Current Problems in Clinical Virology

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PREFACE

Now that virological investigations may influence clinicians directly in their diagnoses as well as the management of patients with virus diseases, many hospitals are making arrangements to have patients investigated virologically and some are actively developing their own clinical virology departments.

This book, written by authors experienced in the application of virology to clinical problems, a branch of medicine which has undergone considerable development in recent years, attempts to highlight problems which may be encountered in everyday practice. It should therefore be useful to clinicians, including general practitioners (one chapter is written by a general practitioner), as well as to those microbiologists who are running or developing their own clinical virology laboratories. In addition, some chapters will be particularly relevant to problems encountered by Medical Officers of Health. Eight of the chapters are entirely virological but the other two on 'Gastroenteritis' and 'Tropical Infections which may present in Temperate Climates', although containing some virology, are rather broader in their scope.

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Chapter 1

ACUTE RESPIRATORY VIRUS INFECTIONS OF CHILDHOOD

P. S. GARDNER

Introduction

Until a few years ago, acute virus respiratory infection of childhood was only studied in terms of the infecting agent. These studies were of enormous epidemiological importance but of limited practical use. Now the physical, chemical, antigenic and biological properties of viruses must be investigated with a view to the future use of antiviral agents. Viruses should be seen against a background of the diseases they are believed to cause and in relation to the immune responses they evoke.

Respiratory virus diseases have been studied in many ways and hospital admissions have proved a profitable source of aetiological information (Gardner *et al.*, 1960; Chanock *et al.*, 1961; Elderkin *et al.*, 1965; Holzel *et al.*, 1963 and 1965). These studies are selective, as they include only the child who is very ill or who has a social problem requiring admission to hospital.

Other studies have been conducted on acute respiratory virus infection occurring in the home. One of the first of these, though not specifically designed as such, was the 1,000 family study carried out in Newcastle upon Tyne initiated by Sir James Spence. This gave a wealth of information about the illnesses from which all children suffer (Miller *et al.*, 1960). Dingle, Badger and Jordan (1964) started an ambitious programme to study illness in a community of civilian families in Cleveland over a period of years. Laboratory investigations were carried out but so far have only included influenza and adenovirus, which account for less than 10 per cent of the everyday illnesses found in the family.

Other workers thought this problem could best be studied by examination of patients in general practice and useful information has been gathered by Banatvala, Anderson and Reiss (1964) and the Medical Research Council Working Party on Acute Respiratory Virus Infection

(1965). It is only by considering all the various facets involved in the epidemiology of virus disease that we can begin to appreciate the complexity of the problem of respiratory virus infection.

AN HISTORICAL REVIEW OF RESPIRATORY VIRUSES

The first of the respiratory viruses to be isolated was influenza A, by Smith, Andrewes and Laidlaw in 1933 using ferrets; influenza B was discovered by Francis (1940a), and Magill in 1940, while influenza C was not discovered until 1949 by Taylor.

Advances in understanding the aetiology of respiratory disease followed rapidly from the introduction of tissue culture techniques by Enders, Weller and Robbins in 1949. One of the first benefits was the discovery of adenoviruses as a latent infection of tonsils and adenoids by Rowe and his colleagues in 1953. The association of adenoviruses with illness in military recruits was first reported by Hilleman and Werner in 1954.

The next step came in 1958 with the simultaneous discovery, by Chanock and his colleagues in the United States and by Beale *et al.*, in Canada, of the first member of the parainfluenza viruses. These viruses had originally been called 'haemadsorption viruses' because they were identified by using guinea-pig red blood cells which adsorbed on to the monkey kidney cells on which these viruses were growing.

In 1956, a virus was isolated from a chimpanzee with coryza by Morris, Blount and Savage and accordingly named the 'chimpanzee coryza agent'. The association of this virus with illness, both severe and mild, in human infants was made by Chanock and his colleagues in 1961. The virus has since been renamed 'respiratory syncytial' (R.S.) virus from the appearance it produces on tissue culture cells, where it forms giant cells and syncytia (Figs. 1 and 2). It is strange that a virus originally discovered in a chimpanzee should prove to be the most important and lethal of all respiratory virus pathogens capable of infecting infants.

