some detail, and Zwartouw's analysis shows DNA to account for 3.2% by weight of the vaccinia virus particle. Total lipid which accounts for 5% by weight of the virus particle includes cholesterol 1.2%, and phospholipid 2.1%, as well as neutral fat. 0.1%, RNA and trace amounts of copper, flavin and biotin have been detected in vaccinia virus preparations but these substances are now believed to be impurities and not integral components of the virus particle. Likewise, earlier claims for the presence of enzymes in the vaccinia virus particle have not been substantiated.

Although enveloped by a membrane, vaccinia, variola, and cowpox viruses are ether stable, but Zwartouw has recently shown that vaccinia virus suspended in dilute buffer is inactivated by ether at 37°C although not at 4°C. Viruses of the vaccinia-variola-cowpox

group are resistant to 1% phenol and 50% glycerol but are inactivated by formalin, oxidizing disinfectants, and media at pH 3.0.

(c) Physical properties

Viruses belonging to the vaccinia variola-cowpox group are inactivated by heat and ultraviolet irradiation under specified conditions. At room temperature, all these viruses exhibit a high degree of stability and in certain circumstances survive for weeks and months. Vaccinia virus is inactivated by the photodynamic action of light, when combined with certain dyes such as methylene blue.

(d) Biological properties

(1) Haemagglutination

A soluble haemagglutinin produced by vaccinia, variola, and cowpox viruses agglutinates red blood cells from some, but not all, fowls. The soluble haemagglutinin, which is easily separable from the virus particle, is a lipoprotein particle about 50 m μ in diameter. Experimental evidence indicates that the lipid moiety is the haemagglutinating principle on which the protein component confers serological specificity. The red cell receptor involved, although not yet investigated in detail, is distinct from the sialomucoprotein receptor employed by myxo- and paramyxoviruses.

Haemagglutination and haemadsorption tests are routinely employed for the detection of virus, and the production of anti-haemagglutinin antibody during the course of infection makes the haemagglutination-inhibition test particularly suitable for serological diagnosis and virus identification. Serologically, the haemagglutinins produced by vaccinia, variola, and cowpox viruses are identical.

(2) Reactivation

The phenomenon of reactivation, discovered by Fenner in 1959, is characteristic of poxviruses. Vaccinia virus inactivated by heat, and inoculated simultaneously with an infective heterologous virus belonging to the Family Poxviridae, is reactivated to produce infective vaccinia virus. Heat inactivation is supposed to leave the virus

genome intact but to damage some essential protein components. Joklik believes that one of the damaged proteins is the protein responsible for 'uncoating' the virus particle, and that its replacement by the related reactivating virus allows the replication of vaccinia virus to proceed.

(e) Antigenic composition

The antigenic relationship between variola, vaccinia, and cowpox viruses is very close indeed and only very minor differences can be detected by refined serological techniques. Five different antigens have been defined, and agar-gel diffusion techniques indicate that some of these consist of multiple antigenic components.

(1) *LS antigen*

The LS antigen is produced in infected tissues and is easily separable from the virus particles. This soluble antigen consists of two components, the L component which is heat labile at 60°C, and the S component which is heat stable at 90°C. Antibodies to the soluble LS antigen are produced in infected animals and react in complement-fixation and precipitation tests but not in neutralization tests.

The soluble LS antigen of vaccinia virus is firmly adsorbed to the virus surface, where it is serologically reactive but not essential for infectivity. In purified preparations, it dissociates from the virus particles on storage; but in unpurified preparations, Westwood and his colleagues locate it beneath the smooth outer envelope which is characteristic of unpurified vaccinia virus particles.

(2) *X agglutinin*

This antigen is an integral part of the virus particle, and is distinct from the LS antigen present on the virus surface. It is responsible for the agglutination of virus particles by homologous immune serum.

(3) *NP antigen*

Alkaline extraction of poxviruses yields an internal nucleoprotein antigen, the NP antigen, which is common to all viruses belonging to the Family Poxviridae. Immunization of animals with NP antigen

stimulates a homologous antibody response but does not confer immunity to infection.

(iv) Soluble haemagglutinin

The soluble haemagglutinins produced by vaccinia, variola, and cowpox viruses are antigenically identical and produce haemagglutination-inhibiting antibodies in infected patients or animals. These antibodies although useful for diagnostic purposes do not confer immunity to infection.

(v) Immunizing antigen

The antigen responsible for the production of neutralizing antibodies and for the conferment of immunity is associated with infective virus particles but has not yet been isolated.

(f) Cultivation

(1) Experimental animals

One of the characters by which vaccinia, variola, and cowpox viruses may be distinguished is their host range.

Variola. Although rabbits and mice are useful experimental animals, monkeys are the only animals which are readily susceptible to variola introduced by inhalation or by intradermal inoculation.

In rabbits, intratesticular passage is required for serial propagation but non-transmissible lesions may be produced after intradermal inoculation of the rabbit skin. The production of keratitis after inoculation into the rabbit cornea was once a popular diagnostic test, and typical inclusions may be found in the corneal epithelium.

Serial propagation of variola in suckling mice after intracerebral or intraperitoneal inoculation provides another useful laboratory method.

Vaccinia. The host range of vaccinia virus is wider than that of variola, it includes calves, sheep, and rabbits, as well as small laboratory animals such as mice, rats, guinea-pigs, and hamsters.

Strains of vaccinia maintained by intradermal passage in rabbits tend to be less pathogenic and neurotropic than strains which have

been maintained by intratesticular and intracerebral passage. The latter strains, which are referred to as neurovaccinia, are highly neurotropic and readily produce meningo-encephalitis in inoculated animals. Similarly, repeated intracerebral passage of vaccinia in mice produces strains which are highly neurotropic in these animals.

Cowpox. Cowpox virus infects cattle and man as well as rabbits, mice, guinea-pigs, and monkeys. Unlike vaccinia, cowpox cannot be passaged intracerebrally in experimental animals.

(ii) *Chick embryos*

Inoculation of vaccinia, variola and cowpox viruses on to the chorioallantoic membranes of 10–13-day-old chick embryos produces pock lesions within 48–72 hours. Vaccinia virus, particularly neurovaccinia strains, produces pocks which are usually larger and more haemorrhagic than those produced by variola, but not as large or as haemorrhagic as those produced by cowpox (Fig. 4).

After prolonged passage in chick embryos, pathogenicity for animal hosts may be altered.

(iii) *Tissue culture*

A number of different types of cell in culture are susceptible to infection with vaccinia, variola, and cowpox viruses. These include monkey kidney, HeLa, and chick embryo cells. Cytopathogenicity, produced by vaccinia in 24–48 hours and somewhat more slowly by variola, is characterized by focal cell degeneration and the formation of syncytial giant cells (Fig. 70). Two to six hours after infection with large doses of poxviruses a toxic cytopathogenic effect may be observed, characterized by cell rounding, which is not due to the production of infective virus. It has been suggested that this type of cytopathogenic effect is due to an incomplete cycle of replication, although it can also be produced by inactivated virus.

In stained preparations of infected tissue culture cells, typical eosinophilic intracytoplasmic inclusion bodies may be seen which consist of aggregations of virus particles embedded in a matrix of cellular origin.

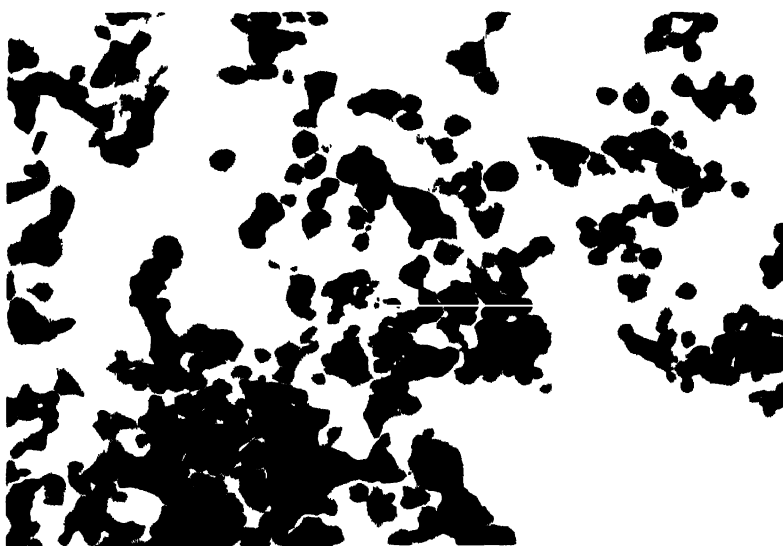
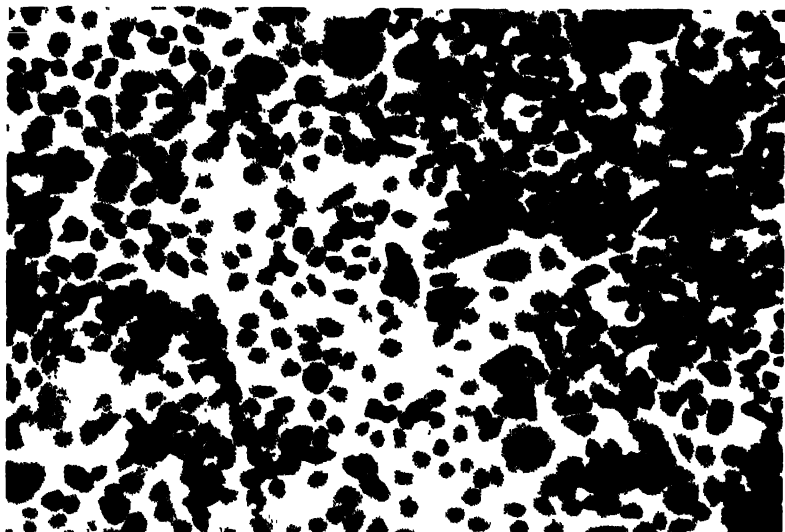


Fig. 70. Cytopathogenic effects of vaccinia virus in HeLa cells, stained with haematoxylin and eosin.

(a) Normal HeLa cells

(b) HeLa cells infected with vaccinia virus.

CHAPTER 27

Poxvirus Infections

II. *Poxvirus variolae*—Smallpox

Smallpox is an acute exanthematous disease of viral origin which, although no longer prevalent in this country and Europe, remains endemic and epidemic in certain parts of Asia, Africa, and South America. Though known for thousands of years in the Far East, it was not until the seventeenth century that the disease became widespread in England. From that time until the middle of the nineteenth century, smallpox was one of the most important causes of death in this country. Subsequently, the disease became less prevalent and was eventually eliminated with the aid of Jennerian vaccination.

Clinical Features

(a) *Variola major*

(i) *Toxaemic phase*

After an incubation period of 12 days, or less commonly 7-16 days, the acute onset of pyrexia, headache, malaise, prostration, vomiting, backache, and limb pains heralds the onset of smallpox. This phase, which lasts 3-4 days, is referred to as the initial, pre-eruptive phase, or toxaemic phase, in which the typical focal rash of smallpox is not yet present. Rarely, a prodromal erythematous or petechial rash, often marked in the inguinal or axillary regions, makes its appearance. Leucopenia and relative lymphocytosis are characteristic of the blood picture at this stage.

(ii) *Eruptive phase*

Three to four days after the onset of the toxaemic phase, a fall in the patient's temperature, the eruption of a typical focal rash, and some

clinical improvement, mark the beginning of the eruptive phase. The rash appears on the face, arms, palms of the hands, and upper part of the back, and then on the legs and plantar surfaces of the feet. Usually fully distributed within 24 hours, several days may be required before the characteristic centrifugal distribution of the rash is complete. Typically, the lesions are least numerous on the trunk, thighs, and upper arms, and closely packed on those areas of skin subject to pressure or irritation by rings or tight clothing.

Commencing as macules, the focal lesions of the rash rapidly become papular and, within a day or two, vesicular. The vesicles develop into pustules by the fifth or sixth day of the rash, when there is a secondary rise of temperature proportional to the severity of the disease. In the pustular phase, which is not usually due to secondary bacterial infection but is a natural development in the course of the disease, the face may become swollen and oedematous and constitutional symptoms may be severe, sometimes leading to delirium and coma. In benign cases, polymorphonuclear leucocytosis replaces the leucopenic blood picture at this stage. Finally, about 8 days after the appearance of the rash, the pustules begin to dry up and form scabs, which separate after 3-6 weeks.

Variola major may occur in varying grades of clinical severity and as many as nine different clinical types have been analysed by Dixon. Presumably, the degree of severity depends on the virulence of the organism and the resistance of the host, of which the latter may be modified by natural immunity, a previous attack of the disease, or by previous vaccination.

(iii) *Clinical types*

Fulminating type. This type is the result of an overwhelming infection and is nearly always fatal within 3-4 days of onset, before the focal rash appears. If the patient survives more than 48 hours, haemorrhages appear in the skin and bleeding may occur from any or all the orifices of the body.

Malignant type. The toxæmic phase may not be unduly severe in malignant smallpox, but the focal rash is accompanied by generalized facial erythema which gives way to diffuse confluent vesiculation; extensive lesions in the mucous membranes of the mouth and

upper respiratory tract lead to dysphagia, and further weakening of the patient's condition. The secondary fever is particularly marked, and ecchymoses and haemorrhages from various orifices hasten the patient's deterioration. The generalized vesicular rash covers large areas of the body surface, and if confluent denotes a poor prognosis in which a mortality rate of about 70% may be expected. The prognosis is better if the rash is semi-confluent; under these conditions the mortality rate is about 25%. When recovery ensues, a crisis occurs about the fourteenth or fifteenth day.

Benign type. In spite of a well developed focal eruption there is much less toxæmia and no tendency to haemorrhage in the eruptive phase of benign smallpox. Nevertheless, a mortality rate of about 20% may be expected if the rash is confluent, and 10% if semi-confluent; when the rash is less widespread, the mortality may be as low as 2%.

Mild type. Mild cases are characterized by the paucity of focal lesions which may number less than 100, and in the mildest cases the toxæmic phase is not followed by any rash at all, variola sine eruptione. Mild smallpox is most likely to occur in those protected by previous vaccination.

(b) Variola minor (alastrim)

Variola minor is a mild form of smallpox clinically indistinguishable from mild cases of variola major. The virus of variola minor, although antigenically identical with that of variola major, is recognized as a stable variant because of its permanent attenuated character, and its inability to form pocks on the chorioallantoic membrane when incubated at 38·2°C.

The origin of variola minor is unknown. It was first imported into this country in 1919 and remained endemic here until 1934. Between 1923 and 1934 more than 80,000 cases were reported but the mortality was low.

Complications of Smallpox

(a) Secondary infection

Secondary bacterial infection of the skin lesions may occur and is not uncommon in tropical areas.

(b) Bronchopneumonia

Bronchopneumonia is always a hazard in the severely ill patient, particularly in the very young and the very old.

(c) Ocular complications

Lesions in the eye and cornea may lead to permanent scarring and blindness, particularly if there is secondary bacterial infection.

(d) Central nervous system

Encephalomyelitis may occur as a complication of both variola major and variola minor, and is clinically and pathologically the same as that following vaccination. It occurs about the tenth day after onset of the disease and is characterized by increasing drowsiness, speech disorders, and sometimes by flaccid or spastic paralyses. Post-mortem, perivascular demyelination in the central nervous system is prominent.

(e) Infection in utero

Abortion is a common sequel to smallpox in pregnant women. In the later stages of pregnancy, the foetus may acquire infection in utero and develop the disease at birth or a few days afterwards.

Pathology

The first changes which occur in the skin lesions are capillary dilatation, plasma cell infiltration of the papillary layer of the corium, and some epithelial proliferation. In the middle epithelial layers, some cells become swollen and vacuolated, and eventually degenerate. Interstitial oedema leads to the formation of vesicles in areas where epithelial cells have been destroyed. The roof of the vesicle is formed of the stretched upper layers of epithelial cells, and strands of reticulum loculate the vesicle itself. The base of the vesicle is formed by compression of the deeper epithelial cell layers, which sometimes give way so that the lesion extends into the corium. Varying degrees of haemorrhage may accompany these changes in the malignant types of the disease. The entry of polymorphonuclear leucocytes into the vesicle fluid converts the lesions into pustules,

which are not, as was previously thought, the result of secondary bacterial infection. Eventually the lesions dry up and scab over. Affected epithelial cells contain intracytoplasmic eosinophilic inclusions (Guanieri bodies) surrounded by a clear unstained halo, and virus particles can be recognized in the vesicle fluid.

Internally, the liver and spleen are always enlarged. Haemorrhagic ulceration of the mucous membranes of the mouth, pharynx, respiratory and alimentary tracts are found in the fulminating and malignant types of smallpox, and haemorrhages may be found in other organs.

Pathogenesis

The virus of smallpox is believed to gain entry via the upper respiratory tract and to multiply in the cells of the reticulo-endothelial system during the long incubation period. Two routes for the transfer of virus from the respiratory tract to the organs of the reticulo-endothelial system are possible. Virus may be transported from the site of implantation after phagocytosis by reticulo-endothelial cells. Alternatively, Fenner's studies in mice with ectromelia or mousepox suggest that initial multiplication of virus takes place at the site of implantation, and in the local lymphatic tissues, before release into the blood stream and distribution to the organs of the reticulo-endothelial system.

At the end of the incubation period, when sufficient virus has been produced in the organs of the reticulo-endothelial system, virus is discharged into the blood stream producing a secondary viraemia and clinical onset of symptoms. From the blood stream, virus is distributed to the target organs, namely the skin and mucous membranes, where the virus multiplies and produces the typical focal lesions. As the eruption develops, the patient's condition begins to improve, virus disappears from the blood, and neutralizing antibody appears in the circulation. Normally, isolation of virus from the blood is not possible after the first or second day of illness, but in the more severe types of illness viraemia is prolonged and the prognosis grave.

The secondary rise of temperature which occurs during the stage

of pustulation is probably due to the absorption of toxic products resulting from tissue necrosis.

Epidemiology

(a) Source of infection

Man is the only naturally susceptible host, in whom clinical, sub-clinical, and missed cases provide the sources of infection.

Patients are non-infective during the incubation period but become so with onset of clinical illness. Epidemiological investigations indicate that patients are most infective on third or fourth day of the disease when the rash appears. This may be due to the appearance of lesions in the mouth and upper respiratory tract, which ulcerate and cause gross contamination of the saliva and respiratory secretions.

In the later stages of the disease, rupture of vesicles and pustules, and shedding of scabs, may make the skin an element in dissemination of virus. It is unlikely that unruptured vesicles or pustules provide an important source for dissemination, but virus is present in scabs which remain infective after separation for considerable periods of time. Although scabs are not now regarded as an important source of infection, the patient must be isolated until all scabs have disappeared.

(b) Transmission of infection

(i) Direct

In the toxæmic or early eruptive phases of smallpox, the disease is spread by droplet infection. Infection may also be spread by direct contact with the patient, and to pathologists and morticians by contact with an infected corpse.

(ii) Indirect

The patient's skin and respiratory secretions may contaminate his clothes and bedlinen. When these are moved or shaken, virus is released into the air, in the form of infected dust particles, and may be inhaled by some unfortunate victim.

Smallpox virus is known to survive for periods of several months

in infected fabrics, and infection may be transferred to persons handling them, especially laundresses, chambermaids and members of the patient's family. In these cases, infection is probably acquired by inhalation of infected dust, and the risk can be lessened by the use of dampened materials. Some outbreaks of infection in mill-workers are believed to have been imported in contaminated bales of cotton and spread by inhalation of infected dust.

Occasionally, contact with infected fomites has been suspected in the spread of infection, but food has never been incriminated. In tropical countries, flies have been thought to act as mechanical vectors of infection after settling on the lesions of a smallpox patient.

(c) Distribution

Smallpox is endemic in Asia, Africa, and South America, but not in this country or Europe. Hence, outbreaks in the United Kingdom are now always due to importation of infection from an endemic area. The risk of importation has increased in recent years with the extension of popular air travel. Previously, the duration of a sea journey from endemic areas to the United Kingdom exceeded the incubation period of smallpox, any cases of smallpox on board were therefore brought to light before the ship's arrival. Nowadays, air travel is so rapid that patients may arrive from endemic areas in the course of the incubation period and be missed on entry.

The last outbreak of variola major in this country occurred in 1962; there were then six instances of importation from Pakistan which resulted in 66 cases and 26 deaths. Since then, no outbreaks of smallpox were reported until 1966 when 44 cases of variola minor, originating from an unknown source, occurred.

Prophylaxis

(a) Routine control

(1) Endemic areas

Where smallpox is endemic, eradication of the disease depends on mass vaccination and revaccination programmes. Efficient public health services to control the spread of the disease when and where it occurs are also necessary.

(u) Non-endemic areas

Mass vaccination for prophylaxis in non-endemic countries, where the incidence of smallpox is very low or non-existent, is a controversial issue which is discussed in the next chapter. Although indiscriminate vaccination is considered by many to be unnecessary, all agree that persons exposed to a higher than normal risk should be vaccinated and revaccinated every 3 years. Those in the high risk category include medical and public health personnel, port and airport workers, the armed forces, morticians, and their families.

Essentially, control of smallpox in non-endemic areas depends on preventing its importation from regions where it is endemic. Often, patients travelling by air arrive in the course of the incubation period and go unrecognized. For this reason, it is necessary to ensure that all persons arriving from endemic areas have been recently vaccinated, and are in possession of a valid International Certificate of Vaccination. If vaccination is primary, it must have been performed not less than 8 days prior to travel. Some countries require certificates from all persons arriving, whether from endemic areas or not.

Facilities for early and accurate diagnosis of infected persons arriving at ports and airports, and the ready availability of public health services to control an outbreak as soon as it is recognized, are further essential requirements for the control of smallpox.

(b) Control of an outbreak*(1) The patient*

All cases and suspected cases of smallpox must be notified immediately to the Medical Officer of Health and the source of infection sought. Patients are isolated until all the scabs have disappeared, preferably in a special Smallpox Hospital provided for the purpose. The patient's discharges and articles with which he has been in contact must be burnt or sterilized, and at the termination of the illness the patient's bedding is steam sterilized and his room washed down and disinfected with formalin.

(ii) Contacts

Close contacts are traced as soon as possible, vaccinated, given a

course of methisazone, and kept under surveillance for 16 days. Any contact developing suspicious symptoms is immediately isolated.

(iii) Vaccination

Mass vaccination, although often demanded, is usually uncalled for; it places a strain on the public health personnel and services which prevents them from dealing efficiently with those at greatest risk. Moreover, mass vaccination carries with it the risk of post-vaccinal complications, which may give rise to more ill-health than the outbreak of smallpox itself. Mass vaccination is only indicated when a number of cases from an unknown source are occurring in a community and the situation cannot be controlled. In other situations, contacts are vaccinated according to the following scheme of priority, and revaccinated if no reaction is visible on the third day of inspection.

Class I contacts. Members of the same family, persons who have been in contact with the patient or his home environment, and persons who worked in close proximity to the patient during the early stages of the illness. These are immediately vaccinated, given a course of methisazone, and examined daily for 16 days. If vaccination is performed late in the incubation period, the use of gamma-globulin as well as methisazone may be considered.

Class II contacts. Persons who have been in the same house or workplace but have not been in close proximity to the patient or his immediate environment. These are immediately vaccinated and given a course of methisazone.

Class III contacts. Persons from the same locality who have had no contact with the patient, his home, his immediate environment or with any Class I or Class II contacts. In general, vaccination of Class III contacts is not important and every effort should be concentrated on Class I or Class II contacts.

Medical and Public Health personnel. Ideally, doctors, nurses, and public health personnel will have been vaccinated within the previous 3 years. If not, they should be vaccinated without delay.

Laboratory Diagnosis

Speedy diagnosis is an important element in the control of smallpox; usually this can be accomplished on clinical grounds and confirma-

tion is provided by laboratory investigations. When the diagnosis is in doubt, laboratory investigations are essential. Ideally, isolation of the virus or evidence of its presence in specimens from the patient are required for diagnosis. Less satisfactory is the demonstration of antibody to variola in the patient's serum, although this may be the only evidence available for retrospective diagnosis or for the diagnosis of variola sine eruptione.

(a) Direct microscopy

(i) Light microscopy

Variola virus particles, suitably stained, are just visible in the light microscope. Smears of aspirated vesicle fluid, or scrapings made from the base of lesions in the maculopapular or vesicular stage, may be stained with Gutstein's methyl violet or Paschen's stain and examined for virus particles. If present, they provide rapid provisional confirmation of the diagnosis and exclude varicella and herpes simplex in which few, if any, particles would be seen; of course, the viruses of variola, vaccinia, and cowpox cannot be differentiated by this method. If no virus particles are found, the diagnosis of smallpox is not excluded and further tests must be performed.

(ii) Electron microscopy

The now almost routine availability of electron microscopes in virological laboratories, and the introduction of negative staining techniques, has made electron microscopic examination of lesion material an important and necessary diagnostic procedure. Variola virus can be recognized in material from lesions at any stage of development, and, although morphologically identical to the viruses of vaccinia and cowpox, variola virus is distinguishable, on morphological grounds, from the viruses of herpes simplex and varicella. In a recent outbreak, electron microscopic confirmation of diagnoses was available within 10 minutes of the receipt of specimens.

(b) Demonstration of variola antigen in lesion material

Several tests are employed for the demonstration of specific antigen in lesion material, none however distinguish between the antigens of variola, vaccinia, or cowpox.

(i) Fluorescence microscopy

Material obtained from lesions at any stage of development may be used as antigen, fixed on a slide, and stained with fluorescent antibody by direct or indirect methods. Stained smears, with appropriate controls, are then examined in the light microscope using ultra-violet light.

(ii) Gel-precipitation test

Variola antigen in lesion material may be identified in agar gel precipitation tests, and in many cases a positive result may be obtained in a few hours. Concentrated suspensions of material taken from lesions at any stage of development are tested with antivaccinal or smallpox convalescent serum, by the micro-gel diffusion technique on microscope slides. With strong reagents, a line of precipitate may appear after 2 hours incubation in a humid atmosphere at room temperature, and may be complete after 5–6 hours. Materials from lesions in the early and late stages of development may fail to precipitate, so that negative results do not exclude the diagnosis of smallpox and indicate further tests. Of course, appropriate antigen and normal serum controls are included in the test which was introduced by Dumbell and Nizamuddin.

(iii) Complement-fixation

Variola antigen in material taken from lesions at any stage of development may be identified by complement-fixation tests with appropriate antisera. Although longer and more complicated to perform than the gel-precipitation test, the complement-fixation reaction is much more sensitive.

(c) Virus isolation

Virus isolation is the most sensitive and decisive test for the diagnosis of smallpox; it is necessary to confirm the results of other tests which give a more rapid answer, and for complete identification of the causative organism.

In the toxæmic phase of the disease, virus may sometimes be isolated from the blood, particularly if the 'buffy coat' is used as the inoculum. This is most likely in the fulminating and malignant types of infection; in milder cases virus is isolated from the blood

less frequently and may only be present in the blood stream for a few hours at the onset of infection. If virus is isolated from the blood after the second day of the disease, a severe viraemia, poor host resistance, and a very grave prognosis, are indicated.

In the early stages of the pre-eruptive phase of the disease, virus may be isolated from the saliva, but more usually virus is isolated from the focal lesions at any stage of development.

The culture techniques used are:

(1) Chick embryos

Blood specimens or lesion material, to which antibiotics have been added, are inoculated onto the chorioallantoic membranes of 11–12-day-old chick embryos. Typical pock lesions are formed after 48–72 hours (Fig. 4b), and may be distinguished by the experienced virologist from those produced by the viruses of vaccinia and herpes virus. The failure of varicella virus to produce lesions on the chorioallantoic membrane makes pock production an important diagnostic criterion in differentiating the viruses of smallpox and chickenpox. An added advantage of chorioallantoic membrane inoculation is the possibility of differentiating the viruses of variola major and variola minor. Nizamuddin and Dumbell observed that the virus of variola minor fails to produce pocks on the chorioallantoic membrane when eggs are incubated at 38.2°C, whereas pock production by the virus of variola major is unaffected.

The identity of the lesions on the chorioallantoic membrane is finally confirmed by serological tests. Extracts of the infected membranes may be tested by complement-fixation, haemagglutination-inhibition, agar-gel precipitation, or neutralization, but none of these tests are capable of differentiating between the viruses of variola, vaccinia, or cowpox.

(2) Tissue culture

Human and monkey kidney cells, as well as HeLa and other human cell lines, may be used for virus isolation. Cytopathogenic effects, which are at first focal, appear in 2–4 days and later become generalized. Although cytopathogenic effects are produced more slowly by variola virus than by vaccinia and cowpox viruses, virus

growth may be recognized 1–2 days after inoculation by the appearance of intracytoplasmic eosinophilic inclusion bodies in stained preparations, and by haemadsorption and fluorescent antibody techniques.

(d) Serological tests

A rise in antibody titre of four-fold, or more, occurring between the acute and convalescent phases of the disease, is diagnostic. The antibody produced does not distinguish between variola and vaccinia, and either antigen may be used for serological tests. The serological techniques employed are haemagglutination-inhibition, complement-fixation, neutralization, and precipitation. Neutralizing and haemagglutination-inhibiting antibodies appear during the first week of the clinical illness but complement-fixing antibodies appear later, about the tenth day of illness in the unvaccinated and the sixth day in the vaccinated. Precipitating antibody appears after the first week of the disease and is not usually present in post-vaccination sera.

Often, only one serum specimen is available which makes interpretation of the results more difficult. In those not recently vaccinated, a haemagglutination-inhibition titre of 1:160 in a single serum specimen, taken during the eruptive phase of the disease, or a complement-fixation titre of 1:20 in a specimen taken after the tenth day of illness, is considered significant. Neutralizing antibodies persist for some years after vaccination, and only high titres of neutralizing antibody in a single specimen are therefore considered significant in the diagnosis of smallpox.

Treatment

The treatment of smallpox remains symptomatic. Methisazone (N-methylisatin- β -thiosemicarbazone), although shown to have an antiviral effect against variola virus in experimental systems, and to be beneficial in preventing infection in man, has little or no therapeutic effect in smallpox.

CHAPTER 28

Poxvirus Infections

III. *Poxvirus vaccinia*

Vaccination against Smallpox

The practice of variolation, in which immunity to smallpox is produced by inoculating smallpox material and inducing a mild attack, was well known in China and the Far East many centuries ago. Lady Mary Wortley Montague, the wife of a British Ambassador to Turkey, introduced the practice into England in 1721 and, in spite of occasional deaths from smallpox, it quickly became established.

About this time, Jenner (1749-1823) became acquainted with the popular observation that cowpox, which sometimes spread from the cow's udder to the hands of a milker, rendered those infected immune to a subsequent attack of smallpox. On 14 May 1796, Jenner carried out an historic experiment in which he inoculated an 8-year-old boy, James Phipps, with material obtained from a cowpox lesion on the hand of a milkmaid, Sarah Nelmes. Seven weeks later, the boy was found to be immune to challenge with smallpox material. After some early vicissitudes, the safer practice of vaccination, as it came to be called, soon replaced the more dangerous practice of variolation which became illegal in 1840. Vaccination became compulsory in 1853 and remained so until 1947.

Vaccine Lymph

Originally, vaccine lymph was obtained from cowpox lesions on the udders of diseased cows, but, with few exceptions, the vaccine lymph used between 1798 and 1881 was supplied by human vaccinia lesions. Because of the gradual loss of potency following arm-

to-arm passage and the dangers of transferring syphilis and other infectious diseases, a Royal Commission recommended the use of vaccine lymph produced in calves, and this source of supply became general in 1899.

The origins of most vaccine strains now in use are shrouded in mystery; the one currently issued by the Lister Institute in the United Kingdom was received from Cologne in 1907, and is reputedly a calf-adapted strain of variola derived from a Prussian soldier who fell victim to smallpox in the Franco-Prussian war of 1870.

Preparation of Vaccine Lymph

Although often referred to as calf lymph, the product of the Lister Institute is now prepared in sheep, and its potency, which may be lost by repeated passage in the same animal, is maintained by alternate passage in sheep and rabbits. Before inoculation, sheep are held in quarantine for 2 weeks and the skin of the abdomen and inner side of the thighs is then shaved, thoroughly washed, and rinsed in sterile distilled water. The skin is now scarified and the virus inoculum is applied. Four to five days later, the animal is sacrificed and, after post-mortem examination has ensured that the animal is free of disease, the skin is thoroughly washed and dried before the vesicle fluid is collected by curettage.

The crude vaccine pulp is treated with twice its weight of 1% phenol and the mixture allowed to stand at 22°C for 48 hours to reduce bacterial contamination. The concentration of phenol is now reduced to 0.4% by the addition of glycerine, and the pulp is sieved and stored at -10°C for 4-6 weeks. Before issue, the vaccine must be demonstrably free of bacterial pathogens and its bacterial count must be less than 5 per mg of lymph. In addition, its potency must compare satisfactorily with a standard vaccine in its ability to produce lesions in the rabbit skin and pocks on the chorioallantoic membrane. A satisfactory vaccine should contain 10^8 PFU/ml, and its field performance in primary and revaccination should be observed from time to time.

Recent Developments

Although sterile vaccine can now easily be prepared in chick embryos, egg produced vaccine has not replaced animal vaccine lymph. It is less thermostable and no more efficient than the well-tried animal vaccine lymph. Vaccines grown in tissue culture are also, at present, under investigation.

The most notable advance in the last decade has been the introduction, by Collier, of a satisfactory freeze-dried vaccine preparation which is more stable than liquid vaccine at higher temperatures. This is an important advantage in tropical climates, and other situations where low temperature storage is a problem.

Animal vaccine lymph used for vaccination is one of the safest immunizing agents at present in use, but the occasional complications which ensue have stimulated attempts to produce vaccines inactivated by formalin or ultraviolet light. Antibody responses are produced by these vaccines, but their efficiency in the face of smallpox infection, although not yet fully evaluated, is almost certainly less than that of orthodox live vaccine; recipients of inactivated vaccine challenged a few weeks later with live vaccine produced an accelerated reaction. Inactivated vaccine may prove to be useful for vaccinating those with conditions that predispose to post-vaccinal complications, especially if it is used to produce partial immunity before subsequent vaccination with live vaccine.

Technique of Vaccination

Almost any part of the body may be, and is, used for vaccination, but the best site is on the upper arm at the level of the insertion of the deltoid, behind the midline. The lower limb, popular with the female sex for aesthetic reasons, produces more severe lesions and lymphadenitis than the arm, and is not recommended.

Ether or acetone may be used to clean the skin, if time is allowed for complete evaporation, but gentle washing with soap and water is eminently satisfactory. Indeed, if the skin is clean no preparatory cleansing is necessary. At the chosen site, a drop of vaccine lymph is placed on the skin and inoculated by the multiple pressure

technique. Pressure is applied to the side and point of a Hagedorn needle held parallel to the skin surface, which is held under slight tension (Fig. 71). For primary vaccination, about 10 such pressures are applied in an area about $\frac{1}{8}$ inch in diameter, but 30 pressures are

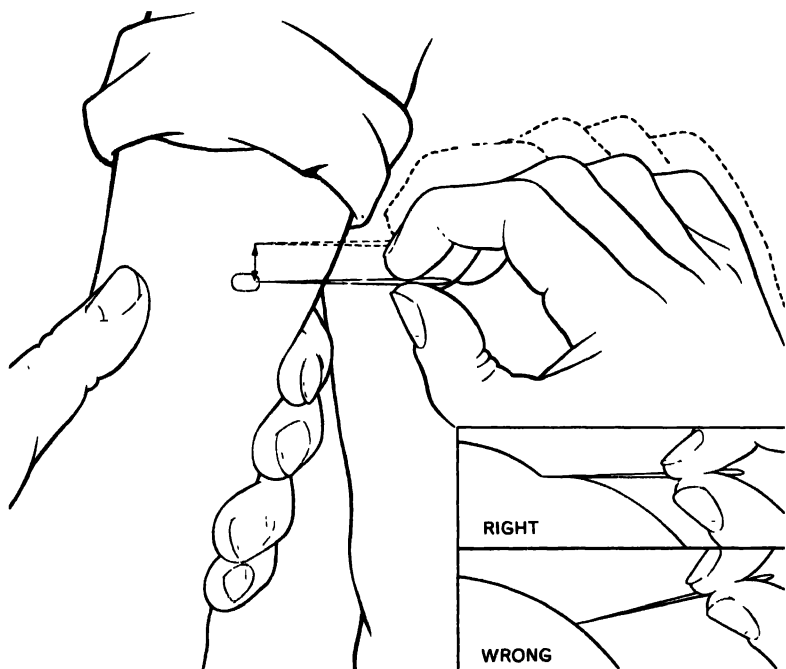


Fig. 71. Technique of smallpox vaccination. The multiple pressure method of vaccination showing the up and down motion of the side of the needle [from Ministry of Health Memorandum on Vaccination against Smallpox, 1962 (by permission of the Controller, H.M.S.O.)]

recommended for revaccination. Breaching of the epidermal layers, only, is required and any form of deep penetration which involves the corium or draws blood is to be avoided (Fig. 72). Excess vaccine is allowed to dry or is wiped clean with sterile gauze; no dressing is required although a light non-occlusive sterile dressing may be used if preferred. Until a scab is formed, the vaccination should be kept dry to prevent the spread of virus.

If the scratch technique is used, a scratch about $\frac{1}{8}$ inch long is made with a cutting needle through a drop of vaccine lymph. A breach in the epidermal layers is all that is required and the drawing of blood

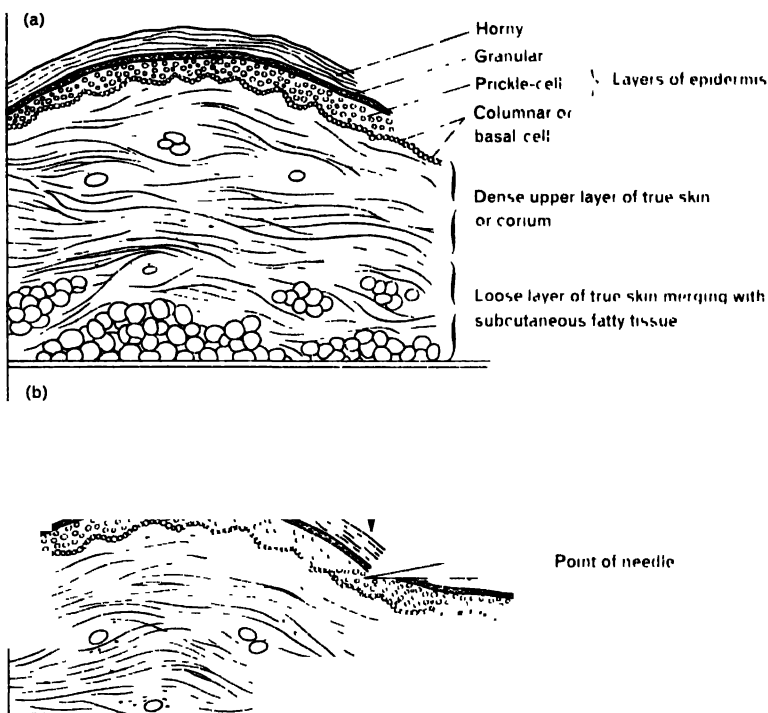


Fig. 72.

(a) Diagrammatic section of the skin on the arm

(b) The needle, held parallel to the skin, being pressed against the skin surface and entering it slightly

[From Ministry of Health Memorandum on Vaccination against Smallpox, 1962 (H.M.S.O), by permission of the Controller]

is unnecessary. Traumatic incisions which used to be practised in order to produce severe lesions, are both undesirable and superfluous.

Development of the Lesion

(a) Primary vaccination

In those who are susceptible and have not received any previous vaccination, a papule appears on the third day which assumes the form of a vesicle surrounded by a zone of erythema on the fifth day. Originally, bluish-white in colour, the vesicle becomes yellow and pustular by the eighth or ninth day. After a day or two, the pustule begins to dry up and a scab is formed on the eleventh or twelfth day (Fig. 73); the scab is shed about the twenty-first day and leaves a permanent scar. By the time the lesion reaches the pustular stage the patient may begin to feel unwell with pyrexia, malaise and painful axillary lymphadenitis. These symptoms are associated with a transient viraemia, and there may be some splenic enlargement.

(b) Revaccination

(1) *Accelerated reaction*

If some years have elapsed since primary vaccination, immunity to vaccinia virus, and presumably also to variola, may be so low that a primary vaccination reaction is induced in response to revaccination. More usually, revaccination gives rise to the accelerated response in which the development and maturation of the lesion is speeded up by several days. The papule appears within 2 days, becomes a vesicle on the third or fourth day and a pustule on the fifth or sixth day, scab formation is usually complete by the seventh or eighth day.

(11) *Immediate reaction*

Local reactions in which raised irritating zones of erythema, without vesiculation, are produced within 48 hours, and which regress in a few days, are more difficult to assess. They may signify immunity or merely represent an allergic response to protein material present in the vaccine lymph. Significantly, immediate reactions are easily elicited by inactivated vaccine lymph in those sensitized by previous vaccination. Because allergic responses of this type cannot be differentiated, with any certainty, from those due to immunity, the W.H.O. Expert Committee on Smallpox recommends that imme-

diate reactions be now termed 'equivocal reactions'. It further recommends the term 'Major reaction' for any vesicular or pustular lesion, scab, or ulcer which becomes evident within 6-8 days of vaccination or revaccination.

Because an 'equivocal reaction' may be produced by impotent vaccine or by faulty technique, persons reacting in this way should



Fig. 73. Reaction to primary vaccination.
(a) Pustule.
(b) Scab formation.

be revaccinated to ensure the potency of the vaccine. If the reaction remains 'equivocal' immunity may be inferred but cannot be guaranteed.

(iii) No reaction

No reaction in response to vaccination is not evidence of complete immunity, as is sometimes assumed. Faulty technique or loss of vaccine potency are more likely explanations, and in those who have not been previously vaccinated, the only ones. Vaccination of these individuals must be repeated, twice if necessary, and preferably at an alternative site. If vaccination continues to be unsuccessful, it should be repeated after an interval of 1 or 2 months, when for no apparent reason it is often successful. Occasionally, individuals are encountered in whom repeated vaccination attempts are unsuccessful, but they are not immune. Indeed, cases are on record of individuals in whom vaccination was repeatedly unsuccessful but who succumbed to smallpox shortly afterwards.

Duration of Immunity

Immunity to smallpox after vaccination is not as long lasting as was previously thought, and is much less effective than that produced by a natural attack of smallpox. The actual duration of immunity is variable, but it probably persists at a high level for about 5 years; subsequently, it declines and a state of partial immunity may be maintained for as long as 20 years. Partial immunity is not always sufficient to prevent infection, but usually suffices to reduce the severity of the disease and prevent a fatal outcome. The maintenance of a high level of immunity in those at special risk requires revaccination every 3 years, and International Certificates of Vaccination remain valid for 3 years only.

Complications

(a) Post-vaccinal encephalitis

Encephalitis which develops 10–14 days after vaccination was not recognized as a clinical entity in this country until 1912. The condi-

tion became more prominent in the 1920s and as many as 90 cases were reported in the period 1926–28. Since then, the incidence has declined and only three cases were reported in 1965, one of which was fatal.

(i) *Clinical features*

The condition begins acutely about 10–14 days after vaccination and is characterized by pyrexia, headache, vomiting, drowsiness, and occasionally spastic or flaccid paralyses or signs of meningeal irritation. The overall mortality of this complication is about 50%. Although no abnormality is found at the site of vaccination, perivascular demyelination and perivascular cuffing with inflammatory cells and microglia are characteristic lesions in the central nervous system.

(ii) *Risk*

The risk from post-vaccinal encephalitis in the United Kingdom was previously highest in young adults receiving vaccination for the first time, and lowest in infants under 1 year of age. Recently a change in this pattern of behaviour has been noted; from data acquired during the period 1951–57 Wynne Griffith estimates the mortality from post-vaccinal encephalitis after primary vaccination to be highest in infants under the age of 1 year. In this age group, there were 15.4 cases and 9.0 deaths per million of those vaccinated compared with 26.5 cases and 5.3 deaths per million of those vaccinated for the first time after the age of 15 years. The risk of post-vaccinal encephalitis after revaccination although less is still present. During the years 1951–57, there were seven cases and two deaths per million of those revaccinated after the age of 15 years. Of the three cases reported in 1965, two occurred in revaccinated adults.

(iii) *Aetiology*

The number of isolations of vaccinia virus from the brain or cerebrospinal fluid of cases of post-vaccinal encephalitis have been too few for the virus to be of aetiological significance. Nor has any other virus, activated as the result of vaccination, been isolated. Because the pathological lesions are similar to those of encephalitis

following measles and other infectious diseases, and to the lesions produced in animals by immunological experiments, many consider the condition to have an allergic origin. It is significant that demyelinating encephalopathy may be produced experimentally by injection of nerve tissue suspensions, especially in the presence of adjuvants, and by injections of vaccinia virus in the presence of phosphatides of neural origin.

(b) Generalized vaccinia

Very occasionally, a generalized eruption develops about 6–14 days after vaccination, and the lesions rapidly pass through the recognized papular, macular, vesicular, and scabbing stages. Although the reason for this generalization is not known, it has been suggested that a delayed antibody response may be responsible.

(c) Eczema vaccinatum

When generalized vaccinia occurs in those with infantile eczema, and sometimes in adults with atopic eczema, a serious and sometimes fatal condition known as eczema vaccinatum develops. The condition, originally described as Kaposi's varicelliform eruption, is manifested by a generalized eruption which is particularly severe in the areas of eczematous skin, by systemic symptoms, and by generalized lymphadenitis. Sometimes, even though the eczema itself is inactive, the eruption is particularly severe in the abnormal areas of the skin.

Many of these cases, if not the majority, result from vaccinal infection accidentally acquired from a contact who has been recently vaccinated. Often the contact is familial, and infants or adults with eczema should be kept away from such contacts for at least 21 days after they have been vaccinated. Hyperimmune antivaccinal γ -globulin has been recommended for both prophylaxis and therapy of these cases.

(d) Vaccinia gangrenosa

Very rarely, the vaccinal lesion progresses to form a deep destructive ulcer which shows no evidence of healing, and which is surrounded by satellite vesicles. The condition, which may spread to

other parts of the body via the blood stream and which is highly fatal, occurs most often in those suffering from hypo- or agammaglobulinaemia. Administration of antivaccinal γ -globulin, and methisazone (isatin β -thiosemicarbazone), has been effective in treatment. Sometimes, however, amputation of the affected limb has to be resorted to.

(e) Autogenous vaccination

Additional lesions may develop in other parts of the body if virus is transferred from the site of vaccination by injudicious handling of the original lesion.

(f) Heterogenous vaccination

In the absence of due care and attention, virus may be transferred from the site of vaccination and infect a contact. Eczematous individuals are particularly susceptible to heterogenous vaccination.

(g) Sepsis

The once not uncommon complication of staphylococcal and streptococcal infection has now been eliminated by the use of sterile instruments and vaccine lymph free of demonstrable pathogens. For the same reasons post-vaccinal tetanus is now also a rarity, but occlusive dressings which may provide anaerobic conditions should be avoided.

Contraindications to vaccination

Routine vaccination of those known to be susceptible to complications should be avoided; included in this category are those with dysgammaglobulinaemia, eczema, leukaemia, skin sepsis, malignant disease, and those receiving cortisone therapy. Where there has been close contact with smallpox, other considerations apply and there is no absolute contraindication to vaccination, but antivaccinal γ -globulin should be administered at the same time. The prophylactic use of antivaccinal γ -globulin in combination with methisazone may now be considered in preference to vaccination in these circumstances.

Vaccination in the first trimester of pregnancy does not produce congenital defects, but MacArthur has shown that the incidence of still-birth is higher in mothers vaccinated during pregnancy than in those unvaccinated. Moreover, a few cases of generalized vaccinia in stillborn and live infants delivered of mothers vaccinated in the first and second trimesters have been reported. It would thus seem imprudent to vaccinate women at any time during pregnancy, except in the face of a real risk of smallpox.

As a general rule, a period of three weeks is allowed to elapse between vaccination against smallpox and any other immunization procedure. Where yellow fever and smallpox vaccinations are required together, the former should be given at least 4 days before the latter.

Routine Vaccination

From 1853 until 1947 infant vaccination was compulsory in the United Kingdom, and there is no doubt that this policy played an important part in limiting the morbidity, mortality, and spread of smallpox, and in achieving its final eradication. Nevertheless, our attitude to infant vaccination and to mass vaccination in general has been subject to reappraisal in recent years. The risk of mortality from smallpox is now so low that the risk of death from vaccination has begun to outweigh that of the disease it seeks to prevent. In 1965, the most recent year for which figures are available, there were no deaths from smallpox, but three children, all under the age of 2 years, died after primary vaccination, which was performed in 378,822 children under the age of 4 years.

The concern aroused by these figures is increased by the realization that immunity to smallpox produced by vaccination is not, as was previously thought, life long. It begins to wane after the first few years and, in the absence of revaccination, becomes minimal or non-existent after 20 years. True, vaccination at some time in life reduces the risk of death from smallpox, but the actual degree of reduction depends on the interval since vaccination was last performed. Clearly, to maintain a high degree of herd immunity, and to prevent the spread of smallpox in the community, vaccination in

infancy without revaccination at 5 or 10 year intervals is not sufficiently adequate. Wynne-Griffith, quoted by Dixon, calculates that compulsory vaccination at 10 year intervals would require 4·5 million vaccinations a year, entailing a risk of 30 fatal complications per million and a cost which would be quite uneconomic. In non-endemic areas where the risk of smallpox is small, and where outbreaks of imported smallpox can be adequately contained by public health control, this would be a wholly unreasonable price to pay.

The long held assumption that primary vaccination before the age of 12 months carries less risk than primary vaccination in late childhood, adolescence, or young adult life has recently been challenged in the light of fresh data. Wynne-Griffith's figures for the United Kingdom in the years 1951-58, quoted by Dixon, show the mortality from primary vaccination in those under the age of 1 year to be 15·4 per million, compared with mortality rates of 5·3 and 1·9 per million in those primarily vaccinated after the age of 15 years and in the 1-4 year age group, respectively. The optimum age for primary vaccination is now considered to be in the second year of life.

It is generally assumed, and rightly, that the risks of revaccination are less than those following primary vaccination; they are not, however, negligible. Wynne-Griffith's figures for 1951-58 show that revaccination after the age of 15 years was responsible for a mortality of 3 per million whereas the mortality from primary vaccination in the same age group was 5·3 per million. In the event of revaccination, which is usually required at some time during life if only for the purposes of travel, statistics show that those primarily vaccinated between the ages of 1 and 4 years have a decided advantage with respect to morbidity and mortality from complications. This fact provides the validity for recommending primary vaccination in this age group. The presumed more rapid immune response to revaccination, especially if required during the incubation period of smallpox, is an added advantage, although Dixon is doubtful of its benefit because no beneficial effects have been revealed in mortality statistics.

For the reasons stated above, there is now a large body of opinion among experts which considers the routine vaccination of small

children in countries free of smallpox to be unnecessary. This is not yet official policy in the United Kingdom where primary vaccination in the second year of life, and revaccination at 8–12 years of age, is recommended.

Of course, where smallpox is endemic and the risk of death from smallpox is significantly higher than that from vaccination, mass vaccination and revaccination programmes are essential for the eradication of smallpox.

In non-endemic countries, vaccination and routine revaccination is required for those at special risk. These include doctors, public health workers, hospital personnel, port and airport workers, and their families. H.M. forces and travellers to and from endemic areas are also required to be vaccinated and to be in possession of an International Certificate of Vaccination, which remains valid for 3 years.

CHAPTER 29

Poxvirus Infections

IV. *Molluscovirus hominis* Molluscum Contagiosum

Molluscum contagiosum is a nodular form of skin disease caused by a virus belonging to the Family Poxviridae.

Properties of the Virus

(a) Morphology

In morphology, the virus of molluscum contagiosum resembles other members of the poxvirus group; it is a brick-shaped particle measuring approximately 230×320 m μ which consists of a dense central nucleoid, believed to contain DNA, and a limiting double membrane.

(b) Antigenic composition

A soluble, heat labile complement-fixing antigen has been prepared from infected skin lesions, which is antigenically distinct from those produced by other members of the poxvirus group.

(c) Cultivation

The virus is not cultivable in experimental animals or in chick embryos. Some cytopathogenic effects have been detected in HeLa and some primary human cell cultures, but they are not serially transmissible and probably represent a toxic effect rather than virus replication.

Clinical Features

Molluscum contagiosum usually occurs in children and young adults, and is characterized by the formation of pink or pearly-white wart-like nodules in the skin. The lesions are distributed over the various parts of the body, except the palms of the hands and soles of the feet, and usually persist for several months. Suppuration and breakdown of the lesions may occur after secondary bacterial infection.

Pathology

Proliferation, hyperplasia, thickening, and degeneration of the epidermis, characterize the lesions in which infected epithelial cells become greatly enlarged and develop large intracytoplasmic, eosinophilic, hyaline inclusion bodies, about 20–30 μ in diameter, which displace the nucleus to one side. The inclusions consist of masses of virus particles enclosed in a loculated protein matrix, which if digested by trypsin leaves the virus particles enmeshed in a gelatinous mass.

Epidemiology

Man is the only known susceptible host, from whom virus is spread by direct contact and by infected fomites. Handling of infected sports equipment and clothing have been reported to be responsible for some cases.

Treatment

There is no specific antiviral treatment.

CHAPTER 30

Herpesvirus Infections

I. *Herpesvirus hominis* Herpes Simplex

The genus *Herpesvirus* includes three medically important species; *Herpesvirus hominis*, the virus of herpes simplex; *Herpesvirus varicellae*, the virus of chickenpox and herpes zoster; and *Herpesvirus simiae*, the B virus of monkeys which occasionally attacks man. Four common properties characterize these viruses and account for their inclusion in the genus *Herpesvirus*; they show marked affinity for ectodermal tissues, produce vesicular lesions, form type A intranuclear inclusions, and are readily isolated from infected tissues.

Properties of *Herpesvirus hominis*

(a) Morphology

The morphology of *H. hominis* has been elucidated by Wildy and his colleagues from electron microscopic studies of negatively stained preparations. The particle is approximately 150-200 m μ in diameter and its capsid is composed of 162 capsomeres arranged in icosahedral symmetry (Fig. 25). The capsomeres are elongated structures, about 120-135 Å in length and 90-100 Å in diameter, which are either hexagonal or pentagonal in cross section. To conform with the geometrical conditions of icosahedral structure, the capsomeres placed at each of the 12 apices of the icosahedron are pentagonal and the remaining 150 capsomeres hexagonal. Inside the capsid is a roughly spherical core, about 750 Å in diameter, which contains the viral DNA. In the complete particle, the capsid is sur-

rounded by an envelope derived from the host cell membrane during the process of maturation and release.

Particles representing various stages of completeness have been observed in negatively stained preparations. Complete particles described above are accompanied by particles devoid of envelopes, termed 'naked' particles, and both enveloped and 'naked' particles may occur with or without the central DNA core; these are referred to as 'full' and 'empty' particles, respectively. Both 'naked' and enveloped particles are believed to be infective but particles lacking the DNA core cannot be. It is estimated that only 1 in 10 of the virus particles is infective.

(b) Chemical and physical properties

H. hominis is heat labile and inactivated by ultraviolet and X-rays. Carbohydrate, protein, phospholipid, and DNA provide the chemical constituents of the virus particles which are inactivated by ether and other detergents.

(c) Antigenic composition

The antigenic composition of *H. hominis* has been studied by neutralization, complement-fixation, and agar-gel diffusion techniques.

Conventional neutralization tests make clear the antigenic homogeneity of *H. hominis* strains, but the recent use of the refined technique of neutralization kinetics has revealed minor antigenic differences between strains.

At least two antigens are detected by complement-fixation techniques, one of which represents the virus particles and the other a soluble complement-fixing antigen distinct from them. Four separate antigenic components have been separated from the soluble antigen by agar-gel diffusion and density gradient centrifugation techniques.

The recent demonstration that sera from patients convalescent from varicella or zoster contain significant amounts of antibody which fixes complement with *H. hominis* suggests an antigenic cross reaction between these two viruses.

(d) Cultivation

(i) *Experimental animals*

H. hominis was first isolated in the rabbit cornea by Gruter in 1912, and later by Lowenstein who published the results in 1919. The rabbit therefore holds an historic place among susceptible laboratory animals but guinea-pigs, mice, and hamsters may also be used.

Rabbits and guinea-pigs. Inoculation of virus into the cornea of these animals produces keratoconjunctivitis which heals in the course of a week or two, often leaving residual corneal opacities. Certain neurotropic strains produce encephalitis following corneal inoculation, but most strains are encephalitogenic after intracerebral inoculation. After intradermal inoculation, herpetic vesicles are formed at the site of inoculation and sometimes the virus spreads to the central nervous system along the peripheral nerves.

Mice. Suckling mice are highly susceptible to herpetic infection. Inoculation by the intracerebral or intraperitoneal route produces weakness, paralysis, abdominal distension, cyanosis, and death in 3–5 days. After inoculation, the virus is generally distributed to the brain and other organs via the circulation.

Adult mice are less susceptible, but spastic paralysis, convulsions, and death from encephalitis may occur after intracerebral inoculation.

(ii) *Chick embryos*

Inoculation of *H. hominis* onto the chorioallantoic membranes of 11–12-day-old chick embryos leads to the formation of small white pock lesions, about 1–2 mm in diameter, which develop in 36–48 hours (Fig. 4d). On initial isolation, two or three blind passages may be necessary before the typical pock lesions appear.

Inoculation into the yolk sac or amniotic cavity of 8–9-day-old chick embryos leads to embryonic death and virus growth in the amniotic and allantoic cavities, but these routes are not generally used.

(iii) *Tissue culture*

A wide variety of tissue cultures are susceptible to *H. hominis*. Most

susceptible are cultures of rabbit kidney cells from 3-week-old rabbits, but primary cultures of human embryo kidney and human amnion cells are satisfactory, although individual amnions vary in their susceptibility. HeLa cells, other continuous lines, and human diploid cells, are less susceptible and therefore less suitable for primary isolation; they are nevertheless widely used because of their ready availability. Chick embryo fibroblasts are also susceptible, but monkey kidney cells are relatively insusceptible to *H. hominis*.

H. hominis produces two types of cytopathogenic effect in monolayer cultures. In one, foci of cells proliferate, round up and fall away from the glass leaving lacunae in the cell sheet; eventually the whole cell sheet may become involved. In the other, large multinuclear giant cells predominate. The type of cytopathogenicity produced is believed to depend on the virus strain, and the frequent combination of both types indicates that strains of *H. hominis* are often heterogeneous (Fig. 74).

Clinical Manifestations of Infection with Herpesvirus hominis

Clinical syndromes produced by *H. hominis* may be divided into those which follow primary infection and those which are recurrent clinical manifestations of persistent infection.

(a) Primary herpetic infections

(i) *Primary infections of the skin and mucous membranes*

Acute herpetic gingivostomatitis. This is the most familiar clinical manifestation of primary herpetic infection and usually occurs in children between the ages of 1 and 6 years. It is characterized by inflammation of the gums, a vesicular eruption on the mucous membranes of the mouth and tongue, a mild degree of pyrexia, and submaxillary and submandibular lymphadenopathy. The vesicles soon collapse and form characteristic yellowish-white plaques which quickly ulcerate leaving typical punched-out ulcers in the mouth. The condition subsides after 7–10 days, and the patient develops a significant antibody response.

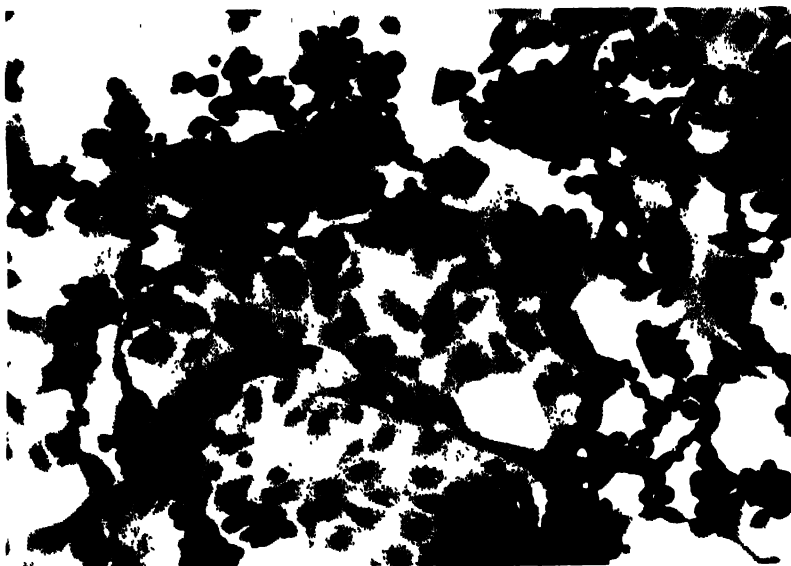
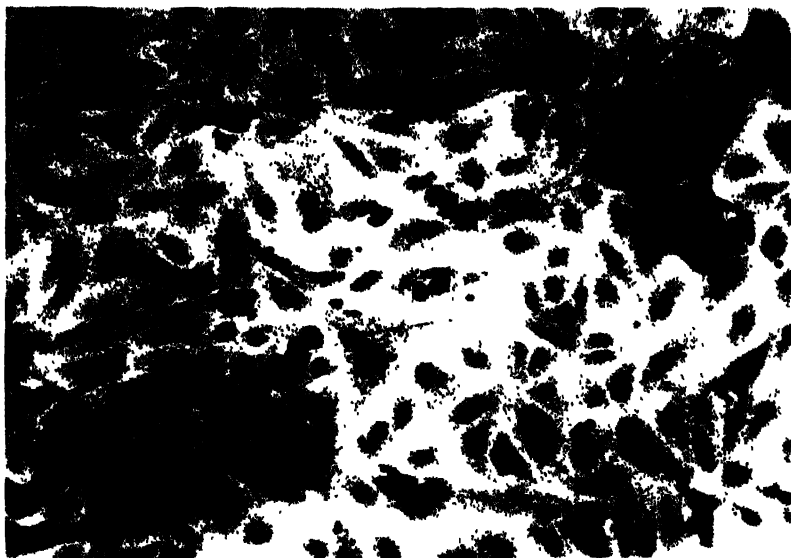


Fig. 74. Cytopathogenic effect of *Herpesvirus hominis* in human embryo kidney (HEK) cells, stained with haematoxylin and eosin.
 (a) Normal HEK.
 (b) HEK infected with *Herpesvirus hominis*.

Many cases previously diagnosed as Vincent's angina, in which the anaerobic organisms *Borrelia vincenti* and *Fusiform fusiformis* were thought to be causal, would today be diagnosed as acute herpetic gingivostomatitis. The presence of both these organisms, which are normal inhabitants of the mouth, in large numbers in oral herpetic infections is due to the presence of necrotic tissue.

Acute herpetic gingivostomatitis has to be distinguished from herpangina due to coxsackie A viruses. The lesions in herpangina are few in number and are situated in the faucial area, whereas herpetic lesions are more numerous and are situated in the anterior regions of the mouth and tongue. Coxsackie infections are also characterized by their short duration and by their definite seasonal incidence in summer.

Eczema herpeticum (*Kaposi's varicelliform eruption*). This condition occurs in children with infantile eczema, in whom the virus is believed to gain entry through the damaged skin producing crops of vesicles in the areas of eczema and the surrounding skin.

Primary herpes genitalis. Primary acute herpetic vulvovaginitis, accompanied by fever and inguinal lymphadenopathy, is one of the manifestations of primary herpetic infection which may occur in children and, less frequently, in adults.

Herpetic infection of the male genitalia is rare, but vesicles on the glans, prepuce, or urethral meatus appear when it occurs.

(ii) *Primary herpetic infections of the eye*

Herpetic keratoconjunctivitis. Primary herpetic infection of the eye when confined to the conjunctiva is characterized by unilateral, non-purulent, follicular conjunctivitis accompanied by local lymphadenitis and mild malaise, and often by vesicles on the eyelids and lips. In about two-thirds of the cases, the cornea becomes involved and an acute punctate keratitis develops which may go on to form a dendritic ulcer.

(iii) *Primary herpetic infections of the central nervous system*

Acute encephalitis. Acute encephalitis usually occurs in newborn infants as part of a fulminating generalized herpetic infection, in

which lesions are distributed in various organs of the body including the liver, adrenals, and often the skin.

Acute necrotizing encephalitis. This condition occurs, without any evidence of cutaneous infection, in older children and adults. Acute cerebral softening and haemorrhagic necrosis, particularly of the frontal and temporal lobes of the brain, occur. Sometimes the signs and symptoms resemble those of a space occupying lesion.

Aseptic meningitis. About 1% of cases of aseptic meningitis are due to herpetic infection. Very occasionally, the condition is associated with herpetic stomatitis.

(iv) Generalized herpetic infection of the newborn

Primary herpetic infection of the newborn, or occasionally of older children suffering from malnutrition and debility, may take the form of a fulminating generalized infection characterized by jaundice, respiratory distress, convulsions, and circulatory collapse. The condition is sometimes accompanied by a vesicular eruption of the skin and mucous membranes. Post-mortem, lesions are found in many internal organs of the body, including the liver, spleen, adrenals, and brain.

(v) Traumatic herpes

Primary herpetic infection may be acquired through an area of traumatized skin; vesicles appear in the traumatized area and may spread centripetally, accompanied by local lymphadenitis. The formation of herpetic whitlows following infection of minor traumata in the region of the fingernails has been described by Stern and his colleagues.

(b) Recurrent herpetic infections

(1) Herpes simplex

After primary infection, some patients develop recurrent eruptions of herpetic vesicles. These eruptions, which commonly occur at the mucocutaneous junctions of the lips and on the mucous membranes of the mouth, are often preceded by severe local irritation and pain. The vesicles pass quickly through a pustular and scabbing stage to complete healing.

The recurrent eruptions are usually precipitated by some non-specific factor, so that herpetic lesions may develop during the course of an unrelated pyrexial illness, on exposure to sunlight, during menstruation, or as the result of gastric disturbances or emotional disorders. The predisposing factor is not, however, always obvious.

(ii) Recurrent herpetic keratitis

Recurrent herpetic keratitis may be unilateral or bilateral and involves only the cornea, sparing the conjunctiva. Often starting as a superficial punctate keratitis, the infection frequently involves the deeper layers of the cornea and leads to the formation of a dendritic ulcer. Because of the dangers of corneal ulceration and scarring, recurrent herpetic keratitis may have serious consequences.

(iii) Herpes genitalis

Recurrent herpes is sometimes manifested by recurrent vulvovaginitis in females and by recurrent herpetic vesicles on the genitalia in males.

Pathogenesis

(a) Natural history

Serological investigations clearly differentiate between the two main types of herpetic infection; primary infection, which occurs in non-immunes, and recurrent infection, which occurs in those with a high and stable level of antibody. Burnet and Williams have interpreted this observation to explain the natural history of the disease, and their views have now been generally accepted. They suggest that primary infection is usually acquired in childhood and that afterwards, although antibodies develop during the course of convalescence, virus is not eliminated but remains latent in the tissues, probably at the site of infection. The exact form in which the virus persists in the latent state is not known but reactivation of virus from time to time, by various non-specific stimuli, leads to periodic recurrent infection.

The close resemblance between the natural history of herpetic

infection and the induction of lysogenic bacteriophages by non-specific stimuli is striking. But no proviral form of *H. hominis* has yet been experimentally demonstrated.

Although the mechanism of symbiosis between *H. hominis* and its host cell has not yet been elucidated, the intracellular survival of the

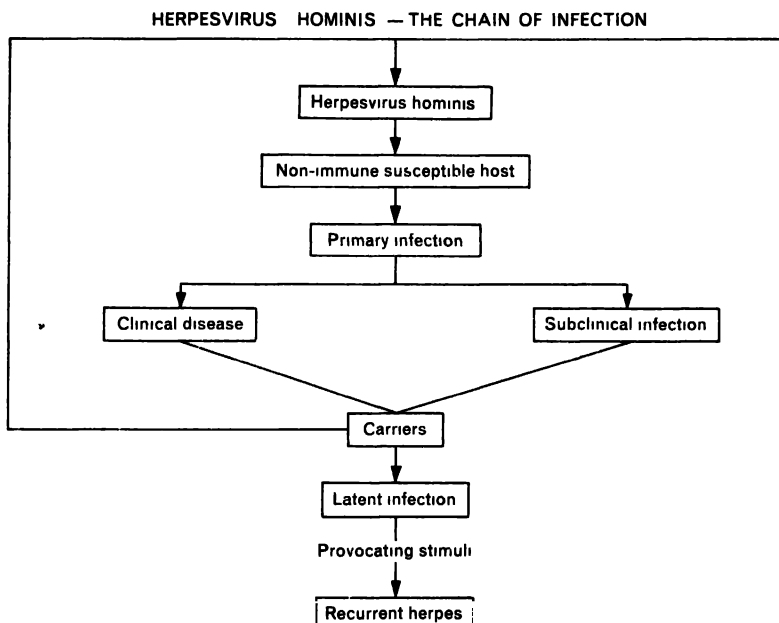


Fig. 75. Herpes simplex—chain of infection.

virus in an immune environment has been demonstrated in tissue culture. Moreover, its ability to spread from cell to cell without release into the extracellular environment, observed in tissue culture, may explain the survival of the virus in patients with high levels of serum antibodies.

(b) Spread to the central nervous system

The mechanism of central nervous system invasion is not yet completely understood. Blood stream spread is evident in the newborn,

in whom encephalitis is usually accompanied by generalized herpetic infection, and in suckling mice, in which blood stream spread has been experimentally demonstrated. In contrast, spread of *H. hominis* to the central nervous system via the peripheral nerves has been demonstrated in experimental animals; possibly, the virus invades the Schwann cells and proceeds by cell to cell transfer. This suggests to some that the localized lesions which sometimes occur in the frontal and temporal lobes of the brain arise from virus which has travelled along the trigeminal and olfactory nerves from the buccal and nasal mucous membranes.

Pathology

(a) Cytopathogenic effect

The development of intranuclear inclusion bodies and the formation of multinucleate giant cells has been studied in tissue culture. The inclusion body begins to develop 6–10 hours after infection and is first basophilic and Feulgen positive. Later, probably after extrusion of virus from the nucleus, the inclusion becomes Feulgen negative, eosinophilic, and draws away from the nuclear membrane, to become surrounded by a halo and form a typical Cowdry type A inclusion (Fig. 78).

The large multinucleate giant cells, characteristic of herpetic infection, are formed by dissolution of the cell membranes of contiguous cells and fusion of their cytoplasm. The nuclei, which develop typical type A inclusions, collect in the centre of these cells. Some strains of *H. hominis* produce small giant cells in tissue culture; these cells usually contain fewer than 10 nuclei and are the result of nuclear division without corresponding cell division.

(b) Cutaneous lesion

The pathology of the herpetic vesicles is typical of the herpetic lesions which may occur in various tissues. Infected cells of the basal and prickle cell layers proliferate, become oedematous and undergo 'balloon degeneration'. Marked intercellular oedema occurs and a vesicle is formed, in which the roof is composed of a stretched layer of prickle and epithelial cells, and in which the contents consist of

fluid, cell debris, giant cells, leucocytes, and fibrin. Cells showing typical 'balloon degeneration' and intranuclear inclusions may be found in the floor and sides of the vesicle, and there is a marked inflammatory reaction in the corium (Fig. 77). In mucous membranes, the roofs of the vesicles degenerate to form yellowish-white plaques which slough and leave typical punched-out ulcers.

(c) Lesions in the central nervous system

Localized areas of softening and haemorrhagic necrosis occur in the brain. Infected neuronal and glial cells swell, form intranuclear inclusions, necrose and undergo phagocytosis by mononuclear cells. Perivascular cuffing with mononuclear cells, meningeal engorgement, and exudation of mononuclear cells into the subarachnoid space, are also evident.

Epidemiology

Infection with *H. hominis* is very widespread and man provides the only known naturally susceptible host. Primary infection is usually acquired in infancy or childhood, but is sometimes delayed until adult life. Rarely, primary infection is acquired at birth from lesions in the mother's birth canal, or occasionally from infected attendants. If so, rapid dissemination of the virus and severe fulminating infection with widely distributed lesions often results. More usually, because of natural passive immunity, primary infection is not acquired until 1–6 years of age. In this age group, the majority of primary infections are subclinical and only a small proportion, variously estimated as 1–10%, develop clinical manifestations of primary infection. In nearly all those infected, either clinically or subclinically, virus persists and may give rise to periodic recurrent infections.

Those with primary or recurrent infections provide a considerable reservoir from which virus may be spread. After primary infection, virus may continue to be excreted for several weeks, and it may be noted that Buddingh and his colleagues recovered the virus from 20% of children under 2 years of age who showed no clinical evidence of herpetic infection, as well as from 2.5% of asymptomatic

adults. From these sources, and from those suffering from recurrent infections, virus is excreted in the oral and nasal secretions, as well as in the stools, and may be spread by direct contact, indirect contact through infected fomites, or by droplet infection. The earlier acquisition and more widespread distribution of primary infection in communities with low social, economic, and hygienic standards suggests that the faecal-oral route of spread may be of some importance. Osculation is also thought to play an important part in the spread of herpetic infection and the frequency of primary infection in children and young adults, the periods of life when osculatory embraces are customary, supports this view. Occasionally, adults may become infected by venereal infection.

Immunity

During the first few months of life, newborn infants are protected from primary infection by naturally acquired passive immunity. Towards the end of the first year, passive immunity declines, antibody titres fall to undetectable levels, and infants become susceptible to infection.

Complement-fixing and neutralizing antibodies appear 4-7 days after primary infection and reach a maximum during the second and third week. Contrary to commonly held assumptions, Buddingh, his colleagues, and others, have shown that antibody levels drop after primary infection and are boosted by recurrent infections which may be clinical or subclinical. The permanently high levels of antibody found in about 80% of the adult population suggest that repeated exogenous or endogenous infections are the rule.

Recovery from herpetic infection is associated with the development of delayed hypersensitivity to one of the soluble antigens of *H. hominis*, and a delayed hypersensitivity element in the production of herpetic lesions has been suggested.

Laboratory Diagnosis

(a) Virus isolation

Virus is readily isolated from swabs taken from lesions in the mouth, eye or elsewhere. Vesicle fluid itself is a rich source of virus, and,

where possible, should be obtained by perforating a vesicle with a glass capillary tube or syringe. Any material removed is expressed in a small volume of broth saline or growth medium containing antibiotics. In cases of stomatitis or labial herpes, saliva provides a good source of virus, as does cerebrospinal fluid in cases of aseptic meningitis and encephalitis. Occasionally, virus may be isolated from the blood.

Three susceptible host systems may be used for isolation; chick embryos inoculated by the chorioallantoic membrane route; suckling mice inoculated intracerebrally or intraperitoneally; and tissue cultures of susceptible cells, preferably rabbit kidney, human embryo kidney or susceptible human amnion cells. The growth of some strains in one host and not another makes it advisable to inoculate more than one host for initial isolation.

Variola and vaccinia, which may be confused with herpes, may be distinguished by the nature of the pocks which their viruses produce on the chorioallantoic membrane, and by serological typing of the virus isolated.

(b) Direct microscopy

(i) Electron microscopy

The demonstration of typical herpes virus particles in negatively stained specimens of vesicle fluids has recently been introduced as an aid to rapid diagnosis. Although *H. hominis* is indistinguishable from *H. varicellae* by this technique, it is easily distinguished from the viruses of variola and vaccinia.

(ii) Fluorescent antibody techniques

The fluorescent antibody technique to demonstrate the presence of viral antigens in sections or smears of infected tissues is another method which has been used for rapid diagnosis.

(iii) Stained preparations

Smears taken from herpetic lesions and stained with haematoxylin and eosin may be searched for giant cells and type A intranuclear inclusions, the presence of which indicates infection with a member of the genus *Herpesvirus*.

(c) Serological tests

Sera drawn from patients in the acute and convalescent phases of a primary infection reveal a four-fold rise, or more, of neutralizing and complement-fixing antibody. In contrast, acute and convalescent sera from patients with recurrent herpes contain high levels of antibody and no rise in titre is detectable.

Recently, significant antibody responses to *H. hominis* have been detected in patients infected with varicella or zoster. Serological tests alone may therefore be misleading although neutralization tests appear to be more specific than complement-fixation tests in this respect.

Control**(a) General measures**

Patients with clinical manifestations of herpes should beware of passing on infection by droplet infection, osculation or other forms of direct or indirect contact.

Some control of recurrent herpes may be achieved by attention to the exciting stimuli, when these are known. Thus dark glasses may be used to prevent eye lesions induced by sunlight, and tranquillizers may be used to control emotional disorders. Attempted control and treatment of respiratory infections may also be beneficial.

(b) Vaccines

The administration of killed *Herpesvirus* vaccines for the prevention of recurrent herpes has been advocated by some but is of little or no benefit. This is not surprising in view of the already high levels of antibody in patients with recurrent herpes. Repeated vaccination with vaccinia virus has also been claimed to prevent recurrent herpes, but its effect is probably psychological rather than immunological and it is not recommended.

Treatment

In addition to the general symptomatic measures indicated in herpetic infections, some specific antiviral methods of treatment have

recently been introduced and are under trial. Of these, local applications of antiserum and interferon have not proved successful but the compound 5-iododeoxyuridine, introduced by Kaufman, has been more effective. This compound blocks the incorporation of thymidine, thereby interfering with the correct formation of DNA, but recent evidence indicates that the compound may also interfere with the maturation of the virus particle.

Successful treatment of superficial, but not deep, corneal lesions in animals and man with 5-iododeoxyuridine has been reported, but the results have not always been confirmed and the compound remains on trial. It is administered locally in the form of drops, hourly during the day and 2 hourly during the night, for 5 days or until the lesion has healed. Drug resistant strains have already been encountered.

Attempts to treat cutaneous lesions with the compound applied in various media have proved unsuccessful, but recently MacCallum and Juel-Jensen reported more favourable results when it was applied in a 5% solution in dimethyl sulphoxide. Although the vehicle itself had some beneficial effect, these authors attributed the success of the compound in this medium to more effective contact with infected cells.

II. *Herpesvirus simiae* B Virus of Monkeys

H. simiae is a natural pathogen of monkeys in whom it causes a disease very similar to herpes simplex in man. Morphologically, and in other ways, it resembles *H. hominis* and the two viruses are antigenically related.

Very occasionally, man may become infected after a monkey bite or from infection of a wound with infected monkey kidney tissue culture cells. In these patients, after an incubation period of 10–20 days, a highly fatal encephalitis or encephalomyelitis develops. To

prevent infection, monkey handlers should wear protective clothing and beware of monkeys with stomatitis or other suspect lesions. Prevention of overcrowding in monkey cages helps to prevent the spread of the disease in monkey colonies.

CHAPTER 31

Herpesvirus Infections

III. *Herpesvirus varicellae* Chickenpox and Herpes Zoster

Chickenpox is a well-known epidemic disease of childhood. Its viral aetiology was established in 1917 by Paschen, who observed elementary bodies in vesicle fluid. The many observations of intranuclear inclusion bodies in infected cells also supported a viral aetiology, but it was not until 1952 that Weller and Stoddard succeeded in isolating the causative virus in cultures of human embryonic tissues.

Properties of the Virus

(a) Morphology

The morphology of *Herpesvirus varicellae* is similar, if not identical, to that of *H. hominis*. Details of the negatively stained particle have been described by Almeida and her colleagues; they showed the complete virus particle to be about 200 m μ in diameter, and to consist of a nucleocapsid surrounded by an envelope, from which short filamentous processes about 8 m μ long project. The capsid is composed of 162 hollow, elongated capsomeres arranged in icosahedral symmetry, and resembles that of *H. hominis* (Fig. 26).

(b) Chemical and biological properties

Although inactivated by ether, little else is yet known about the chemical or biological properties of *H. varicellae*.

(c) Antigenic composition

Although satisfactory antisera have not yet been prepared in experimental animals, the antigenic composition of *H. varicellae* has been

studied by neutralization, complement-fixation and agar-gel diffusion techniques, using human sera obtained from patients convalescing from varicella or herpes zoster. In this way, the antigenic identity of viruses isolated from varicella and zoster has been established, and a possible antigenic relationship between *H. varicellae* and *H. hominis* has been demonstrated.

The complement-fixing antigen is a soluble antigen, distinct from the varicella virus particle, which is found in the extracellular fluid of infected tissue cultures. In vesicle fluids, Taylor-Robinson has detected three distinct antigens by agar-gel diffusion techniques, and precipitating antigen was also detected in extracellular tissue culture fluids.

(d) Cultivation

No laboratory animal is susceptible to *H. varicellae* nor is the virus cultivable in chick embryos; however, a number of tissue cultures are now known to support the growth of the virus.

Primary cultures of human embryonic tissues and human amnion cells, as well as the continuous HeLa cell line, are suitable for virus cultivation; monkey kidney cells are less susceptible but may be used, especially for tissue culture adapted virus. Characteristically, a focal cytopathogenic effect appears about 4–5 days after inoculation, but 6–7 weeks may be required before the cell monolayer is completely destroyed. Affected cells become swollen, rounded, and refractile, and sometimes develop fine cytoplasmic processes. As the foci spread radially, the cells at the centre of the focus degenerate and undergo necrosis. Multinucleate giant cells also are characteristic of tissue cultures, especially epithelial cell cultures, infected with varicella (Fig. 76).

Unlike most viruses, *H. varicellae* is retained within the host cell and is not extruded into the extracellular fluid. Consequently, varicella virus cannot be subcultured by transfer of infected culture fluids, but only by transfer of infected viable cells.

Clinical Features

Chickenpox is characterized by the acute onset of mild pyrexia, malaise, and a generalized cutaneous eruption. The rash, which

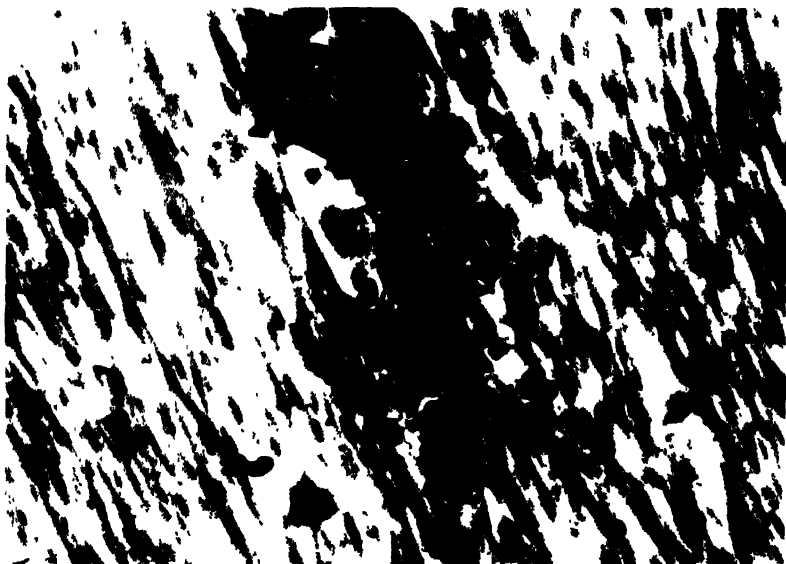


Fig. 76. Cytopathogenic effects of *Herpesvirus varicellae* in human diploid fibroblasts, stained with haematoxylin and eosin.

(a) Normal human fibroblasts.

(b) Human fibroblasts infected with *Herpesvirus varicellae*.

develops in crops over a period of 24–48 hours, is first maculopapular, becoming vesicular within a few hours. After 3–4 days, the vesicles pass through a pustular stage, form crusts, and heal with the formation of scabs which persist for 2–3 weeks. In contrast to smallpox, the lesions tend to be distributed centripetally and are more profuse on covered than exposed parts of the body; rarely, the lesions may be of the haemorrhagic or bullous variety. Generalized lymphadenopathy is common and is particularly marked in the suboccipital region. In adult cases, which are uncommon, constitutional symptoms are more marked and may precede the rash by a day or two.

Complications

In a minority of patients one of the following complications may occur.

(a) Encephalitis

About 4–10 days after the onset of the eruption, neurological signs and symptoms of encephalomyelitis may occur. Although most patients recover without sequelae, permanent damage and death may supervene. It is not yet certain if the condition arises by direct invasion of the central nervous system or by an allergic response to infection.

(b) Pneumonia

Primary varicella pneumonia, which is distinct from the secondary bacterial pneumonias that sometimes complicate varicella, is a rare condition which affects adults rather than children and which is fatal in about 20% of cases. The sputum, which is often bloody, may contain cells which exhibit typical type A intranuclear inclusions. Radiological examination reveals bilateral nodular pulmonary infiltration, and lesions are found in the lungs, and sometimes in other organs, post-mortem.

(c) Pyogenic infection

Pyogenic infection of the skin lesions is the most common complication of chickenpox. Infection may lead to abscess formation, sup-

purative lymphangitis, cellulitis, and sometimes even septicaemia and death.

Pathogenesis

So far, the pathogenesis of chickenpox has not been studied by virological methods. From its relatively long incubation period of 10–21 days, average 14–17 days, and from analogy with other exanthemata, the pathogenesis of chickenpox is believed to resemble that of experimental mouse-pox (ectromelia), studied by Fenner. Virus, supposed to be transmitted by droplet infection, is believed to gain entry via the mucosa of the upper respiratory tract. After some multiplication at the site of infection and in the draining lymph glands, the virus is distributed by the blood stream to the internal organs of the reticulo-endothelial system, particularly the liver and spleen, where further multiplication takes place. At the end of the incubation period, a secondary viraemia distributes the virus to the target organ, namely the skin, where lesions make the presence of virus clinically manifest.

Pathology

The cutaneous lesions of chickenpox resemble those of herpes simplex. Marked intercellular odema, and balloon degeneration of infected cells in the basal and prickle cell layers, lead to the formation of vesicles roofed by stretched epidermal and prickle cells. Multinucleate giant cells and type A intranuclear inclusions provide other essential histological features of the lesion (Figs 77 and 78). The vesicle fluid contains cell debris, fibrin, polymorphonuclear leucocytes, and phagocytic cells.

Epidemiology

(a) Distribution

Chickenpox is an epidemic disease of childhood, exhibiting its maximum incidence between the ages of 2 and 6 years; by the age of 15 years, about 70% of the population have acquired infection. World wide in its distribution, chickenpox occurs periodically in

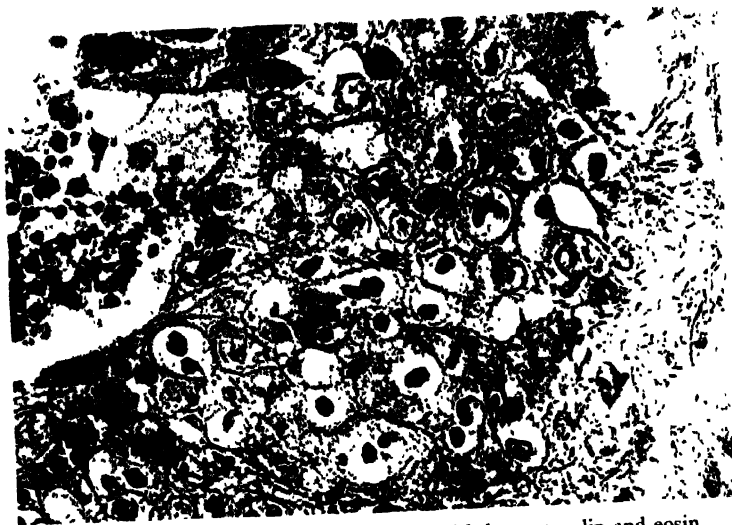


Fig. 77. Section of zoster vesicle, stained with haematoxylin and eosin, showing part of the wall and vesicular debris. Note the balloon cells and intranuclear inclusions. (High power, light microscopy.) The lesions of herpes simplex are similar.

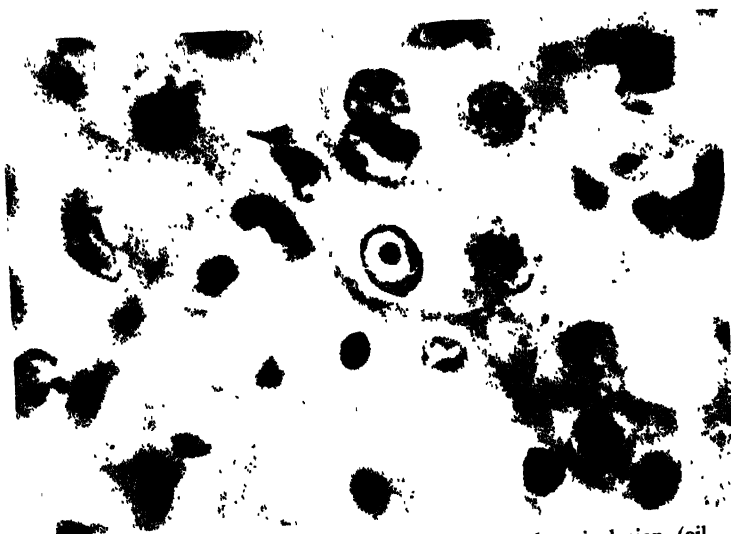


Fig. 78. Zoster vesicle—Balloon cell and intranuclear inclusion (oil immersion, light microscopy). Similar changes are produced by *Herpesvirus hominis*.

epidemics, usually in the winter months; although the incidence is high its mortality remains low.

(b) Source of infection

Man is the only naturally susceptible host, in whom infection may be either clinical or subclinical. Clinical cases, which form the majority, are infective from a few days before the rash until about 5 days after its onset. During this time, virus is believed to be excreted in the respiratory secretions and from lesions which sometimes occur in the mucous membrane of the mouth. Although infective virus may be found in the vesicle fluid within 3 days of its first appearance, spread of virus from unruptured vesicles is unlikely and it is assumed that pustular, crusted and scabbed lesions are non-infective. In contrast, virus may well spread from vesicles which rupture in the early stages.

Another important source of infection is provided by patients who are suffering from herpes zoster, which is simply another clinical manifestation of infection with *H. varicellae*.

(c) Transmission of infection

The main route of spread is believed to be by droplet infection. Transfer by direct or indirect contact with skin lesions in the early stages of chickenpox is not held to be an important mode of spread, although this mode of transfer from lesions of herpes zoster may be.

Immunity

It is well known that an attack of chickenpox leads to lifelong immunity, and that second attacks are extremely rare. Antibodies appear about 4–7 days after the onset of the disease and reach maximum levels within about 3 weeks. Complement-fixing antibody falls to undetectable levels within a few months but neutralizing antibody persists at low levels for long periods.

Laboratory Diagnosis

Although not usually called for, laboratory diagnosis may be required to distinguish varicella from variola, or to confirm the

diagnosis of herpes zoster in patients who develop muscular paralysis.

(a) Virus isolation

Virus may be isolated from the vesicles of both varicella and herpes zoster if specimens are taken within 3 days of their appearance. Primary cultures of human amnion provide the host cells of choice and focal cytopathogenic changes can usually be detected in 4–5 days.

The failure of *H. varicellae* to produce pocks on the chorio-allantoic membrane of the chick embryo distinguishes it from the viruses of variola, vaccinia, and herpes simplex. In addition, its lack of pathogenicity for suckling mice and its failure to haemadsorb in infected tissue cultures provide useful criteria by which *Herpesvirus varicellae* may be distinguished from *Herpesvirus hominis* and variola virus, respectively.

(b) Direct microscopy

The presence of multinucleate giant cells and type A intranuclear inclusions in smears or sections of cutaneous lesions help distinguish both *H. varicellae* and *H. hominis* from variola virus. Recently, electron microscopy of vesicle fluid has become another important and rapid means of differentiating between these viruses. Indirect fluorescent antibody techniques for the detection of varicella antigen in vesicle fluid have also been introduced as a rapid method of diagnosis.

(c) Serological tests

In atypical cases of varicella or zoster, vesicle fluid may not be available for virus isolation and retrospective confirmation of the diagnosis by serological tests may then be required. A rise in antibody titre of four-fold or more, occurring between the acute and convalescent phases of the disease, is diagnostic. A significant rise of antibody titre in cases of zoster is not, however, always demonstrable because high titres of antibody are often present in acute phase sera.

Neutralization tests are not generally employed because of the

difficulty of obtaining cell-free virus in tissue culture. Complement-fixation tests, using soluble complement-fixing antigen prepared from tissue culture fluids, are however available.

Prophylaxis

Isolation of the patient from susceptible contacts for 1 week after the onset of the disease is advisable, but quarantine measures are not indicated. γ -Globulin from human immune serum may modify but not prevent chickenpox in susceptibles, if administered within 3 days of contact. It is not however indicated except in those exposed to special risk, including those with blood dyscrasias or eczema, those in receipt of steroid therapy, and in neonates in whom severe generalized infection may occur.

HERPES ZOSTER

The view that chickenpox and herpes zoster are different clinical manifestations of infection with the same aetiological agent was first put forward by von Bokay in 1909. He observed, as many others have done since, that children in contact with herpes zoster frequently develop chickenpox. In recent years, virological studies have amply confirmed von Bokay's hypothesis and clearly established the identity of the viruses causing chickenpox and zoster.

Clinical Features

Herpes zoster is a localized cutaneous eruption which occurs in adults who have often suffered from varicella in childhood. Constitutional symptoms are not often prominent and the lesions, which are nearly always unilateral, are distributed in areas of skin corresponding to the distribution of sensory nerves arising from one or more segments of the spinal cord. Areas of skin innervated by the thoracic and cervical segments of the cord are the ones most commonly affected, but areas innervated by cranial nerves, particularly the trigeminal nerve, are sometimes involved.

The cutaneous lesions appear in a localized maculopapular eruption, which becomes vesicular within 24 hours and pustular after a few days. The pustules soon dry up and form scabs which separate in 2–3 weeks. Pain and parasthesiae in the affected area are prominent features, which often precede the eruption by a few days and may persist for weeks or months afterwards.

Complications

(a) Lower motor neurone paralysis

Lower motor neurone paralysis is a recognized but infrequent complication of herpes zoster which indicates involvement of anterior horn cells. The paralysis, which may be temporary or permanent, most commonly affects the upper limbs or muscles innervated by the cranial nerves. The well known Ramsey-Hunt syndrome, in which facial palsy is accompanied by lesions in the external auditory meatus, follows involvement of the seventh nerve.

(b) Generalized vesicular eruption

Occasionally a generalized vesicular eruption, resembling varicella, follows the original zoster vesicles. This complication, which usually occurs in elderly patients, is presumably due to entry of the virus into the blood stream and its distribution to other sites.

(c) Meningo-encephalitis

This is a rare complication in which virus has, on occasion, been isolated from the spinal fluid.

Pathogenesis

Although clinical, epidemiological, and virological evidence now leave no doubt that the viruses of zoster and varicella are identical, the pathogenesis of herpes zoster remains essentially unknown. Nevertheless, several hypotheses have been put forward. Of these, the one put forward by Garland, and others, that zoster follows re-activation of varicella virus which has remained latent in the tissues after varicella infection, is the most attractive. The absence, in most

cases, of any history of contact with zoster or varicella, together with the occurrence of zoster in partially immune individuals, its lack of seasonal incidence similar to that of varicella, and its similarity to herpes simplex, support Garland's hypothesis.

After an attack of varicella, virus is believed to persist in a latent form in the neurones of the posterior root ganglia until activated by some known or unknown stimulus. It is well known that zoster may be precipitated by trauma, injections of heavy metals, certain febrile illnesses, and by certain malignant conditions. After reactivation, the virus presumably travels along the nerve fibres to the skin and perhaps centripetally to the cord as well. Although this hypothesis remains unproven, it is of interest to note that Cheatham found evidence of infection in posterior root ganglia, and other organs, in a patient who died 17 days after varicella infection.

An alternative hypothesis, that zoster represents the reaction of a partially immune host to exogenous reinfection with *Herpesvirus varicellae*, has been put forward by some. In support of this hypothesis, the occasional cases of zoster which are followed by generalized eruptions are held to represent a breakdown of this immunity. The absence of any history of contact in most cases of herpes zoster is against the hypothesis of exogenous reinfection, which if it occurs must be acquired from asymptomatic carriers of *H. varicellae*.

Cheatham suggested that exogenous virus gains entry via the peripheral nerves of the skin and mucous membranes of the alimentary tract, and travels centripetally to the posterior root ganglia and cord. Some of the older observers drew attention to the mild upper respiratory illnesses which often precede an attack of zoster, suggesting that the virus gains entry via the mucous membranes of the upper respiratory tract.

Pathology

The cutaneous lesions of herpes zoster are pathologically the same as those of varicella, and virus may be recovered from them in the early stages (Fig. 77). Lesions found in the posterior root ganglia are marked by lymphocytic infiltration and, sometimes, haemorrhage; localized meningeal inflammation occurs in the region of the pos-

terior roots and cord segments involved. When the posterior horn cells are affected, lesions similar to those seen in poliomyelitis are found; the similarity is even closer in those occasional cases complicated by muscular paralysis, in which the anterior horn cells are involved. Degeneration and cellular infiltration of the affected sensory and motor nerve fibres follow the neuronal changes.

Epidemiology

Herpes zoster occurs sporadically in adults who usually have had no contact with varicella or zoster. The condition is not very infectious except to those who are susceptible to varicella, namely infants and young children. The absence of any history of contact in most cases supports the hypothesis that the disease is due to reactivation of latent *H. varicellae*.

Immunity

The neutralizing and complement-fixing antibodies produced in varicella and zoster are identical. Although sera drawn in the early stages of zoster usually contain antibody, sometimes in high titre, those drawn in the early stages of varicella are usually devoid of antibody; sera drawn in the convalescent phases of both zoster and varicella contain high titres of antibody. This conforms to the supposition that zoster occurs in those who are immune or partially immune to *H. varicellae*.

Treatment

Treatment is symptomatic and no specific antiviral therapy is available.

CHAPTER 32

I. Cytomegalovirus Infections

Cytomegalovirus hominis Cytomegalic Inclusion Disease

The genus *Cytomegalovirus* is one of the genera belonging to the Family Herpesviridae. Viruses assigned to this genus resemble those of the genus *Herpesvirus* in their morphology and their ability to form intranuclear inclusions, but they differ from them in their peculiar affinity for salivary glands.

Infection of older children and adults is quite widespread but usually inapparent. In contrast, infection of the newborn, usually acquired in utero, results in a fatal generalized infection. Although cytomegalic lesions have been recognized since the beginning of the century, and a viral aetiology was suggested by Glahn and Pappenheimer in 1925, it was not until 1956 that the causative virus was isolated in cultures of human tissues by Smith, and independently by both Rowe and Weller, and their colleagues. Significantly, a viral aetiology of a similar condition occurring in guinea-pigs was proved by Cole and Kuthner as long ago as 1926.

Properties of the Virus

(a) Morphology

Smith and Rasmussen have found negatively stained cytomegalovirus particles to resemble closely the particles of *Herpesvirus hominis* and *Herpesvirus varicellae*. They found the capsid to be composed of 162 capsomeres similar in morphology and arrangement to those of herpesviruses; the nucleic acid core was often missing and this may have accounted for the fact that only 1 in 1000

particles was infective. The nucleocapsid of some particles was surrounded by an envelope (Fig. 79). The diameter of the virus particle was estimated by Smith and Rasmussen to measure about 113 m μ .



Fig. 79. Negatively stained enveloped cytomegalovirus particle [from H. T. Wright Jr, C. R. Goodheart, and A. I. Ielousis (1964) *Virology* 23, 419-24 (Academic Press Inc., New York and London)].

(b) Chemical and physical properties

Cytomegaloviruses are DNA viruses which are heat labile, and are destroyed at pH 4.0 and by ether. Although originally difficult to

preserve at low temperature, successful preservation at -60°C may be achieved in the presence of sorbitol.

(c) Antigenic composition

The failure to produce satisfactory antisera by artificial immunization of experimental animals has hampered the study of human cytomegaloviruses. Nevertheless, the use of human immune sera in neutralization and complement-fixation tests has proved satisfactory. The reaction of sera from infected infants with homologous but not heterologous strains, and the often broader response of sera from older children and adults, suggest that more than one antigenic type of human cytomegalovirus may exist.

(d) Cultivation

Cytomegalovirus hominis grows only in fibroblast and myometrial cells of human origin, preferably in roller tube cultures. Primary cultures of human embryonic tissues, and human diploid cells are suitable sources of fibroblasts. Epithelial cells are not susceptible although they are affected in the diseased patient.

In culture, typical foci of cytopathogenicity appear after 5–24 days, depending on the size of the virus inoculum, and two months may be required before degeneration spreads through the whole culture. The foci are characterized by enlarged rounded cells which develop dark refractile granules and degenerate, leaving behind some granular debris. In stained preparations, large intranuclear inclusions, usually eosinophilic, may be seen, and hyaline inclusion bodies or masses of granules may be observed in a paranuclear position in the cytoplasm.

On initial isolation, little or no virus is released into the extracellular fluid, and subculture is best achieved by transfer of infected cells. After adaptation to tissue culture, when extensive cytopathogenic effects develop, subculture by transfer of infected fluids is possible.

Clinical Features

(a) Intrauterine infection

After intrauterine infection, the disease becomes clinically manifest

at birth, or may be delayed for a few weeks or months. Cytomegalic inclusion disease in the newborn is usually generalized, and is associated with hepatosplenomegaly and jaundice, thrombocytopaenic purpura, cerebral involvement which leads to microcephaly, optic atrophy, and other cerebral disorders. The disease, which is often associated with prematurity, is usually fatal within a few days or weeks and may lead to stillbirth. In those who survive, mental retardation is now recognized as one of the complications.

It is possible that some cases of generalized infection in infants under the age of 6 months are acquired post-natally.

(b) Post-natal infection

(i) Localized infection

Five to ten per cent of infants over the age of 6 months examined at routine autopsy exhibit a focal form of the disease, manifested by the presence of pathognomonic cytomegalic cells in the salivary glands. These infections are not accompanied by any clinical symptoms and occur in normal children. Serological and virological evidence indicates that inapparent infections are common in older children and adults.

(ii) Generalized infection

Generalized infection is very rare in adults or in children over 4 years of age. When it occurs, it usually complicates some underlying malignant or other chronic debilitating condition. Lesions are found in the lungs, liver, and adrenals, and the condition often presents clinically as a case of interstitial pneumonitis.

Recently, a condition resembling infectious mononucleosis, in which abnormal liver function tests were associated with a significant rise of complement-fixing antibody to cytomegalovirus and a negative Paul-Bunnell test, has been reported by Klemola and Kaariainen from Finland. These authors have also reported transfer of the condition through the medium of large blood transfusions given during the course of open heart surgery.

Pathogenesis

Although cytomegalic inclusion disease in the newborn and early in

the post-natal period has long been thought to follow intrauterine infection, it is only recently that the virological studies of Medearis have confirmed what was previously suspected. He recovered virus not only from the urine and sputum of infants with the disease but also from the urine of 40% of their mothers. This and the placental cytomegalic lesions, described by Cochard and his colleagues, suggest that maternal virus is spread by the blood stream and reaches the foetus through the placenta. Probably, the maternal infection in these cases is primary, and, in spite of prolonged viraemia, is eventually controlled, since no mother has yet given birth to a second infant with the disease. Significantly, a history of mild upper respiratory infection during pregnancy is sometimes obtained from mothers who give birth to children with cytomegalic inclusion disease.

The disastrous effect of cytomegalovirus infection in the newborn, compared with the asymptomatic and seemingly harmless infections of older children and adults, provides a further example of enhanced susceptibility of newborns to virus infection.

Pathology

The lesions of cytomegalic inclusion disease are characterized by the presence of greatly enlarged epithelial cells, which may reach 25–40 μ in diameter. These cells display large intranuclear inclusions which may be eosinophilic, or basophilic, and small basophilic intracytoplasmic inclusions about 2–4 μ in diameter (Fig. 8o).

When infection is generalized, lesions are found in the kidney, liver, lung, pancreas, central nervous system, salivary glands, and other organs of the body. Focal necrosis and inflammatory changes may be found in the liver and brain.

The excretion of virus in the urine and saliva of those inapparently infected suggests that their kidneys as well as their salivary glands are infected.

Epidemiology

Serological tests indicate that intrauterine infection is rare but that postnatal infection is widespread, although nearly always inapparent. Rowe and his colleagues, in the United States, have

shown that most infants are born with passively acquired immunity; this gradually wears off so that by the age of 6 months most have become susceptible to infection. Serological evidence of infection becomes increasingly frequent after 2 years of age; it is present in 53% of sera representing the age group 18-25 years, and in 81% of

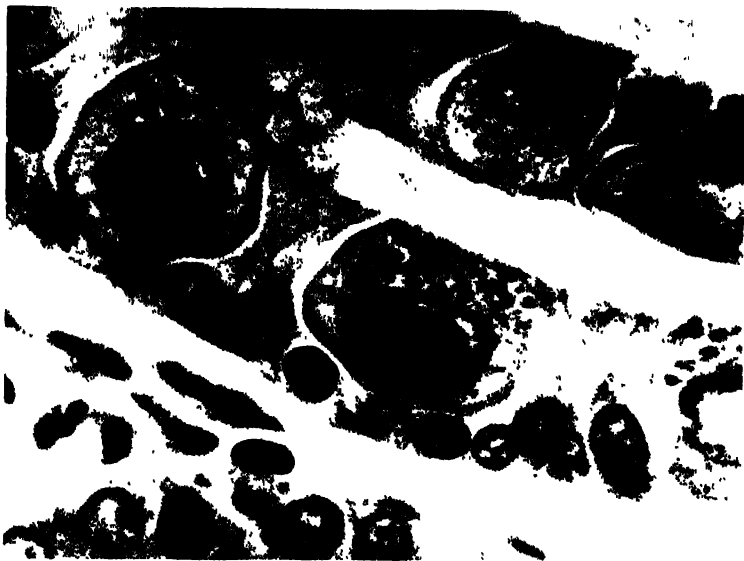


Fig. 80. Two duct epithelial cells from the submaxillary gland showing typical nuclear and cytoplasmic inclusions produced by cytomegalovirus [from J S. Nelson and J.P. Wyatt (1959) *Medicine* 38, 223-41 (The Williams & Wilkins Co., Baltimore, U.S.A.)].

sera representing adults over the age of 35 years. A similar incidence of infection has been found by Stern and Elek in this country.

Although infection after 6 months of age is usually inapparent, virus is excreted in the urine and saliva for periods of several months. From these sources, and from those with clinical disease, virus presumably spreads by direct and indirect contact, and by droplet infection. The studies of Rowe and his colleagues on a group of children admitted to a nursery institution in the United States indicate that about 10% of children under 4 years of age in the

general population are carriers and excrete virus. In closed communities, where opportunities for cross infection are increased, the proportion of carriers and virus excretors may be twice or three times that in the general population.

Laboratory Diagnosis

(a) Virus isolation

Virus may be isolated from throat swabs, or more frequently from the urine. Specimens should be inoculated as soon as possible, or after minimal storage at 4°C, into roller tube cultures of human fibroblasts derived from human embryonic tissues or human diploid cells. Any virus isolated is identified by the pathognomonic cytopathogenic effects produced by cytomegaloviruses, although these may be delayed for a month or more if the virus inoculum is small.

(b) Direct microscopy

In some cases, typical cytomegalic cells with intranuclear inclusions are excreted in the urine. These are sought for in smears made of fresh urine centrifuged concentrates, stained with haematoxylin and eosin. Smears from several specimens of urine are examined because excretion of cytomegalic cells may be intermittent. Direct cytological examination of the urine is a very much less sensitive method of diagnosis than virus isolation.

(c) Serological tests

The use of serological tests is limited by the difficulty of providing adapted strains for neutralization tests, and by the antigenic differences which exist between the various prototype strains of human cytomegalovirus. Nevertheless, neutralization and complement-fixation tests have been used for epidemiological investigations. The neutralization test has generally been more sensitive than the complement-fixation test but recent improvements in the preparation of the complement-fixing antigen have made the latter test more sensitive.

Control

Where a case of cytomegalic inclusion disease occurs in the nursery of a maternity unit, the patient should be removed from contact with other newborn infants.

Treatment

There is no specific antiviral treatment for the condition.

Animal Cytomegaloviruses

Histopathological lesions characteristic of cytomegalovirus infection have been observed in the salivary glands of guinea-pigs, mice, and monkeys, and causative viruses have been isolated from them. These viruses, like those recovered from humans, show remarkable host specificity and can be isolated only in tissues derived from the homologous host. The viruses from each of these species are antigenically distinct and there is no cross reaction between them, with the exception of a relationship between the monkey and human strains.

CHAPTER 33

Rabiesvirus Infection

I. *Rabiesvirus canis*—Rabies

Rabies, although no longer endemic in the United Kingdom, remains one of the most dreaded of all virus diseases. Essentially a disease of dogs and wild carnivores, rabies is occasionally transmitted to man by the bite of an infected animal. Galtier and Pasteur, who were pioneers in the study of the disease, appreciated the infective nature of the condition, but it was not until 1903 that Paul Remlinger showed the causative agent to be a filterable virus.

Properties of the Virus

(a) Morphology

Two forms of *Rabiesvirus* particles have been seen in the electron microscope; one, spherical in shape and variously estimated to be 80–200 m μ in diameter, and the other, elongated rod-like particles, 120–300 m μ in length and 60–100 m μ in width. The spherical particles, when negatively stained, are morphologically similar to myxoviruses. They consist of an inner, flattened double helical filamentous component, about 100 Å in diameter, surrounded by an outer membrane from which protrude radially orientated projections about 10 m μ long (Fig. 81).

(b) Chemical and physical properties

The failure of bromodeoxyuridine to inhibit rabiesvirus replication indicates that the nucleic acid component of rabiesvirus is RNA. The inactivation of the virus by ether and sodium deoxycholate suggests that its envelope is lipid in nature, and provides further evidence of similarity to the myxoviruses.



Fig. 81. Negatively stained rabiesvirus particles; the upper particle is intact and the lower particle disrupted [from J.D. Almeida, A.F. Howatson, L. Pinteric and P. Fenje (1962) *Virology* 18, 147-51 (Academic Press Inc., New York and London)].

The virus is highly sensitive to inactivation by heat, ultra-violet light, formalin, β -propiolactone, and phenol. Even at room temperature, the virus is highly labile unless protected by glycerol or a stabilizing protein. For preservation, lyophilization or storage at -60°C is best.

(c) Antigenic composition

Two antigenic components are produced in infected tissues; a soluble antigen distinct from the virus particles, and a viral antigen which represents the virus particles themselves. From the soluble antigen, Neurath and his colleagues have separated two components by differential centrifugation whose sedimentation constants are 23S and 10S respectively. Possibly, these two components represent the two precipitation lines produced by rabiesvirus in gel-diffusion tests. Although the soluble antigen does not produce any neutralizing antibody in experimental animals, it represents the major complement-fixing antigen of rabiesvirus preparations.

Only slight antigenic differences between strains, of doubtful significance, have so far been revealed by neutralization, complement-fixation, gel-diffusion, and cross protection studies.

(d) Cultivation

(i) *Experimental animals*

Rabbits, hamsters, guinea-pigs, mice, and the natural hosts, are susceptible to experimental inoculation. Of these, the laboratory mouse, which is susceptible to inoculation by almost any route, is the most convenient. After intracranial inoculation, mice develop paralysis and die within 5–30 days.

When first isolated from natural human or animal hosts, rabiesvirus preserves its natural properties and is referred to as street virus. After adaptation to experimental animals by prolonged serial intracranial passage, a virus strain with altered properties is produced, and is referred to as fixed virus. It differs from street virus by its shorter incubation period in experimental animals, 3–6 days compared with 21 days, its failure to produce Negri bodies or to multiply in the salivary glands, its reduced pathogenicity for man, its reduced

infectivity when administered by routes other than the intracerebral one, and its altered pathogenicity in experimental animals.

(ii) Chick embryos

Both fixed and street rabiesvirus may be adapted to the chick embryo brain by chorioallantoic inoculation of 5–6-day-old chick embryos, and may be maintained by intracerebral passage in 10–13-day-old embryos. Once egg adapted, strains may be maintained by yolk sac inoculation of 7-day-old chick embryos, and virus recovered from embryo suspensions after 9–10 days incubation.

(iii) Duck embryos

Duck embryo adapted strains, which produce high yields of virus, are now available and provide suitable vaccine strains.

(iv) Tissue culture

Primary cultures of chick embryo fibroblasts, mouse or hamster kidney cells, and dog salivary gland cells, are susceptible to rabiesvirus, but yields are low and cytopathogenic effects are not obviously manifest or are absent. Virus may nevertheless be demonstrated in tissue cultures by indirect methods, which include fluorescent antibody techniques, direct microscopic examination for Negri bodies, interference and mouse inoculation tests.

Similar growth characteristics have been described in continuous lines of mouse ependymal cells, the BHK-21 line of hamster kidney fibroblasts, and in dog kidney cells. Cultivation of rabiesvirus in human diploid cells has also been reported.

Clinical Features

(a) The incubation period

The incubation period following a bite from a rabid animal is approximately 4–12 weeks, although shorter and longer periods have been reported. The length of the incubation period appears to be determined by the site of the bite, its severity, and the amount of virus introduced. In general, the closer the bite to the central nervous system, the deeper its penetration, and the richer the tissue

innervation, the shorter the incubation period. Deep bites of the face are therefore usually associated with short incubation periods, and superficial bites of the extremities with longer ones.

(b) The prodromal phase

At the end of the incubation period, the patient enters into the prodromal phase of the illness which usually continues for 2–4 days, but may last only a few hours. During this time, the patient may experience paraesthetic sensations at the site of the bite, anorexia, headache, sore throat, pyrexia, malaise, insomnia, and a general state of anxiety or depression.

(c) The acute phase

Towards the end of the prodromal phase, the patient becomes hypersensitive to sensory stimuli which provoke spasm of the laryngeal and pharyngeal muscles and lead to increasing difficulty of respiration and deglutition. Attempts to drink water give rise to such painful spasm of the pharyngeal and laryngeal muscles that eventually the very sight of water is sufficient to bring on an attack. It was this feature which once gave the name hydrophobia to the disease. Gradually, the spasms become more frequent and spread to produce generalized convulsive seizures. Excessive salivation, perspiration and dilatation of the pupils, which are characteristic features of the disease, provide evidence of stimulation of the sympathetic nervous system.

During the acute phase of the illness, periods of cerebral excitement alternate with periods of relative tranquillity but the patient, although anxious, remains rational to the end. Death, from respiratory failure in the course of a convulsive seizure, usually occurs within a week, and often within 48 hours. If the patient survives the seizures, progressive paralysis ensues leading to coma and death.

Rarely, the disease may take the form of Landry-type ascending paralysis, especially when it has been transmitted by bats.

Pathogenesis

After implantation, there is little doubt that rabiesvirus reaches the central nervous system by way of the peripheral nerves. The pre-

vention of viral spread to and from the central nervous system, and the prolongation of the incubation period, by section of an inoculated nerve in experimental animals, supports this view. In man, the relationship between the length of the incubation period and the proximity of the site of implantation to the central nervous system is further evidence of nerve fibre spread. So far, no unequivocal evidence that virus spreads by the blood stream has been obtained.

After implantation, virus is believed to gain entry into the terminal nerve fibrils, without prior replication; from here, virus travels up the afferent nerves to that part of the central nervous system responsible for innervating the site of implantation, and later spreads to involve the whole of the central nervous system. In rabid animals, the salivary glands presumably become infected with virus which migrates from the central nervous system along the efferent nerves.

Pathology

Apart from some congestion, the brain, cord and meninges usually appear macroscopically normal. The typical lesions of rabies are, however, revealed by histopathological examination. Focal areas of inflammation, characterized by marked infiltration of mononuclear leucocytes, perivascular cuffing and sometimes small haemorrhages, are found in the brain, cord and ganglia of the central nervous system. Nerve cells in varying stages of degeneration, characterized by vacuolation, chromatolysis, and nuclear degeneration, are also present. When completely destroyed, the nerve cells undergo neuronophagia by inflammatory and microglial cells.

The characteristic intracytoplasmic Negri inclusion bodies are found in both degenerating and undamaged cells, and sometimes two or three Negri bodies are found in the same neurone. They are most conspicuous in the cells of Ammon's horn of the hippocampal lobe but in man they are also common in the cerebral cortex, cerebellum, and medullary nuclei. They are eosinophilic inclusions, about 10 μ in diameter or sometimes larger, which contain one or more basophilic granules.

They do not contain complete virus particles but histochemical

examination of Negri bodies in tissue culture cells indicates that they consist of rabiesvirus antigen and a small amount of RNA.

In infected animals, and in some human cases, the submaxillary salivary glands are affected. Degeneration of the acinar epithelium and cellular infiltration of the glands has been observed. Evidently, non-nervous tissue *in vivo* as well as in tissue culture is able to support the growth of rabiesvirus.

Epidemiology

Rabies is enzootic among several species of carnivores in nearly all parts of the world. Included among these species, which provide reservoirs of infection, are dogs, cats, wolves, foxes, jackals, skunks and mongooses. Birds may also be affected but are only very rarely responsible for rabies in man. Transmission of infection from one of the usual reservoirs to a tangential non-biting host, such as man, results in death of the host and a break in the chain of infection. In the natural biting host, however, excretion of virus in the saliva of rabid animals, and the high density of susceptible animals, ensures the maintenance of the disease in the reservoir species.

The dog, which is the species in closest contact with man, provides the usual source of human infection. In turn, dogs usually

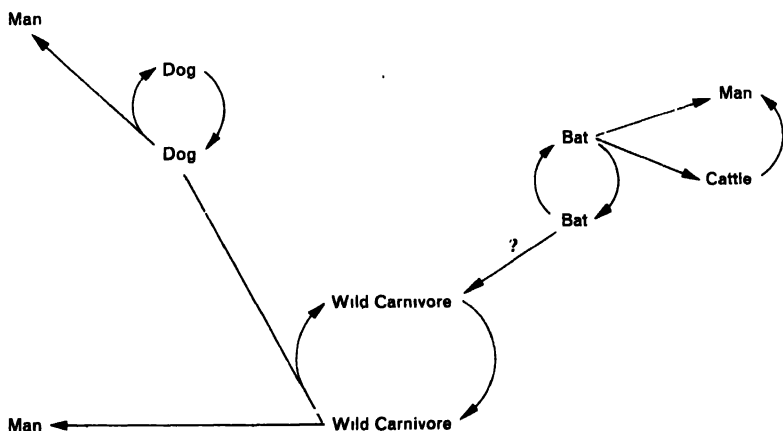


Fig. 82. Epidemiology of rabies.

acquire infection from another rabid dog or from one of the wild animal species in whom the disease is maintained. Rabid dogs either become wildly excited or succumb quietly to progressive paralysis.

In Central and South America, the vampire bat is now known to be an important reservoir of infection, from which the disease is transmitted mainly to cattle in whom serious losses have been reported. Infection of vampire bats may be asymptomatic, and insectivorous bats have also been found to carry the disease in the U.S.A. Human rabies acquired from bats often takes the form of an ascending paralysis.

Rabies has been eliminated from the United Kingdom through effective quarantine measures, but the disease remains a world problem. According to the World Health Organization, 1453 people in 97 countries died of rabies in 1962, and 496,915 received prophylactic treatment for the disease. Furthermore, rabies is still widespread among wild animals in Europe, Asia, Africa and America. In 1963, 2660 wild animals in West Germany were found to be infected with rabies, and 2071 of these were foxes.

Any population increase in the animal reservoir species leads to an increased incidence of the disease which periodically reaches epidemic proportions. Control of epizootics are therefore best controlled by reducing and maintaining the size of the reservoir below a certain critical level.

Laboratory Diagnosis

Laboratory confirmation of a clinical diagnosis may be required in atypical cases in which there is no history of exposure. More often, laboratory investigation is required to establish the diagnosis of rabies in an animal responsible for a bite, so that an appropriate decision regarding prophylactic treatment can be taken to protect the bitten patient. Animals which are obviously rabid are sacrificed and their brains examined for evidence of rabies. Animals not showing any symptoms of rabies are isolated and observed for 10 days, during which time isolation of virus from specimens of saliva may be attempted. Animals who develop rabies or die during the

observation period are examined post-mortem and their brains searched for evidence of the disease.

(a) Direct examination

Post-mortem, the diagnosis of rabies is confirmed by the presence of Negri bodies, and other characteristic histopathological changes, in specimens of Ammon's horn of the hippocampus, and sometimes in the cerebral cortex, cerebellum, medulla, and mid-brain.

(i) Negri bodies

The direct microscopic examination of impressions or smears made from appropriate specimens and suitably stained for Negri bodies is a quick, convenient, and standard method of diagnosis. A small percentage of specimens from infected animals do not display Negri bodies but yield virus after mouse inoculation. Specimens not revealing direct microscopic evidence of rabies should therefore be checked by virus isolation or fluorescent antibody techniques.

(ii) Fluorescent antibody test

The fluorescent antibody test, introduced by Goldwasser and Kissling in 1958, represents an important advance in the diagnosis of rabies. It has the advantages of rapidity and accuracy, and is as sensitive as the mouse inoculation test.

Impressions, smears, or tissue sections, from appropriate specimens are stained, on a slide, with the γ -globulin fraction of anti-rabies serum, which has been prepared in hamsters or guinea-pigs and labelled with a fluorescent dye. Positive specimens, which are always compared with positive and negative controls, display masses of rabies antigen, which may vary from the size of a small particle to that of a Negri body.

(b) Virus isolation

(i) Mouse inoculation

Virus isolation may be attempted from the saliva during life, and from brain and salivary gland suspensions after death. Specimens, suitably diluted and with added antibiotics, are inoculated intracerebrally into suckling or infant mice. Mice developing symptoms

are sacrificed and their brains examined for Negri bodies and fluorescent antigen. Although 5–18 days usually elapse before symptoms develop, sufficient mice are inoculated to allow the sacrifice and examination of one or two every day, from the second day after inoculation, to provide a more rapid diagnosis. Mice are observed 30 days before a negative result is accepted.

(ii) Tissue culture

Although rabiesvirus will grow in various tissue culture systems and may be detected by fluorescent antibody techniques as soon as 14 hours after infection, previous adaptation of virus to tissue culture is usually required rendering the method unsuitable for routine diagnosis.

(c) Serological tests

Although serum-neutralization, complement-fixation and fluorescent-antibody tests may be used to estimate the level of serum antibody, they are not generally applicable in diagnosis.

Prophylaxis

(a) Control of reservoir and sources of infection

(i) Non-endemic countries

Control in non-endemic countries is adequately maintained by preventing the importation of infected animals. To this end, all dogs entering the United Kingdom are strictly quarantined in special kennels for 6 months before admission. In spite of occasional protests by caninophiles, the efficacy of this measure in keeping the country free of rabies more than justifies its retention.

(ii) Endemic countries

Control of wild-life reservoirs. Where rabies is enzootic among wild animal species, epizootics develop when the population density becomes unduly great. Control is achieved by reducing the population density of the affected species to a level below which an epizootic can no longer be supported. In these circumstances, complete eradication is not necessary.

In Latin America, where vampire bats spread infection to cattle and occasionally to man, control of the bat population has been attempted.

Control of domestic animal reservoirs. Rabid animals, stray animals, and those bitten by rabid or suspectedly rabid animals, should be destroyed. Dogs should be muzzled and leashed, and the keeping of dogs generally discouraged. Importation of dogs should not be allowed unless they have been quarantined for 6 months or, if vaccinated, 45 days.

Vaccination of domestic animals. In addition to general control measures, mass vaccination of the dog population and other susceptible domestic animals is a most important measure in the control of rabies in endemic countries. For dogs, immunization with the Flury LEP live attenuated strain, produced in chick embryos, is recommended. Immunization is commenced at 3 months of age and repeated every 3 years; any restrictive measures on dogs may be relaxed 30 days after vaccination. Although the Flury LEP strain is perfectly safe in dogs it may be pathogenic for other species, the Flury HEP strain is therefore recommended for immunization of cats and cattle. Inactivated vaccines may also be used (see Table 11).

Table 11. Rabies vaccines

Vaccine	Virus strain	Preparation in:	For use in:
Live attenuated	Flury LEP Low egg passage strain (40-60 passages)	Chick embryo	Dogs
	Flury HEP High egg passage strain (> 180 passages)	Chick embryo	Dogs, cattle, cats
	Fixed virus (Semple)	Rabbit C.N.S.	Man and animals
Inactivated	Duck embryo adapted Pasteur fixed virus strain	Duck embryo	Man
Partially inactivated	Pasteur fixed virus strain (Fermi vaccine)	Sheep C.N.S.	Man

(b) Control of rabies in man**(i) Before exposure**

Persons in certain occupations, who are exposed to a higher than normal risk, should receive routine immunization. These include laboratory staff working with rabies, dog handlers, and veterinary surgeons. The recommended course of vaccination in these individuals is three doses of inactivated vaccine at monthly intervals, followed by a booster dose 6 months later. One month after the booster dose, the patient's serum is tested by the indirect fluorescent antibody technique for the presence of antibody. If the antibody response has been unsatisfactory, the booster dose is repeated until antibody appears. Thereafter, booster doses should be repeated every 2-3 years.

(ii) After exposure

Local prophylaxis. The wound is encouraged to bleed, thoroughly cleaned with soap and water, rinsed and thoroughly irrigated with 1:500 or 1:1000 benzylkonium chloride or with a quaternary ammonium compound. Chemical corrosives, once considered to be an important part of treatment, are no longer considered necessary, except perhaps in deeply penetrating wounds. The wound, which is left unsutured, is infiltrated with high-titre antirabies serum, after the patient has been tested for hypersensitivity.

Appropriate steps for preventing tetanus and other infections known to follow dog bites must also be taken. Local application of antibiotics or suitable antiseptics are therefore necessary.

Systemic prophylaxis. Patients bitten by rabid or suspectedly rabid animals are immediately immunized, actively and passively, before laboratory confirmation of the presence of rabies in the biting animal is received. If rabies later proves absent from the biting animal, the course of immunization may be interrupted.

The treatment of patients bitten by healthy animals will depend on circumstances, particularly on the severity of the bite, the mode of its infliction, and the presence of rabies in the area. Passive immunization with hyperimmune serum as soon as possible after exposure, and observation of the animal in isolation, is the best course to adopt. If the animal becomes ill, active immunization of the

patient is commenced and laboratory confirmation of rabies in the sick animal sought. Some advocate active and passive immunization as soon as possible after exposure if the bites are severe, and especially if the head and neck are involved.

Immunization

(a) Active-immunization

(i) *Inactivated vaccines*

Pasteur's classic work on the treatment of exposed patients with antirabies vaccine, from 1885 onwards, holds a well known and honoured place in the history of immunization. After demonstrating that rabies-infected spinal cords of rabbits become less virulent during the course of desiccation, Pasteur inoculated his first patient on 6 July 1885 with great success. The patient, Joseph Meister aged 9 years, had been badly mauled by a rabid dog. Inoculation was begun with the least virulent cord material, which had been dried for 14 days, and was continued with cord material of increasing virulence administered daily for 11 days. This method of treatment was subsequently modified and eventually replaced by vaccines inactivated with phenol.

Fermi-type vaccine is prepared from central nervous system tissue of infected sheep, in which virus is partially inactivated with phenol at 22°C so that a small amount of active rabiesvirus remains. In contrast, Semple-type vaccine is prepared from central nervous system tissue of infected rabbits, in which virus is completely inactivated by phenol at 37°C. Both these vaccines sometimes give rise to severe and even fatal neuroparalytic reactions, which are believed to be auto-allergic in origin and are characterized by nerve cell destruction and, occasionally, by demyelination.

Potent vaccines, the use of which is claimed to be free of complications, have been prepared from infected suckling mice or rats and inactivated by ultraviolet light and phenol, respectively. So far, however, these vaccines have received only limited trial.

The commercial preparation of suitable inactivated vaccines from the Flury strain, grown in chick embryos, has been prevented by the low yields obtained. Higher yields of rabiesvirus have however

been obtained in duck embryos, and recently a satisfactory vaccine inactivated by β -propiolactone has been prepared in this host. The use of this vaccine has much reduced the incidence of neuro-paralytic complications, but has not yet completely eliminated them.

(ii) Live attenuated vaccines

The attenuated Flury strains, prepared in chick embryos, fail to multiply in human tissues and are therefore unsuitable for use in man; nevertheless, they provide suitable live attenuated vaccines for animals. Research on vaccines prepared from virus grown in tissue culture is continuing but, so far, no vaccine suitable for use in humans has been developed.

(iii) Administration of vaccines

Inactivated vaccines are administered, as soon as possible after exposure, by subcutaneous injection in the abdominal wall. A dose equivalent to 2 ml of a 5% tissue-suspension is repeated daily for at least 14 days, and if the risk is very high daily injections are continued for 20–30 days. Booster doses, 10 and 20 days after the last daily dose, are now considered essential, especially when active and passive immunization have been administered together.

The more rapid antibody determinations now made possible by the indirect fluorescent antibody technique allow the course of active immunization to be fitted to the antibody response. Thus, a poor antibody response to a minimum course of immunization may be recognized and the course prolonged.

(b) Passive immunization

Of course, not everybody bitten by a dog develops rabies, it is therefore difficult to assess the efficacy of vaccine in preventing the onset of the disease. Most of the evidence is circumstantial and recent small controlled trials indicate that, at best, vaccine is only partially effective in preventing the disease. In contrast, the experiments of Habel, and Koprowski and his colleagues, as well as later clinical trials, have clearly established the marked efficacy of rabies hyper-immune serum in preventing the onset of the disease, if administered

within 24 hours of exposure. Administration of hyperimmune serum is only partially effective 24–72 hours after exposure, and after 72 hours it is ineffective.

The most effective protection after exposure is provided by vaccination supplemented by passive immunization, using the gamma-globulin fraction of rabies hyperimmune horse serum. As soon as as possible after exposure 50–100 units/kg are injected intramuscularly, preferably within 24 hours and not later than 72 hours. Before use, the patient's sensitivity to the antiserum is ascertained and desensitizing procedures commenced if necessary. Although a high incidence of serum reactions has been noted, these are not usually serious enough to override the use of hyperimmune serum in so serious a disease. If available, serum prepared in a non-equine species may have to be resorted to.

Because passive immunization with hyperimmune serum may inhibit the antigenic effect of simultaneously administered vaccine, booster doses of vaccine given 10 and 20 days after the last daily dose are required to maintain the active antibody response.

Treatment

No specific antiviral treatment is available. Symptomatic treatment is provided by heavy sedation, antispasmodics, and the use of muscle-relaxing drugs.

CHAPTER 34

Viral Hepatitis

I. Virus A—Infective Hepatitis

Infective hepatitis, which is endemic and epidemic in all parts of the world, is a common disease which becomes especially prevalent in times of war. Its viral aetiology, now well established, has been extremely difficult to prove because no experimental animal, with the possible exception of chimpanzees and marmosets, is susceptible to infection.

The Virus

(a) Cultivation

(i) *Human volunteers*

In the absence of a susceptible experimental animal, the first experimental transmission of the disease was established in human volunteers by Vogt, in 1942. Originally, the disease was transmitted by oral administration of duodenal fluid obtained from a patient with infective hepatitis; since that time, the presence of the aetiological agent in the blood and faeces of patients suffering from infective hepatitis has been clearly established in human volunteers. The passage of the infective principle through bacteriological filters left little doubt of its viral nature.

(ii) *Tissue culture*

Early claims reporting isolation of infective hepatitis virus in embryonated eggs have not been confirmed, nor has it been possible to grow the virus in conventional types of tissue culture, generally available in the laboratory. Recently, Richtsel and his colleagues isolated a singular group of cytopathogenic viruses from the serum and faeces of patients with infective hepatitis, using Detroit-6 cells.

These cells, which were originally derived from human bone marrow, are malignant epithelial-like cells. More recently, using improved techniques and a clone of Detroit-6 cells sensitive to the virus, this team of investigators has fully confirmed its original observations and established that tissue cultured virus gives rise to clinical hepatitis in volunteers, and stimulates the production of specific antibody.

Attempts by other laboratories to isolate infective hepatitis virus in Detroit-6 cells have produced conflicting results. But the recent isolation of virus in Detroit-6 cells from several cases of infective hepatitis in Melbourne, by Cole, and in Roumania, by Lazlo and his colleagues, suggests that the failure of some laboratories may have been due to the technical difficulties which attend the culture of these cells. The aetiological significance of this new group of viruses has not been readily accepted, and further confirmation of the work of Richtsel and his colleagues will be required to establish it unequivocally.

Identification of the causative virus of infective hepatitis has been further confused by the isolation of adenoviruses, echoviruses, coxsackieviruses, and a virus closely resembling the paramyxoviruses, from patients with the clinical syndrome of infective hepatitis. But in no case has the causal relationship between the isolated virus and the hepatic condition been established. Probably, they are unrelated and represent the fortuitous isolation of adventitious viruses from cases of infective hepatitis. Alternatively, the viruses isolated are responsible for specific clinical syndromes in which hepatic involvement is part of a generalized disease.

(b) Properties of the virus

The virus isolated by Richtsel and his colleagues has been examined in the electron microscope. In purified preparations, it is a very small particle about 15 m μ in diameter, and therefore one of the smallest human pathogens yet described.

Examination of clinical specimens has shown the virus to be very stable, resisting heat at 60°C for 30 minutes and surviving at -20°C for more than 2 years. The virus is also resistant to ether, and to chlorine in concentrations of 1 part per million.

Clinical Features**(a) Pre-icteric phase**

The onset of infective hepatitis is characterized by mild or moderate pyrexia, malaise, lassitude, headache, and the gastro-intestinal symptoms of anorexia, nausea, vomiting, and sometimes diarrhoea. Lymphadenopathy, particularly of the posterior cervical glands, an enlarged tender liver, and an enlarged spleen, may be found towards the end of this stage of the disease, as well as in the icteric phase.

At the end of the pre-icteric phase, which usually lasts 1–8 days, but may last as long as 2–3 weeks, about half the patients develop jaundice and proceed to the icteric phase of the disease. In those without jaundice, hepatic involvement is revealed by abnormal liver function tests.

(b) Icteric phase

A marked symptomatic improvement is often evident towards the end of the pre-icteric phase but gastro-intestinal symptoms return a day or two before the onset of jaundice. Jaundice, with the attendant bilirubinaemia and light-coloured stools, reaches a maximum in 10–14 days and usually lasts 4–6 weeks; rarely, jaundice may persist for 3–4 months. Recovery from the disease is the rule, but acute or subacute necrosis of the liver may prove fatal in approximately 1 in 1000 cases.

In a few cases, relapses occur which almost always result in complete recovery, although rarely chronic hepatic disease may supervene. Chronic hepatic disease, in which post-necrotic scarring of the liver occurs following an attack of infective hepatitis, carries a grave prognosis. It is more common in older patients, and particularly in females at certain times of endocrine change as at puberty, during pregnancy, and after the menopause.

(c) Subclinical and inapparent infections

A number of infections with the virus of infective hepatitis are anicteric, subclinical, or inapparent, and produce few or no clinical signs or symptoms. Although clinically unrecognized, infection may be revealed by abnormal liver function tests. Subclinical and in-

apparent infections are believed to be particularly common in children.

Pathogenesis

It has long been known from experiments on human volunteers that the incubation period of the disease is 2–8 weeks, and that virus is present in the blood and faeces of volunteers in the pre-icteric and icteric phases of the disease. These observations have now been supplemented by the classic studies of Krugman and Ward in children at the Willowbrook State School, New York. They have shown that virus is present in the stools towards the end of the incubation period, about 2–3 weeks before the onset of jaundice, and persists until about 1 week after the onset of jaundice. On two occasions, virus has been recovered from the stools of infants with chronic hepatitis 5 and 15 months after onset. Viraemia has been demonstrated during the pre-icteric phase of the disease and shown to persist until about 3 days after the onset of jaundice; it has also been demonstrated in a case of inapparent infection.

From the limited evidence made available by studies on human volunteers, certain facts have now been established. Infective hepatitis is characterized by a long incubation period, towards the end of which virus appears in the stools and persists there until about 1 week after the onset of jaundice. It further appears that inapparent and anicteric cases outnumber those in whom jaundice is manifest; moreover, virological and pathological evidence indicates that infective hepatitis is a generalized infection. From this, and epidemiological evidence discussed below, it has been suggested that the pathogenesis of infective hepatitis resembles that of poliomyelitis. Thus it is suggested that infection is initiated in the alimentary tract, and that viraemia may occur later and lead to generalized infection. Less frequently, if the target organ is affected, jaundice occurs, representing, like paralysis in poliomyelitis, a relatively uncommon event in an otherwise common infection.

This concept of the pathogenesis of infective hepatitis is based on observations which are necessarily incomplete, but the introduction of virological methods of study will undoubtedly contribute to the final elucidation of the problem.

Pathology

The hepatic lesion of infective hepatitis is characterized by focal necrosis of liver cells and periportal mononuclear cell infiltration. Kupffer cells become swollen, and bile thrombi are present in the canaliculi. Normally, evidence of hepatic regeneration, manifested by the irregular shape of the hepatic cells and the prevalence of mitoses and cytoplasmic basophilia, soon appears. Eventual return to normal hepatic architecture is the rule unless necrosis of parenchymal cells has been severe and the reticulin framework damaged; post-necrotic scarring leading to chronic hepatic disease may then occur. If fatal acute hepatic necrosis develops, there is complete destruction of parenchymal cells and no evidence of cellular regeneration.

Although extrahepatic lesions in fatal cases of infective hepatitis have been described, it was only recently that Conrad and his colleagues described lesions in the stomach, small intestine, and kidneys, of non-fatal cases occurring in military personnel in Korea. These observations indicate that infective hepatitis is a generalized systemic disease in which the liver is not the only organ affected.

Epidemiology

(a) Occurrence and distribution

Infective hepatitis is endemic in all parts of the world and outbreaks of epidemic character occur from time to time. The disease tends to be most prevalent in the autumn and winter months.

(b) Source of infection

Man is the only naturally susceptible host, in whom subclinical and inapparent infections, as well as icteric and anicteric cases of the disease, provide the sources of infection. The disease is most common but least severe, and often inapparent, in children under 15 years of age. Because infection is often undetected in members of this age group they provide a dangerous source of infection.

(c) Transmission of infection

(i) *Faecal-oral route*

Infection of volunteers by the oral route and excretion of virus in the faeces suggests that the faecal-oral pathway is the most likely route of transmission. Suggestions that the disease may be spread by droplet infection have not been confirmed. The excretion of virus in the stools from 2–3 weeks before the onset of jaundice and for at least 1 week afterwards defines the period, probably commencing before the onset of the clinical disease, during which the patient is infectious.

Transmission via the faecal-oral route may be by direct or indirect contact. In most outbreaks, infection is spread by close contact with infected patients, although the means by which the virus is spread from one person to another is not precisely known. Other outbreaks have been traced to sewage contaminated water, various foods contaminated by food handlers, and to oysters and clams bred in sewage contaminated water.

In common with other infections spread by the faecal-oral route, outbreaks of infective hepatitis are particularly severe in closed communities where conditions of poor sanitation, overcrowding, and low standards of personal hygiene prevail. Institutions for mental defectives and military camps are therefore subject to large outbreaks. Recent reports indicate that hospital personnel in close contact with infective hepatitis patients are exposed to a high risk of infection, particularly in paediatric wards.

McCollum has drawn attention to the resemblance between the epidemiological characteristics of infective hepatitis and those of poliomyelitis; it is suggested that infective hepatitis is primarily an alimentary infection which is more widespread than the incidence of jaundice makes evident. Like poliomyelitis, infective hepatitis was common among allied military personnel in the Middle East during World War II, although it was uncommon in the indigenous populations. Theoretically, and paradoxically, the better immunity of the latter can be ascribed to their lower hygienic standards, which would facilitate the spread of an infection transmitted by the faecal-oral route and hence lead to a high incidence of infection in children

and to subsequent immunity. In contrast, childhood infections of this type would be less common in societies with more advanced hygienic standards, and a high proportion of susceptibles would be found in older age groups, in whom infection is more commonly associated with clinical symptoms and jaundice. This hypothesis explains the increased incidence of infective hepatitis in allied troops serving in the Middle East, and is supported by the recent shift in age incidence of the disease, from childhood to adult life, which has been noticed in the U.S.A. and Sweden. It may be noted that no change in the age incidence of the disease has yet occurred in the United Kingdom.

(n) Parenteral transmission

The prolonged viraemia which occurs towards the end of the incubation period, and in the pre-icteric and icteric stages of the disease, makes the transfer of infective hepatitis by infected blood possible. Transfer of infective hepatitis, which must be clearly distinguished from serum hepatitis, may be effected parenterally by blood or blood products, and by contaminated syringes or instruments. Although it is unlikely that infective hepatitis is commonly spread in this way, a serious outbreak of infective hepatitis recently occurred in a renal dialysis unit, in which infection was transferred to medical attendants, laboratory workers, and other patients.

Immunity

After an attack of infective hepatitis, a state of immunity develops which is generally accepted to produce life-long protection against a second attack. Nevertheless, in Krugman and Ward's series 4·6% developed a second attack within 2-16 months, although it is not known if these recurrences were endogenous or additional exogenous infections.

The production of antibody against the virus isolated from infective hepatitis has been demonstrated by Rightsel and his colleagues, using a modified neutralization test. In this test, convalescent serum is incubated with the host cells at 37°C before challenge virus is added. The effect measured is therefore cell-blocking rather than

direct neutralization of the virus, but its specificity and the rise in titre after infection indicate its antibody nature. Surprisingly, antibody produced in response to experimental infection with this virus disappears within one year, whereas antibody produced after natural hepatitis persists for several years. Moreover, in experimental infections, virus and antibody are present together in the same serum specimen.

Among the non-specific factors known to affect the outcome of the disease are malnutrition, endocrine changes, debility, and age.

Laboratory Diagnosis

Routine laboratory diagnosis of infective hepatitis by virus isolation or by serological tests is not yet available, and discussion of the non-specific liver function tests, which are widely employed in the diagnosis of the disease, is not within the province of a virological textbook.

Recently, a haemagglutination test has been described by Havens, and others, the value of which remains to be determined. Seventy per cent of sera from clinical cases of viral hepatitis, particularly those from patients in the early stages of the disease, have been found to agglutinate erythrocytes from day-old chicks. In contrast, only a small percentage of sera from normal people, or from patients suffering from other hepatic conditions, haemagglutinated. The destruction of the haemagglutinin at 60°C. in 30 minutes, and the agglutination of day-old chick erythrocytes by sera from patients with infectious mononucleosis, make it unlikely that the haemagglutinin is the infective hepatitis virus. The identification of the haemagglutinin in the γ -globulin fraction of the serum, and other evidence, suggests that it is a heterophil antibody, distinct from that which occurs in infectious mononucleosis.

Control

The control of infective hepatitis, which is almost certainly spread by the faecal-oral route, demands the same prophylactic measures employed in preventing any other enteric infection.

(a) General measures

Scrupulous attention to personal hygiene, particularly for those engaged in food-handling and in water undertakings, is essential. Likewise, provision of pure water supplies and the proper disposal of sewage is necessary.

(b) Control of patients and contacts

Excretion of virus before the onset of the disease and during the pre-icteric phase, when the diagnosis is often obscure, together with the relatively high proportion of subclinical and inapparent infections, makes isolation and quarantine measures of little use in controlling the spread of infection in the community, or in the patient's family.

The risk to hospital personnel of acquiring infective hepatitis from patients has recently been emphasized, patients admitted to hospital should therefore be isolated and their excretions concurrently disinfected. Patients should be considered infectious until at least 1 week after the onset of jaundice. Although quarantine of contacts is unnecessary it may be prudent to exclude them from food handling.

During the course of an outbreak, attempts should be made by epidemiological investigation to ascertain the vehicle of infection, if any, and the appropriate steps taken. In this connection, it may be noted that the usual methods of chlorinating water are probably ineffective against the virus of infective hepatitis.

Since it is known that infective hepatitis can be transmitted by blood and blood products, the precautions employed in the control of serum hepatitis (see below) should be observed. Syringes, needles and other instruments likely to become contaminated should be of the disposable type, or be sterilized by autoclaving, or by hot air at 180°C for 1 hour. Patients with a history of infective hepatitis should not be accepted as blood donors.

(c) Passive immunization

The long incubation period of infective hepatitis makes possible the modification of the disease, in contacts, by passive immunization with pooled human γ -globulin. The administration of γ -globulin

does not prevent infection but modifies the signs and symptoms, and may prevent them completely if given as soon as possible after exposure. γ -globulin has been reported to be effective in preventing jaundice if administered at least 6 days before the onset of symptoms.

Passive immunization usually wears off after 6–8 weeks, but may persist for several months. When long-lasting, Stokes and his colleagues suggest that the immunity is acquired by subclinical infection occurring under the cover of passive immunity, i.e. it is the result of active and passive immunity.

In this country, supplies of γ -globulin are very limited but its use may be considered in institutional outbreaks, and for debilitated individuals or pregnant women in intimate contact; if adequate supplies of γ -globulin are available, family contacts should also be passively immunized. Although providing some protection for the individual, γ -globulin does not prevent the spread of virus in the community; indeed, by increasing the number of subclinical cases it may even facilitate it. Its use in the control of epidemics, even if sufficient γ -globulin was available, is therefore questionable.

Treatment

Treatment of infective hepatitis is symptomatic, and no specific antiviral treatment is yet available.

II. Virus B—Serum Hepatitis

Serum Hepatitis resembles infective hepatitis in its clinical, laboratory and pathological features but is distinguished by its very long incubation period, its insidious onset, and by the absence of any virus excretion. It tends to be more severe than infective hepatitis and is associated with a poorer prognosis; possibly, this is because serum hepatitis more often occurs in older and debilitated patients.

Epidemiology

Serum hepatitis is transmitted parenterally and never, like infective hepatitis, by the faecal-oral route. It is acquired after administration of blood or blood-products, and, rarely, after transplantation of human tissues. It may also be transmitted by the use of a contaminated syringe and needles, or by instruments which penetrate the skin or mucous membranes.

(a) Transmission by blood and blood products

The greatest danger of serum hepatitis arises from administration of pooled human plasma. Attack rates of up to 12% have been reported after plasma transfusions, to which a number of unrecognized subclinical anicteric cases of serum hepatitis, now known to occur, must be added. The risk is related to the number of donors contributing to the pool and is reduced significantly if the number of donors is limited. Unsterilized plasma products, such as fibrinogen and thrombin, also carry a high risk, but γ -globulin prepared by the Cohn cold ethanol fractionation technique is free of virus.

Transfusion of whole blood carries a much lesser degree of risk, estimated at less than 1%. The more blood transfused, the greater the risk becomes, because of the increased number of donors required.

Convalescent human serum used for therapeutic or prophylactic purposes, and prophylactic products containing human serum, are other important vehicles of transmission. Indeed, it was the severe outbreaks which occurred after administration of yellow fever vaccine, containing human serum, which brought the condition to general notice in World War II.

(b) Transmission by contaminated syringes, needles and instruments

The danger of transmission by syringes and needles contaminated with infected blood is evident from the large outbreaks which have occurred in clinics administering large numbers of injections with common syringes and needles. The disease has also occurred after the use of imperfectly sterilized dental equipment, and even after

tattooing. Among hospital and laboratory personnel, accidental self-inoculation by contaminated apparatus or laboratory glassware is an occupational hazard.

(c) Source of infection

It is well known that asymptomatic carriers of serum hepatitis virus occur, and that inapparent infection followed by prolonged viraemia may occur after inoculation. Stokes has estimated the carrier rate in the U.S.A. to be between 2 and 4%. Although these carriers provide a significant reservoir of infection, the biological survival of serum hepatitis virus in the community is difficult to explain if transmission is dependent entirely on artificial inoculation.

Two hypotheses have been put forward to explain the biological survival of serum hepatitis virus, both of which suggest a relationship to the virus of infective hepatitis. Havens suggests that the virus of infective hepatitis becomes modified in some patients, and persists as serum hepatitis virus. Significantly, virus has been recovered from a volunteer, as long as 121 days after infection with the infective hepatitis virus isolated by Rightsel and his colleagues. An alternative hypothesis, suggested by Burnet, postulates the transmission of infective hepatitis virus to the foetus in utero and the production of a state of immunological tolerance, allowing the infant to become a long term carrier of the virus variant. Although it is difficult to reconcile this hypothesis with the antigenic difference between the two viruses, transmission of infective hepatitis virus in utero has been demonstrated.

Pathogenesis

No evidence of alimentary tract infection is found during the course of serum hepatitis, nor is any virus excreted in the faeces during the course of the disease. In contrast, viraemia is easily demonstrable, with the aid of human volunteers, during the long incubation period and in the acute phase of the disease. In one instance, viraemia has been demonstrated as long as 87 days before the onset of symptoms. During the convalescent period, viraemia is not so easily demonstrable but prolonged and intermittent viraemia after infection has

been observed. Stokes and his colleagues have described a case in which viraemia was present for as long as 5 years.

Prolonged viraemia in the absence of hepatic involvement has led F.O. MacCallum and other authors to suggest that hepatic damage in both serum and infective hepatitis arises from an immunological reaction between an altered liver antigen and an auto-antibody, rather than from a direct action of the virus.

Properties of the Virus

MacCallum's early filtration studies showed the virus to be less than 26 m μ in diameter. The virus is highly stable, resisting heat at 60°C for as long as 4 hours, and surviving at low temperatures for long periods. It is also resistant to ether and ultraviolet light, but may be inactivated by a combination of ultraviolet light and 0.3% β -propiolactone.

There seems little doubt that the virus of serum hepatitis is antigenically distinct from infective hepatitis virus. No cross immunity is demonstrable between the two viruses, and a dosage of γ -globulin which effectively prevents jaundice in infective hepatitis is less effective in serum hepatitis.

Control

(a) Blood and plasma transfusion

Limitation of unnecessary blood transfusions is an obvious aid to control, and selection of donors is a prudent measure. Although it is not yet possible to detect asymptomatic carriers of serum hepatitis, blood donors who W.H.O. authorities recommend should be rejected include those with a previous history of jaundice, those who have been in contact with infective hepatitis during the preceding 6 months, those whose blood is suspected of responsibility for a case of serum hepatitis, and those who have received a transfusion. Narcotic addicts, who have high carrier rates, should also be excluded.

No satisfactory or acceptable method of inactivating serum hepatitis virus in plasma has yet been found. Plasma should not therefore

be used if other satisfactory substitutes are available. If plasma transfusion is unavoidable, it is recommended that not more than two donors should contribute to any plasma pool.

Syringes and needles used for parenteral injections or venipuncture, transfusion equipment, and any instrument which penetrates the skin, should be of the disposable type, or adequately sterilized in the autoclave, or in the hot air oven at 180° for 1 hour, before use.

The use of γ -globulin, although less effective in preventing jaundice in serum hepatitis than in infective hepatitis, is beneficial if given in adequate dosage soon after transfusion and again 1 month later. The limited supplies of γ -globulin available prevent its routine administration after transfusion, but its use may be considered for those at high risk, like aged and debilitated patients and those requiring repeated and extensive transfusions.

Table 12. Distinctive features of infective and serum hepatitis

Feature	Infective hepatitis	Serum hepatitis
Incubation	2-8 weeks	8-26 weeks
Onset	Acute	Insidious
Age	More common in children	All ages
Spread	Faecal-oral route; parenteral, occasionally	Parenteral
Evidence of alimentary tract infection	Present	Absent
Virus excretion	Present	Absent

CHAPTER 35

Arbovirus Infections

I. Arboviruses General Description

Arthropod-borne viruses, better known by the abbreviated term arboviruses, comprise those viruses which are transferred from one vertebrate host to another by blood sucking arthropod vectors. In nature, arboviruses are maintained in various species of vertebrates, in whom infection is characterized by a stage of viraemia which may or may not produce clinical signs and symptoms. The vector feeds on vertebrate blood which, if infected, transmits the virus to the arthropod tissues, where it multiplies and eventually reaches the salivary glands. Henceforward, the arthropod vector remains infected for life, and the virus may be transmitted in the saliva to new vertebrate hosts through the bite by which the vector takes its feeds. In contrast to some viruses which are transmitted by insects acting as mechanical vectors, arboviruses actually replicate in the arthropod tissues which serve as an important link in the chain of virus survival.

A wide variety of extrahuman mammalian and avian species act as natural hosts for arboviruses. In appropriate ecological circumstances, the arthropod vector may leave its natural host to feed on and infect man. The incidental use of man as a source of a blood-feed may be considered a biological disaster, unnecessary for the maintenance of the virus in nature. With the exception of sandfly fever, dengue, and urban yellow fever, for which man is an important natural host, transfer of infection to man is inimical to virus survival. Incapacitated and very often killed by the infection, man is usually unable to serve as a source for further human infection or as a link in the natural chain of arbovirus transmission; consequently, infection of man brings the epidemiological cycle to an end.

Although only three groups of arthropods (mosquitos, ticks, and sandflies) are responsible for the transfer of arbovirus infections to man, 51 different arboviruses are known to be pathogenic for man. In addition about 200 more arboviruses are known which are not pathogenic for man. With so rich an aetiological spectrum, encompassing a diversity of clinical and epidemiological features, only a brief outline of arbovirus infections can be attempted here.

Properties of Arboviruses

(a) Morphology

The genus *Arbovirus* comprises a morphologically heterogeneous group of viruses, only a few of which have been studied in any detail by electron microscopy. With the exception of dengue and one or two other viruses which are rod-shaped particles, all appear to be spherical in shape and range from approximately 20-125 m μ . All the arboviruses affecting man, and many others, appear to consist of a dense core surrounded by a lipid envelope from which, in some strains, radially orientated projections protrude.

(b) Chemical and physical properties

All arboviruses so far studied have proved to be RNA viruses, and infective RNA has been extracted from some of them. With few exceptions, arboviruses are inactivated by ether and sodium desoxycholate, indicating the presence of a lipid component. Trypsin and other proteolytic enzymes inactivate arboviruses belonging to antigenic Group B but not those belonging to Group A.

Arboviruses are unstable at room temperature and at acid pH, but may be stored satisfactorily at -70°C or in the lyophilized state, particularly in protein media.

(c) Biological properties

Haemagglutinating and complement-fixing components can be extracted from infected tissues, and are present in infected tissue culture fluids. These components have been identified as the infective virus particles but some arboviruses produce soluble haemagglutinins also.

Red cells of geese or day-old-chicks are used for arbovirus haemagglutination, although fowl and sheep red cells, which are less sensitive, may also be used. The optimal temperature for haemagglutination by most arboviruses is 37°C, but some strains haemagglutinate best at room temperature. Marked pH dependence is also characteristic of arbovirus haemagglutination, and most viruses belonging to antigenic group A haemagglutinate best at pH 6·6-6·3, whereas those belonging to group B haemagglutinate best at pH 6·3-6·8. Non-haemagglutinating variants of haemagglutinating strains sometimes occur and may cause confusion.

(d) Antigenic composition

By means of complement-fixation, haemagglutination-inhibition, and, in some cases, neutralization tests, arboviruses have been classified into more than 20 groups. Most viruses belong to antigenic Groups A, B, or C or the Bunyamwera group. The others are grouped into a number of smaller groups, and many remain ungrouped. Within each group, strain-specific antigens are best detected by intracerebral neutralization tests in suckling mice, or by neutralization tests in tissue culture.

(e) Cultivation

(i) Experimental animals

Intracerebral inoculation of suckling mice is the most sensitive method for primary isolation of arboviruses. Signs of encephalitis are produced in about 5 days and the infected brain provides a rich source of virus. Inoculation of suckling mice by other routes or inoculation of weanling mice, although possible, is not always successful.

(ii) Chick embryos

Some arboviruses may be isolated by chorioallantoic inoculation of 13-day-old chick embryos, or by yolk-sac or intraembryonic inoculation of 8-10-day-old chick embryos. Many antigenic Group B viruses produce pocks on the chorioallantoic membrane.

(iii) Tissue culture

In general, cells in culture are less sensitive than suckling mice for primary isolation of arboviruses, but may be used advantageously for the growth of some arbovirus strains after adaptation. Tissue culture techniques, if successful, are especially convenient for providing quantities of virus for use as antigens, or for the preparation of vaccines.

Chick embryo, primary hamster kidney, BHK-21 (baby hamster kidney cell line), and HeLa cells, are widely used for the cultivation of arboviruses, which vary not only in their affinity but also in their pathogenicity for different types of tissue culture cells. Some strains replicate in tissue culture but do not produce cytopathogenicity, and others may produce cytopathogenicity only after several passages.

Experiments in tissue culture have revealed that arboviruses replicate in the cell cytoplasm and mature at the cell surface in a manner similar to that of myxoviruses.

Clinical Syndromes

(a) Encephalitis

A number of arboviruses localize predominantly in the brain producing encephalitis or meningo-encephalitis. Some infections cause only a mild aseptic meningitis but others give rise to coma, paralysis and death.

(b) Systemic undifferentiated fevers

Many arbovirus infections produce comparatively mild illnesses, characterized by fever, malaise, headache, muscle and joint pains, and occasionally a rash.

(c) Haemorrhagic fevers

Yellow fever and some of the tick-borne infections are characterized by haemorrhagic manifestations in addition to fever, headache, malaise, and muscle and joint pains. In yellow fever, jaundice due to severe liver damage accompanies the haemorrhagic syndrome. These infections are often serious and sometimes fatal.

Transmission

Three Families of arthropods, only, are responsible for transmission of disease to man; mosquitos belonging to the Family Culicidae, ticks belonging to the Family Ixodidae, and sandflies belonging to the Family Psychodidae. In general, there is no relationship between the antigenic group of a virus, its arthropod vector, or the clinical syndrome it produces; however, all Group A viruses are mosquito-borne.

Laboratory Diagnosis

Laboratory infections with arboviruses, in which virus gains entry by inhalation or trauma, are not infrequent, and may be very severe and even fatal. Extremely strict safety precautions are therefore necessary in those laboratories where arboviruses are investigated, and immunization of laboratory personnel against those viruses for which vaccines are available should be the rule.

(a) Virus isolation

Viraemia is usually present during the first few days of infection and virus may be isolated from the blood during this period, but in St Louis and Japanese B encephalitis viraemia is short-lived and virus can only rarely be isolated from the blood.

In tick-borne encephalitis, and rarely from some mosquito-borne encephalitides, virus may be recovered from the cerebrospinal fluid. In fatal cases of encephalitis, virus may be recovered from the brain and other organs, post-mortem. In one infection, Venezuelan equine encephalitis, virus can be recovered from the nasopharynx in throat swabs or washings.

Specimens which cannot be inoculated immediately may be stored in an alkaline, protein containing medium at 4°C for a few hours, or at -70°C for longer periods.

For primary isolation, intracerebral inoculation of suckling mice is the method of choice. Mice are observed for 14-21 days for signs of encephalitis; sick mice are sacrificed, and their brains homogenized to form 10% suspensions which are then passaged intracerebrally in further mice until a sufficiently high titre is obtained

to provide virus for further tests. If no signs of illness are produced by the first passage, at least one further passage is made before a negative result is accepted. Once isolated, some strains can be adapted to tissue culture or chick embryos to provide material for serological and other tests.

If necessary, the viral nature of the isolate may be confirmed by passage after filtration through bacteriological filters. Further evidence of identification is obtained from its sensitivity to ether and sodium desoxycholate, and the presence of haemagglutinins. The antigenic group is next determined by haemagglutination-inhibition or complement-fixation tests with group reacting immune sera; the type specificity of the isolate is then defined by neutralization tests with selected antisera. Sometimes, the experimental host range of the isolate must be ascertained before it can be identified.

New arboviruses which differ antigenically from known reference strains are still being isolated. The burdensome and specialized investigation of these strains is now conducted in special W.H.O. reference laboratories.

(b) Serological tests

Because the stage of viraemia in arbovirus infections is often short-lived, it is not always possible to confirm the diagnosis by virus isolation. Serological tests then provide the only means of laboratory diagnosis; they may also be required to demonstrate a homologous antibody response to any virus isolated, thereby confirming its aetiological role, or for epidemiological serum surveys.

More than one type of serological test for the detection of antibody may be necessary, because different types of antibody vary in their times of appearance and periods of persistence. Other difficulties may be encountered if the patient has previously been infected with an antigenically related strain; the already high titre of group-reacting antibody may obscure the antibody response to an infecting strain and may make specific serological identification difficult.

(1) Neutralization

Of the three main types of antibody produced in response to arbovirus infections, neutralizing antibody is usually the first to appear,

is the most specific, and persists for the longest period. It appears during the first week of infection, sometimes within a few hours, and reaches a peak within about 3 weeks; thereafter, the antibody titre falls but persists at a moderate level for a prolonged period and, sometimes, even for life. Because of its persistence, neutralizing antibody is the best indicator of previous infection, and neutralization tests are the most useful for conducting a retrospective epidemiological investigation.

Arbovirus neutralization tests are usually performed by the constant serum-varying virus technique, and the neutralization index calculated. Intracerebral inoculation of the serum-virus mixtures into suckling mice is the technique of choice, but tissue culture, plaque neutralization, and haemadsorption-inhibition, tests may be used with tissue culture adapted strains.

Sera which have been stored for any length of time, even at 4°C, lose some of their neutralizing capacity, because of the destruction of a heat-labile 'accessory factor'. To avoid anomalous results, test sera are heated at 56°C for 30 minutes and the heat-labile 'accessory factor' is restored, before testing, by the addition of fresh normal monkey or human serum.

(ii) Complement-fixation

Complement-fixation antibody usually appears 1-2 weeks after neutralizing antibody and reaches its maximum titre a week later; thereafter, the level quickly falls reaching low or undetectable levels within a year.

(iii) Haemagglutination-inhibition

Haemagglutination-inhibiting antibody, which is the least specific of the three types of antibody, appears shortly after the neutralizing and before the complement-fixing antibodies. It reaches its maximum titre in 3-4 weeks and thereafter falls to more moderate levels, which may persist for long periods.

Before testing, sera should be treated with kaolin and extracted with acetone to remove non-specific inhibitors of arbovirus haemagglutination, absorption with red cells to remove naturally occurring agglutinins is also required. Recent evidence that kaolin

removes early (19S) antibody should be borne in mind when testing early convalescent sera.

Control

Control and prevention of arbovirus infections depends on the control of the arthropod vector, immunization of the exposed human population, and, if possible, the control of any extrahuman reservoirs of infection. Ideally, eradication of the vector is required, but only rarely is this practical; reduction of the vector population below a 'critical density' has proved sufficient in the control of some arbovirus infections. Eradication and density reduction of the vector may be achieved by chemical and physical treatment of the arthropod and its breeding grounds. Drainage of mosquito breeding grounds, control of ticks by scrub and pasture clearance, and rodent control, are further important measures for the control of vector populations.

So far, it has proved uneconomic and impractical to control any of the extrahuman reservoirs of infection. The human host may, however, be protected from vectors in endemic areas by the use of protective clothing and bed nets, the screening of living quarters, and the use of insect repellants. Vaccination is a further means of protecting the susceptible human host but, so far, only the vaccine against yellow fever is of proven value. Vaccines against some other arboviruses have been and are being developed but none has yet been fully evaluated.

Therapy

There is no specific antiviral therapy for arbovirus infections.

CHAPTER 36

Arbovirus Infections

II. Mosquito-borne Arbovirus Encephalitides

Encephalitides due to Arboviruses of Antigenic Group A

(a) Western equine encephalitis (WEE)

(i) Clinical features

Mild cases present with the symptoms and signs of aseptic meningitis; these are fever, headache, vomiting, and pain and stiffness of the neck and back. In more severe cases, drowsiness, stupor, and coma supervene, and in children convulsions frequently occur. Death occurs in about 3% of cases, otherwise recovery occurs after a week or two, and sequelae are uncommon except in children.

(ii) Pathology

The meninges are congested and oedematous, and microscopic examination reveals infiltration with mononuclear cells. In the central nervous system, lesions are found mainly in the basal ganglia, pons, and cerebellum, and are characterized by focal neuronal destruction, petechial haemorrhages, perivascular cuffing, endothelial proliferation, and areas of demyelination associated with mononuclear infiltration.

(iii) Epidemiology

WEE is endemic in the Western United States and Canada, as well as in Central and South America; the disease usually becomes prevalent in the summer months, and epidemics occasionally occur.

The primary reservoir of infection is in wild birds, in whom the disease is asymptomatic and the viraemia prolonged. Small rodent species may also act as reservoirs. The principal vector is *Culex tarsalis*, although other species of mosquito have also been incriminated. Normally, the vector transmits infection from bird to bird, but under favourable conditions it may transmit infection to man or horses which serve as tangential hosts, unnecessary for the cycle of transmission which is brought to an end.

The majority of WEE infections in man are inapparent and probably only 1 in 1000 infections actually produces clinical symptoms.

(iv) Control

Mosquito control and eradication programmes have reduced the incidence of infection in affected areas. Horses, in whom the mortality from WEE is high, can be protected by formalin-inactivated vaccines, prepared from virus grown in chick embryos. Such vaccines have not been recommended for general use in man, but laboratory workers who are exposed to undue risk should be vaccinated.

(b) Eastern equine encephalitis (EEE)

(i) Clinical and pathological features

EEE is one of the most severe of the arthropod-borne encephalitides, and mortality in some outbreaks is as high as 60%. The signs and symptoms are the same as those of WEE but are much more severe, often producing death in 5 days. The disease occurs mainly in children, and mental deficiency, paralysis, and convulsions are common sequelae in those who survive.

The pathological lesions in the central nervous system do not differ from those found in WEE.

(ii) Epidemiology

EEE is endemic, and occasionally epidemic, in the Eastern United States and Canada, and in Central and South America. The primary reservoir is believed to be in wild birds, and outbreaks in the human

population are often preceded by severe epizootics in horses and pheasants. Several species of mosquito have been incriminated as vectors which transmit infection from the primary reservoir to man and horses; vertebrate species serve as tangential hosts in whom the cycle of transmission is not maintained.

In contrast to WEE, the ratio of inapparent to clinical EEE infection is low and is estimated to be about 50:1. Control measures are the same as for WEE.

(c) Venezuelan equine encephalitis (VEE)

(i) Clinical features

Patients infected with VEE present with signs and symptoms of mild aseptic meningitis, which lasts only a few days and is rarely fatal. Signs of encephalitis occur only in a minority of cases. A unique feature of VEE, which distinguishes it from other arbovirus encephalitides, is the presence of virus in the throat from which it may be isolated in the early stages of the disease.

(ii) Epidemiology and control

VEE is endemic in Venezuela and other parts of South America, and in Trinidad. The primary reservoir of infection is unknown, but some rodent species have been suspected. Almost certainly, infection is transmitted by mosquitos to horses and man, but transmission by contact or droplet infection is also possible. Clinically inapparent infections sometimes occur.

In the absence of an accurate knowledge of the virus ecology, or of a suitable vaccine, control of VEE is a difficult task.

Encephalitides due to Arboviruses of Antigenic Group B

(a) Japanese B encephalitis

(i) Clinical features

Japanese B encephalitis is one of the more serious forms of arbovirus encephalitis. Prodromal symptoms of nausea, anorexia, and drowsiness, lead in a few days to the acute onset of high pyrexia and the symptoms and signs of meningo-encephalitis. Restlessness, speech defects, pareses, delirium, and sometimes coma, are prominent

signs; in fatal cases, coma deepens and death supervenes. In different outbreaks, the mortality rate has varied from 30–90%. In those who recover, the temperature falls by lysis after about 5 days and recovery from the acute phase is complete in about 2 weeks. Mental impairment, personality changes, and various forms of paresis, are common sequelae, especially in children.

In contrast to some other arbovirus infections, the virus of Japanese B encephalitis is rarely isolated from the blood or cerebrospinal fluid.

(ii) Pathology

The changes in the central nervous system are similar to those seen in other arbovirus encephalitides but neuronal degeneration and neuronophagia are more prominent. Purkinje cells in the cerebellum are particularly involved.

(iii) Epidemiology

Japanese B encephalitis is endemic in Japan, China, Malaya, and other parts of South-East Asia, where epidemics, in which there is a high incidence of infection in children, are frequent. The primary reservoir of infection is in pigs and wild birds, and the principal vector of transmission is *Culex tritaeniorhynchus*, although other species of mosquito have also been incriminated. When infected mosquitos become particularly numerous, man and horses are infected as tangential hosts in whom the cycle of transmission is not maintained.

(iv) Control

The extensive distribution of *C. tritaeniorhynchus* makes its control an impractical proposition. Formalin-killed infected mouse brain vaccine has been under trial, and some degree of protection in Japanese children has been claimed.

(b) Murray Valley encephalitis

The virus of Murray Valley encephalitis, although immunologically distinct, is antigenically very closely related to the virus of Japanese B encephalitis. The disease itself resembles Japanese B encephalitis,

and, like it, has a high rate of mortality and is followed by a high incidence of sequelae.

The primary reservoir of infection is in the wild birds of Northern Australia, and the vector of transmission is *C. annulirostris*. Infection is brought south by the migration of wild birds, and the disease may then be transmitted to fowl and water birds which become temporary reservoirs of infection. Man is a tangential host in whom the cycle of transmission is not maintained.

(c) St Louis encephalitis

(i) Clinical features

The virus of St Louis encephalitis is another virus closely related to that of Japanese B encephalitis. Most infections are inapparent, and clinical infections, which are generally mild and without sequelae, occur only in a minority of cases. In a small percentage of patients, usually the elderly, infection is more severe and signs and symptoms of meningo-encephalitis, similar to other arbovirus encephalitides, are produced. As with Japanese B encephalitis, virus is rarely recovered from the blood of patients with St Louis encephalitis.

(ii) Epidemiology and control

St Louis encephalitis is endemic in the Western and Central United States, particularly in Florida, and in the Caribbean islands and South America. The primary reservoir of infection is probably in wild birds and rodents, and the principal vector is *C. tarsalis*. In the Central United States, where the disease is mostly urban in distribution, *C. pipiens* and *C. quinquefasciatus* are the main vectors. Man is a tangential host in whom the cycle of transmission is not maintained.

Control of the disease depends on the control of the relevant mosquito vectors, where possible.

(d) Ilheus

This is a very mild or inapparent mosquito-borne encephalitic infection which occurs in Central and South America, and in Trinidad.

CHAPTER 37

Arbovirus Infections

III. Mosquito-borne Arbovirus Fevers

Fevers due to Mosquito-borne Arboviruses of Antigenic Group A

These fevers are dengue-like conditions which occur in tropical or semi-tropical geographical areas. They are characterized by the sudden onset of fever, headache, and malaise, together with muscle and joint pains. An irritating maculopapular rash and lymphadenopathy often complete the syndrome.

(a) **Chickungunya**

This condition which occurs in South and East Africa exhibits a high morbidity and negligible mortality. The primary reservoir of infection has not yet been identified, although primates are suspected; *Aedes aegypti* and several other species of mosquito are the vectors of transmission. The disease has recently been found in India and South-East Asia.

(b) **O'Nyong-Nyong**

O'Nyong-Nyong fever occurs in Uganda and East Africa, and is caused by a virus closely related to chikungunya. It is transmitted by *Anopheles funestus* from an unknown reservoir.

(c) **Mayoro**

Mayoro fever occurs in Trinidad and South America. The vector is *Mansonia venezuelensis*, and the reservoir is unknown.

(d) Semliki Forest and Sindbis

Although these viruses have not yet been recovered from infected persons, the presence of antibodies to them in human sera, from India and Africa, suggests that they are capable of human infection.

**Fevers due to Mosquito-borne Arboviruses
of Antigenic Group B****(a) Dengue****(i) Clinical features**

Dengue fever is usually a mild or inapparent infection which develops after an incubation period of 5–8 days. Sometimes, prodromal symptoms of headache, malaise, and anorexia, of a few days duration, precede the acute phase of the disease which is ushered in by a sudden rise of temperature. Severe pains in the joints and limbs, conjunctivitis, photophobia, punctate erythema over the knees and elbows, and relative bradycardia, are characteristic manifestations of the first phase of the illness. After 3–5 days, the second phase characterized by a generalized irritating morbilliform or scarlatiniform rash appears, sometimes accompanied by lymphadenopathy. Recovery is usually the rule but convalescence is prolonged.

Four antigenic types of dengue virus producing the typical dengue syndrome have been recognized. In recent years, recurrent epidemics due to dengue viruses types 3 and 4 have occurred in Thailand and the Philippines, which have been characterized by a more severe haemorrhagic form of the disease in children of oriental origin. In this form, the disease is insidious in onset and presents with fever, headache, nausea and vomiting which may resolve after a few days. If not, haemorrhagic manifestations including severe cutaneous ecchymoses, epistaxis, haematemesis, and melaena, develop, together with a maculopapular rash and marked abdominal pain and tenderness. In a high proportion of cases, circulatory collapse and shock develop leading to a fatal outcome.

(ii) Pathology

Pathological lesions are mainly confined to the small blood vessels

which show evidence of endothelial proliferation, perivascular oedema, and mononuclear infiltration. In fatal cases, oedema and haemorrhagic effusions into the serous cavities, and other organs, is evidence of a gross increase in vascular permeability; hepatic necrosis is also sometimes seen.

(iii) Epidemiology

Dengue is widely distributed in tropical and semi-tropical areas, including Africa, India, the Middle East, and South-East Asia. In contrast to other arboviruses, the only known reservoir of dengue infection is man, although an extrahuman reservoir of infection in monkeys is suspected but not yet demonstrated. The principal vector is *Aedes aegypti*, but other species of mosquito have also been incriminated.

Because man serves as the principal reservoir, the disease spreads rapidly in the human population and the incidence of infection is high. In highly endemic areas, dengue is a disease of children and visiting susceptibles; most of the indigenous adult population are immune from previous infection. Reinfection with dengue viruses of heterologous antigenic type may occur.

(iv) Control

Eradication of the mosquito vector has eliminated the disease in some areas. Where this has not been practicable, various methods of mosquito control are important in preventing the spread of the disease. Live attenuated virus vaccines have been used experimentally with some success, but have not yet been used routinely.

(b) West Nile fever

(i) Clinical features

West Nile fever is a dengue-like illness which often occurs in summer epidemics in Africa, the Middle East, and South-East Asia. After an incubation period of 3–6 days, there is a sudden onset of high fever, headache, drowsiness, myalgia, conjunctivitis, maculopapular rash, and lymphadenopathy. The disease is mild, and uncomplicated recovery usually occurs in 2–3 weeks. In elderly

patients, meningoencephalitis may occur which sometimes proves fatal. In contrast, infection of children is often inapparent.

(ii) Epidemiology and control

The primary reservoir of infection is probably in wild birds, and the disease is transmitted by *Culex molestus* and *Culex univittatus*, although other species of mosquito have also been incriminated. Man serves as a tangential host in whom the cycle of transmission is not maintained. Mosquito control is an important prophylactic measure.

(c) Other fevers

Wesselbron and Spondweni viruses are other mosquito-borne Group B arboviruses of very minor importance which are found in various parts of Africa, and may cause fever in man.

**Fevers due to Mosquito-borne Arboviruses of
other Antigenic Groups**

(a) Group C arboviruses

Group C arboviruses which may cause mild fever in man, particularly forestry workers, have been isolated in Brazil. The various viruses are known by the exotic names of Oriboco, Caraparu, Apeu, Murutucu, Morituba, and Itaquí. The primary reservoirs of infection may be in rodents, and several species of mosquito are probably involved in transmission.

(b) Bunyamwera group

Three viruses of the Bunyamwera group cause mild fevers and inapparent infections in various parts of Africa. These are the Bunyamwera, Ilesha, and Germiston viruses. Another member of the group, the Guaroa virus, causes a similar infection in Colombia and Brazil.

(c) Bwamba, Catu, and Oropouche viruses

Similar mild fevers are caused by Bwamba virus in East and Central West Africa, and by Catu and Oropouche viruses in Brazil and Trinidad.

(d) Rift Valley fever virus

Rift Valley fever virus is a widespread cause of enzootic hepatitis in sheep and cattle in Central and Southern Africa. Men in close contact with infected animals may acquire infection, leading to a typical arbovirus fever. Some human infections are thought to be acquired by inhalation of virus through the handling of infected tissues.

The primary reservoirs of infection are in sheep, rodents, and other vertebrates; several species of *Aedes* and *Eretmapodites* have been incriminated as vectors. A formalin-inactivated tissue cultured virus vaccine has been effective in protecting laboratory workers.

CHAPTER 38

Arbovirus Infections

IV. Mosquito-borne Arbovirus

Haemorrhagic Fever

Yellow Fever

Yellow fever is the most important of all arbovirus infections. Although often clinically inapparent, or producing no more than a mild fever, it is best known in its severest form which presents as an acute fever followed by jaundice, haemorrhage, and albuminuria. Yellow fever is a disease confined to the tropical and subtropical regions of Africa and America, and the elucidation of its aetiology and epidemiology represents one of the most notable achievements of modern medicine.

Believed to have originated in West Africa, the disease, together with its mosquito vector *Aedes aegypti*, was introduced into the American continent by unhygienic sailing ships plying the slave trade between Africa and America. From 1648 onwards, devastating epidemics of yellow fever occurred in the Southern United States, Central America, and the West Indies.

Carlos Finlay, a physician resident in Havana, was the first to suggest, in 1881, that yellow fever was transmitted by mosquitos; his concept was proved correct in 1900 by the work of the United States Army Yellow Fever Commission working in Cuba under the command of Walter Reed. In the light of its findings, Gorgas introduced anti-mosquito measures and eradicated the disease by 1901. The Reed Commission, besides successfully contributing to the control of yellow fever, established the aetiological agent of the disease as a filterable virus. This was the first conclusive proof of viral aetiology for any human disease.

The very great contribution of the Walter Reed Commission can only be fully appreciated when the danger to which members of the Commission exposed themselves is realized. Suffice it to say, that Lazear, a member of the Commission, sacrificed his life after experimental self-inoculation. Later, yellow fever claimed the lives of no less than six participants of the Rockefeller Foundation's yellow fever programmes in West Africa and in Central and South America; they were Cross, Stokes, Noguchi, Lewis, Young, and Hayne.

Properties of the Virus

(a) Morphology

Bergold and Weibel have examined the virus by electron microscopy and found it to be a spherical particle, about 38 m μ in diameter, which consists of a dense core enveloped by a membrane (Fig. 83).

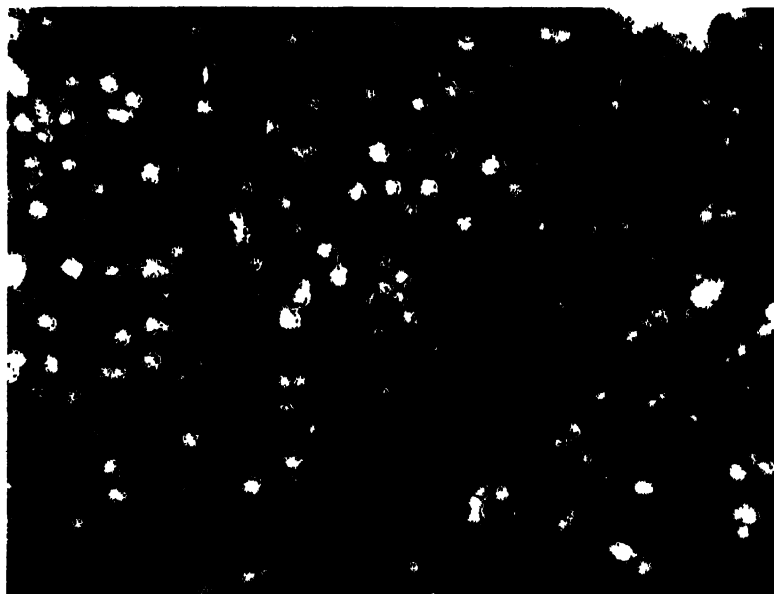


Fig. 83. Electron micrograph of yellow fever virus particles, grown in KB cells [from E.H.Bergold and J.Weibel (1962) *Virology* 17, 554-62 (Academic Press Inc., New York and London)].

(b) Chemical and biological properties

Yellow fever is an RNA virus, and, like other arboviruses, haemagglutinates. In addition to the haemagglutinating virus particles, a soluble haemagglutinin and complement-fixing antigen are produced.

(c) Antigenic composition

Yellow fever virus is antigenically a Group B arbovirus. Although the American, African, and vaccine strains of yellow fever virus are immunologically very closely related, minor antigenic differences have been described.

(d) Cultivation**(i) Experimental animals**

Transmission of the Asibi strain of yellow fever virus to rhesus monkeys, in 1927, marked the beginning of laboratory investigations devoted to yellow fever virus. These investigations were greatly facilitated, from 1930 onwards, by Theiler's adaptation of the virus to white mice by the intracerebral route.

Naturally occurring yellow fever virus is pantropic, and its pathogenicity in experimental animals is assessed in terms of its viscerotropism and neurotropism. In rhesus monkeys and man, the virus is highly viscerotropic, producing considerable viraemia and death from liver necrosis; some degree of neurotropic affinity is, however, exhibited by all strains. In contrast, the neurotropic affinity of the virus is predominant after intracerebral inoculation into white mice, and its neurotropic pathogenicity is enhanced by serial intracerebral mouse passage. Neurotropic strains which have been serially passaged in mice or monkeys, by the intracerebral route, exhibit decreased viscerotropism for rhesus monkeys.

Suckling mice succumb to encephalitis, after intraperitoneal as well as intracerebral inoculation of yellow fever virus.

(ii) Chick embryos

Strains adapted to mice, intracerebrally, or to tissue culture can be readily passaged in chick embryos. In contrast, isolation of unmodified strains in chick embryos is difficult.

Large scale production of tissue culture adapted virus for the preparation of vaccine is effected in chick embryos. Although chorioallantoic membrane inoculation may be used, higher virus yields are produced by inoculation directly into the embryo, in which high concentrations of virus are produced in the brain and muscles. Seven- to nine-day-old embryos are inoculated intra- or peri-embryonically, and the infected embryos are harvested after 4 days incubation. The harvested embryos are macerated and virus is extracted from the pulp of embryonic tissue.

(iii) Tissue culture

Theiler and his colleagues cultivated yellow fever virus in cultures of chick and mouse embryo tissues, more than 30 years ago, and obtained virus variants with altered tissue affinities. One of these variants, the 17D strain, after prolonged passage in mouse embryo and chick embryo tissues, proved to be non-pathogenic for monkeys and suitable for human vaccination.

Some adapted strains of yellow fever virus are cytopathogenic for HeLa, KB, chick embryo, and other cells.

Clinical Features

(a) Stage of infection

After an incubation period of 3-6 days, the stage of infection commences and clinical symptoms erupt with the sudden onset of fever, headache, nausea, vomiting, myalgia, conjunctivitis, malaise, and marked cutaneous erythema. During this phase, which lasts about 3 days, viraemia is present, and virus is most likely to be recovered from the blood at this time. When the infection is mild, the disease may not progress beyond this stage and recovery ensues. If, however, the infection is severe, the patient passes into the second stage, the stage of remission.

(b) Stage of remission

This stage, which is marked by a fall in temperature and by some improvement of the patient's condition, varies in duration from a few hours to a few days. Soon, however, the patient passes into the third and final stage, the stage of intoxication.

(c) Stage of intoxication

With the onset of the third stage, the temperature rises again, the pulse is slow, jaundice appears, and albuminuria is marked. Soon, haemorrhagic manifestations make their appearance; these include haematemesis, giving rise to the characteristic 'black vomit', melaena, uterine haemorrhage, epistaxis, and bleeding gums. In fatal cases, which are frequent, delirium and coma, indicating cerebral involvement, precede the fatal outcome. Non-fatal cases proceed to recover after about 3-4 days, and there are no sequelae. The mortality rate in different outbreaks varies from 10-40%.

Pathology

The principal lesion is in the liver, which is enlarged and exhibits fatty degeneration and necrosis. Microscopically, the degeneration and necrosis are seen to start in the mid-zonal area of the lobules and extend so that few recognizable liver cells are eventually left. Aggregates of necrotic cells which form acidophilic masses, known as Councilman bodies, constitute typical elements of the lesion. Outstanding, is the absence of any inflammatory reaction.

The kidneys are swollen and congested, and microscopic examination reveals cloudy swelling and necrosis of the tubular epithelium, and granular debris and casts in the lumina; there is little evidence of any inflammatory reaction.

Haemorrhagic manifestations are commonly found in other organs, particularly in the stomach and intestines.

Epidemiology

Two epidemiological cycles of yellow fever infection are now recognized. The urban yellow fever cycle in which infection is maintained in man and transmitted by *Aedes aegypti*; and the sylvatic cycle, in which infection is maintained in wild animal host reservoirs, particularly monkeys, in the jungle forests of Central and South America, and of Africa. In the sylvatic cycle, the principal vector of transmission is *Haemagogus spegazzinii*, which occasionally attacks men working in the jungle and gives rise to the jungle or sylvan type of yellow fever.

(a) Yellow fever in America

(i) Urban yellow fever

Urban yellow fever, which was endemic and epidemic in Central America and the Southern United States for centuries, is transmitted from man to man by *Aedes aegypti*. Its urban habitat is determined by the strictly human host reservoir and by the vector's preference for human domestic surroundings. Intensive anti-mosquito measures have now succeeded in eradicating this form of the disease from endemic areas of America.

(ii) Jungle or Sylvan yellow fever

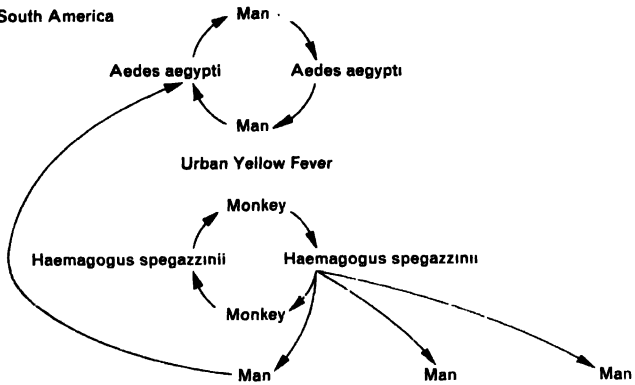
The reappearance, from time to time, of small outbreaks of yellow fever in Central and South America, after all the endemic foci had been eradicated, was for long a puzzling feature. Its explanation was provided by the observations made by Soper and his colleagues in Brazil, in 1934. These workers described outbreaks of yellow fever occurring in rural areas adjacent to the jungle forests of Brazil and Colombia, in which *Aedes aegypti* was nowhere in evidence; moreover, they obtained serological evidence of infection in monkeys inhabiting these areas. It is now established that the primary host reservoir of jungle or sylvan yellow fever is in primates, and that the principal vector of transmission is the forest mosquito *Haemagogus spegazzinii*.

The forest mosquito, which is normally the vector of transmission within the primary host reservoir, occasionally bites people who live in close proximity to the jungle forests or work in them. Woodcutters are particularly affected because *H. spegazzinii*, which normally inhabits the forest canopy, is released in large clouds at ground level when trees are felled. Rarely, when haemagogus mosquitos are particularly prevalent, epidemics in which the mosquito transmits infection from man to man may occur in forest regions.

Once acquired, jungle yellow fever can be transferred by a peripatetic patient in the early stages of the disease to urban communities, with the aid of the urban vector *A. aegypti*; in this way an urban epidemic may be set up and maintained (Fig. 84a). Although the eradication of *A. aegypti* is important for the control of urban

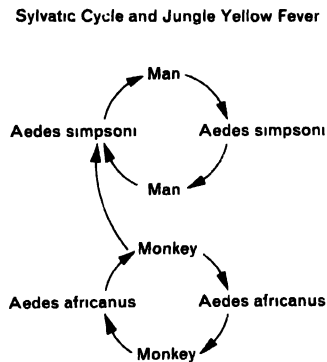
Epidemiology of Yellow Fever

(a) Central and South America

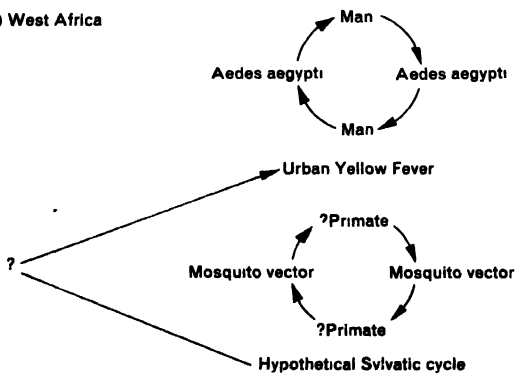


(b) Africa

(i) East Africa



(ii) West Africa



yellow fever, the disease can never be completely eliminated because of the sylvatic cycle of infection.

(b) Yellow fever in Africa

Yellow fever is endemic in Africa in an area bounded in the North by latitude 15°N and in the South by latitude 10°S. In the Western part of this area, urban yellow fever, in which *A. aegypti* is the vector, is both endemic and epidemic. In the Eastern part, where the great rain forests are found, *A. simpsoni* is the principal vector of transmission from man to man, although other species of mosquito have also been incriminated. In this region, an extrahuman primate reservoir of the disease is thought to exist in the jungle, and infection is probably transmitted from monkey to monkey by *A. africanus*. The transfer of infection from monkey to man is mediated by *A. simpsoni*, which is a semi-domesticated species breeding in the vicinity of human habitations, located near the edge of the forest. Originally transmitting infection from monkeys, which maraud the plantations or which inhabit the edge of the forest, *A. simpsoni* subsequently transmits infection from man to man (Fig. 84b).

A sylvatic cycle and jungle reservoir of yellow fever probably also exists in West Africa, but is so far unproven.

Laboratory Diagnosis

(a) Virus isolation

Virus isolation is the diagnostic procedure of choice; blood is collected from the patient as early as possible in the disease, preferably during the first 3–4 days, and is inoculated intracerebrally into suckling mice or a susceptible strain of adult white mice. The isolate is identified by neutralization tests with homologous anti-serum.

(b) Serological tests

In primary infections, a four-fold rise in titre of specific antibody is demonstrable in serum specimens drawn in the acute and con-

Fig. 84. Epidemiology of yellow fever

(a) Central and South America.

(b) East and West Africa.

valescent phases of the disease. Neutralization, complement-fixation or haemagglutination-inhibition tests may be employed. A previous infection with another Group B arbovirus may obscure the specific nature of the antibody rise.

(c) Post-mortem diagnosis

When the diagnosis during life is in doubt, and for epidemiological purposes, specimens obtained by post-mortem viscerotomy or after autopsy are examined histologically for the typical lesions of yellow fever.

Control

(a) Mosquito control

The work of Gorgas and the Reed Commission proved that urban yellow fever could be controlled and indeed eliminated by the eradication of the *A. aegypti* vector. Even reduction of the *A. aegypti* population to a 'critical level', or below, is sufficient for the control of yellow fever. The recognition of a permanent sylvatic reservoir, and the ever-present danger of reintroduction of the disease from other endemic areas, makes the continuation of strict mosquito control a necessity in areas at risk.

Complete eradication of the *A. aegypti* vector has been achieved in Brazil and Bolivia, and its eradication from other parts of the American continent and the Caribbean in order to prevent its reintroduction into the freed territories is now the policy of the Pan American Health Organization.

The complete eradication of the widely distributed *A. aegypti* and other mosquito vectors in Africa is not yet possible. But anti-mosquito measures in cities, ports, airports, travel routes and other inhabited areas are of extreme importance in control of the disease. When infection occurs, it is important to prevent access of mosquitos to any patient within the first 3 days of infection.

The eradication of the vector and reservoir of the sylvatic cycle of yellow fever is clearly not possible, and control of the infection in those exposed must rest on vaccination.

(b) Vaccination

Two live attenuated virus vaccines are available; the French

neurotropic strain, attenuated by mouse brain passage, and the 17D strain, attenuated in tissue culture and prepared in chick embryos. The former has the advantage of inoculation by cutaneous scarification, and is therefore most convenient for use in mass immunization campaigns in remote areas. Occasional adverse encephalitic complications, however, make the 17D strain preferable, in spite of the necessity of subcutaneous inoculation. The 17D vaccine produces long-lasting immunity, and International Regulations accept vaccination with it as valid for 6 years from the tenth day after vaccination.

Vaccination is recommended for all persons exposed to infection, through residence in endemic areas or through occupation, e.g. medical personnel, laboratory workers, and travellers to and from endemic area.

(c) International control measures

Some parts of the world, especially India, which are free of yellow fever, are nevertheless plagued with *A. aegypti*. The introduction of yellow fever into these areas, where conditions are favourable for the spread of the disease, is an ever-present hazard, particularly as a result of air travel. Aircraft may transfer infected mosquitos from endemic to non-endemic areas, and human cases may be transferred during the incubation period of the disease. To protect against these possibilities, International Sanitary Regulations require effective mosquito control to be practised at airports, ports, and their surrounding regions, both in endemic areas and in areas where conditions for spread of yellow fever are favourable. In addition, they require disinsectization of aircraft and ships arriving in these areas, and valid International Certificates of Vaccination against yellow fever from all passengers, including transit passengers, arriving from endemic areas of Africa or South America. W.H.O. further requires international reporting of outbreaks of the disease.

Primates arriving from endemic areas are quarantined for 7 days from the date of leaving a yellow fever area.

Treatment

There is no specific antiviral treatment for yellow fever.

CHAPTER 39

Arbovirus Infections

V. Tick-borne Arbovirus Infections

Tick-borne Arbovirus Encephalitides

All tick-borne arboviruses causing encephalitis belong to the antigenic Group B.

(a) Russian spring-summer encephalitis

(i) *Clinical features*

After an incubation period of 8–14 days, there is a sudden onset of fever, headache, nausea, vomiting, photophobia, coma, and occasionally convulsions. Flaccid paralyses of the shoulder girdle, and sometimes the limbs, occur and may be permanent. In severe cases, the bulbar centres and the cervical cord may become involved and the vital centres affected. The mortality rate is 20–30%.

(ii) *Epidemiology*

The disease has been prevalent during the last 30–40 years, mainly in the Eastern part of the Soviet Union where it occurs in spring and early summer when ticks are numerous. The principal vectors are *Ixodes persulcatus* and *Ixodes ricinus*, and the primary reservoir is believed to be in small rodents. From this reservoir, men working, visiting or building in the virgin forest lands of endemic regions are infected as tangential hosts, in whom the cycle of transmission is not maintained.

Goats feeding in tick-infested pastures become infected and excrete the virus in their milk. As a result many outbreaks have occurred as a result of drinking infected goats' milk.

(iii) Control

Protection from tick-bites by the use of protective clothing, insect repellants, and the spraying of tick-infected areas with DDT, is important in endemic areas. Formalin-inactivated infected mouse brain vaccines have been used extensively in the Soviet Union, with success; milk-borne infections are easily prevented by pasteurization of milk.

Laboratory infections are common, serious, and sometimes fatal, so that all people working with this or closely related viruses should be immunized.

(b) Central European encephalitis

This disease is clinically and epidemiologically similar to Russian spring-summer encephalitis, but tends to be less severe. It is common in Czechoslovakia, and occurs in other parts of Eastern and Central Europe. The causative virus is closely related to that of Russian spring-summer encephalitis but is antigenically distinguishable.

(c) Louping-ill

Louping-ill is an acute encephalomyelitis of sheep, characterized by pareses and cerebellar ataxia, which occurs in Scotland and the North of England. In sheep, infection is transferred by the tick vector *Ixodes ricinus*, but the primary reservoir of infection may be in some other mammalian species. Men in close contact with infected sheep may become infected, and laboratory workers and butchers who handle infected material may become infected via the respiratory route.

The causative virus is antigenically very closely related to the virus of Central European encephalitis, and the infection in man is clinically indistinguishable from this disease. A formalin-inactivated infected sheep or mouse brain vaccine is available.

Tick-borne Arbovirus Haemorrhagic Fevers**(a) Haemorrhagic fevers due to arboviruses of antigenic group B****(i) Kysanur Forest disease**

This disease, which has only recently come to light in the Kysanur

Forest of India, is characterized by the sudden onset of fever, headache, myalgia, conjunctivitis, vomiting, diarrhoea, and sometimes respiratory symptoms. Severe cases are associated with haemorrhagic manifestations, including haemorrhage from the alimentary tract and epistaxis. Infection is very often mild, but recovery and convalescence may be prolonged, and the fatality rate is 1-10%.

The reservoir of infection is in monkeys and rodents, and the tick vector is *Haemaphysalis spinigera*. Men working in the forest areas become infected as tangential hosts in whom the cycle of transmission is not maintained.

(ji) Omsk haemorrhagic fever

This infection, which is clinically very similar to Kysanur Forest disease, occurs in Siberia, other parts of the Soviet Union, and in northern Roumania. The primary reservoir is in rodents, and the vectors of transmission are *Dermacentor pictus* and *Dermacentor inorcinatus*. A formalin-inactivated infected mouse brain vaccine has been developed.

(b) Haemorrhagic fevers due to antigenically ungrouped arboviruses

(i) Crimean haemorrhagic fever

This infection, which occurs in Crimea and Central Asia, is characterized by a generalized petechial rash and symptoms and signs which resemble those of other tick-borne arbovirus haemorrhagic fevers. Mild infections are common but mortality rates of 2-15% have been reported.

The reservoir of infection is in hares, and possibly birds, and the tick vector is *Hyalomma marginatum*. Man is a tangential host in whom the cycle of transmission is not maintained.

(ii) Colorado tick fever

This is acute dengue-like fever occurring in the Rocky Mountain area of the United States. Usually a mild disease, haemorrhagic and encephalitic complications may occur in children. The reservoir of infection is in small rodents, and the tick vector is *Dermacentor*

andersoni. Man is infected as a tangential host in whom the cycle of transmission is not maintained.

VI. Phlebotomus-borne Arbovirus Fevers

(a) Sandfly fever

Sandfly fever, which occurs commonly in the Mediterranean area, India, and the Far East, is an influenza-like illness characterized by the sudden onset of high temperature, myalgia, conjunctivitis, photophobia, and retro-orbital pains. The temperature, which may rise to 104°F, subsides by lysis in 3–4 days, and the disease is never fatal.

Two antigenic types of Sandfly fever virus are now recognized, known as the Neapolitan and Sicilian types. The only known reservoir of infection is in man, and the sandfly vector is *Phlebotomus papatasi*; an animal reservoir is suspected.

Summary of Arbovirus Infections

Table 13. Arbovirus encephalitides

Clinical syndrome	Disease	Antigenic group	Extrahuman reservoir	Vector	Geographical area
ENCEPHALITIS	Western Equine (WEE)	A	Wild birds Horses ? Small rodents	MOSQUITO	Canada U.S.A. Central and S. America Trinidad
	Eastern Equine (EEE)		Wild birds Horses Pheasants		Canada U.S.A. Central and S. America Trinidad
	Venezuelan Equine (VEE)		? Rodents Horses		Central and S. America Trinidad
	Japanese B	B	Pigs Wild birds		Japan China S.E. Asia
	Murray Valley		Wild birds		Australia New Guinea
	St Louis		Wild birds Rodents		U.S.A. Caribbean S. America
	Ilheus		Birds		Central and S. America Trinidad
	Russian Spring Summer		Rodents Goats	TICK	Eastern U.S.S.R.
	Central European		Rodents Goats		Eastern-Central Europe
	Louping Ill		Sheep		Scotland and N. England

Table 14. Arbovirus fevers

Clinical syndrome	Disease	Antigenic group	Extrahuman reservoir	Vector	Geographical area
F E V E R	Chikungunya	A	?	M O S Q U I T O	E. and S. Africa
	O'Nvong-Nyong		?		E. Africa
	Mayaro		?		Central and S. America
	Semliki Forest		? Monkeys		Trinidad
	Sindbis		Wild birds		Africa
					Africa
					India
					S.E. Asia
	Dengue	B	None known ? Monkeys		Africa
					India
					Middle East
					S.E. Asia
	West Nile		Wild birds		Africa
					Middle East
					S.E. Asia
	Oriboca Caraparu Apeu Murutucu Marituba Itaquí	C	Rodents		Brazil
	Bunyamwera	Bunyamwera	? Monkeys		E. and S. Africa
	Ilesha		?		Africa
	Germiston				S. Africa
	Guaroa	Others			S. America
	Bwamba		Monkeys		Africa
	Catu		Monkeys		Brazil
	Oropouche		Monkeys		Brazil
					Trinidad
	Rift Valley	Ungrouped	Rodents Sheep		Central and S. America
	Sandfly fever	Ungrouped	None	P H L E B O - T O M U S	Middle and Far East
					India
Y E L L O W F E V E R	Yellow fever	B	Monkeys	M O S Q U I T O	Central and S. America
H A E M O R - R H A G I C F E V E R	Kysanur Forest disease	B	Monkeys Rodents	T I C K S	Central Africa
	Omsk haemorrhagic fever		Rodents		India
	Crimean haemorrhagic fever	Ungrouped	Horses ? Birds		U.S.S.R. N. Roumania
	Colorado tick fever		Rodents		U.S.S.R.
					U.S.A.

CHAPTER 40

Psittacosis–Lymphogranuloma– Trachoma Group of Organisms

I. General Description

Classification

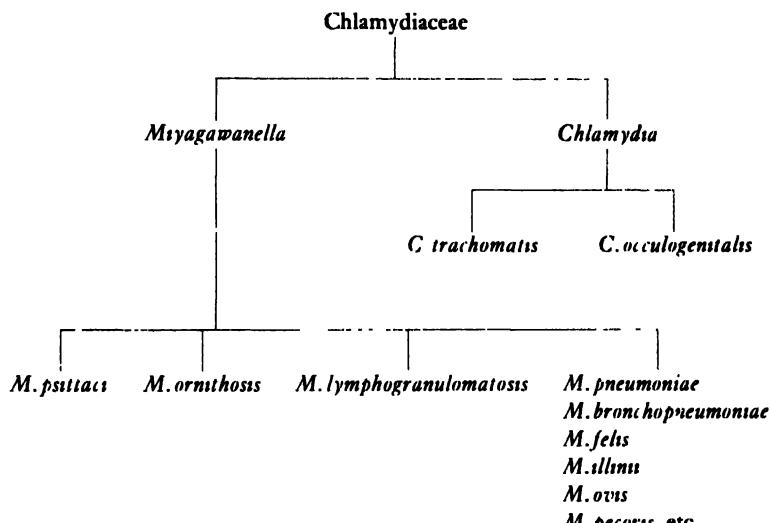
The psittacosis–lymphogranuloma–trachoma (PLT) group of organisms, previously characterized as viruses because of their filterability and obligate intracellular parasitism, differ from true viruses in a number of their properties. They multiply by binary fission, do not undergo morphological eclipse after cell penetration, incorporate both RNA and DNA, and are sensitive to antibiotics. Moreover, these organisms are limited by an outer membrane, which closely resembles the bacterial cell wall in chemical and physical properties; the membrane was shown by Allison and Perkins to contain muramic acid, a hexosamine found almost exclusively in bacterial cell walls. For these reasons, organisms of the PLT group are now considered to be more closely related to *Rickettsia*, and are assigned to the Order Rickettsiales, in the Family Chlamydiaceae.

Two genera of the Family Chlamydiaceae are recognized; the genus *Miyagawanella* or *Bedsonia*, the members of which are easily cultivated in various experimental hosts in the laboratory, and the genus *Chlamydia*, which includes the aetiological agents of trachoma and inclusion conjunctivitis, only recently isolated in chick embryos.

The various species of the genus *Miyagawanella* are differentiated according to their host reservoirs, avian, animal, or human, although all are related by a common group antigen. The medically important species are *M. psittaci*, the causative organism of psittacosis, and *M. ornithosis*, the causative organism of ornithosis, both of which survive in avian host reservoirs; and *M. lymphogranulomatosis*, the

causative organism of lymphogranuloma venereum, which is a strictly human parasite. Other species with mammalian host reservoirs are of less importance in medicine, but include the agents of meningo-pneumonitis (*M. pneumoniae*), mouse pneumonitis (*M. bronchopneumoniae*), feline pneumonitis (*M. felis*), bovine encephalomyelitis (*M. pecoris*), and enzootic abortion of ewes (*M. ovis*).

Chlamydiaceae of Medical Importance



Properties of Chlamydiaceae

(a) Morphology

All the organisms representing the several species of *Miyagawanella* and *Chlamydia* are morphologically indistinguishable from each other. In the light microscope, smears of heavily infected tissues reveal numerous small dense particles, 200–300 mμ in diameter, which are the elementary bodies. After staining with Giemsa, the elementary bodies, which are the infectious particles, may be seen both intra- and extracellularly, often in clumps or chains. Less numerous large granular particles, about 1 μ in diameter, with

different staining reactions, are also seen intra- and extracellularly, singly or in clumps. These are the non-infective initial bodies, which represent the vegetative form of the organism that precedes the formation of elementary bodies in the developmental cycle.

In the electron microscope, the elementary bodies are seen to consist of an electron dense central portion or nucleoid, surrounded

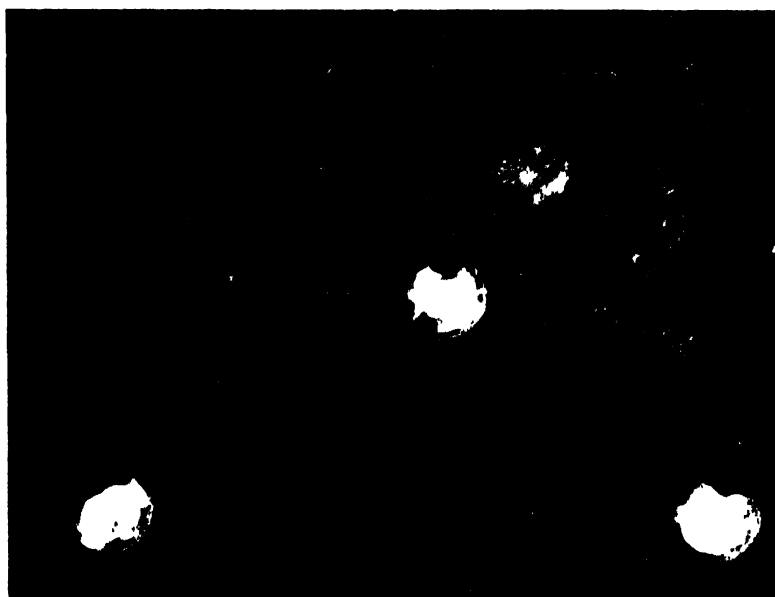


Fig. 85. Trachoma elementary bodies [electron micrograph by Dr R. Valentine, reprinted from, L. H. Collier, S. Duke-Elder, and B. R. Jones (1958) *Brit. J. Ophthalm.* 42, 705-20 (British Medical Association, London, by permission of the authors, editors, and publishers)].

by a halo composed of the flattened less dense outer membrane (Fig. 85). The halo is probably an artefact produced by preparation for electron microscopy, and this artefact probably accounts for the larger diameter, about 400 m μ , of organisms measured in the electron microscope. In the normal hydrated state, the particles are probably spherical in shape.

(b) The developmental cycle

The course of events following the introduction of elementary

bodies into the host cell is not yet properly understood, and some controversy remains as to the exact mechanism of replication. Nevertheless, all members of the Family Chlamydiaceae exhibit similar patterns of development, differing in a few details only, the original elucidation of which we owe mainly to Bedson.

The elementary body, representing the infective particle, is rapidly adsorbed to the cell surface, and is ingested by viropexis. After ingestion, there is a marked drop in titre of cell-associated infective material, which has been interpreted by some as evidence of an eclipse phase; but Litwin has shown it to be due to the decreased infectivity of the initial bodies formed after ingestion. The titre of cell-associated infective material remains constant during a long latent period, during which no new infective material is produced. This period may last 15-24 hours, depending on the organism and host cell system under study, during which time the organisms undergo a series of morphological changes while retaining their morphological integrity.

After ingestion, elementary bodies are enclosed in pinocytotic vesicles bounded by host cell membrane, and no change is observed for about 5 hours. At the end of this time, elementary bodies enlarge to form initial bodies, about $0.8\ \mu$ in diameter, in which the dense nucleoid material, visible by electron microscopy, becomes dispersed and reticulated. The initial bodies, easily identified by light microscopy, represent the non-infective, or weakly infective, vegetative multiplying form of the organism and are associated with intensive RNA synthesis (Fig. 86a).

In the course of the next few hours, the initial bodies coalesce in intracytoplasmic vesicles to form yet larger inclusion bodies, easily seen in the light microscope. Towards the end of the latent period, the initial bodies divide by binary fission to form somewhat smaller particles, often arranged in clusters, which eventually become numerous and closely packed within enlarged vesicular inclusion bodies. Finally, at the end of the latent period, which may last up to 24 hours, the small dense elementary bodies, about 200-300 m μ in diameter and formed by shrinkage of larger particles, make their appearance (Fig. 86b).

Coincident with the appearance of elementary bodies, there is a



Fig. 86. *M. psittaci* in impression preparations of mouse spleen.

(a) Colony of large forms (initial bodies) spread out in making the preparation, 16 hours after infection.

(b) Elementary bodies—48 hours after infection.

[From S.P. Bedson and J.V.T. Gostling (1954) *Brit. J. exp. Path.* 35, 299-308.]

sharp rise of intra- and extracellular infective material, which continues exponentially for 10–15 hours and reaches a maximum at 30–40 hours. At the same time, the number of elementary bodies continues to increase as the number of initial bodies decreases, so that a large number of elementary bodies and a few initial bodies in various stages of development fill the inclusion at the end of the developmental cycle. After 24–48 hours, when the inclusion is packed with elementary bodies, the host cell breaks down and the particles are released into the environment.

Some chemical studies have revealed that DNA, RNA, and protein are synthesized at different periods in the developmental cycle. This has led some to postulate a viral mode of replication by assembly of subunits; the morphological evidence, however, prevents this view from being generally accepted.

(c) Chemical and physical properties

RNA and DNA, in approximately equal amounts, large amounts of lipid, in the form of lecithin and neutral fat, and protein, are present in the elementary bodies. Although the organisms possess enzymes capable of synthesizing certain proteins and nucleic acids, they lack the mechanisms required for the production of metabolic energy; hence, their obligate intracellular parasitism.

PLT organisms are not preserved well on storage; they lose their infectivity within a few days at 4°C, and in a few weeks or months at –70°C. They are best preserved by lyophilization from suspensions in skimmed milk or 7.5% dextrose. The organisms are heat labile, and are inactivated by formalin, phenol, and ultraviolet light.

(d) Biological properties

(i) *Haemagglutination*

Soluble haemagglutinins separable from the elementary bodies are produced by *Miyagawanella* in chick embryos. These haemagglutinins agglutinate red cells from mice and certain fowls. Only those fowl red cells which are agglutinable by vaccinia and variola viruses are, in fact, haemagglutinated; the haemagglutinin, like that of vaccinia and variola viruses, is a phospholipoprotein in which the

phospholipid moiety is the haemagglutinating factor upon which the protein moiety confers antigenic specificity.

(ii) Toxicity

Some strains, grown in the chick embryo yolk sac, produce a species specific toxin which is a property of the elementary body itself. The toxic effect is recognized by its lethal effect in mice injected intravenously, and is distinguished from death due to actual infection by its rapid onset, within 18–30 hours, and the inability of antibiotics to suppress it. The toxin is antigenic and the effect may be neutralized by the appropriate antiserum.

(e) Antigenic composition

Organisms belonging to the Family Chlamydiaceae possess two complement-fixing antigens. One, which is heat stable, resists boiling, and is destroyed by periodate and lecithinase, is a group-specific antigen common to all members of the Family. The other, which is heat labile, alkali soluble, and destroyed by papain, is species specific and responsible for the production of neutralizing antibody. Both antigens are probably present in the cell wall, and Ross and Gogolak believe them to be lecithin-nucleoprotein complexes. The species specific antigen is composed of at least 11 different components, the qualitative and quantitative distribution of which is believed to underly the antigenic specificity of the different species of Chlamydiaceae.

(f) Cultivation

(i) Experimental animals

Mice. Most organisms of the genus *Miyagawanella* can be isolated in mice by one or other route. Virulent strains produce pneumonia and death in a few days, and less virulent strains death in 8–20 days, after intranasal inoculation. Elementary bodies are present in smears from the consolidated lungs.

After intraperitoneal inoculation, the toxic effect may kill mice within 2–3 days, producing few visible pathological lesions, but death occurs more often after 5–15 days from infection. Post-mortem, fibrinous exudate in the abdomen, enlargement of the

liver and spleen, and areas of hepatic necrosis are characteristic; microscopically, numerous elementary bodies are seen in the cells of the liver and spleen. For isolation purposes, mice which survive are sacrificed after 3 weeks and examined post-mortem; emulsions of liver and spleen are subpassaged at least twice before a negative isolation result is accepted.

Intracerebral inoculation produces paralysis in 24-48 hours and death in 3-10 days, from meningo-encephalitis. Elementary bodies are found in the inflammatory cells of the meningeal exudate.

Guinea-pigs. Organisms from mammalian host species are lethal to guinea-pigs within 5-10 days of intraperitoneal inoculation, whereas organisms from avian hosts are usually avirulent.

(ii) *Chick embryos*

Yolk-sac inoculation. Yolk-sac inoculation of 7-day-old chick embryos is the method of choice for isolating Chlamydiaceae. Embryonic death occurs within 3-8 days of inoculation, and elementary bodies are numerous in impression smears made from yolk sacs washed free of yolk material.

Chorioallantoic membrane inoculation. Chorioallantoic membrane inoculation of 10-day-old chick embryos with infective material produces pock-like lesions and death of the embryo within 3-8 days.

Allantoic inoculation. Allantoic inoculation of 9-10-day-old chick embryos produces high yields of organisms in the allantoic fluid, and death of the embryo in about 4 days.

(iii) *Tissue culture*

Low yields of organisms may be recovered in tissue cultures of HeLa cells, monkey kidney cells, L-cells, and chick embryo cells.

CHAPTER 41

Psittacosis—Lymphogranuloma— Trachoma Group of Organisms

II. *Miyagawanella psittaci* Psittacosis

Psittacosis is primarily a disease of psittacine birds, including parrots, parakeets, and budgerigars. From these sources, infection is sometimes transmitted to man in whom clinical or subclinical psittacosis may develop.

Clinical Features

After an incubation period of 4–15 days, and sometimes longer, illness commences with the acute onset of headache, pyrexia, malaise, myalgia, and bradycardia, followed by non-productive cough, alimentary disturbances, insomnia, restlessness, and sometimes delirium.

Physical examination may yield evidence of patchy pneumonic consolidation, particularly at the lung bases, but often the pneumonic involvement is first revealed by X-ray examination. Characteristically, the constitutional disturbance is disproportionate to the extent of the pulmonary involvement. Myocarditis, encephalitis, and thrombophlebitis may complicate the infection, and rose spots sometimes appear on the skin. In fatal cases, increasing respiratory distress, cardiovascular collapse, and generalized toxaemia terminate the illness, after about 10–18 days. The majority of patients, however, recover following a lytic fall of temperature in the second or third week. A long convalescence is required, and relapses sometimes occur, particularly after inadequate antibiotic therapy.

Severe forms of psittacosis, in which the mortality prior to the introduction of antibiotics was 20–30%, are now known to represent only a minority of human psittacosis infections. Serological evidence indicates that infection is frequently inapparent or produces only a mild illness, resembling mild influenza.

Pathology

Post-mortem, the lungs exhibit areas of greyish-red consolidation in which the alveoli are filled with fibrinous exudate, mononuclear cells, and degenerated alveolar cells, in which inclusions and elementary bodies may be seen.

Enlargement and congestion of liver and spleen provide evidence that psittacosis is a generalized infection with an affinity for the reticulo-endothelial system. Elementary bodies are found in the macrophages of the spleen and in the Kupffer cells of the liver, which shows areas of focal necrosis. Cardiac involvement is revealed by the presence of cloudy swelling in the myocardium and subendocardial inflammatory exudate in the region of the heart valves. The brain and spinal cord are congested and oedematous.

Epidemiology

The reservoir of infection is in psittacine birds, in whom infection is usually symptomless although widespread. The organism is excreted in the faeces and nasal secretions, particularly of immature and sick birds; from these sources, young birds acquire infection in the nest, although congenital infection may be possible. After infection, the organism is carried for long periods and, therefore, persists in infected aviaries; any adverse change of environmental conditions, such as overcrowding, dietary deficiency, or cold, will affect the host-parasite relationship adversely, and convert latent into overt disease, with greatly increased excretion of the organism. Infected excreta contaminate bird feathers and dust which convey infection from bird to bird and from bird to man by aerial transfer, by direct contact, or by handling of infected birds. Droplet infection is also possible. In these circumstances, it is hardly surprising that

psittacosis is principally a disease of bird-keepers and bird-dealers. Often, outbreaks in man are preceded by epizootics in recently imported birds which have been transported under bad conditions.

Transmission of infection from person to person is rare, but the disease has been spread to nurses and other medical attendants in close contact with patients. Likewise, infection of laboratory personnel engaged in psittacosis investigations is a very real, well-known, and not infrequent hazard. After infection, man may excrete the organism in the sputum for long periods but such carriers do not appear to be dangerous sources of infection.

Diagnosis

(a) Virus isolation

The highly infectious nature of the psittacosis agent demands special precautions to prevent laboratory infection, and examination of specimens should be confined to specialized laboratories equipped for the purpose.

Sputum, blood, throat washings, vomit, or pathological specimens, from untreated patients provide suitable specimens for isolation. Before inoculation, specimens are treated with a tyrothricin-sulphadiazine-streptomycin antibiotic mixture to reduce bacterial contamination; exposure to this mixture for less than 24 hours does not affect the psittacosis organism. Specimens should be stored frozen if there is any delay between collection and inoculation.

Mice are inoculated intranasally, but may also be inoculated intraperitoneally or intracerebrally. Characteristic post-mortem lesions are sought, and tissue smears are stained for elementary bodies. Some diagnostic confusion may be caused by the activation of latent mouse pneumonitis agent after the inoculation procedure. This may be avoided by the use of chick embryos.

Seven-day-old chick embryos are inoculated by the yolk sac route. Death of the embryo usually occurs in 3-8 days, and elementary bodies are sought in impression smears of the yolk sac.

After isolation, identification of the organism is confirmed by characterizing its antigenic specificity in complement-fixation,

neutralization and cross immunity tests. Sometimes precise identification requires pathogenicity tests in various species of experimental animal.

(b) Serological tests

Serological methods of diagnosis are preferred in routine laboratories because of the hazards of working with infective material.

(i) Complement-fixation test

In routine work, the group-specific complement fixation test is usually used. A four-fold rise in titre of group-specific antibody, occurring between the acute and convalescent phases of the disease, is diagnostic. Because low titres of group-specific antibody are sometimes found in the sera of normal persons, a titre of at least 1 : 32 in a single serum specimen is required for diagnosis. Treatment with antibiotics may delay the development of the antibody response, and specimens obtained later in convalescence must then be tested.

Species-specific complement-fixation tests may be used in appropriate cases.

(ii) Haemagglutination-inhibition test

Although less reliable than complement-fixation tests, haemagglutination-inhibition, using the group-specific soluble haemagglutinin, may be used.

Control

(a) In birds

Where avian psittacosis is not endemic, as in the United Kingdom, importation of psittacine birds must be forbidden. Psittacine birds in the pet industry must be kept in disease-free conditions, designed to prevent those factors like overcrowding, malnutrition, and cold, which are known to favour the spread of the disease.

When psittacosis is endemic in an aviary, birds may be freed from infection by feeding them tetracycline antibiotics. In this way, the infection may be cleared, and perhaps eventually eliminated.

(b) In man

Human cases must be strictly isolated and the source of infection traced. Birds suspected of being the source of infection are killed and transmitted to the laboratory, after immersion in 2% lysol, and preferably in the frozen state. The presence of infection in the bird is confirmed by post-mortem appearances, virus isolation, and serological tests. All infected birds are destroyed, and their cages and environments thoroughly disinfected.

Treatment

M. psittaci, the causative agent of psittacosis, is sensitive to penicillin, chloromycetin, and the tetracycline group of antibiotics. Chlortetracycline is the drug of choice but should not be used in conjunction with penicillin, which antagonizes its action in the therapy of psittacosis. Treatment must be continued for at least one week after the temperature returns to normal.

III. *Miyagawanella ornithosis* Ornithosis

The widespread distribution of psittacosis-like infection in various species of non-psittacine birds, which is occasionally transmitted to man, is now recognized. The disease, which is indistinguishable from psittacosis, is referred to as ornithosis, and the causative organism, which differs only slightly from *M. psittaci*, is *M. ornithosis*.

The reservoirs of ornithosis infection are in pigeons, ducks, chickens, turkeys, pheasants, and other avian species. In this country, pigeons kept in captivity are the main sources of ornithosis; human infection is thus found in pigeon fanciers and those concerned with carrier and racing pigeons. In Glasgow, as recently as 1963, Grist and McClean found 54% of feral pigeons to be infected,

although they did not appear to serve as an important source of human infection.

The clinical, pathological and epidemiological characteristics of psittacosis and ornithosis are the same, and the principles of their diagnosis and treatment are identical. Slight differences between the respective organisms may, however, be noted. *M. ornithosis* recovered from pigeons, chickens, and ducks is less pathogenic for mice, inoculated by the intraperitoneal route, than *M. psittaci*, and isolates from turkeys and egrets are pathogenic for guinea-pigs by the intraperitoneal route. Minor antigenic differences between *M. ornithosis* and *M. psittaci* occur, and some avian immune sera fail to fix complement directly with homologous *M. ornithosis* antigen. These sera are tested by indirect complement fixation tests in which antibody content is measured by inhibition of complement fixation between the antigen and an immune serum from a heterologous species.

IV. *Miyagawanella louisianae*, *Miyagawanella illinii* Human Pneumonitis

Occasional outbreaks of human pneumonitis, from which *Miyagawanella* of high virulence have been recovered, have been reported from San Francisco, Louisiana and Chicago. In these outbreaks, infection was spread from fatal cases only, and no contact with birds was established.

The organisms were originally thought to have become adapted to man, and to no longer require an avian host, but the recovery of similar organisms from egrets and pigeons suggests that the outbreaks may have had an avian origin. This view is supported by the absence of any detectable avian source of infection in more than 25% of patients with psittacosis or ornithosis.

CHAPTER 42

Psittacosis–Lymphogranuloma– Trachoma Group of Organisms

V. *Miyagawanella*

lymphogranulomatosis

Lymphogranuloma Venereum

Lymphogranuloma venereum is a venereal disease, most frequent in tropical climates; it is characterized by a vesicular lesion on the genitalia and suppurative inguinal adenitis. The causative organism is *Miyagawanella lymphogranulomatosis*.

Clinical Features

5–21 days after venereal exposure, the primary lesion, in the form of a small papule or vesicle which soon ulcerates, appears on the genitalia; it may regress without further development and may then be overlooked, especially in the female. Rarely, the primary lesion may present as urethritis, or as a lesion in the mouth or eye.

From the primary lesion, the organism spreads, in the course of the next few days or weeks, to the draining lymph glands, where painful enlargement, suppuration, and sinus formation ensue. In males, the inguinal glands are usually affected, but in females, and males whose primary lesion is in the anorectal region, it is the pelvic lymph glands which are affected.

Pyrexia, headache, anorexia, and joint pains may occur in the acute phase of infection; later, infection may become generalized and affect other lymph nodes and the spleen. Elephantiasis of the genitalia, particularly in women, follows scarring of the draining

lymph glands; later sequelae which follow spread of the organism through the perirectal tissues are proctitis, rectal stricture and vagino-rectal fistula.

The Organism

The causative organism, *M. lymphogranulomatosis*, resembles *M. psittaci* in morphology, development, and other properties, although its species specific antigen is distinct. It is less pathogenic for mice by the intraperitoneal route than *M. psittaci* although very toxic by the intravenous route; it is also less pathogenic for birds.

Pathology

The lesions are characterized by inflammatory exudate of large numbers of mononuclear and plasma cells, which may contain inclusions and elementary bodies; a few polymorphs and eosinophils may also be seen. The additional presence of epithelioid cells and giant cells, together with the proliferation of fibrous tissue, form lesions which resemble those of tuberculosis. Tissue necrosis and suppuration is also characteristic.

Epidemiology

Lymphogranuloma venereum occurs in all parts of the world, and is most common in the Southern U.S.A., Africa and the Mediterranean littoral. Its presence in these areas is believed to reflect the poor social conditions rather than the warm climate. In the United Kingdom, the disease is becoming more common as the result of increased immigration.

Man is the only known host reservoir of infection, in whom the organism is nearly always spread by venereal contact, although indirect contact with contaminated clothing or other articles is possible. Medical personnel have also become infected by direct contact with patients' lesions. Inapparent infection as the source of the disease is possible.

Diagnosis

(a) Direct examination

Smears of pus, when available, are stained with Giemsa or Machiavello's stain and examined for the presence of elementary bodies.

(b) Isolation of the organism

Isolation is not used as a routine method of isolation, but may be attempted by inoculating infected material into the yolk sacs of 7-day-old chick embryos, or into the brains of mice. The identity of the isolate may be confirmed by toxin-neutralization and other serological procedures.

(c) Serological tests

(i) The Frei test

The Frei test is based on a delayed hypersensitivity reaction which becomes positive after infection with *M. lymphogranulomatosis*. 0.1 ml of a standard preparation of *Miyagawanella* group-specific antigen, prepared from *M. lymphogranulomatosis*, or other *Miyagawanella*, grown in the chick embryo yolk sac, is injected intradermally into the flexor surface of the forearm; normal yolk sac material is injected into the flexor surface of the other forearm as a control. A raised papule measuring 6 mm or more in diameter, which appears in 48 hours and is larger than any papular reaction produced by the control, is considered a positive reaction.

The reaction becomes positive between 1 week and several months after infection, and remains positive for many years, if not for life. Negative reactions in the early stages of infection may become positive later, but false negative reactions may occur in those with tuberculosis, or syphilis, and in those receiving cortisone therapy. Because the group-specific antigen is the test material usually used, the Frei test is also positive in the later stages of psittacosis.

(ii) Complement-fixation

Complement-fixation, using the group-specific complement-fixing antigen, is considered by many to be the method of choice for

laboratory diagnosis. Ideally, a four-fold rise of complement-fixing antibody, occurring between the acute and convalescent phases of the disease, is required for diagnosis. This is not usually possible in patients seen for the first time in the later stages of the disease, in whom the diagnosis has to be made on a single serum specimen. In single serum specimens, a titre of 1 : 32 or greater is considered to be diagnostically significant. Although similar reactions are displayed by sera from patients with psittacosis; lymphogranuloma and psittacosis are such distinct clinical entities that no confusion arises.

In lymphogranuloma venereum, both the Frei and complement-fixation tests are usually positive, but sometimes one or other may be negative. In the early stages of infection the complement-fixation test may become positive before the Frei test. Occasional instances in which a strongly positive Frei test is present in the absence of complement-fixing antibody are suggestive of quiescent infections.

Control

Control measures are the same as those advocated for other venereal diseases.

Treatment

Sulphadiazine and the tetracycline group of antibiotics are both therapeutically effective against *M. lymphogranulomatosis*, but complications due to fibrosis require surgical treatment.

CHAPTER 43

Psittacosis–Lymphogranuloma– Trachoma Group of Organisms

VI. *Chlamydia trachomatis* Trachoma

Trachoma is an infective keratoconjunctivitis which was known to the physicians of Greece, Rome, and China in ancient times. Today, the disease is widespread in Africa and the East, where it is the most common cause of blindness. The World Health Organization estimates that 400 million people suffer from the disease at the present time, of whom 20 million are blind or defective in sight.

Long known to be a contagious disease, it was not until 1907 that Halberstaedter and von Prowazek established the infective nature of the condition, by transmitting the infection to apes. During long years of study, several people claimed to have isolated the causative organism in tissue culture or chick embryos but their claims were not confirmed. In 1957, Tang and his colleagues in China successfully isolated the organism in chick embryos by repeated yolk sac inoculation in the presence of streptomycin, and their work has been fully confirmed. Since that time, progress in the understanding, control and treatment of trachoma has been rapid.

The Aetiological Agent

The elementary bodies of the trachoma organism were identified by Halberstaedter and von Prowazek in 1907, and the larger initial bodies were recognized by Lindner in 1909. Neither differs in size, morphology or staining characteristics from those of other members of the PLT group of organisms. The developmental cycle of the

organism was described in 1934 by Thygeson who drew attention to its similarity with that described in psittacosis by Bedson and Bland. These similarities, and the development by patients with trachoma of complement-fixing antibodies reacting with the organisms of psittacosis and lymphogranuloma venereum, served to establish the close relationship between these organisms and that of trachoma; the similar sensitivity of all these organisms to antibiotics has since substantiated this relationship. Because laboratory cultivation of the trachoma organism was originally impossible, it was assigned by Rake to a separate genus, *Chlamydia*.

Properties of the Aetiological Agent, *C. trachomatis*

(a) Chemical and physical properties

The organism is stable for 24 hours or more, at temperatures of 0–4°C, depending on the suspending medium, but is rapidly inactivated by heat at 56°C. Freezing or lyophilization partially inactivates the organism, but the residuum may be preserved at –60°C for 6 months or by lyophilization from suspensions in skimmed milk.

The organism is destroyed by formalin, phenol or 30% ether.

(b) Biological properties

(i) *Toxin*

Mice, which are not usually susceptible to infection with the organism, die from toxæmia in 2–18 hours after intravenous injection of concentrated suspensions of yolk sac origin. Previous immunization of mice with trachoma vaccine protects them from death due to toxæmia, and protection tests of this type have revealed some antigenic differences between strains.

(c) Antigenic composition

Evidence that the *Miyagawanella* group-specific antigen is shared by the trachoma organism is provided by the reactions between sera from trachoma patients and the group-specific antigen of *M. lymphogranulomatosis*, and by the reactions between sera from lymphogranuloma patients and suspensions of trachoma organisms.

Antibodies produced in experimental animals by immunization with trachoma organisms also react with *Miyagawanella* group-specific antigen.

So far, no species-specific complement-fixing antigen of trachoma has been unequivocally demonstrated. Bernkopf, however, has produced evidence of species specificity in fluorescent-antibody and micro-agglutination tests, with the aid of absorbed sera.

Specific neutralization antibodies to trachoma are extremely difficult to detect in laboratory tests. The most sensitive method is that of Furness and his colleagues, based on the reduction of the number of inclusions produced by a tissue culture adapted trachoma strain under standard conditions.

(d) Cultivation

(i) *Chick embryos*

The success of Tang and his colleagues in isolating the trachoma organism in the chick embryo yolk sac may be ascribed to their attention to three important details. The prevention of bacterial contamination by the use of streptomycin, in place of penicillin to which the organism is sensitive; incubation in an atmosphere of saturated humidity at a temperature of 35°C, the temperature of the normal conjunctival sac; and serial propagation by multiple blind passages.

For isolation, conjunctival scrapings are collected in 0.5–1.0 ml amounts of broth-saline containing streptomycin 0.4 mg per ml, other antibiotics to which the organism is insensitive may be added if necessary. After 30 minutes at room temperature, 0.5 ml is inoculated into the yolk sacs of 6–8-day-old chick embryos. Embryos which die within 48 hours are discarded, but those which die after 3 days are harvested, and smears of their yolk sacs are examined for the presence of elementary bodies. If death has not occurred by the seventh day of incubation, the yolk sac is harvested and passaged; as many as six passages may be required to establish the organism in the yolk sac. Eggs vary in their susceptibility to infection, and are sometimes completely resistant for no obvious reason.

After prolonged passage in eggs, strains may lose their pathogenicity for primates.

(ii) Tissue culture

With few exceptions, trachoma fails to grow in tissue culture. Only one strain, the Tang TE/55 strain, which has been adapted to mice by the intracerebral route and is non-pathogenic for primates, has been serially propagated in the FL line of human amnion cells, by Bernkopf and his colleagues. This strain has also been grown in HeLa and ERK cells, in which it forms typical inclusions and large yields of infective elementary bodies.

Experiments by Gordon, Quan, and Trimmer suggest that resistance of cells in tissue culture to trachoma infection arises from difficulties of adsorption and penetration. They showed that organisms forced into chick embryo host cells, by centrifugal force, replicate and produce typical inclusions, and that, using this method, the organisms could be maintained in tissue culture for two or three passages.

(iii) Experimental animals

In general, monkeys and apes infected by the conjunctival route are the only animals susceptible to trachoma infection. The exceptional Tang egg-adapted TE/55 strain has, however, been grown in mice inoculated by the intracerebral route, and this intracerebrally adapted strain can also infect suckling mice inoculated by the intranasal and intraperitoneal routes.

Clinical Features

The incubation period of naturally occurring trachoma is difficult to assess, but experimental infections produce signs of disease in 2–12 days. The first sign of infection is inflammation of the upper tarsal conjunctiva, which may be symptomless in the early stages, or lead to irritation, lacrimation and mucopurulent discharge. Later, papillary follicular hypertrophy in the upper conjunctiva leads to the formation of red and white spots. Soon, the cornea reveals signs of superficial keratitis accompanied by subepithelial infiltration and vascularization, and the formation of pannus.

The second stage of the infection is one of florid inflammation, in which the lesions of the first stage are accentuated and the pannus

spreads across the cornea. The first and second stages of the disease may continue for several months or years before the third stage of scarring and cicatrization sets in. This stage may persist for several years, and leads to increased visual disability, blindness, deformity of the eyelids, and interference with tear function. Finally, the fourth stage, that of healing, in which scar tissue contracts and deformities become accentuated, brings the course of the disease to an end. Secondary bacterial infection with *H.aegypticus*, *N.gonorrhoeae*, and other organisms, is common, causing marked aggravation of the trachomatous condition and lesions.

The course of trachoma may last several months or several years, during which there are periods of exacerbation and remission. The disease tends to be less severe in children, in some of whom it may heal spontaneously, whereas the condition and its cicatricial sequelae are usually more serious in adults.

Pathology

In trachoma, infection with *C.trachomatis* is limited to the superficial epithelial cells of the conjunctiva and cornea, which alone exhibit the typical inclusion bodies. The organism is absent from the subepithelial tissues in which there is a marked inflammatory reaction, although some have postulated the production of a diffusible toxin to account for the subepithelial inflammatory response.

In the conjunctiva, marked proliferation as well as necrosis and exfoliation of epithelial cells is seen. The inflammatory reaction of the subepithelial tissues is marked by capillary dilatation and infiltration with lymphocytes, plasma cells, and mononuclear cells, and by the formation of follicles. Follicles are scattered aggregations of lymphocytes, mononuclear cells, and histiocytes, surrounded by fibroblasts; eventually, cells at the centre of the follicle degenerate and undergo necrosis, so that the follicle either ruptures externally or is replaced by scar tissue. Proliferation of connective tissue accompanies the subepithelial inflammatory reaction, and leads to the fibrosis, scarring, and eyelid deformities, associated with the disease.

The earliest change in the cornea is the development of avascular punctate epithelial and subepithelial keratitis. Later, vascularization of the cornea, and cellular infiltration of the subepithelial corneal tissue, form the pannus, whose spread produces haziness of the cornea and impairment of vision. The pannus may eventually regress with some clearing of the cornea, but the formation of scar tissue often leads to permanent corneal opacities and blindness.

Epidemiology

Trachoma is endemic in Africa, the Middle East, India and other parts of Asia, as well as in parts of South America and in certain Indian reservations in the United States. It is essentially a disease of underdeveloped areas, where its spread is facilitated by poverty, dirt and squalor. Trachoma is particularly common in dry, dusty desert areas, where irritation of the eye by sand particles predisposes to infection. In the worst affected areas, the incidence of infection may be as high as 90–95%.

In areas of high incidence, infection is usually acquired in childhood, frequently from the mother and sometimes at birth. The disease is highly communicable in the acute but not the chronic phase; it is spread by direct contact, flies, and water, and particularly by the use of common washbasins, towels, and handkerchiefs. Secondary bacterial infection, which increases the amount of conjunctival exudate, facilitates the transmission of infection.

Accidental infection of laboratory and medical personnel may occur if proper precautions are not observed.

Laboratory Diagnosis

(a) Direct examination

Direct examination of epithelial scrapings is a routine, rapid, reliable, and important method of laboratory diagnosis. Slides are made of epithelial scrapings, taken from diseased areas of the conjunctiva, stained with Giemsa and examined for inclusion, elementary and initial bodies. These are numerous in the acute stages, but difficult, if not impossible, to find in the chronic stages of the disease.

The inclusion body is an intracellular mass of elementary and initial bodies, in varying proportions, suspended in a fluid carbohydrate matrix. Elementary bodies, which may be extracellular in the acute phase of the disease, stain bluish-red with Giemsa; inclusion bodies stain blue with Giemsa (Fig. 87), and brown with Lugol's iodine which stains the carbohydrate matrix (Fig. 88). The recent introduction of the fluorescent antibody technique for the detection of inclusions is a definite advance and, when properly used, has proved to be a more sensitive means of diagnosis than routine staining, or isolation of the organism.

The intracytoplasmic inclusions of trachoma and inclusion conjunctivitis are indistinguishable, but trachoma inclusions are more numerous in the upper tarsal conjunctiva and fornix. Moreover, the

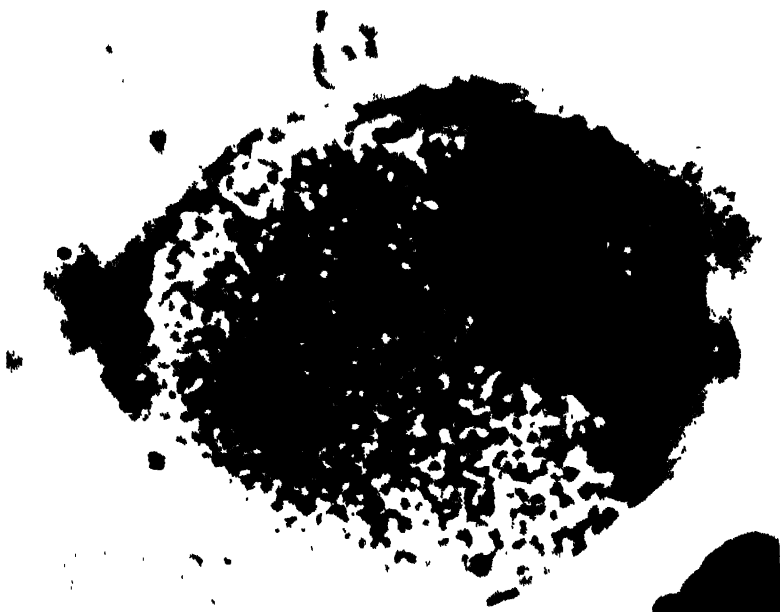


Fig. 87. Trachoma inclusion body, occupying most of the cytoplasm, packed with elementary bodies; Giemsa/May-Grunwald stain [from Shiona Sowa, J. Sowa, L. H. Collier, and W. Blyth (1965) *M.R.C. Special Report Series* No. 308. Trachoma and Allied Infections in a Gambian Village (by permission of the Controller, H.M.S.O.)].

diagnosis of trachoma may be confirmed by the characteristic histological appearance of exudate expressed from trachoma follicles. When stained with Giemsa, this reveals large numbers of macrophages, a few plasma cells, lymphocytes, degenerating lymphoblasts, cell debris, and occasional multinucleated epithelial cells.



Fig. 88. Trachoma inclusion body stained with iodine [from Shiona Sowa, J. Sowa, L. H. Collier, and W. Blyth (1965) *M.R.C. Special Report Series No. 308* (by permission of the Controller, H.M.S.O.)].

Smears of conjunctival exudate collected in the acute stages of trachoma and inclusion conjunctivitis are characteristically rich in polymorphonuclear leucocytes. In contrast, exudate from true viral conjunctivitis is characterized by the presence of mononuclear cells. Gram stained smears from trachoma cases may reveal evidence of secondary bacterial infection, the causative organisms of which can be identified by bacteriological culture methods.

(b) Isolation of the organism

Although the organism can be isolated in the chick embryo yolk sac, the method is not yet sufficiently rapid or reliable for routine diagnostic purposes. Nevertheless, it may be noted that the organism has been isolated from specimens in which inclusions were not found.

(c) Serological tests

The antibody response in trachoma is generally poor, because localization of the organism in the conjunctival epithelium provides a barely sufficient antigenic stimulus. Nevertheless, attempts have been made to provide serological methods of diagnosis by complement-fixation, micro-agglutination and fluorescent antibody tests, using purified elementary body suspensions, but none have proved sufficiently reliable for diagnostic purposes.

Immunity

The absence of a potent antibody response in trachoma is correlated with a poor degree of immunity. Repeated infections after clinical cure may therefore occur in heavily infected environments.

Control**(a) General measures**

For control, and the eradication of trachoma from affected areas, improvement of economic and social conditions, improved standards of hygiene and sanitation, and facilities for early diagnosis and adequate treatment of the disease, are essential.

(b) Vaccine

The possibility of reinfection after clinical cure, in highly infected environments, makes the development of a suitable vaccine, inducing long-lasting immunity to the disease, highly desirable. Purified elementary body suspensions, grown in the chick embryo yolk sac, provide suitable vaccines in both the living and formalin-inactivated state. The moderate antibody response and limited immunity produced in response to these vaccines may be enhanced by the addition

of adjuvants. In recent field trials, some decrease in the incidence of infection in vaccinees has been noted, but the beneficial effects are reported to be short-lived. Although an exaggerated response to experimental challenge infection has been noted in some immunized volunteers, probably as the result of a hypersensitivity reaction, no reactions of this sort have been noted after natural infection of vaccinees. It remains to be seen if the control of trachoma by vaccination can be achieved. If improvement of economic, social and hygienic conditions could be effected, vaccination methods of control would probably be rendered unnecessary.

Treatment

The organism is highly sensitive to tetracycline and sulphonamides, and less sensitive to penicillin, erythromycin and chloramphenicol. Full doses of oral sulphonamides for 2–3 weeks, combined with local administration of tetracycline into the conjunctival sac twice daily for 1–3 months, is the therapeutic regimen of choice. In mass campaigns, where medical facilities are poor and regular treatment impracticable, other regimens of treatment may be necessary.

Antibiotic treatment instituted in the early phases of the disease is entirely successful, but initiation of treatment in the later stages, although limiting infection, does not prevent the deformities due to scarring, for which surgical treatment may be necessary.

Cortisone has no therapeutic activity but its administration may reactivate trachoma. It has therefore been used as a provocative agent to produce inclusions, exudate and clinical activity for the purposes of diagnosis, and as a test of cure.

VII. *Chlamydia oculogenitalis* Inclusion Conjunctivitis

Inclusion conjunctivitis is a follicular conjunctivitis caused by an organism, *Chlamydia oculogenitalis*, which is very closely related to

C. trachomatis but differs from it in clinical and epidemiological characteristics.

Properties of *C. oculogenitalis*

C. oculogenitalis is indistinguishable from *C. trachomatis* in its morphological, serological, and biological properties, but is marked by its affinity for urogenital as well as conjunctival epithelium. Because of their similarity, the organisms of trachoma and inclusion conjunctivitis are sometimes referred to as the TRIC group of organisms.

C. oculogenitalis can be cultivated in the chick embryo yolk sac, but not in tissue culture or in experimental animals other than primates. An exceptional egg-adapted strain, LB1, has however been serially propagated in tissue culture, and, like the analogous trachoma strain TE/55, can be serially propagated in mice inoculated by the intracerebral route.

Clinical Features

Infection in the newborn, sometimes known as inclusion blennorrhoea, becomes manifest 5-15 days after birth. It is characterized by diffuse papillary conjunctivitis and copious purulent discharge, which resolves in a few weeks or months.

In adults, signs of acute papillary or follicular conjunctivitis appear 3-4 days after infection. In contrast to trachoma, the condition is most marked in the lower tarsal conjunctiva and fornix where it produces irritation, photophobia and mucopurulent discharge. The disease is relatively short in duration, and unlike trachoma does not produce scarring, pannus or corneal complications. Even if untreated, inclusion conjunctivitis resolves in a few weeks or months.

Infection of the urogenital tract can cause benign urethritis in the male, and symptomless cervicitis in the female.

Pathology

The conjunctival epithelium exhibits many inclusions, necrosis, and hypertrophy. Plasma cell and lymphocyte infiltration of the

subepithelial tissues, and follicle formation, occur. The follicles, unlike those of trachoma, do not undergo necrotic degeneration, nor is there any fibrosis or scarring.

Epidemiology

The reservoir of infection is in the mucosa of the adult urogenital system. Infection is therefore easily transmitted to the newborn from an infected mother; in adults infection is maintained, through the urogenital pathway, by venereal contact, or by water. Infection is conveyed to the eye from an infected maternal birth canal, or by contaminated fingers or water, particularly from swimming pools contaminated by infected urine or genital secretions; eye to eye transmission although possible is unusual.

Laboratory Diagnosis

Diagnostic procedures are the same as for trachoma. Giemsa stained epithelial scrapings reveal extracellular elementary bodies and typical inclusions, which are more numerous than in trachoma.

Urethral exudate from the male, and cervical scrapings from the female, may be examined for the confirmation of urogenital infection. From these sources, the organism of inclusion conjunctivitis may be confused with that of lymphogranuloma venereum, but the pathogenicity of the latter for mice, its production of inclusions only in monocytes, and the absence of any iodine-staining carbohydrate matrix from these inclusions, serve to distinguish it. Similar distinctions have to be made on the rare occasions when conjunctivitis is caused by the agent of lymphogranuloma venereum.

Control

Inclusion blennorrhœa of the newborn is not prevented by the Crédé or penicillin methods of prophylaxis. Its control, and that of inclusion conjunctivitis, depends on the limitation of adult urogenital infections, in which disinfection of swimming pools by adequate chlorination of the water is important.

Treatment

Oral administration of sulphonamides in the usual doses, or local application of tetracycline four times daily for 2 weeks, is rapidly effective.

CHAPTER 44

Miscellaneous Infections

I. Cat-scratch Fever

Cat-scratch fever is a mild disease, characterized by fever, malaise, and lymphadenitis, in which a history of a cat-scratch or cat-lick a few days before the onset of the illness is obtained in about 50% of cases.

The Aetiological Agent

The aetiological agent is believed to be a virus, which Mollaret and his colleagues claim to have transmitted to cercopithecus monkeys by subcutaneous infection of human lymph node material. All attempts to isolate the aetiological agent in other species, chick embryos or tissue cultures have failed.

Stained sections of infected human and monkey lymph nodes have revealed structures resembling the elementary bodies of *Miyagawanella*, and some authors have suggested that the causative organism belongs to this group. But the failure of the cat-scratch fever agent to grow in chick embryos, its resistance to antibiotics, and the negative reactions of patients convalescent from cat-scratch fever to the Frei test, is against this view.

Clinical Features

After an incubation period of 2–14 days, a papule which becomes pustular, and which may ulcerate, develops at the site of the scratch. This primary lesion is soon followed by inflammation and suppuration of the draining lymph glands, which may discharge bacteriologically sterile pus. The early stages of the disease are associated

with pyrexia and malaise, and sometimes erythematous rashes and generalized lymphadenopathy develop. The condition may take several weeks to resolve, and encephalitic complications have been observed.

Laboratory Diagnosis

Although patients with cat-scratch fever do not react to the Frei test, an intradermal skin test based on similar principles has been developed. Antigen, prepared from purulent material discharged from patients with the disease, is inactivated by heat, and injected intradermally. Patients with cat-scratch fever produce an area of erythema and induration at the site of injection.

Epidemiology and Control

The infective agent is probably present in the saliva of infected cats, who do not usually suffer from any overt disease, and is transmitted to man by scratching, biting, licking, or contact. Some cases have been reported in which no history of contact with cats has been obtained.

Treatment

The efficacy of treatment with chlortetracycline and other antibiotics is equivocal, although some authors report a beneficial effect.

CHAPTER 45

Miscellaneous Infections

II. Lymphocytic choriomeningitis

Lymphocytic choriomeningitis (LCM) is a natural infection of mice which is occasionally transmitted to man, in whom it is a cause of aseptic meningitis. Natural infection of house mice leads to chronic inapparent infection but infection of laboratory mice is usually fatal.

The Virus

(a) Morphological and chemical properties

The diameter of the virus particle, estimated by filtration and ultracentrifugation studies, is approximately 40–60 m μ . The virus is sensitive to ether but the nature of its nucleic acid component has not yet been established. Its precise classification is therefore not yet possible.

(b) Cultivation

(i) *Experimental animals*

Mice, guinea-pigs, and monkeys are susceptible. Five to 12 days after intracerebral infection, mice develop signs of central nervous system disease and die within a few days of the onset of illness. Post-mortem, inflammation and perivascular infiltration of lymphocytes are found in the central nervous system, liver and spleen.

The natural inapparent infection of house mice has been explained by the experiments of Traub, Hotchin, and their colleagues. They showed that infection of mice in utero, or within a few hours of birth, led to a retardation of growth from which the majority recovered within a week or so. Recovered mice appeared normal but carried high titres of virus in their blood, and in various organs, without developing antibody. Apparently, under these conditions

infection leads to immunological tolerance allowing persistent tolerant infection with the virus. Persistently infected mice are immune to challenge, in spite of the absence of antibody, and produce litters of healthy mice which are also persistently infected. Hotchin's experiments suggest that the acute LCM disease which occurs in adult mice is the result of immunological reactivity and delayed hypersensitivity reactions.

(ii) Chick embryos

LCM multiplies on the chorioallantoic membrane of chick embryos without producing specific lesions. The virus also multiplies in the yolk sac without affecting the embryo.

(iii) Tissue culture

Cytopathogenic effects are produced in chick embryo fibroblasts and KB cells. Growth, without cytopathogenic effects, occurs in some other cell types.

Clinical Features

Initially, infection produces an influenza-like illness from which many patients recover without further symptoms. Others, after a few days of clinical improvement, develop the signs and symptoms of acute aseptic meningitis from which recovery in about 2 weeks is the rule. During this stage, the cerebrospinal fluid is under slightly increased pressure, and exhibits a slight elevation of protein content and an increased lymphocyte cell count which varies between a few hundred and several thousand per mm³. Rarely, severe meningo-encephalitis occurs which may prove fatal. About 10% of human infections are subclinical or inapparent.

Laboratory Diagnosis

(a) Virus isolation

In the early febrile period of the disease, virus is present in the blood, cerebrospinal fluid, urine, and nasopharyngeal secretions. Blood or throat washings collected in the early stages, or cerebrospinal fluid collected in the meningeal phase, is inoculated intracerebrally into

suckling or weanling mice. After 5–12 days, mice develop central nervous system disturbances and convulsions, and rapidly die. Virus recovered, post-mortem, from mouse brain, liver and spleen is identified by neutralization and complement-fixation tests with specific antisera.

It is essential that mice used for isolation of LCM must be from colonies proved free of LCM infection, and strict precautions are necessary to prevent spread of virus from experimental mice to handlers and other animals.

(b) Serological tests

A four-fold rise in titre of antibody to LCM, occurring between the acute and convalescent phases of the disease, is diagnostic. Complement-fixing antibodies to a virus specific soluble antigen, prepared from infected guinea-pig spleen, appear within about 3 weeks of the onset of illness and quickly decline thereafter. Neutralizing antibodies appear later, reach a peak 4–8 weeks after the onset of illness, and persist for some time. Sera are therefore tested for neutralizing antibody at 4 and 8 weeks after infection.

Epidemiology and Control

The primary reservoir of infection is in house mice, in whom epizootic foci persist for long periods because of the life-long carriage of LCM, and its transplacental transmission to the newborn. Virus excreted in the urine, faeces and nasal secretions of infected mice may contaminate food or dust, through which infection may be transferred to man. Infection is therefore usually found in those inhabiting mouse infested dwellings, or in laboratory workers.

Virus is believed to gain entry into the human host by inhalation into the respiratory tract. Arthropod vectors have been suggested but not proved. Virus is not transmitted directly from man to man, but concurrent disinfection of the patients' excreta, which may contain virus, is required.

Efficient rodent control is sufficient to prevent the spread of the disease.

Treatment

There is no specific antiviral treatment.

CHAPTER 46

Antiviral Agents

I. Antibiotics and Chemotherapy of Virus Infections

No true virus is sensitive to the antibiotic and chemotherapeutic agents which have proved so successful in the treatment of bacterial infections. But a few substances claimed to have some clinically useful activity have been reported, and the search for antiviral agents is a subject of active investigation at the present time. A formidable barrier to the discovery of antiviral substances is the intimate relationship between virus and host cell, making it difficult to affect one without the other. Indeed, until a few years ago, the search for antiviral agents was thought to have little chance of success. The recent demonstration of virus specific enzymes essential to the course of virus replication, and the introduction of N-methylisatin- β -thiosemicarbazone and 5-iodo-2-deoxyuridine into clinical use, have now led to a more optimistic view of the future.

Although only very few compounds have any clinically useful activity, a number of naturally occurring and synthetic substances are known to have some antiviral activity in experimentally infected tissue cultures. But their potency is usually so low and their toxicity to the host cell so high that their trial in man is precluded. Nevertheless, a consideration of some of these substances, and their mode of action, is useful for illustrating the approaches being pursued in the search for clinically useful antiviral agents.

By the time signs and symptoms of many virus infections have become clinically manifest, virus is already widely distributed in the body and virus replication has reached its maximum; the therapeutic potentiality of even a potent antiviral agent is therefore often

questioned. But, the prompt administration of such an agent in the early stages of infection, or during the preliminary viraemia characteristic of some diseases, cannot fail to have a significant effect on the course of an infection. Nevertheless, there is little doubt that antiviral agents would prove most useful as chemoprophylactics, to prevent infection after exposure.

Chemoprophylactic agents which are immediately effective in preventing the initiation of infection would have a great advantage over vaccines for the prevention of disease. In diseases like influenza, which occur in short-lived epidemics and whose onset is unpredictable, the administration of the agent would be required only when there was known danger of an epidemic and its administration would need to be continued only for so long as the danger lasted. In contrast, many vaccines, which confer short-lived protection and require repeated administration to maintain immunity, are given in vain if an epidemic fails to materialize or if the epidemic strain of the organism is antigenically different from the one incorporated in the vaccine.

Theoretically, viruses are susceptible to attack before and after entry into the host cell, and at any stage in the infective cycle. An antiviral substance may thus be active at any stage of infection, and combinations of substances active at different stages of the infective cycle may eventually prove to be more efficacious than any one alone.

Agents active against Extracellular Virus

A variety of chemical substances, including protein denaturing agents, detergents, lipid solvents, and others, inactivate extracellular virus. None, however, is suitable for internal use as a chemotherapeutic agent.

Agents preventing Adsorption

(a) Specific antibody

Virus adsorption to the host cell surface is prevented by homologous antibody, which attaches to specific antigenic sites on the virus surface and blocks the virus receptors. Once inside the cell, virus is

unaffected by antibody, which is incapable of penetrating the host cell membrane. For this reason, hyperimmune serum or γ -globulin, which are often effective in preventing infection after exposure, are usually of little therapeutic benefit.

(b) Neuraminidase

The destruction of specific receptor sites on the host cell surface may prevent virus adsorption. The destruction of receptors for influenza viruses may be achieved by using the enzyme neuraminidase, whose protective effect after local administration to eggs and mice infected with influenza virus was demonstrated by Stone many years ago. So far, this approach has not found any clinical application.

(c) Mucoprotein receptor analogues

A number of naturally occurring mucoproteins, present in normal animal sera and glandular secretions, resemble the host cell and red cell myxovirus receptor substances in chemical structure, and inhibit influenza virus haemagglutination and adsorption to the host cell surface. Some are thereby capable of preventing infection, and normal horse serum, which contains the potent γ -inhibitor of influenza virus haemagglutination, is very effective, after intranasal administration, in preventing Asian (A_2) influenza infection in mice.

These substances have not yet found any clinical application, but the chemical analysis and manipulation of the horse serum γ -inhibitor molecule, now known to be an α_2 -macroglobulin, could lead to the production of more active inhibitory substances with wider ranges of activity.

Agents preventing Penetration

(a) Adamantanamine hydrochloride (amantadine)

Adamantanamine hydrochloride, described by Davies and his colleagues, has been found to have some inhibitory activity against influenza viruses A and C, parainfluenza 1, and rubella virus in experimental systems, but is without effect on influenza B and other parainfluenza viruses. The drug, which acts only in the very early

stages of infection, is believed to act by interfering with virus penetration.

Adamantanamine hydrochloride confers partial protection, of no very great degree, on mice experimentally infected with influenza virus. Clinical trials have shown the drug to confer some protection against influenza in man, but its low potency and its toxicity when administered in full dosage make its adoption as an effective chemotherapeutic agent unlikely.

Agents inhibiting Intracellular Virus Replication

A number of substances which interfere with the synthesis of nucleic acid and protein inhibit virus replication; most of them are not virus specific and poison the host cell as effectively as they inhibit virus replication. A few substances which, for qualitative or quantitative reasons, selectively inhibit the synthesis of viral nucleic acid or protein are however known.

(a) Inhibitors of DNA synthesis

(i) 5-Iodo-2-deoxyuridine (IDU)

5-Iodo-2-deoxyuridine, synthesized by Prusoff in 1959, is an inhibitor of DNA synthesis which is capable of inhibiting certain DNA viruses, particularly *Herpesvirus hominis*. As an analogue of thymidine, IDU becomes incorporated into the DNA molecule, and also inhibits the formation of DNA polymerase. Its apparent viral specificity probably depends on the very high rate of synthesis of viral DNA and virus specific enzymes soon after infection, which presumably allows the drug to be directed towards viral rather than cellular DNA and its polymerases. Viral DNA in which IDU has replaced thymidine functions abnormally, and fails to initiate infection or produce mature virus.

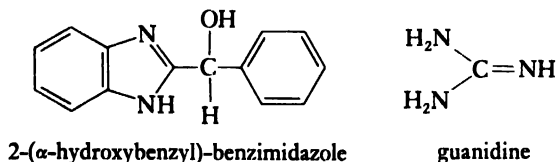
Herrmann demonstrated the antiviral effect of IDU in tissue culture, and Kaufman showed the compound to be therapeutically effective in herpetic and vaccinia infections of the cornea. Although too toxic for systemic administration in man, local application of IDU is beneficial in the treatment of superficial herpetic keratitis. Local application of IDU ointments to dermal herpetic lesions in

man is of doubtful efficacy, but MacCallum and Juel-Jensen have reported some beneficial effect from local application of IDU in 5% solutions in dimethyl sulphoxide.

IDU-resistant strains of *Herpesvirus hominis* have been produced experimentally and after clinical treatment. These strains remain susceptible to another DNA inhibitor, cytosine arabinoside, which interferes with the incorporation of cytosine, and is active against a number of DNA viruses in tissue culture. Although effective, it is more toxic than IDU, even after local administration, and its use in man is therefore precluded.

(b) Inhibitors of RNA synthesis

(i) 2-(α -Hydroxybenzyl)-benzimidazole (HBB) and guanidine



The antiviral effect of the benzimidazole group of compounds has been extensively investigated by Tamm and his colleagues. The most active of these benzimidazole compounds is HBB which inhibits the formation of RNA polymerase and thus interferes with the synthesis of RNA. The compound, in doses which are non-toxic to the host cells, inhibits the multiplication of poliovirus, coxsackieviruses, and most echoviruses in tissue culture. Mutants which are resistant or dependent on HBB have been produced.

The action of guanidine is similar but not identical to that of HBB. It is more active than HBB against poliovirus but less active against coxsackieviruses and echoviruses; mutants which are resistant to one drug are not necessarily resistant to the other. Neither HBB nor guanidine has been shown to have any prophylactic effect in experimental animals, possibly because of the rapid development of drug-resistant mutants.

(ii) Actinomycin D

Actinomycin D is one of a number of antibiotics isolated from actinomycetes by Waksman and Woodruff; in small doses, it selectively inhibits DNA-dependent RNA synthesis by forming stable complexes with the DNA helix. The inhibition of host cell DNA-dependent RNA synthesis eventually proves to be cytotoxic, and cells in culture die within 24–48 hours. Nevertheless, actinomycin D has been shown to inhibit the growth of some DNA viruses, including vaccinia, *Herpesvirus hominis*, and SV 40. In contrast, actinomycin D permits the growth of small RNA viruses and sometimes even enhances it; presumably, these viruses replicate independently of host cell DNA. The growth enhancement which is sometimes apparent may be due to the inhibition of interferon synthesis which has been demonstrated in the presence of actinomycin D.

Curiously, some of the larger, more complex RNA viruses are inhibited by actinomycin D. These include Rous sarcoma virus, and *Myxovirus influenzae*. *Paramyxovirus multiforme* is actinomycin-sensitive in some cell systems but not in others. Reovirus, whose RNA is double stranded, is also inhibited by actinomycin D, but only by doses which inhibit cell RNA synthesis by more than 90%. Presumably, sensitive RNA viruses pass through a host cell DNA-dependent event in the early stages of virus replication.

Actinomycin D is a useful experimental tool for the study of viral and host cell RNA synthesis and mechanisms of virus replication, but it has no clinical or therapeutic application in virus diseases.

(iii) Interferon

The phenomenon of viral interference, in which infection of a cell with one virus inhibits the multiplication of another, has been known for more than 30 years. It was not until 1957, however, that Isaacs and Lindenmann discovered interferon, the substance which is produced by virus-infected cells and mediates the interference phenomenon.

Interferon, which inhibits virus replication, is produced in cells infected with a number of DNA and RNA viruses, it is also produced by inactivated virus and by some ribonucleic acid preparations from

sources foreign to the host cell. Viruses vary in their susceptibility to interferon and in their ability to produce it; moreover, the activity of interferon is limited by its cell specificity, evident from its greater activity in the type of cell in which it is produced than in others. The mode of action of interferon is not yet clear but it is believed to affect the formation of viral messenger RNA, an intermediate metabolic component common to both RNA and DNA viruses.

Interferon is effective only when it is present at the time of virus inoculation, its potential is therefore in the field of prophylaxis rather than therapy. Locally administered, it is effective in protecting rabbits from the effects of intradermal or intraocular vaccinia infection. Systemically administered, its protective effect in mice is very low indeed, although the more concentrated preparations now available may prove to be more potent.

In man, locally administered interferon has been shown to prevent the formation of a lesion after smallpox vaccination, but local interferon treatment was ineffective against respiratory infections. The low activity of interferon, the difficulty of its preparation, its viral and cellular specificities, and the need for its administration before virus infection, make any hope of its immediate practical use as a therapeutic agent remote.

Recently, a ribonucleoprotein, helenine, and a macromolecular polyanionic polysaccharide, statolon, isolated from two species of *Penicillium*, have been shown to stimulate interferon formation in mice and tissue cultures, protecting them against virus infection. When purified preparations become available, trials in man may be expected.

(c) Inhibitors of protein synthesis

(i) *N-Methylisatin- β -thiosemicarbazone*

Soon after the activity of thiosemicarbazone compounds against mycobacteria had been demonstrated, Hamre and his colleagues found *p*-aminobenzaldehyde 3-thiosemicarbazone to be active against vaccinia virus in eggs and mice. Later, isatin- β -thiosemicarbazone was shown to be more active by Thomson and his col-

leagues, and the compound N-methylisatin- β -thiosemicarbazone (Methisazone or Marboran), synthesized by Bauer and Sadler, proved to be even more active.

The mode of action of methisazone is not precisely known, but it has no effect on the synthesis of viral DNA and has been shown to act late in the virus replication cycle. According to Appleyard and his colleagues, the formation of some but not all the vaccinia virus antigens are prevented by an effect on messenger RNA. Many authors have observed the formation of morphologically abnormal, immature, non-infective vaccinia virus particles devoid of any central nucleoid structure; some effect on the assembly of mature virus particles therefore seems possible.

Methisazone, administered before or within 48 hours of virus inoculation, has a considerable protective effect in mice, but is without effect once the disease has become manifest. The compound is therefore of prophylactic rather than of therapeutic value. Nevertheless, its high activity in experimental animals prompted trials in man. In recent clinical trials in Madras, carried out by Bauer and his colleagues, methisazone was successful in preventing smallpox in those who had been in recent contact with infection. Bauer reports that in 2283 contacts treated with methisazone, there were six cases of smallpox and two deaths, compared with 105 cases of smallpox and 18 deaths in 2526 untreated contacts. Similar results have been obtained in recent alastrim outbreaks in Brazil. Evidently, smallpox contacts can now receive immediate protection by the administration of methisazone. Vaccination, because of the relatively high inoculating dose of vaccinia virus, is little affected by systemic doses of methisazone, and is recommended as an adjunct to methisazone treatment to confer protection after the effect of the drug has worn off.

Although without effect in the treatment of clinical smallpox, methisazone is beneficial in the treatment of eczema vaccinatum and vaccinia gangrenosa. The dosage recommended is 200 mg/kg followed by 50 mg/kg 6 hourly for eight doses; using this dose, vomiting is a disturbing but not serious side effect.

Another thiosemicarbazone compound, M & B 7714 or 4-bromo-3-methylisothiazole-5-carboxaldehyde thiosemicarbazone, has also

been shown to be active as a smallpox prophylactic, but is less effective than methisazone.

Recently, Bauer and Apostolov have reported that methisazone is active against adenoviruses in tissue culture, at a late stage in the infective cycle.

CHAPTER 47

Oncogenic Viruses

I. Introduction

RNA Oncogenic Viruses

Introduction

More than 60 years ago, in 1908, Ellerman and Bang succeeded in transmitting a malignant condition, erythromyeloblastosis of chickens, by passage of cell-free filtrates. The importance of this observation, which suggested a viral aetiology for a fowl leukaemic condition, was overlooked at the time, because neither the malignant nature of leukaemia nor the importance of viruses was then generally recognized; nor would many admit the relevance of this observation for the understanding of similar conditions in man. A few years later, in 1911, Peyton Rous successfully transmitted certain chicken sarcomata by passage of cell-free filtrates, and proposed a viral aetiology for malignant tumours. The work of Peyton Rous created some excitement at the time, but the failure to repeat it in species other than the chicken soon cast doubt on the role of viruses as carcinogens in man.

Subsequently, a sufficient number of important observations were made to keep interest alive and prevent the complete abandonment of the virus theory of cancer. The viral aetiology of the human wart, which although not malignant is a human tumour, was demonstrated by Ciuffo in 1907 and by Wile and Kingery in 1919. In 1932, Shope isolated a virus from benign fibromata of wild cottontail rabbits; this virus produced similar benign tumours in

domestic rabbits, but they usually regressed in a few weeks and never became malignant. Of more interest was Shope's isolation of the rabbit papilloma virus, in 1933, from benign cutaneous papillomata of wild cottontail rabbits. This virus produced papillomata in domestic rabbits which, in contrast to those in wild rabbits, sometimes became malignant. The tumours produced in wild rabbits were rich in infective virus but those produced in domestic rabbits yielded little or no infective virus, although virus specific antigen was sometimes demonstrable by appropriate immunological techniques. The loss of infective virus after tumour induction was originally an ill-understood phenomenon, but is now known to be a characteristic of DNA tumour viruses.

The growing evidence in support of a viral aetiology of cancer was supplemented by Lucké's isolation of a virus responsible for a frog renal adenocarcinoma, in 1934, and by Bittner's evidence that some mouse mammary adenocarcinomas were viral in origin. Bittner's hypothesis has since been fully confirmed, but it was by no means generally accepted when first proposed in 1936.

In the last 15 years, the introduction of new virological techniques, particularly those of tissue culture and the use of suckling mice and hamsters, has revolutionized the field of oncogenic virology. The modern phase of cancer virus research begins in 1951 with Gross's very significant discovery of the mouse leukaemia virus, followed by Stewart and Eddy's isolation of the intriguing polyoma virus, in 1957. The unequivocal evidence that viruses cause cancerous conditions in animals, and the more recent evidence that certain human adenoviruses are oncogenic in hamsters, has convinced prevailing scientific opinion that at least some human malignancies may be caused by viruses. The revolution of scientific opinion which has taken place is well illustrated by the career of Peyton Rous. His work which was originally greeted with healthy scepticism was rewarded nearly 60 years later, in 1966, by the award of the Nobel prize to the veteran scientist at the age of 80.

Oncogenic viruses do not differ from other viruses in their general properties, and like them may be divided into two groups, RNA and DNA viruses, each of which is characterized by particular properties.

RNA Viruses

(a) Avian leucosis and Rous sarcoma viruses

Several neoplastic conditions of the chicken haematopoietic system are caused by viruses; these include erythroblastosis, myeloblastosis, and visceral lymphomatosis. The viruses causing these conditions are closely related both antigenically and morphologically, and are now referred to as avian leucosis viruses or as the avian leucosis complex. The avian leucosis viruses are themselves closely related to Rous sarcoma virus (RSV), and recent work indicates that the relationship is much closer than was originally thought.

(i) *Properties of avian leucosis viruses and RSV*

For description, the viruses of avian leucosis and Rous sarcoma may be considered together.

Morphology. The virus particles are spherical in shape, about 80–120 m μ in diameter, and consist of a dense inner core or nucleoid, 35–45 m μ in diameter, surrounded by an outer lipid envelope. Radially orientated spikes project from the envelope, and the inner core consists of a helical structure similar to the nucleocapsid of myxoviruses. The morphological resemblance to myxoviruses is striking, but avian leucosis viruses do not haemagglutinate.

Chemical Properties. Avian leucosis viruses are composed of RNA, protein, and lipid, and they are easily inactivated by ether, chloroform, deoxycholate, detergents, and heat.

Antigenic properties. Avian leucosis viruses are antigenically closely related, but some differences are detectable in neutralization tests. A group complement-fixing antigen, which may be part of the viral core, is common to all viruses of the group.

Biological properties. Intracellular virus replication takes place in the cell cytoplasm, and the particles mature at the cell-surface by budding through the cell membrane which becomes incorporated in the virus surface (Fig. 89). The host origin of the virus envelope is evident from the acquisition of certain enzymic and antigenic properties specific for the host cell.

(ii) *Virus-host cell relationships*

RSV is remarkably effective in producing malignant growths in

chickens; after subcutaneous inoculation in the wing, a tumour may be formed in 5–6 days and involve the whole wing in 14 days; eventually, the tumour metastasizes and kills the chicken. The sarcoma which develops is rich in infective virus, but virus is also found in some normal tissues. Avian leucosis virus is likewise found



Fig. 89. Budding of avian leucosis virus from an epithelial cell in the oviduct of an infected Chicken [from H. S. Di Stefano and R. M. Dougherty (1965) *Virology* 26, 156–59 (Academic Press Inc., New York and London)].

to replicate in normal tissues but produces neoplastic transformation only in undifferentiated cells of the haematopoietic system. Clearly, cell factors are important influences in determining a neoplastic outcome after infection with avian tumour viruses.

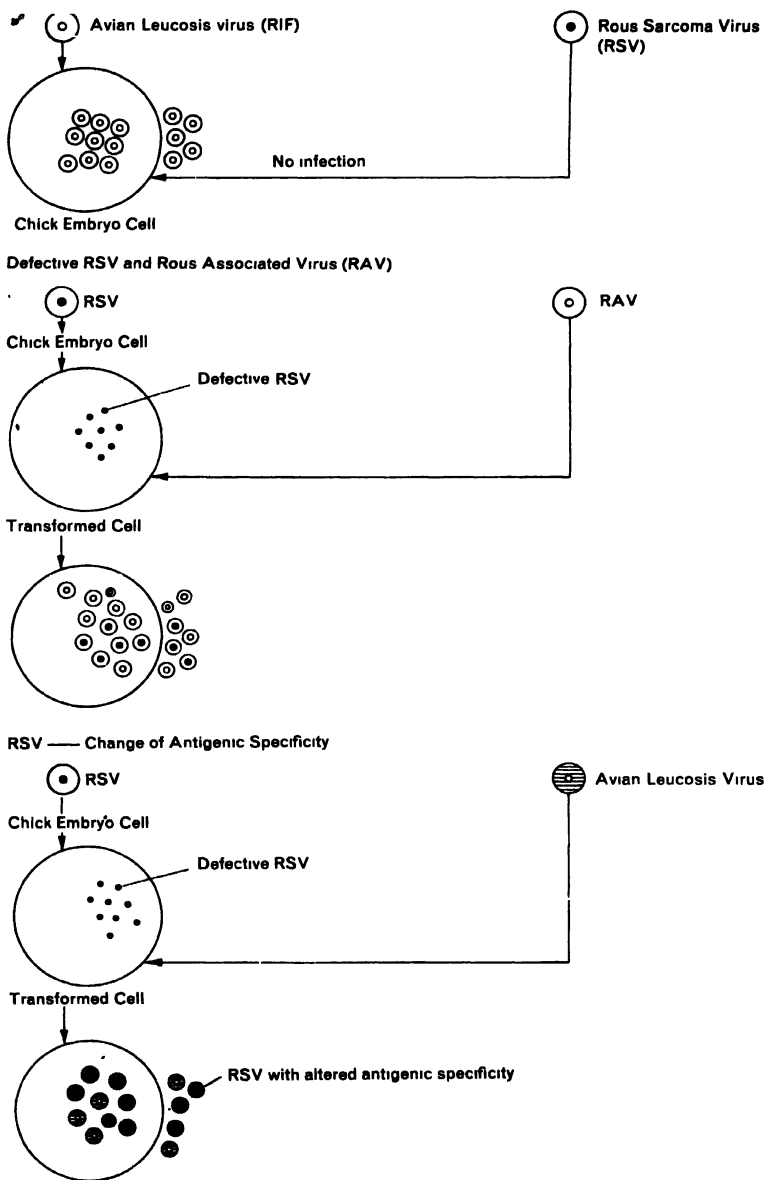
The use of tissue culture techniques has greatly facilitated our understanding of the avian tumour virus-host cell relationship. Chick embryo cell monolayers infected with RSV undergo morphological transformation and take on malignant characteristics. The infected fibroblasts, originally elongated, become spherical in shape and their cytoplasm becomes refractile; the cells lose the property of contact inhibition and multiply without restraint, so that heaps of transformed cells or microtumours are formed in about 7 days. Each of these microtumours is apparently produced by a single infective unit, their formation may therefore be used as a method of RSV assay.

In contrast to the usual virus-host cell relationship, RSV infected cells are not killed but survive and continue to produce infective virus. Indeed, infected cells remain viable for much longer periods than uninfected cells.

(iii) Resistance inducing factor (RIF) and defective RSV

During the course of their studies on RSV, a very intimate and complex relationship between this virus and avian leucosis virus has been brought to light by Rubin, Temin, Hanafusa, and their colleagues. The resistance of some chick embryo cells to infection with RSV was found to be associated with infection by a resistance inducing factor (RIF), later identified as avian leucosis virus (Fig. 90). Subsequently, some standard strains of RSV were found to be contaminated with avian leucosis virus which produced leucosis in chickens and, acting as an RIF, produced resistance to infection with RSV. The avian leucosis virus in question, unlike the RIF originally described, was found to be antigenically identical with RSV, and therefore called Rous associated virus (RAV).

Further investigation showed that cells transformed by RSV do not produce infective RSV unless inoculated with RAV at the same time or soon after the original RSV inoculation. Thus RSV alone, although able to transform cells, cannot produce complete infective virus without the aid of a 'helper' virus; it is said to be defective. Presumably, only the RSV genome is required for neoplastic transformation, but the presence of the RAV genome is necessary for the synthesis of the RSV protein coat. RAV, which acts as a 'helper'



ig. 90. RSV, RIF, and Avian leucosis virus relationships.

virus if inoculated at the same time or soon after RSV, acts as an RIF conferring resistance to RSV if present before RSV is inoculated (Fig. 90).

RAV is not the only strain of avian leucosis virus which acts as 'helper' to RSV, several different strains can act in this way. Surprisingly, the antigenic specificity of the mature infective RSV produced is determined by, and is identical with, the helper virus, and can be varied at will by changing the helper virus (Fig. 90). Moreover, only the antigenically homologous helper virus can serve as the resistance inducing factor to RSV infection.

The resistance of some chickens and their tissues is not always explicable by the presence of RIF, sometimes it is determined by genetic factors. Nevertheless, ducks, turkeys, and other birds are now known to be susceptible to RSV, and certain strains of RSV will produce sarcomata in certain mammals, including rodents and newborn rhesus monkeys. In these species, serial passage of tumours is possible only with viable cells and not with cell-free filtrates, suggesting that RSV in mammalian tissues is defective.

Avian leucosis infection of chickens is extremely common and is responsible for widespread outbreaks of visceral lymphomatosis. Its widespread distribution makes it not only commercially and economically important but also relevant to the production of live virus vaccines in chick embryos. The introduction of a potentially oncogenic virus into the human population carries a hypothetical risk, which makes it desirable to prepare live virus vaccine in embryos from leucosis-free flocks only.

(b) Mouse leukaemia viruses

Efforts to demonstrate a virus responsible for leukaemia in mammalian species proved fruitless until 1951, when Gross, after many unsuccessful attempts, isolated mouse leukaemia virus by inoculating newborn mice with cell-free leukaemic material. A long latent period of several weeks or months was originally required for the development of leukaemia, but this was later reduced after serial passage of the virus in mice increased its potency. Mouse leukaemia virus, originally isolated from spontaneous mouse leukaemia, was subsequently isolated from mouse leukaemias induced by irradiation.

tion and even from the normal issues of healthy animals. Gross's original leukaemia virus has since been shown to produce all types of leukaemia and lymphoma in rats as well as mice.

Since 1951, 14 different mouse leukaemia viruses have been described which probably represent different strains of a single or only few types of virus. All are the same or nearly the same in physical, pathological and morphological properties, but there is some evidence that they differ serologically.

(i) Properties of mouse leukaemia virus

Morphology. Mouse leukaemia virus is spherical in shape, about 100 m μ in diameter, and consists of a dense nucleoid structure surrounded by a lipid envelope.

Chemical and biological properties. Mouse leukaemia viruses are RNA viruses which are inactivated by ether. They replicate in the cell cytoplasm and mature at the cell surface, through which they bud, acquiring part of the host cell membrane which becomes incorporated in their surface. In vivo, the virus replicates in normal as well as leukaemic cells of the haematopoietic system.

Natural transmission of leukaemia virus to newborn mice may take place by way of the placenta, milk, or, in limited degree, by contact. The pathogenic effect of the virus is modified by genetic, nutritional and hormonal factors in the host; thymectomy at birth is also known to reduce the incidence of the lymphatic type of leukaemia.

(ii) Murine sarcoma virus (MSV)

Recently, some of the Moloney strains of mouse leukaemia virus have been shown to be contaminated with a virus producing sarcomata in newborn mice, rats, and hamsters. This virus, now called murine sarcoma virus (MSV), produces foci of transformed cells in tissue cultures of mouse embryo cells simultaneously infected with mouse leukaemia virus. The development of these foci of transformation is prevented by previous infection of the mouse embryo cells with mouse leukaemia virus or by the presence of mouse leukaemia virus antiserum. Evidently, the relationship between MSV and mouse leukaemia virus is very similar to that be-

tween RSV and avian leucosis virus. Murine sarcoma virus appears to be a defective virus which may be converted into the mature infective form with aid of a 'helper' virus, in this case mouse leukaemia virus, in mouse embryo tissue culture cells.

The action of MSV differs from that of RSV in several important respects. In both experimental animals and tissue cultures, neoplastic transformation is not produced by MSV alone but requires the aid of 'helper' virus; in experimental animals the 'helper' virus is eventually lost and only defective MSV is produced. For this reason, virus induced murine sarcomata cannot be transmitted by cell-free filtrates, and infective MSV can only be recovered by culturing sarcoma cells in the presence of leukaemia infected mouse embryo cells. Under these conditions, the defective MSV provides the sarcoma virus genome and the leukaemia 'helper' virus the protein coat.

(c) Bittner virus or mouse mammary tumour agent

In 1936, long before the present emphasis on tumour virus research, Bittner described what is now recognized as a mammalian oncogenic virus. He demonstrated the transmission of certain mammary adenocarcinomas of mice from one generation to the next by an agent present in the mother's milk. After transfer of the agent to the newborn, a long latent period of 6–12 months is required before the production of tumours.

Not all mouse mammary tumours are due to Bittner virus, and virus is only one of the factors involved in those which are virus induced. Hormonal and genetic factors are important additional elements combining to produce a tumorigenic result. Some evidence indicates that the virus induces small, potentially malignant, hyperplastic nodules in the mammary glands which are transformed into adenocarcinomas by hormonal stimulation in mice of the right genetic constitution.

(i) *Properties of the Bittner virus*

Morphology. Virus particles, about 100 m μ in diameter, and some smaller particles, are seen in the electron microscope. The virus particles consist of a dense inner core surrounded by a lipid

envelope from which radially orientated projections protrude.

Chemical and biological properties. Bittner virus is an RNA virus which replicates in the cell cytoplasm, and matures by budding through the host cell membrane, part of which is incorporated in the virus surface during the process of maturation and release. So far, the virus has not been recovered in tissue culture, but organ cultures of mouse embryo mammary gland have proved susceptible. Serial transplantation of the tumour, in vivo, may result in the loss of infective virus without any loss in the malignant properties of the tumour.

CHAPTER 48

Oncogenic Viruses

II. DNA Oncogenic Viruses

DNA viruses now known to be oncogenic in experimental animals are polyoma virus, simian virus 40, and some adenoviruses of human and monkey origin.

Polyoma Virus

(a) Effect in experimental animals

Gross observed the sporadic production of parotid gland tumours in mice inoculated with mouse leukaemia filtrates. Similar observations were later made by Stewart, who described the production of a wide variety of carcinomata and sarcomata by leukaemic filtrates in mice. At first, transmission of these tumours by cell-free filtrates proved difficult because, as has now become apparent, they contained little or no infective virus. It was not until 1957 that Stewart and Eddy succeeded in isolating the causative virus, and produced it in high titre in tissue cultures of monkey kidney and mouse embryo cells. The virus recovered proved to be distinct from the leukaemia virus, and, because of the wide variety of tumours it produced, was named polyoma virus.

The conditions for tumour production established in experiments with mouse leukaemia virus and Bittner's mouse mammary tumour virus apply also to polyoma virus. Mice must be inoculated at or within 2 days of birth, and a long latent period, of 3-9 months, is required for the development of tumours. In due course, carcinomata and sarcomata of divers histological types appear in several

organs and tissues, but of these parotid gland tumours are the most common (Fig. 91).

There is now little doubt that a single virus entity is responsible for the different types of tumour produced, and that polyoma virus is oncogenic in rats, hamsters, and ferrets, as well as mice. Very little infective virus is produced in these polyoma tumours, and virus may disappear altogether after a few serial tumour transplantations.



Fig. 91. Polyoma virus tumours in a mouse [courtesy of Dr Sarah E. Stewart, National Institutes of Health, U.S.A.].

Serial transmission by cell-free filtrates is therefore always difficult and later impossible.

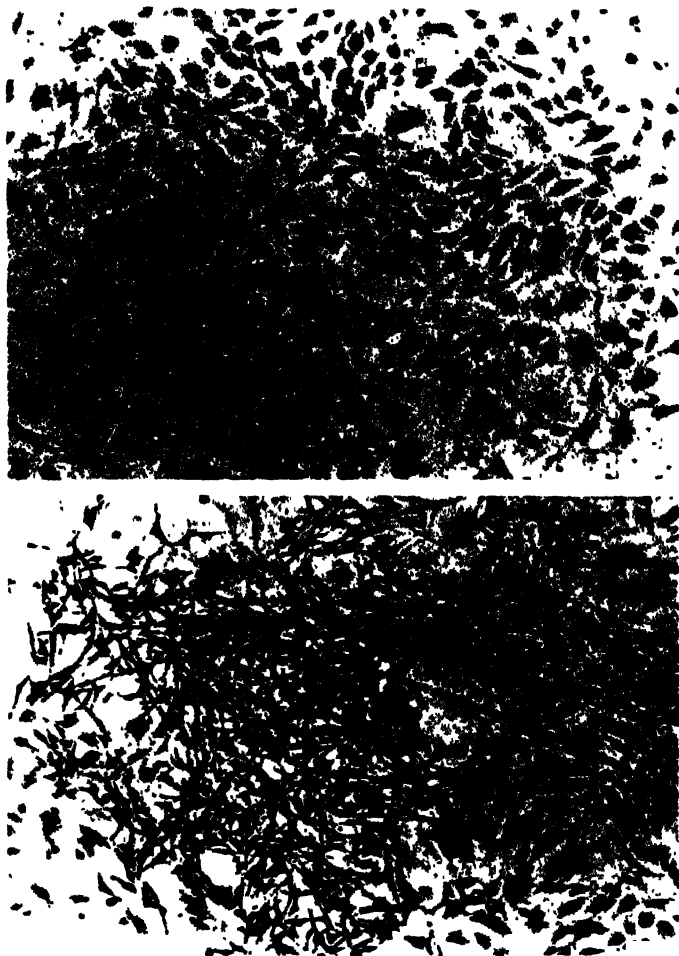


Fig. 92. Transformation of cells by polyoma virus.

(G) Normal hamster embryo liver cells.

(I) Hamster embryo liver cells transformed by polyoma virus.

[From Dan Medina and Leo Sachs (1965) *Virology* 27, 398-408 (Academic Press Inc., New York and London)].

(b) Effect in tissue culture

Two quite distinct effects are produced by polyoma virus in mouse embryo cells. Most cells support replication of the virus in the nucleus, and undergo degeneration and necrosis with the release of infective virus particles. In contrast, a proportion of cells survive, become transformed, and do not produce infective virus. Polyoma transformed cells take on all the properties of malignant cells; they



Fig. 93. Negatively stained polyoma virus particles [from S.S.Breese Jr (1964) *Virology* 24, 125-26 (Academic Press Inc., New York and London)].

persist indefinitely in subculture, they lose the property of contact inhibition, they multiply without restraint so that irregular heaps of cells are formed in the monolayer, and they produce tumours when transplanted back into experimental animals.

In cultures of hamster fibroblasts only the transforming effect of the virus is seen (Fig. 92). A small amount of infective virus is sometimes produced, and this is thought to result from a cytopathic effect on a few isolated cells.

(c) Properties of polyoma virus

(1) *Morphology*

Polyoma virus is about 45 m μ in diameter and spherical in shape; it consists of a DNA core and a capsid made up of capsomeres, believed to be 42 in number, which are arranged in icosahedral symmetry (Fig. 93). Elongated particles and particles in which the DNA core is missing are sometimes seen.

(u) *Chemical and biological properties*

Polyoma virus is a DNA, ether stable virus which is particularly resistant to chemical and physical agents, including heat (Fig. 94).

Red blood cells of several species are agglutinated by polyoma virus which, although it is not endowed with the neuraminidase enzyme, adsorbs to the sialomucoprotein myxovirus haemagglutination receptors. Haemagglutination and haemagglutination-inhibition tests may therefore be used for virus and antibody assay.

All strains of polyoma virus so far recovered are antigenically homogeneous.

(d) Epidemiology

The observations of Huebner, Rowe, Hartley, and others, on the distribution of polyoma virus in the field are relevant to our understanding of the behaviour of tumour viruses in nature. They found polyoma virus widely distributed in wild mice and laboratory stocks, but as a commensal organism not producing tumours. Although infected carrier mice excrete virus in the saliva, faeces, and urine, so that the environment becomes heavily contaminated, the virus is



Fig. 94. Electron micrograph of DNA from two polyoma viruses [from L. E. Caro (1965) *Virology* 25, 226–36 (Academic Press Inc., New York and London)].

not transmitted across the placenta or in the milk. Absence of infection in the first few days of life, when the young are protected by maternal antibody, may therefore be inferred. Presumably, young mice acquire infection by inhalation or ingestion at a time when the development of immunological competence serves to reduce the tumorigenic potential of the virus.

Simian Virus 40 (SV 40)

(a) Effect in experimental animals

SV 40 is a simian virus commonly found as a contaminant in rhesus monkey kidney cell cultures, in which it is not cytopathogenic. Sweet and Hilleman recognized the virus because of its cytopathogenic effect in African green monkey kidney cell cultures. Some species of primate are commonly infected with the virus but no oncogenic or other clinical disease is produced by natural infection.

In contrast, inoculation of the virus into newborn hamsters produces sarcomata after a latent period of 4–8 months. Infective virus is usually but not always absent from these tumours.

Before the oncogenic potential of SV 40 was discovered, many live and inactivated polio vaccines were prepared in monkey kidney cultures contaminated with SV 40. The virus, which is resistant to formalin, has therefore been given inadvertently to millions of individuals, and it is known that children continued to excrete SV 40 for 5 weeks after ingestion of live polio vaccine. Fortunately, no untoward effects have so far been observed. All monkey kidney cultures used in the preparation of virus vaccines are now screened for the presence of SV 40 and excluded if infected.

(b) Effect in tissue culture

SV 40 produces no cytopathogenic effects in rhesus monkey kidney cells, although it often contaminates them. In contrast, infection of African green monkey kidney cell cultures leads to vacuolation of the cell cytoplasm in 3–4 days and to the development of small plaques of degenerated cells in the monolayer. Infection of cells from other species, including those from hamster, calf, mouse, pig, and rabbit, and, significantly, those of human origin, gives rise to cell transformation leading to the acquisition of properties characteristic of malignant cells. Transformed cells are abnormal in morphology and chromosomal configuration, lose the property of contact inhibition, survive indefinitely, grow at an accelerated rate in unrestrained fashion so that irregular heaps of cells form in the monolayer, and produce tumours when transplanted back into susceptible animals.

The transformation of human fibroblast cells, which takes place in several phases, has been studied in some detail by Koprowski and his colleagues. After infection, stimulation of cell growth is the first change to be noticed but within a few days some cells are seen to degenerate and necrose. The remaining cells remain unchanged for 8–16 weeks when they suddenly begin to proliferate in a rapid and irregular manner. This proliferative phase, during which infective virus is produced, continues for 15–33 weeks ending in a stage of crisis, in which almost the entire culture degenerates. A few cells

which survive begin to proliferate rapidly and take on all the characteristics of malignant cells, but they do not produce any infective virus. Cells infected towards the end of their *in vitro* lifetime go through the transformation process in about half the time normally required.

Infective virus is not usually recoverable from tumour or transformed cells, but may be recovered from tumour cells seeded onto a feeder layer of cells susceptible to SV 40, e.g. African green monkey kidney cells. Presumably, the virus in tumour cells is defective and incapable of full expression.

(c) Properties of SV 40

SV 40 is approximately 40 m μ in diameter, and consists of a DNA core enclosed in capsid made up of 42 capsomeres arranged in icosahedral symmetry. It is resistant to ether, and relatively resistant to other chemical and physical agents, including heat.

Unlike polyoma virus, SV 40 does not agglutinate red blood cells.

Adenoviruses

Trentin was the first to observe that adenovirus type 12 produced sarcomata when injected into newborn hamsters. Since then, a number of adenoviruses, including types 18, 31, 7, 3, 14, and 16, have been found to be oncogenic in newborn hamsters. In common with other oncogenic viruses, tumours are produced only if adenoviruses are introduced into the newborn, and a long latent period of about 90 days is required before tumours develop. None of the adenovirus tumours produce infective virus.

No *in vitro* transformation of tissue culture cells by adenoviruses has been reported, although the normal infective cycle leading to characteristic adenovirus cytopathogenic effects and the production of infective virus takes place in many cell types.

Adenoviruses represent the first viruses of human origin which are demonstrably oncogenic in experimental animals. This raises the question of their oncogenic potentiality in the natural human host, a problem which is discussed in Chapter 50.

Virus-specific Tumour Cell Antigens

Infective virus is not usually produced by virus induced tumour or transformed cells, but the identification of virus-specific antigens in these cells indicates that at least a part of the virus genome persists in them.

(a) The transplantation antigen

Adult mice which have suffered from inapparent polyoma infections reject transplants of virus-free polyoma tumours. This type of immunity, discovered by Sjogren and his colleagues in Sweden, and Habel in the U.S.A., is demonstrable only by transplant challenge and proves to be of the homograft type. It develops only in response to tumour growth or inoculation of live virus, and is mediated by lymphocytes and not humoral antibodies. Subsequent work has shown that polyoma induced tumour or transformed cells produce a polyoma specific transplantation antigen at the cell surface. Immunity produced by this antigen does not prevent transplantation of tumours induced by heterologous viruses.

Analogous virus-specific transplantation antigens have now been demonstrated in tumours produced by SV 40, and adenovirus type 12, and in tissue culture cells transformed by SV 40. The production of new virus-specific cellular antigens is evidently a property common to all the DNA oncogenic viruses.

(b) The virus-specific complement-fixing T-antigen

Virus induced tumour or transformed cells produce a virus-specific complement-fixing T-antigen in the cell nucleus which is quite distinct from the transplantation antigen. The T-antigen reacts with a humoral antibody present in the sera of animals bearing tumours induced by the homologous virus. Cells of different species transformed by the same virus produce immunologically identical complement-fixing T-antigens, but the T-antigens produced in cells of the same species transformed by different viruses are immunologically distinct.

The T-antigen, which is not found in the mature infective virus particle, plays no part in establishing resistance to tumour transplantation. It has recently been found in infected cells during the

early part of the normal virus replication cycle, and is believed to represent a virus-specific enzyme which is required for virus synthesis but is not incorporated in the mature virus particle.

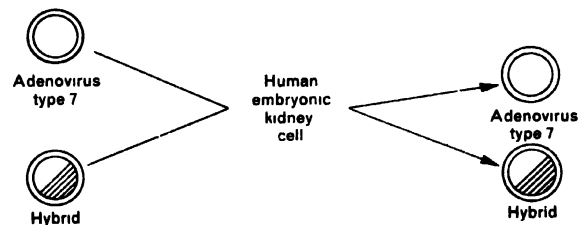
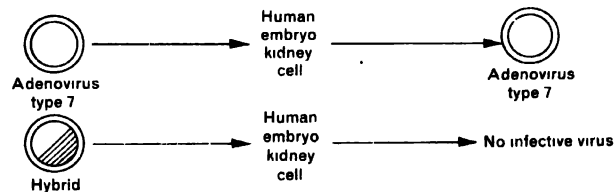
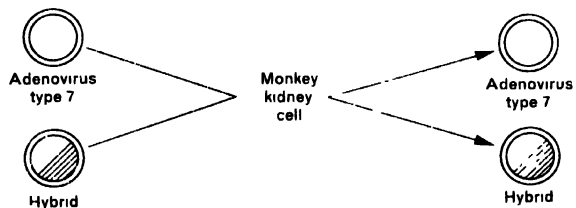
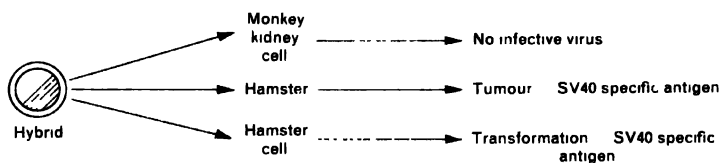
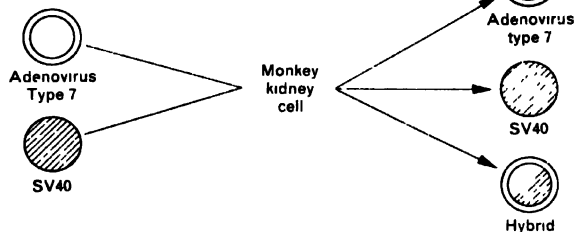
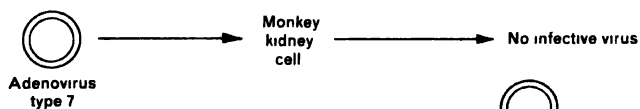
SV 40-Adenovirus Relationships

A curious relationship between SV 40 and adenoviruses has recently been uncovered by both Huebner and Rapp, and their colleagues, in the course of experiments with a strain of adenovirus type 7 adapted to monkey kidney cells. The adapted strain was found to be contaminated with SV 40, and was purified by passage in the presence of SV 40 antiserum. Now free of SV 40, the adapted strain, surprisingly, continued to elaborate SV 40-specific T-antigen and to produce tumours in hamsters. Its neutralization by adenovirus type 7 antiserum but not by SV 40 antiserum suggested that the adapted strain was a hybrid, in which part of the SV 40 genome had become incorporated in an adenovirus type 7 capsid.

Further study of the adapted strain in human and monkey kidney cells revealed it to be a mixture of the SV 40-adenovirus hybrid and mature infective adenovirus type 7. In Rowe and Baum's experiments, human embryo kidney cells supported the replication of adenovirus type 7 particles but not the hybrid; the defectiveness of the latter was revealed by the production of both mature adenovirus type 7 and hybrid particles after dual infection (Fig. 95). Monkey kidney cells failed to support the replication of either adenovirus type 7 or the hybrid, but did so after dual infection. Evidently both the hybrid and adenovirus type 7 particles are defective in monkey kidney cells; after dual infection each aids the other as a 'helper' virus and they become mutually dependent (Fig. 95). Adenovirus type 7 which fails to grow in monkey kidney cells does grow when 'helped' by the adenovirus type 7-SV 40 hybrid or by SV 40 virus itself. Boeyé, Melnick, and Rapp have obtained similar results.

Other types of adenovirus can act as 'helpers' for the production of adenovirus-SV 40 hybrids in monkey kidney cells. When they

Fig. 95. Adenovirus type 7-SV 40 relationships: diagrammatic representation.



do, Rapp, Butel, and Melnick have shown that the capsid of the hybrid is exchanged for one provided by the type of adenovirus used as a 'helper'. Thus adenovirus type 2, which is non-oncogenic, is capable of providing a capsid for the hybrid in place of that provided by adenovirus type 7 (Fig. 96). This phenomenon, by means of

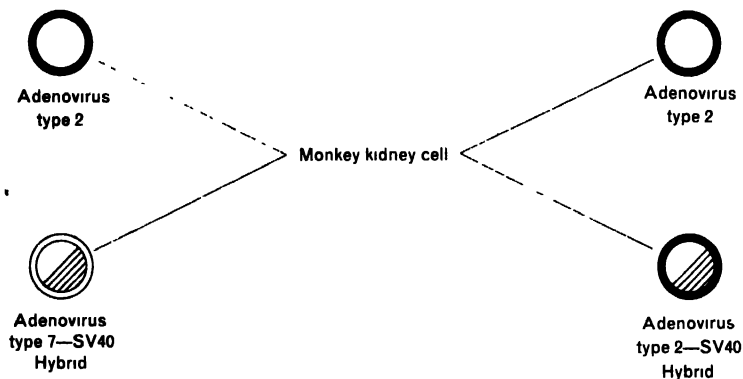


Fig. 96. Adenovirus transcapsidation.

which an oncogenic virus can be provided at will with the antigenic specificity of a non-oncogenic virus, is known as transcapsidation. Clearly, the phenomena of hybridization and transcapsidation may play a significant role in viral oncogenesis, and deserve the closest study.

CHAPTER 49

Oncogenic Viruses

III. Virus-Host Relationship

The viral aetiology of certain benign and malignant tumours of various animal species is now beyond doubt. Either RNA or DNA viruses may be responsible, but their properties and virus-host relationships, summarized in Table 15, differ significantly.

Table 15. Properties of RNA and DNA tumour viruses

Property	RNA viruses	DNA viruses
1. Tumours	May occur naturally, e.g. Avian leucosis, Rous sarcoma	No evidence of natural occurrence. Only produced artificially in experimental animals
2. Infective virus in tumours	Infective virus produced	Infective virus not usually produced
3. Effect in tissue culture	(a) Transformation, usually with production of infective virus (b) No cytopathogenic effect	(a) Transformation without production of infective virus (b) Cytopathogenic effect with release of infective virus
4. Maturation	In cell cytoplasm	In cell nucleus
5. Release	Budding at cell surface	Cell lysis
6. Morphology	Resemble myxoviruses. Nucleo-capsid enveloped by lipoprotein membrane	All exhibit icosahedral symmetry. No membrane

Experimental Systems

The relationship between oncogenic viruses and their hosts has been established from two main lines of experimental approach. First, from the study of virus induced tumours in the intact animal, and later, at the cellular level, by the study of the phenomenon of transformation in tissue culture cells. Transformation rarely occurs spontaneously but is frequent after infection with oncogenic viruses; it is considered analogous to the induction of neoplasia in intact animals. Certainly, transformed cells display all the properties of malignant cells. They produce tumours in genetically compatible hosts, which are indistinguishable from those produced by the homologous virus. They display an enhanced rate of growth, develop immortality, and no longer possess the property of contact inhibition. In culture, their irregular and unrestrained patterns of growth produce heaping up of cells in an otherwise regular monolayer. Chromosomal abnormalities, which may or may not be of significance, are not usually seen immediately after transformation but develop after serial propagation.

The Role of Viral Nucleic Acid

Stoker's experiments have shown that a single virus particle is sufficient to induce transformation, although its probability of doing so is very low. The transforming abilities of the virus are thought to be markedly influenced by the particular physiological state of the host cell and the mitotic phase of its nucleus. The transforming and oncogenic potential of tumour viruses, like the infective principle of other viruses, resides in the nucleic acid moiety. DNA from polyoma and SV 40 viruses and RNA from mouse leukaemia virus, freed from viral proteins, have been shown to transform cells in culture and produce tumours in experimental animals. Indeed, it has been calculated that only 1 molecule of polyoma DNA is required to produce cell transformation. Clearly, the factor responsible for oncogenicity must be encoded somewhere in the viral nucleic acid.

The absence of infective virus from tumours and transformed cells, induced by DNA viruses, suggests that not all the virus genome

or nucleic acid is required to produce the neoplastic change. Even cells transformed by RSV, which normally produce infective virus, are now known to be transformed by a defective cycle of virus replication in which infective virus is not produced without the aid of a 'helper' virus. Cogent evidence has therefore been adduced for supposing that a defective cycle of virus replication is a necessary prerequisite for transformation of cells. The defective cycle may result from a physical defect in the viral genome, which makes it deficient in the information required for the synthesis of the viral protein coat and for virus maturation. Alternatively, the viral genes which control viral protein synthesis and virus maturation may be repressed in some way and prevented from expressing themselves.

Transformed or tumour cells which do not normally produce infective virus retain the capacity to do so. Superinfection of polyoma transformed cells with homologous virus leads to cell lysis and the production of infective virus, and cells transformed by defective RSV can produce infective virus with the aid of an appropriate 'helper'.

Persistence of the Viral Genome

The presence of virus-specific antigens in virus-free but virus induced tumour and transformed cells makes it certain that at least part of the viral genome persists. The resemblance to lysogenic conversion, in which infection with prophage produces new somatic antigens in certain types of bacteria, is striking, but the relationship between tumour virus and transformed cell is demonstrably different from the prophage-bacterial cell relationship. None of the factors which induce the change from prophage to lytic phage is capable of producing infective virus from transformed cells, and, unlike lysogenic bacteria, transformed cells are not resistant to superinfection with homologous virus. Nevertheless, some indirect evidence of the incorporation of viral genes into the host cell genome has been obtained by nucleic acid homology studies.

RNA complementary to SV 40 DNA, prepared *in vitro* by Black, was found to exhibit greater complementarity to DNA prepared from SV 40 transformed cells than from normal control cells.

Similarly, Axelrod and his colleagues demonstrated a greater degree of complementarity between polyoma virus DNA and DNA from polyoma tumour cells than between polyoma virus DNA and DNA from normal cells. Winocour, however, was unable to demonstrate any specific complementarity between messenger RNA from a polyoma tumour and polyoma virus DNA. Some form of integration of host and viral genomes, replicating in synchrony, seems probable at least for DNA oncogenic viruses. If so, the integrated viral gene or genes might operate by stimulating various cellular synthetic pathways, or by releasing them from regulatory restraint imposed by host cell repressor genes. The mechanism of action of RNA oncogenic viruses is at present entirely obscure.

The analysis of base ratios of DNA extracted from some oncogenic viruses has revealed some interesting characteristics. Base ratios of DNA from polyoma, SV 40, and tumorigenic adenoviruses, resemble those of DNA extracted from mammalian host cells more closely than do those of DNA from non-oncogenic viruses. This and the ready transfer of genetic information from one virus to another, which is revealed by the phenomena of hybridization and transcapsidation, make the suggestion of Rapp and Melnick, that a virus may acquire a piece of DNA from the genome of the host cell, at least conceivable. If so, the spontaneous transformation which sometimes occurs in uninfected cell cultures nearing the end of their viability becomes significant, and suggests a cellular origin for the oncogenic potentiality carried by certain viruses.

Immunological Aspects of the Virus-Host Relationship

The immunological response of the host to virus-specific antigens present in virus-induced tumour and transformed cells raises the possibility of immunological mechanisms functioning in virus tumour production. Habel was the first to suggest that immunity to the transplantation antigen plays an important part in the outcome of infection with tumour viruses. The unique susceptibility of newborn animals to virus induced tumours was ascribed by him to their

immunological incompetence. In these animals, tumour cells flourish because any transplantation antigen produced is not recognized as 'foreign', and no rejection of the cells can therefore take place. Conversely, immunologically competent adult animals immediately recognize the transplantation antigen as 'foreign' and promptly reject any tumour or transformed cells.

With the development of immunological competence in neonates, there is some reduction in the degree of tolerance, and the outcome of infection with oncogenic viruses may then depend on the balance between the degree of specific transplantation immunity and the quantity of antigen presented by the tumour. The experiments of Eddy and her colleagues illustrate this balance. They administered large doses of SV 40 virus or irradiated SV 40 tumour cells to newborn hamsters in the early part of the long latent period which follows infection with tumour producing doses of SV 40. By artificially stimulating the development of specific transplantation immunity they inhibited tumour production.

Additional evidence favouring the view that immunological competence plays an essential role in resistance to production of virus induced tumours has been obtained. Thus, increased resistance to polyoma virus tumour production has been conferred on newborn mice by implantation of genetically compatible lymph node cells from normal adult mice. Conversely, polyoma virus tumour production is enhanced in both newborn and adult mice by neonatal thymectomy and by irradiation.

The localization of the transplantation antigen at the cell surface suggests that tumour and transformed cells exhibit a virus controlled structural change at the cell surface. The relationship between this structural change and the physical changes of increased negative charge and loss of contact inhibition, which are known to occur at the cell surface, has not yet been elucidated. It is not improbable, however, that the faculty of uncontrolled cellular growth, characteristic of neoplasia, is the result of changes in the properties of the cell membrane.

CHAPTER 50

Oncogenic Viruses

IV. Viruses and Human Cancer

The viral aetiology of human cancer has been long suspected but so far no virus has been isolated from a human malignant tumour. This does not preclude a viral aetiology; indeed, circumstantial evidence for the viral hypothesis has never been stronger. The oncogenic potentiality of certain viruses in experimental animals makes it reasonable to suppose that man, who is no less susceptible to physical and chemical carcinogens than other species, is subject to the oncogenic effects of specific viruses.

The study of animal virus tumours has revealed very complex virus-host cell relationships which make simple virus isolation procedures unlikely to reveal any causative virus of a human tumour. Except leukaemia, virus induced malignant tumours in mammalian species are characteristically free from infective virus, the probability of recovering infective virus from non-leukaemic human tumours is therefore low. Various indirect methods, suggested by experimental findings in laboratory animals, are more likely to succeed in establishing the viral aetiology of human tumours. Nevertheless, all possible methods of investigation merit consideration and are being actively pursued at the present time.

Virus Isolation

The conditions required for isolation of human tumour viruses in experimental animals have been defined by the study of animal tumour viruses. For successful isolation, the use of newborn animals is imperative and a long period of observation is required before any tumour production becomes evident. Although oncogenic

viruses are not rigidly species specific and have a wider host range than was originally thought, it is significant that newborn hamsters are particularly susceptible. This animal is therefore most favoured for isolation of oncogenic viruses; its susceptibility, and that of other animals, may be further enhanced by immunosuppressive measures, such as neonatal thymectomy and irradiation, which inhibit the development of transplantation type immunity.

Any tumours produced by human material in experimental animals cannot be assumed to result from the original inoculum. The possibilities of spontaneous development of natural tumours in experimental animals, or of activation of latent viruses by experimental manipulation, add to the difficulties of interpretation. The emergence of polyoma virus in the course of experiments with mouse leukaemia is a clear example of this. Possibly, the use of germ-free animals may prove to be an essential requirement for the attempted isolation of viruses from human tumour material, although it is of interest to note that mouse leukaemia virus has been detected in germ-free mice treated with X-irradiation.

So far, adenoviruses are the only viruses of human origin shown to be oncogenic in experimental animals. Viruses which are oncogenic in experimental animals are not necessarily so in their natural hosts, the significance of adenovirus oncogenicity in human disease is therefore questionable. Indeed, the widespread infection of SV 40 and polyoma viruses in their natural host species, without tumour production, suggests that the oncogenicity of adenoviruses may be a strictly laboratory phenomenon which does not occur in nature. The widespread infection of the human population with adenoviruses, including some of the oncogenic types, leads to the conclusion that tumour production, if it occurs at all, must be rare.

Some of the difficulties posed by the use of experimental animals can be overcome by the use of tissue culture methods. Virus transformation of human cells in culture is probably more analogous to tumour production in humans than any other experimental system. For this reason, transformation of human cells by SV 40 is highly significant in view of the one time administration of SV 40 contaminated vaccines. The use of tissue cultures has the added advantage that it makes possible the identification of defective and non-

infective viruses by subtle methods. The use of complex methods, like those required for the demonstration of murine sarcoma virus, may improve the chance of isolating a virus from human tumour tissue. It will be recalled that murine sarcoma virus is only revealed when infected sarcoma cells are co-cultivated with mouse embryo fibroblasts infected with mouse leukaemia virus, which acts as a 'helper'. Possibly, similar combinations of human tissues might be successful in yielding human tumour viruses.

Immunological Methods

The persistence of the viral genome in virus induced tumour and transformed cells is manifested by the production of virus-specific T- and transplantation antigens; immunological methods of identifying viral elements in human tumour cells, or in cells transformed by human tumour extracts, are therefore practicable. Complement-fixation and fluorescent antibody tests are particularly suited for the detection of virus antigens in infected cells.

Electron Microscopy

Electron microscopy for the detection of virus-like particles in human tumour tissue is a method which is now being intensively applied to the study of human cancer. So far, the results have been more suggestive than conclusive, because of the difficulties of interpretation. Virus-like particles are easily confused with cell components and organisms belonging to the genus *Mycoplasma*. Visual identification of virus particles alone is of no aetiological significance, because many viruses are fortuitous 'passengers' unrelated to the tumour. However, the constant association of a particular type of virus particle with a particular tumour would be strongly suggestive, but confirmation by biological experiment would still be necessary before an aetiological relationship could be accepted.

Epidemiological Approach

Attempts to demonstrate that some human tumours are distributed in accordance with the epidemiological characteristics of infective

disease have been made. But the long latent interval between infection and tumour production in animals may be equivalent to a period of years in the human life-span, and the exposure of an individual during this period to a number of carcinogenic factors, known and unknown, so confuses the picture as to make the epidemiological approach of doubtful value. The epidemiological observation most suggestive of a viral aetiology is that of Burkitt concerning the childhood lymphoma syndrome endemic in Central Africa.

Of considerable epidemiological interest is the possibility, raised by Huebner, of the transfer of oncogenic viruses from animals to man. Polyoma virus is known to contaminate grain stored in mouse infested granaries, and to pollute the environment of infected mouse colonies. Infection of man by inhalation or ingestion is thus a possibility; similarly avian leucosis virus, which is widely distributed among domestic poultry, may be transferred to man.

Human Leukaemia

Leukaemia is one of the few oncogenic conditions of animals which yields infective virus. The probability of recovering a virus from the related human condition is therefore correspondingly high, and an intensive search for virus in human leukaemia is currently being pursued in many centres of research. So far, no virus has been isolated, but suggestive evidence of its presence has been obtained in tissue culture and by electron microscopy.

The recovery of a cytopathogenic agent from human leukaemic bone marrow, by Negroni, proved to be a *Mycoplasma* organism and, after some initial excitement, of no aetiological significance. An important lead has, however, been obtained by Plotkin and his colleagues. They inoculated African green monkey kidney cells with material from human leukaemia and subsequently challenged them with Rouse sarcoma virus (RSV). Twenty-five per cent of the human specimens produced interference against RSV, suggesting that a human resistance inducing factor (RIF) is present, which behaves in a manner similar to that of avian leucosis virus.

Evidence supporting the possible viral aetiology of human

leukaemia has been obtained by electron microscopy. Dmochowski and his colleagues reported the presence of virus-like particles in thin sections of lymph nodes from leukaemic patients; since then, particles resembling murine and avian leukaemia viruses have been seen in a greater number of plasma specimens from leukaemic than from normal patients. Although suggestive, the presence of virus-like particles is not proof of a causative role; indeed, there is no unequivocal proof that any of the virus-like particles seen in leukaemic tissues are, in fact, viruses. Evidence of leukaemogenesis in experimental animals or transformation of human cells in culture would be required before a causative role could be accepted.

Burkitt's Tumour—African Childhood Lymphoma

The limited geographical distribution of childhood lymphoma, which is common in several parts of tropical Africa, was first remarked on by Burkitt a few years ago. Epidemiological investigation later showed it to be confined to low lying land, and limited by climatic factors. These considerations and a geographical distribution similar to that of yellow fever led Burkitt to propose a mosquito vector for the disease. If so, the oncogenic agent most likely to be transmitted would be a virus, as suggested by Davies. The high incidence of the disease in early childhood suggests, on the basis of experimental leukaemia studies, infection in very early life followed by tumour formation after a latent period of a few years.

Because of its presumed viral aetiology, numerous attempts have been made to isolate a virus from Burkitt's tumour. Reovirus type 3, *Herpesvirus hominis*, herpes-like viruses, and organisms of the genus *Mycoplasma* have been isolated, or visualized in the electron microscope, but all the evidence suggests that these are 'passenger' viruses rather than the true aetiological agents. Furthermore the recent recognition of childhood lymphoma in the U.S.A., Great Britain and Vietnam has cast some doubt on the theory of arthropod transmission of the disease.

Part 3
Rickettsial Infections

CHAPTER 51

Rickettsial Infections

I. Rickettsiae General Properties

Rickettsial organisms were first observed by Howard Taylor Ricketts in the course of his investigations on Rocky Mountain spotted fever. He described small bacteria-like organisms in the blood of human patients, and in the tissues of the responsible arthropod vector, but was unable to prove their aetiological role before his death from typhus in May 1910. Ricketts had found similar bacteria-like organisms in the blood of patients suffering from Mexican typhus fever (Tarbadillo), and Prowazek later described them in lice fed on typhus patients. The aetiological role of these organisms in typhus fever was finally established by de Rocha-Lima, who named them *Rickettsia prowazeki* in honour of the two investigators who succumbed to the disease in the course of their investigations. A number of different species of Rickettsiae were later identified as the cause of various diseases transmitted by arthropod vectors; many non-pathogenic species, which are found as commensals in the alimentary tracts of insects and arthropods, are also known.

The failure to cultivate Rickettsiae on artificial cell-free media was originally interpreted to indicate their close affinity to viruses. Other properties now relate them more closely to bacteria; these include replication by binary fission, retention by bacteriological filters, sensitivity to antibiotics, possession of some metabolic enzymes, and a nucleic acid composition comprising both DNA and RNA (Table 1).

General Properties

(a) Morphology

Rickettsiae are small coccoid or rod-shaped organisms, about 0.3–0.5 μ long and 0.3 μ wide. Often pleomorphic, long bacillary forms, 1–2 μ in length, as well as diploid and chain forms may be seen. Although poorly stained by ordinary dyes, rickettsiae are gram-negative and exhibit bipolar staining when stained by Ziehl-Neelsen or Machiavello stains.

Electron microscopy shows rickettsiae to possess a limiting membrane, which resembles a bacterial cell wall, and a dense nucleoid which is probably composed of nucleoprotein (Fig. 97).

(b) Chemical and physical properties

The co-existence of RNA and DNA in rickettsial nucleic acid invalidates any claim for the inclusion of rickettsiae in the phylum Virales. Lipid, protein, and carbohydrate, complete their chemical composition. Muramic acid, a constituent characteristic of bacterial cell walls, has been demonstrated by Perkins and Allison in rickettsiae, and serves to emphasize the relationship between rickettsiae and bacteria.

Excepting *Coxiella burnetii*, all rickettsiae are heat labile and easily inactivated by lysol, formalin, and phenol; all are, however, resistant to ether. Infectivity is maintained for only a few days at refrigeration temperatures, but is maintained for long periods when rickettsiae are stored in a medium of skimmed milk at -60°C , or in the lyophilized state.

Because of their size rickettsiae do not pass through bacteriological filters.

(c) Biological Properties

(1) Pathology of rickettsial lesions

The lesions which rickettsiae produce in vertebrate hosts are essentially due to their affinity for vascular endothelium, particularly that of the small blood vessels. Infected endothelial cells proliferate and give rise to thromboses, around which polymorphonuclear leucocytes, lymphocytes, and macrophages aggregate. These localized



Fig. 97. Electron micrograph of thin section through *Rickettsia quintana* [from Susumu Ito and J. W. Vinson (1965) *J. Bact.* 89, 481-95 (American Society for Microbiology)].

perivascular accumulations of inflammatory cells, often associated with petechial haemorrhages, are widely distributed in the various organs, including the skin, myocardium, central nervous system and lungs.

(ii) Rickettsial toxin

Several species of rickettsiae produce specific toxins which are associated only with infective rickettsiae. These toxins, which are not separable from the organisms, are lethal to rats and mice when high concentrations of rickettsiae are injected intravenously or intraperitoneally. The toxic effect is particularly marked on capillaries and leads to vasoconstriction, increased capillary permeability and haemoconcentration. The contribution of the toxic effect to rickettsial lesions in human infections is not yet clear, but the specific toxin-neutralization test provides a useful laboratory tool for identifying the various species of rickettsiae.

(iii) Rickettsial haemolysin

Several species of rickettsiae, including those responsible for typhus and spotted fevers, haemolyse rabbit and sheep red cells. The haemolytic reaction, which is thought to depend on the activity of certain rickettsial enzymes, is inhibited by appropriate antisera and thus provides a suitable serological test of identification.

(d) Antigenic properties

(i) The Weil-Felix reaction

In 1916, before direct serological tests with rickettsial suspensions were possible, Weil and Felix fortuitously discovered a unique serological reaction which was to prove of great practical and theoretical importance in the study of rickettsial infections. They found that a strain of *Proteus*, OX19, recovered from the urine of a typhus patient, was agglutinated by sera from patients with typhus. Although of no aetiological importance in typhus, some *Proteus* strains apparently share common antigens with rickettsiae.

Sera from patients with epidemic or endemic typhus agglutinate *Proteus*, strain OX19; sera from patients with scrub typhus agglutinate *Proteus* strain OXK, but not OX19; and sera from patients

suffering from any of the rickettsial spotted fevers agglutinate *Proteus* strains OX19 and OX2, but not OXK. Sera from patients with Q fever, rickettsialpox or trench fever do not agglutinate any of these *Proteus* strains.

(ii) *Complement-fixation*

Antigenic analysis of rickettsial suspensions by complement-fixation techniques reveals two antigenic components. One, a soluble antigen, is separable from the rickettsial organisms by repeated washing and differential centrifugation; the other is an antigen inseparably bound to the rickettsial organism itself. The heat stable soluble antigen is group specific and defines certain groups of antigenically related rickettsiae; in contrast, the rickettsial antigen is species specific and differentiates the various organisms within each group.

By means of common soluble antigens, two main antigenic groups of rickettsiae are recognized. These are the typhus group of organisms, which includes *R. prowazeki* and *R. typhi*; and the spotted fever group of organisms, which include *R. rickettsii*, *R. conorii*, *R. australis*, *R. siberica*, and *R. akari*. Individually distinct soluble antigens are produced by *R. tsutsugamushi*, the causative organism of scrub typhus, *R. quintana*, the causative organism of trench fever, and *Coxiella burnetii*, the causative organism of Q fever.

(iii) *Agglutination tests*

Direct agglutination of rickettsial suspensions by appropriate antisera is a highly specific but not widely used test. The preparation of adequate quantities of purified antigen from infected yolk sacs is a problem preventing its wider application, but as a micro-agglutination test it is useful in the diagnosis of Q fever.

(iv) *Fluorescent antibody techniques*

Fluorescent antibody techniques, which are species-specific, have recently been introduced, and have become important in the diagnosis of rickettsial infections. A drop of test serum is added to a dried smear of heavily infected yolk sac material on a microscope slide, and a drop of fluorescein-labelled anti-human horse globulin

applied. Antigen-antibody combination is recognized by fluorescence in an ultraviolet light microscope.

(v) *Erythrocyte sensitizing agent (ESS)*

Chang extracted an erythrocyte sensitizing agent (ESS) from rickettsial suspensions heated in alkaline solution. ESS sensitizes human group O cells to agglutination by group specific antibodies present in sera of patients convalescent from infection with organisms of the typhus or spotted fever groups of rickettsiae. ESS is closely related to the soluble complement-fixing antigen, and may be one of its degradation products.

(vi) *Neutralization tests*

Species specific infectivity or toxin-neutralization tests in mice are sometimes but not often used for the identification of rickettsiae.

(e) **Cultivation**

(i) *Experimental animals*

Guinea-pigs. About 1 week after rickettsial infection, guinea-pigs develop a systemic febrile illness which, unless produced by *R. rickettsii*, is not usually fatal. Intraperitoneal inoculation of male animals produces specific scrotal reactions which provide an extremely useful method of preliminary identification. *R. mooseri*, the causative organism of endemic typhus, produces a characteristic periorchitis with marked oedema and erythema of the scrotal tissues; this is sometimes known as the Neill-Mooser reaction. In contrast, *R. prowazeki*, the causative organism of epidemic typhus, does not usually produce any scrotal reaction. The scrotal reaction to *R. rickettsii*, the causative organism of Rocky Mountain spotted fever, is quite characteristic and very severe, it is marked by haemorrhagic oedema, necrosis and ulceration of the scrotal tissues.

Mice. Most species of rickettsia produce inapparent infection or no infection at all after parenteral injection into mice, unless injected in very high toxic concentrations. Intranasal infection, however, often produces rickettsial pneumonia and death.

Virulent strains of *R. tsutsugamushi* and *R. akari*, the causative organisms of scrub typhus and rickettsialpox, respectively, may be pathogenic for mice by the intraperitoneal route, on first isolation.

About 2 weeks after infection, mice become ill with oedema, ascites, pleural effusions, splenomegaly and haemorrhagic pneumonia, and eventually die. Strains of lesser virulence produce similar but less severe signs of infection.

(ii) Chick embryos

Although not usually suitable for primary isolation, rickettsiae isolated in experimental animals may be adapted to chick embryos by serial passage. Five- to seven-day-old embryos are inoculated by the yolk sac route and the yolk sacs are harvested after 6-10 days. After serial passage, the organisms grow well and become lethal to the embryo.

(iii) Tissue culture

A few species of rickettsiae have been grown in tissue cultures of mouse lymphoblast and fibroblast cells, and in chick embryo monolayer cell cultures. The yields are usually small, and the cytopathogenic effects are delayed if they occur at all; these techniques have not therefore been widely applied.

In contrast to viruses, which grow optimally when the host cells are actively metabolizing, the optimal growth of most species of rickettsiae occurs when host cells exhibit low rates of metabolic activity. This suggests that certain intrinsic enzymes, of which one glutamic-aspartic transaminase has been identified, confer some degree of independent metabolic activity on the rickettsial organisms.

Treatment of Rickettsial Infections

All rickettsial infections respond to treatment with the tetracycline group of drugs. Chloramphenicol may also be used but its toxicity makes its use undesirable. Experimental evidence indicates that these antibiotics act as rickettsiastatic rather than as rickettsiacidal agents, suppressing the growth of rickettsiae in the tissues until the body defences are sufficient to overcome them.

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Rickettsial Infections

II. *Rickettsia prowazeki*

Epidemic Typhus Fever

A scourge of mankind for more than two thousand years, classical epidemic typhus fever still reappears in times of war and famine. Within living memory, major outbreaks have occurred in Serbia in 1915, and in Russia during the years 1918-23 when 30 million cases and 3 million deaths were estimated to have occurred. During World War II, serious epidemics broke out in North Africa and in Naples but they were very effectively controlled by the Allied Armies. Inevitably accompanying conditions of human suffering and privation, epidemic typhus was widespread in the infamous German concentration camps.

Aetiological Agent

The aetiological agent of epidemic typhus is *R. prowazeki*, a small gram-negative organism approximately 0.5 μ in diameter, which is found in the cytoplasm of infected cells.

Clinical Features

After an incubation period of 5-23 days, but usually 10-14 days, the disease begins with an acute onset of fever, headache, malaise, conjunctivitis, and generalized myalgia. The temperature reaches a maximum of 102-105°F within 2-3 days, and remains constantly raised until death or recovery takes place. A macular or maculopapular rash appears on the third-seventh day of the disease and

spreads to cover the whole body except the face, palms and soles. The rash is absent in about 10% of cases, but may become haemorrhagic in severe cases.

Photophobia, deafness, stupor, delirium, insomnia, hyperaesthesia, and other symptoms indicate the involvement of the central nervous system, sometimes leading to frank meningoencephalitis. Prostration and toxæmia increase until about the twelfth–fourteenth day, when the disease usually terminates by rapid lysis and recovery, or progresses to a fatal conclusion. Gangrene, pneumonia, or renal insufficiency may supervene in non-fatal cases. In the absence of antibiotic treatment, the case-fatality varies from 10–40%.

Pathogenesis and Pathology

Man acquires infection from an infected louse, which bites its victim and contaminates the wound with infected droppings. The irritation set up is usually countered by scratching, which effectively massages the infected droppings well into the wound. Passage of the organism from dried infected louse faeces through the conjunctiva or respiratory mucous membrane is a possible but improbable mode of infection.

Once in the blood stream, where the organisms are demonstrable in the early febrile period of the disease, *R. prowazeki* is distributed to the small blood vessels, where it multiplies in the vascular endothelium. Infected endothelial cells swell and occlude the blood vessels leading to the formation of thrombi, around which aggregations of inflammatory cells form the typical typhus nodules. Petechial haemorrhages and focal necrosis may accompany the nodules which are found in the skin, brain, myocardium, and other organs.

Epidemiology

Man provides the only reservoir for *R. prowazeki* infection, and epidemic typhus fever is transmitted from man to man by the body louse, *Pediculus corporis*, or less frequently by the head louse, *Pediculus capitis*. Any environment which encourages louse infestation will therefore predispose to outbreaks of epidemic typhus.

Overcrowding, malnutrition, poverty, and circumstances in which people are unable to wash or change their clothing for long periods of time, provide the required conditions. Epidemic typhus is current in Mexico and other parts of South America, Eastern Europe, Africa, and Asia.

Lice become infected by feeding on the blood of typhus patients during the rickettsiaemic febrile period of the disease. After feeding, the louse leaves the febrile patient, or frigid corpse, for the more optimal environmental temperature provided by the normal human host. At the same time, the rickettsiae multiply in the gut epithelium of the louse and are excreted in the faeces before the louse dies, usually 10 days after infection.

Laboratory Diagnosis

(a) Serological tests

Because isolation of typhus rickettsiae is a time consuming exercise, fraught with danger and practical only in specialized laboratories, serological methods of diagnosis using non-infective antigens are preferred. Paired serum specimens taken in the acute and convalescent phases of the disease are required for the demonstration of a significant rise of antibody titre.

The Weil-Felix reaction becomes positive 2–3 weeks after the onset of the disease. Agglutinins for *Proteus* OX19 usually reach titres of 1:160–1:1000, but occasionally these agglutinins fail to develop.

The complement-fixation test is particularly useful when differentiation between epidemic and endemic typhus is required. For this purpose, type-specific rickettsial antigens are essential, but for routine diagnosis the soluble group-specific antigen, which is commercially available, is more commonly used.

Other serological tests described in Chapter 51 are sometimes, but not widely, used.

Antibiotic therapy early in the disease may delay the production of antibody, and this may affect the results and interpretation of serological tests used in diagnosis.

(b) Rickettsial isolation

In specialized laboratories, rickettsial isolation from the patient's blood may be attempted. Specimens, preferably red cell concentrates, are inoculated into guinea-pigs; development of fever without scrotal reaction is indicative of *R. prowazekii*, and intracellular rickettsiae may be demonstrated in smears made from the spleen surface, after sacrifice of the animal.

Identity of the organism is confirmed by demonstrating the development of complement-fixing antibodies in infected guinea-pigs and by cross protection tests with known strains. Serial passage in the guinea-pig host may be necessary before identification is possible. Rickettsial antigens for serological tests and identification may be prepared in the chick embryo yolk sac after adaptation of the isolate.

Prophylaxis**(a) General measures**

Prevention of overcrowding and malnutrition, and provision of facilities for frequent bathing and laundering of clothes, are usually sufficient to prevent louse infestation and typhus fever. Delousing, when required, is an additional requirement which can now be easily achieved by application of DDT to infested persons and their clothes. Lice which have become resistant to DDT may be treated with other available insecticides.

(b) Vaccines**(i) Inactivated vaccine**

Inactivated vaccines for use in medical and military personnel who are particularly exposed in endemic areas, and for general use in face of an epidemic, are available. Cox's vaccine, which is of proven efficacy in preventing morbidity and mortality, is the one recommended; it is prepared in the chick embryo yolk sac and inactivated with formalin. 1 ml. is inoculated subcutaneously and is followed by a second dose 10–14 days later; booster doses are then given at yearly intervals so long as the danger lasts. Allergic reactions in patients sensitive to egg proteins should be guarded against.

(ii) Live attenuated vaccine

A live attenuated vaccine, giving more prolonged immunity, has been developed by Clavero and Gallardo, using the technique of serial egg passage. In use, the vaccine has led to severe reactions and is still under trial.

(c) Patients and contacts

After notification, the patient is isolated until his clothes and environment have been thoroughly deloused with DDT and by other ancillary measures. Contacts should be vaccinated and quarantined for 15 days or until they have been thoroughly deloused.

(d) Epidemics

In small outbreaks, delousing the patient and his contacts, and vaccination of the latter is sufficient to control the epidemic. In more extensive epidemics, delousing of the whole population by insecticide treatment is required. This is easily and rapidly achieved by insufflating DDT powder down the neck, sleeves and trousers of each individual, and into their hair. This technique was used with great success in the typhus epidemic which broke out in Naples during World War II.

Vaccination of the general population is recommended in face of an epidemic.

Treatment

Tetracycline drugs administered until the temperature has returned to normal for several days are highly efficacious.

**RECRUDESCENT TYPHUS OR
BRILL-ZINSSER DISEASE**

Seventy years ago, Brill described a mild sporadic form of typhus, often occurring without a rash, which affected poor East European immigrants to New York. None of them were louse infested, but

many gave a history of classical epidemic typhus in their East European countries of origin. Zinsser suggested that these patients carried a latent form of *R. prowazekii* in their tissues after their initial attack of typhus fever, and that many years later the latent organism was stimulated into activity by unknown factors.

Since its original description by American authors, the syndrome has been recognized in other parts of the world, occurring in endemic areas of Eastern Europe and in immigrants coming from endemic areas. In fact, recrudescence of latent infection is now recognized as a complication of typhus, which may occur 20 or more years after the initial clinical or subclinical attack. In this respect, Price's recovery of *R. prowazekii* from lymph glands of two healthy persons many years after they had left typhus endemic areas is of interest.

Table 16. Epidemic and recrudescent typhus

Characteristic	Epidemic	Recrudescent
History	No previous attack	Previous attack
Course	Severe, often fatal	Mild
Rash	Present in more than 90% of cases	Often absent
Incidence	Epidemic	Sporadic
Vector	Lice	None
Weil Felix reactions	Usually strongly positive	Weak or absent
Complement-fixation test	Positive	Positive

Because recrudescent attacks of typhus are characterized by rickettsiaemia, they serve as sources from which infection may be transferred by lice, if present. Moreover, latent infections account for the persistence of the disease during interepidemic periods, and their stimulation into activity probably represents the starting point of the epidemic eruptions which occur from time to time.

III. *Rickettsia typhi* Endemic or Murine Typhus Fever

A relatively mild form of typhus which occurs in the U.S.A., Mexico, Australia, and other parts of the world has been distinguished from classical epidemic typhus by its occurrence in a non-epidemic and non-contagious form. Its clinical course resembles that of classical epidemic typhus but it is less severe and not often fatal.

Aetiological Agent

The causative organism of endemic typhus is *R. typhi*; it was previously named *R. mooseri* in honour of Mooser who, with others, recognized the disease as a clinical, pathological and epidemiological entity. The organism resembles *R. prowazeki* in its morphological, chemical and physical properties but differs from it in its antigenic, pathogenic, and epidemiological properties.

In guinea-pigs, *R. typhi* produces a febrile response and the 'Neill-Mooser' reaction, in which oedema and erythema of the scrotal tissues develop. At autopsy, adhesions between the testis and tunica vaginalis are found, and smears of the tunica, stained with suitable stains, reveal numerous intracytoplasmic and extracellular rickettsiae. The pathogenicity of *R. typhi* for white rats and mice is another feature which differentiates it from *R. prowazeki*.

R. typhi and *R. prowazeki* produce the same group-specific soluble complement-fixing antigens, and the antibodies they produce are indistinguishable in the Weil-Felix reaction. Their type specific rickettsial antigens are however different, and the organism may be differentiated in type-specific complement-fixation, infectivity neutralization, or toxin-neutralization, tests.

Clinical Features

After an incubation period of 6-14 days, a mild form of typhus fever ensues which usually terminates in recovery after 9-14 days.

Epidemiology

Endemic typhus is a disease associated with rodent infested environments, particularly seaports in the hotter parts of the world. It has been reported from parts of the U.S.A., Africa, Asia, and the Middle and Far East.

Rats form the main reservoir of infection, and the disease is transmitted from rat to rat by the rat flea, *Xenopsylla cheopis*, or by the rat louse *Polyplax spinulosus*. In rats and rat fleas, the disease is mild and not fatal, so that virus is harboured in rats for some weeks and in rat fleas for life. From rats, infection may be transferred to man by the rat flea, which occasionally uses man as an alternative tangential host. Human cases, which are only incidental to the natural cycle of infection, are usually secondary to an enzootic in the rat population. Although several cases may occur simultaneously in the same locality, the disease is not transmitted from man to man.

Evolution of *R. typhi* into *R. prowazeki* during the course of its adaptation to man and his louse parasite has been suggested. No evidence of natural transformation from one species to another has however been obtained.

Prophylaxis

Control of rodent and rat flea populations is sufficient to prevent endemic typhus. The measures used include rat proofing of buildings and food stores, the reduction of rat flea populations by the application of DDT and other insecticides to rat burrows, followed by the use of rat poisons.

Vaccination with killed *R. typhi* vaccines is not necessary except for those particularly exposed, of whom workers in typhus laboratories form an important group.

No isolation or quarantine measures are necessary for patients or contacts.

Treatment

Tetracycline drugs are highly successful in treatment.

Table 17. Epidemic and endemic typhus

Characteristic	Epidemic	Endemic
Course	Severe, often fatal	Mild, low fatality
Incidence	Epidemic	Sporadic
Extrahuman reservoir	None	Rodents
Vector	<i>Pediculus corporis</i> (human body louse)	<i>Xenopsylla cheopis</i> (rat flea)
Organism	<i>R. prowazeki</i>	<i>R. typhi</i>
Weil Felix reaction	Positive with <i>Proteus</i> OX19	Positive with <i>Proteus</i> OX19
Complement-fixation with rickettsial antigen	Specific with <i>R. prowazeki</i>	Specific with <i>R. typhi</i>
Experimental animals:		
(i) Guinea-pig	Fever, no scrotal reaction	Fever + scrotal reaction
(ii) Mice	Inapparent infection	Pathogenic

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IV. *Rickettsia tsutsugamushi* Scrub Typhus

Scrub typhus, sometimes known as tsutsugamushi disease or Japanese river fever, is prevalent in South East Asia and adjacent areas. The causative organism, *R. tsutsugamushi*, is transferred from its rodent reservoir to man by the red mite, *Trombicula akamushi*, which flourishes in the lush undergrowth of wet and humid tropical areas.

Aetiological Agent

R. tsutsugamushi is a small pleomorphic organism which replicates in the cytoplasm of infected cells. It differs from *R. prowazeki* and *R. typhi* in its antigenic specificity, pathogenicity, and epidemiological properties.

R. tsutsugamushi is easily distinguished from organisms belonging to other rickettsial groups by means of its soluble as well as rickettsial antigens, and antibodies produced in human sera in response to infection agglutinate *Proteus* OXK and not OX19 or OX2. Some antigenic heterogeneity has been demonstrated among the various strains of *R. tsutsugamushi* isolated, and this has been held to account for second attacks of scrub typhus which sometimes occur.

The organism is extremely pathogenic for mice, even in small doses, but guinea-pigs are not highly susceptible though they may develop fever without any scrotal reaction.

Clinical Features

After an incubation period of 6–21 days, fever, headache, malaise, conjunctivitis, generalized lymphadenopathy and splenomegaly make their appearance. At this stage a vesicular, erythematous lesion which soon ulcerates and forms a black scab, or eschar, is often observed in the axilla, groin, neck, or trunk, and marks the site of the vector's bite. This lesion takes about 3 weeks to heal and the draining lymph glands are enlarged.

Towards the end of the first week, a macular or maculopapular rash develops on the trunk and sometimes on the extremities which resolves in the next few days. At this stage, cough and X-ray evidence of pneumonitis are common. In the course of the second week of the disease, the signs and symptoms increase in severity, and delirium, stupor and deafness, indicating involvement of the central nervous system, may become manifest.

Recovery, if it occurs, begins after about 14 days with a fall of temperature by lysis, but convalescence is prolonged and central nervous system sequelae sometimes occur. In the absence of antibiotic therapy, the case fatality rate varies from 1–40%.

Pathogenesis and Pathology

Soon after implantation in the wound left by the vector, the organism is believed to be distributed by the blood stream to the endothelium of the small blood vessels. After replication in the skin wound and vascular endothelium, during the incubation period, rickettsiaemia heralds the onset of the disease; nodular lesions resembling those of typhus, but usually less severe, develop in the lungs, heart, brain, lymphatic tissues, and the skin where the lesions eventually lead to the necrosis of the skin epithelium.

Epidemiology

The reservoir of infection is maintained in wild rodents, and possibly also in birds and larger mammals. Transmission of infection from one rodent host to another occurs through the medium of a complicated host-parasite relationship. The vector, *Trombicula akamushi*,

feeds off an infected vertebrate host only in the larval or 'chigger' stage of its life cycle; the nymphal and adult forms of the vector are not parasitic. By transovarial passage of *R. tsutsugamushi*, a reservoir of infection is maintained in the vector population as well as in rodents. In certain circumstances, the larvae parasitize man instead of the usual rodent host and the natural cycle of infection is then brought to an end, because infection is not spread from man to man.

The disease is geographically confined to tropical and semi-tropical areas of South East Asia, where lush overgrown vegetation is favoured by damp and humid climatic conditions. Individuals frequenting these areas, where mite and rodent reservoirs exist, are the only ones to acquire the disease, and the high incidence among troops in South-East Asia during World War II presented a serious military problem.

Laboratory Diagnosis

(a) Serological tests

In the Weil-Felix reaction, sera from about 50% of patients with scrub typhus agglutinate *Proteus* OXK but not OX19 or OX2. Complement-fixation and immunofluorescent techniques, using rickettsial antigens, may also be used to confirm the diagnosis.

(b) Rickettsial isolation

During the febrile period, the causative organism may be recovered from the patient's blood by intraperitoneal inoculation of white mice. Smears taken from the peritoneal or splenic surfaces of mice which have died with peritonitis and ascites, or have been sacrificed after 14–21 days, reveal intracellular rickettsiae when suitably stained. Identification of the organism is confirmed by neutralization and cross protection tests in mice, and by in vitro serological tests using antigens prepared in the chick embryo yolk sac.

Prophylaxis

No suitable vaccine is available, so that control of the disease depends on keeping rodents and mite vectors at bay. Parasitization of

the person by mite vectors is discouraged by impregnating clothes and blankets with mitocidal chemicals, like dimethyl-phthalate and benzyl-benzoate, and by the use of mite repellents, like dimethyl-phthalate and diethyl-toluamide, on exposed skin.

Before the establishment of military camps in endemic areas, all vegetation in the area is cleared by mechanical and other means. The cleared vegetation is then burnt and the area sprayed with a long-acting insecticide and rodent poisons. The camps erected are proofed against rodents.

Treatment

Treatment with the tetracycline group of drugs is very effective, but should be continued for some days after the temperature returns to normal to prevent relapse.

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V. Spotted Fever Group of Rickettsiae Spotted Fevers

RICKETTSIA RICKETTSII— ROCKY MOUNTAIN SPOTTED FEVER

Rocky Mountain spotted fever is an acute febrile typhus-like fever, characterized by a distinctive maculopapular rash. Originally described in the Rocky Mountain area of the United States, the disease is now known to occur in other parts of the U.S.A. and also in South America. The causative organism, *R. rickettsii*, is transmitted to man by ticks, and is closely related to other species of tick-borne rickettsiae producing similar syndromes in other parts of the world.

Aetiological Agent

R. rickettsii was first described by Ricketts; it is a small pleomorphic gram-negative organism, about $0.2\ \mu$ in diameter; sometimes lanceolate forms, about $0.6\text{--}1.2\ \mu$ long, are seen, these often occur in pairs surrounded by a halo and resemble the larger diplococcal forms of *Strept. pneumoniae*; filamentous forms also sometimes occur.

In contrast to other rickettsiae, *R. rickettsii* and other members of the spotted fever group proliferate in the nucleus as well as the

cytoplasm of infected cells, and produce a common group-specific soluble antigen. Infection with this group of organisms produces antibodies which react with *Proteus* OX19 and OX2 in the Weil-Felix reaction but the titre against *Proteus* OX19 is usually higher.

R. rickettsii is differentiated from other members of the group by its type-specific rickettsial antigen, and by its high virulence for guinea-pigs which produces very severe scrotal reactions with necrosis, haemorrhage and ulceration of the scrotal tissues.

Clinical Features

Three to 12 days after a bite from an infected tick, the temperature rises rapidly to 104–105°F and is maintained at the same level for approximately 14 days; in those who recover, the temperature falls by lysis during the third week of the disease. Severe headache, malaise, myalgia, arthralgia, conjunctivitis and photophobia accompany the pyrexia. On the third or fourth day of the disease, a maculopapular rash appears on the extremities and spreads rapidly over most of the body in centripetal fashion. In severe cases, large haemorrhagic ecchymoses, which may ulcerate, develop, particularly over bony prominences.

Irritability and restlessness, which in severe cases give way to deafness, delirium and coma, mark the involvement of the central nervous system. In fatal cases, renal and vasomotor failure also ensue. In the absence of antibiotic therapy, the overall mortality is about 20%.

Pathogenesis and Pathology

Infection is usually acquired through a bite from an infected tick, but the organism may enter through skin which has been contaminated by crushing an offending tick. After entry, the organism is believed to be distributed by the blood stream to the vascular endothelium where it multiplies during the incubation period. Rickettsaemia heralds the onset of the clinical disease, and focal lesions are found in the brain, myocardium, skeletal musculature, skin, lungs,

and kidneys. The focal lesions resemble those found in typhus, but thromboses are more prominent and degenerative changes occur in the muscle coat of the arterioles, leading to rupture of the vessel walls and haemorrhage.

Epidemiology

In the Rocky Mountain area and in the Western United States, the principal vector of the disease is the wood tick, *Dermacentor andersoni*, and the reservoir of infection is in small and large mammalian species, most of which suffer only inapparent infection. The larval and nymphal forms of the immature tick feed on small rodents, rabbits and other species of small wild mammals. Man is usually infected by the adult tick, which normally feeds on large wild and domestic animals including cattle, sheep and deer.

The principal vector in the Eastern United States is the dog tick, *Dermacentor variabilis*, and the main mammalian reservoir of infection is in dogs; in the larval and nymphal stages, immature ticks feed on field mice and other small mammalian species.

In the South American form of the disease, known as Sao Paulo fever, dogs form the main reservoir and the dog tick, *Amblyomma cajennense* and *Rhipicephalus sanguineus*, are the principal vectors.

Transovarial and sexual transmission of the organism, which is harboured for the complete natural life cycle of the tick, makes the tick population an important natural reservoir as well as a vector of *R. rickettsii*. Man acts solely as a tangential host and plays no part in maintaining the natural epidemiological cycle. The incidence of human infection is determined by the proximity of individuals to the appropriate tick population, and is therefore highest in spring and summer when adult ticks are most prevalent.

In ticks, *R. rickettsii* may exist in a non-virulent form, to which virulence is restored by a blood meal, maintenance at 37°C for 24 hours, or by egg passage. It has been suggested that this is the reason why ticks do not infect until they have fed for several hours. Non-infective antigens, which may represent the non-virulent form of the organism, have been demonstrated in infected ticks by Sheppard and Goldwasser.

Rickettsial Infections

Laboratory Diagnosis

(a) Serological tests

The reactions of sera in the Weil-Felix reaction do not always differentiate Rocky Mountain spotted fever from typhus fever. Although agglutinins for *Proteus* OX19 and OX2 develop in response to infection with *R. rickettsii*, the titre of OX19 agglutinins is usually higher. Complement-fixation tests using the group-specific soluble antigen of the spotted fever group are usually adequate to confirm the diagnosis. If accurate identification of the organism is required, complement-fixation tests using type-specific rickettsial antigens are necessary.

The erythrocyte sensitization test and immunofluorescent techniques for the detection of group-specific antibodies are under trial.

(b) Rickettsial isolation

In specialized rickettsial laboratories, attempts may be made to isolate the causative organism from patients' blood. Specimens collected during the early febrile period of the disease are inoculated into guinea-pigs by the intraperitoneal route, and any isolate is identified by its pathogenic effects and by examination of infected tissues. Cross-protection tests and in vitro serological tests, with rickettsial antigens prepared in chick embryos, complete the identification.

Prophylaxis

(a) General measures

In tick-infested areas, protective clothing which prevents access of ticks should be worn. Both the clothing and exposed parts of the body should be treated with tick repellents, such as dimethyl-phthalate or diethyl-toluamide, and any attached ticks should be carefully removed without crushing.

Eradication of tick populations is generally difficult, but land clearing and control of animal reservoirs in inhabited areas may help. Spraying with DDT and other insecticides, and application of DDT powders to animal pets to free them from ticks, is an important prophylactic measure, particularly where dog ticks are the vectors.

(b) Vaccination

Vaccination with killed *R. rickettsii* vaccine, prepared in chick embryos, is nowadays required only for those exposed to high risk of infection, including laboratory workers and those frequenting highly endemic areas.

(c) Patients and contacts

After all ticks have been removed, no isolation of patients or contacts is required.

Treatment

Treatment with the tetracycline group of drugs is very effective if continued for several days after the temperature has returned to normal. For those who are severely ill, cortisone is beneficial in reducing the toxæmic effects of the disease.

**RICKETTSIA CONORII—
FIÈVRE BOUTONNEUSE**

Fièvre boutonneuse is a mild rickettsial fever resembling the more severe Rocky Mountain spotted fever. Originally described in Tunis by Conor and Bruch, in 1910, the disease was later recognized in Marseilles where it acquired the name of Marseilles fever.

Actiological Agent

The causative organism, *R. conorii*, closely resembles *R. rickettsii* in its morphological and biological characteristics. Like *R. rickettsii*, it multiplies in the nucleus as well as the cytoplasm of infected cells, and shares a common soluble antigen with other members of the spotted fever group. *R. conorii* is distinguished by its type-specific rickettsial antigen, and is differentiated from *R. rickettsii* by its lower pathogenicity for guinea-pigs, in whom it produces a febrile reaction and scrotal swelling but no necrotic ulceration.

Clinical Features

A febrile illness, usually milder than Rocky Mountain spotted fever, develops about 5–7 days after a bite from an infected tick; it is characterized by pyrexia, headache, malaise, and conjunctivitis. At the site of the tick-bite, a primary lesion, known as the ‘tache noire’, develops and forms a small ulcer, 2–5 mm in diameter, which displays a black centre surrounded by a zone of erythema. Regional lymphadenitis accompanies the lesion.

Four to five days after the onset of illness, a generalized maculopapular rash appears and persists for 6–7 days. After 10–14 days, the fever subsides and convalescence is usually uneventful.

Pathology

The course of infection and the lesions produced do not differ in any important respect from those of Rocky Mountain spotted fever.

Epidemiology

Fièvre boutonneuse is found in the Mediterranean coastal areas of Europe and the Middle East, in the coastal areas of the Black and Caspian seas, and in India, South America, and Kenya. The reservoir of infection is in dogs and small wild animals, and the vector of transmission is the tick, *Rhipicephalus sanguineus*; in South Africa and Kenya, the tick vectors are *Haemaphysalis leachi* and other ticks infecting dogs. A history of contact with dogs is usual in patients with fièvre boutonneuse.

Transovarial transmission of *R. conorii* and the maintenance of infection throughout the life-cycle of the tick makes the tick population an important reservoir as well as vector of the disease.

Laboratory Diagnosis

(a) Serological techniques

The reactions of sera in the Weil–Felix reaction are similar to those of sera from patients with Rocky Mountain spotted fever. Infection

with a member of the spotted fever group of rickettsiae may be confirmed by demonstrating a rise in titre of complement-fixing antibody against the group-specific soluble antigen; more accurate identification of the infection requires the use of the type-specific rickettsial antigen of *R. conorii* in complement-fixation tests.

(b) Rickettsial isolation

In specialized rickettsial laboratories, attempts may be made to isolate the causative organism; specimens of patients' blood, obtained in the early febrile period of the disease, are inoculated into guinea-pigs, and the identification of any isolate is similar to that described in Rocky Mountain spotted fever.

Prophylaxis

Tick-bites should be avoided by the measures described in the prophylaxis of Rocky Mountain spotted fever. No effective vaccine is available.

Treatment

The tetracycline group of drugs is highly effective in the treatment of fièvre boutonneuse.

R. AUSTRALIS— QUEENSLAND TICK TYPHUS

A febrile illness clinically similar to fièvre boutonneuse was described by Brody in North Queensland, in 1946. The causative organism, *R. australis*, resembles *R. conorii* in its morphological, biological and pathological properties. It shares a common soluble antigen with other rickettsiae of the spotted fever group but is distinguished by its type specific rickettsial antigen.

Man is a tangential host for the organism whose reservoir is in bandicoots, other small marsupials, and wild rodents; the vector is *Ixodes holocyclus* and other varieties of tick, which because of life-long infection serve as reservoirs as well as vectors.

R. SIBERICA— NORTH ASIAN TICK TYPHUS

An infection similar to Queensland tick typhus has been described by Soviet workers in Siberia and the Far Eastern territories of the U.S.S.R.; more recently, the disease has been described in other parts of Russia. The causative organism, *R. siberica*, resembles *R. conorii* and *R. australis* in its morphological, biological, and pathological properties. It possesses the common soluble antigen of the spotted fever group of rickettsiae but is distinguished by its type-specific rickettsial antigen.

The reservoir of infection is in wild rodents and larger animals, and the vectors of transmission are various species of tick belonging to the genera *Dermacentor* and *Haemophysalis*, which also provide additional reservoirs. Man is infected as a tangential host and plays no part in maintaining the natural epidemiological cycle.

R. AKARI—RICKETTSIALPOX

Rickettsialpox is a benign rickettsial infection which was first observed in New York in 1946; it has since been recognized in other parts of the U.S.A. and Russia. The causative organism is *R. akari*, which is maintained in rodent hosts and is transmitted by the rodent mite *Allodermanyssus sanguineus*.

Clinical Features

One to three weeks after a bite from an infected mite, a papular lesion appears at the site of trauma, which soon becomes vesicular, forms a black scab, and sloughs off after about 3 weeks. Regional lymphadenitis accompanies the lesion.

One week after the primary lesion appears, the patient suddenly develops a high fever, headache, malaise, and generalized myalgia, followed in 3–4 days by a generalized rash which usually spares the palms and soles. At first maculopapular in nature, the rash passes through a vesicular stage, which may be confused with varicella,

before scabs are formed and separate without scar formation. The patient recovers in a week or two.

Aetiological Agent

R. akari, although mite-borne, shares the common soluble antigen of the spotted fever group of rickettsiae but is distinguished by its type-specific rickettsial antigen. Morphologically, *R. akari* does not differ from other members of the group, and like them it proliferates in the nucleus as well as the cytoplasm of the infected cell.

In contrast to other members of the spotted fever group, *R. akari* is pathogenic for mice. After intraperitoneal inoculation, it produces generalized illness, ascites, and death. Guinea-pigs are also susceptible and respond with fever and scrotal swelling.

Epidemiology

The reservoir of infection is in mice and rats, and the vector of transmission is the mite, *Allodermanyssus sanguineus*, which commonly infests rodents. Man is a tangential host playing no part in the natural epidemiological cycle.

Rodents and their ectoparasites flourish in unhygienic environments and overcrowded cities, rickettsialpox is therefore a disease associated with overcrowded and poorer parts of urban environs.

Laboratory Diagnosis

(a) Serological tests

Sera from patients with rickettsialpox do not usually react in the Weil-Felix reaction, although agglutinins to *Proteus* OX19 are sometimes produced in low titre. The diagnosis may, however, be confirmed by complement-fixation tests using the group or type-specific soluble and rickettsial antigens, respectively.

(b) Rickettsial isolation

The organism may be recovered from patients' blood, collected during the early febrile period of the disease; intraperitoneal inoculation of mice is the method of choice.

Prophylaxis

Efficient rodent control and the use of miticidal agents in domestic rodent hiding places are the measures required for preventing the disease.

No isolation or quarantine measures for patients or contacts are required.

Treatment

The tetracycline group of drugs is highly effective in treatment of the disease.

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Rickettsial Infections

VI. *Rickettsia quintana* Trench Fever

Trench fever, known by the Germans as Wolhynian fever, is a relatively benign febrile condition which came into prominence during World War I. Little was heard of it again until World War II, when outbreaks occurred among German troops on the Eastern front.

The causative organism is *R. quintana* and the vector of transmission is the human body louse, *Pediculus corporis*; the reservoir of infection is thought to be in the human population.

Aetiological Agent

R. quintana has not been isolated in experimental animals, chick embryos or tissue cultures. The organism may however be seen in stained preparations of infected louse tissues, in which it characteristically occupies an extracellular position (Fig. 98). The infectivity of infected louse tissues has been demonstrated in human volunteers but, so far, little is known about the antigenic composition or properties of *R. quintana*.

Clinical Features

After an incubation period of 10–20 days, there is a sudden onset of fever, headache, conjunctivitis, generalized myalgia, and a short-lived maculopapular rash on the trunk. The disease lasts about 3–5 weeks, and convalescence is sometimes prolonged. Occasionally, the fever is of the relapsing type in which short periods of

pyrexia are separated by afebrile intervals of 3–8 days. Mild and inapparent cases have been observed, and relapses as long as 1–3 years after the original infection have been reported.



Fig. 98. *Rickettsia quintana* in the gut lumen of an infected louse [from Susumu Ito and J. W. Vinson (1965) *J. Bact.* 89, 481–95 (American Society for Microbiology)].

Epidemiology

The reservoir of infection is presumed to be in man and the vector of transmission is the human body louse, *Pediculus corporis*; any environment which encourages louse infestation therefore predisposes to outbreaks of the disease. Lice remain infected for life, but there is no evidence of transovarial transmission.

Infection is believed to be endemic in certain regions, where it is stimulated into activity in suitable epidemiological conditions. Between outbreaks of infection, the organisms appear to remain dormant in the human host, and organisms have been demonstrated in the blood of patients as long as 8 years after infection. This and the occurrence of inapparent infections explains, at least in part, the survival of the organism between outbreaks of infection.

Laboratory Diagnosis

In the absence of any susceptible laboratory animal or suitable serological test, diagnosis can only be confirmed by feeding lice on infected patients. A few days after feeding, extracellular rickettsiae are demonstrable in the louse intestine.

The Weil-Felix reaction is negative; serological tests using rickettsial antigens prepared in lice have been employed, but on a very limited scale.

Control and Treatment

Control of louse infestation and other methods used in the prophylaxis of typhus fever are necessary for the prevention of the disease. Little is known about the efficacy of antibiotic treatment in this disease but it is presumably effective.

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Rickettsial Infections

VII. *Coxiella burnetii*—Q Fever

An acute febrile disease with signs and symptoms of atypical pneumonia was described in Australia, by Derrick, in 1937; because of its uncertain aetiology at that time, Derrick referred to it as Q (query) fever. The disease has since been recognized in many parts of the world including Great Britain.

Burnet and Freeman isolated the causative organism and identified it as a rickettsia in 1939. Because of some atypical properties it has been assigned to a separate genus, *Coxiella*, and the organism is now known as *Coxiella burnetii*.

Aetiological Agent

(a) General properties

Coxiella burnetii, which resembles other rickettsiae in morphology and other properties, exhibits a number of distinctive characteristics. Because of its slightly smaller size, $0.3 \times 0.72 \mu$, the organism passes through filters impermeable to other rickettsiae; it is non-toxic in high concentration; it is not transmitted to man by an arthropod vector; it produces no agglutinins for *Proteus* OX strains; and the clinical syndrome it produces is not characterized by a rash.

(b) Chemical and physical properties

The organism is extremely resistant to heat, desiccation, and chemical agents. Two per cent formaldehyde and 1% lysol are the minimum concentrations required for disinfection, and the organisms remain viable for months at 4°C, and for years at -20° or -60°C.

(c) Biological properties

The organism is pathogenic for guinea-pigs but is not lethal except in high dosage; smaller doses produce a febrile response but no scrotal reaction. Mild, usually non-fatal, peritonitis and splenomegaly are produced in mice, and the organism grows well in the mouse liver and spleen. The organism grows abundantly in the chick embryo yolk sac, usually killing the embryo, and growth has been obtained in cultures of various mammalian and chick embryo cells.

(d) Antigenic composition

Both the soluble and rickettsial antigens produced by *Coxiella burnetii* are distinct from those produced by other rickettsiae. Strains isolated from many parts of the world reveal only very minor antigenic variations, but their reactions with convalescent sera are affected by the phase variation described by Stoker and Fiset. They found that strains well adapted to the chick embryo react with early and late convalescent sera in the complement-fixation test, but that unadapted strains react only with late convalescent sera.

Unadapted strains, said to be in phase I, do not usually react with human convalescent sera; in man, high titres of antibody against phase I organisms usually indicate chronic infection with *Coxiella burnetii*. For the detection of complement-fixing antibodies in human sera, egg-adapted phase II strains are required. Phase II strains are easily converted into phase I by passage in experimental animals.

Clinical Features

After an incubation period of 2-3 weeks, fever, severe headache, sweating, malaise, anorexia, myalgia, and sometimes signs of meningeal irritation suddenly develop. A few days later, dry cough, chest pain, and the expectoration of a limited amount of sputum, which is sometimes blood-stained, herald the development of pulmonary consolidation, which occurs in most but not all patients with Q fever. Characteristically, the physical signs of consolidation are minimal and at variance with X-ray appearances. Enlargement of

the liver and spleen provides evidence of systemic infection in a syndrome which is not confined to the lungs.

The febrile period of the disease continues for about 2 weeks but may persist for 1-2 months. Rarely, chronic infection leads to complications, and cases of hepatitis and subacute bacterial endocarditis due to *Coxiella burnetii* have been reported. In the absence of antibiotic therapy, the mortality of Q fever is about 1%.

A high ratio of subclinical to clinical infections in endemic areas is indicated by serological investigations

Pathology

When death occurs, diffuse lobar pneumonia is usually the outstanding lesion. Intense vascular congestion and abundant alveolar exudate, characterized by mononuclear and macrophage cells, are present, and rickettsiae can be seen in some of the inflammatory cells. Lesions may also be found in other organs, especially the liver.

Epidemiology

(a) Source of infection

Inapparent infection with *Coxiella burnetii* is common in cattle, sheep and goats, as well as in many wild species, including rodents, marsupials and birds in various parts of the world. Many species of tick are also naturally infected with the organism, and probably play an important part in transmitting the infection in wild animal species and probably in sheep. Ticks do not, however, play an important role in spreading infection among domestic ruminants or from them to man.

In Great Britain, infection with *C. burnetii* is endemic in animals kept in the Southern and Midland regions. The large sheep populations in Kent are considered to be important sources of human infection, and there is some evidence that cattle too are infected in sheep rearing areas.

(b) Transmission of infection

Human infections usually occur in those who have close contact with animal livestock or their products. Infected animals excrete

C. burnetii in their milk, urine and faeces, but during parturition organisms are shed in abundance with the placenta and birth fluids. Curiously, infection may remain latent during pregnancy but becomes stimulated into activity near the time of parturition, when rickettsiae become abundant in the placenta and birth fluids.

After excretion, infection is probably spread among cattle and sheep by airborne transmission of infected aerosols or dried particulate matter contaminating the environment. The resistance of the organism to adverse conditions ensures prolonged contamination of the environment; for this reason, transfer of infection by contaminated water or infected pasture is also possible.

Marmion and Stoker consider unpasteurized infected milk to be an important source of infection, but most outbreaks of Q fever seem to have been acquired by inhalation of infected material from contaminated environments. Q fever is therefore an occupational hazard of farmworkers, shepherds, woolsorters, and abattoir workers, particularly in the lambing season when the environment is heavily contaminated. Sometimes the association between infected patients and livestock is indirect, and can be traced to the handling of contaminated clothing, straw, wool, hides or animal carcasses from infected sources.

Transmission of Q fever from one person to another is not usual but does occur; it is a particular hazard for nurses and medical attendants in close contact with patients. Transfer of infection to pathologists at post-mortem examinations and to other staff in hospitals is on record.

Q fever often occurs in newcomers to endemic areas, who have not acquired immunity from previous exposure. Marmion and Stoker suggest that immunity of the local population is acquired in childhood from infected milk, and the high ratio of subclinical to clinical infections certainly indicates that infection is more common than often thought.

Laboratory Diagnosis

(a) Seriological tests

Complement-fixation tests using phase II *Coxiella* antigens is the

technique of choice for demonstrating a significant rise of antibody against *C. burnetii*. An antibody rise against phase I antigen is an important diagnostic aid in recognizing chronic infection with *C. burnetii*.

Direct agglutination tests using coxiella antigens, and slide agglutination tests which require only small quantities of reagents, have been developed. These tests, which have the advantages of high sensitivity and specificity, are capable of detecting antibodies in the early stages of the disease before complement-fixing antibodies appear.

The Weil-Felix reaction is negative for all *Proteus* OX strains.

(b) Rickettsial isolation

In specialized rickettsial laboratories, isolation of *C. burnetii* from patients' blood, collected during the early febrile period of the disease, may be attempted. Specimens are inoculated into guinea-pigs by the intraperitoneal route; infection leads to a febrile response without any scrotal reaction, and the identity of the isolate is confirmed by bleeding the animal 4 weeks later and examining its serum for the presence of antibody specific for *C. burnetii*.

Mice and chick embryos may also be used for the isolation of *C. burnetii*, which can be identified in stained smears of infected tissues made after an appropriate period of incubation. Immunofluorescent techniques may also be used for identifying organisms in infected tissues.

Prophylaxis

(a) General measures

High standards of cleanliness and hygiene in the care of livestock and the disposal of their excreta are important. This applies particularly to the care of parturient animals and the disposal of animal placentas.

Pasteurization of milk is an obvious measure for preventing milk-borne spread of infection.

(b) Vaccines

Formalin-killed vaccines prepared from phase I organisms are effective but are not generally available. Their use may be considered

for those exposed to a high risk of infection, including laboratory workers and non-immune livestock handlers in endemic areas.

(c) Patients and contacts

Patients should be isolated, and their excreta and discharges disinfected. Quarantine of contacts is not required.

Treatment

Antibiotics of the tetracycline group are the drugs of choice, although somewhat less effective than in other rickettsial diseases. For best results, they must be administered as early as possible in the disease and continued for several days after the temperature has returned to normal.

Table 18. Summary of rickettsial infections

Rickettsial group	Rickettsial species	Disease in man	Vector	Main vector species	Reservoir	Weil-Felix agglutinins	Rash	Geographical distribution
Typhus fever group	<i>R. prowazeki</i>	Classical epidemic typhus	Lice	<i>Pediculus corporis</i> <i>Pediculus capitis</i>	Man	Proteus OX19	+	S. America Africa Asia
		Recurrent typhus (Brill-Zinsser)	None	None	Patient	Absent or weak for Proteus OX19	±	Eastern Europe New York and typhus endemic areas
	<i>R. typhi</i>	Endemic or murine typhus	Rat flea	<i>Xenopsylla cheopis</i>	Rats	Proteus OX19	+	U.S.A. S. America Africa Asia Middle East
Tsutsu-gamushi	<i>R. tsutsu-gamushi</i>	Scrub typhus	Red mite	<i>Trombicula akamushi</i>	Wild rodents, mites, and possibly birds	Proteus OXK	+	S.E. Asia
	<i>R. rickettsii</i>	Rocky Mountain spotted fever	Wood tick Dog tick	<i>Dermacentor andersoni</i> <i>Dermacentor variabilis</i>	Rodents, rabbits, larger mammals, and ticks Dogs, rodents, small mammals, and ticks	Proteus OX19 and OX2 Proteus OX19 and OX2	+	Western U.S.A. Eastern U.S.A.

	Sao Paulo fever	Dog tick	<i>Amblyomma capense</i> <i>Rhipicephalus sanguineus</i>	Dogs	Proteus OX19 and OX2	+	S America	
Spotted fever group	<i>R conorii</i>	Fièvre boutonneuse	Brown dog tick	<i>Rhipicephalus sanguineus</i>	Dogs, rodents, and ticks	Proteus OX19 and OX2	+	Mediterranean Coast
			Common dog tick	<i>Haemaphysalis leuclini</i> and others	Dogs, rodents, and ticks	Proteus OX19 and OX2	+	S Africa India
	<i>R australis</i>	Queensland tick typhus	Tick	<i>Ixodes holocyclus</i>	Wild rodents, small marsupials, and ticks	Proteus OX19 and OX2	+	N Queensland Australia
	<i>R sibirica</i>	North Asian tick typhus	Tick	<i>Dermacentor Haemaphysalis</i> various species	Wild rodents, cattle, and ticks	Proteus OX19 and OX2	+	U S S R
	<i>R akari</i>	Rickettsialpox	Rodent mite	<i>Hoodermyssus sanguineus</i>	Rats and mice	None	+	U S A U S S R.
Trench fever	<i>R quiniana</i>	Trench fever	Lice	<i>Pediculus corporis</i>	Man	None	+	Eastern Europe
Coxiella	<i>C. burnetii</i>	Q fever	None for man	Various species may play some part in maintaining animal reservoirs	Cattle, sheep, goats, rodents, and small marsupials	None	--	World wide

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