About this time, Tyrrell and his colleagues (1960) isolated the first members of a new rhinovirus group which had eluded virologists for many years and now accounts for many of the common colds occurring so frequently in all age groups.

Certain members of the Coxsackie and echovirus groups are believed to be associated with respiratory disease and outbreaks associated with them have been reported (Philipson, 1958; Heggie *et al.*, 1960; Kendall, Cook and Stone, 1960).

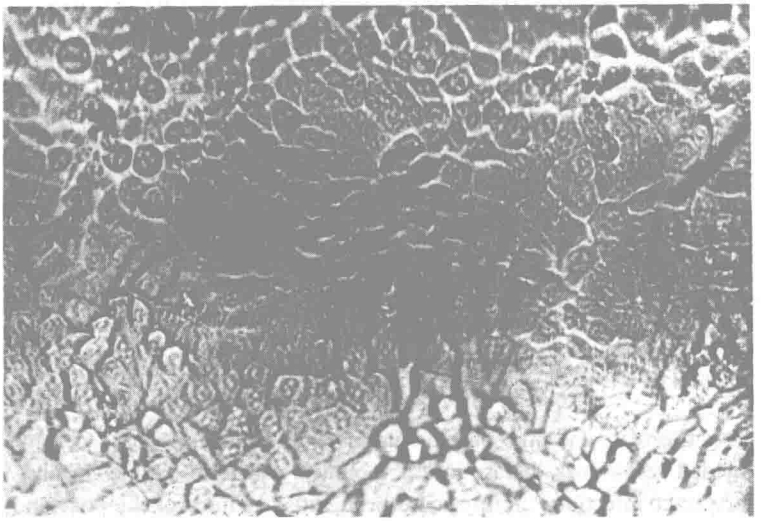

Figure 1. The appearance of uninoculated HeLa cell tissue culture (× 330)

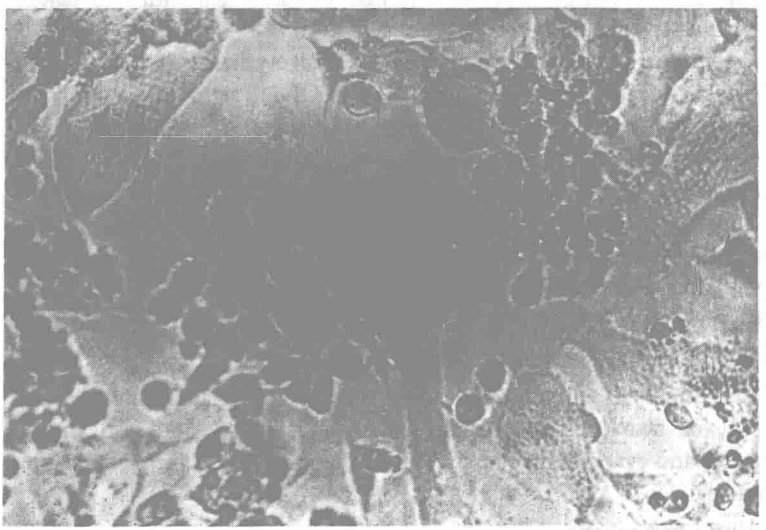

Figure 2. The appearance of HeLa cell culture which has been inoculated with respiratory syncytial virus. The giant cells and syncytia stand out clearly. (× 330)

In 1967, a number of viruses related to avian bronchitis virus and mouse hepatitis virus (McIntosh, Becker and Chanock, 1967) were isolated in human organ culture. Bradburn, Bynoe and Tyrrell (1967) showed that one of these viruses could cause coryza in human volunteers. Its exact relationship to fowl and murine viruses has still to be determined, but it appears that a new family of viruses capable of causing human disease has been discovered. This group has tentatively been called the 'coronavirus group' from their electron microscopic appearance (Almeida *et al.*, 1968).

A CLASSIFICATION OF ACUTE RESPIRATORY INFECTIONS OF CHILDHOOD

Over the years and on repeated studies of hospital admissions, we have divided respiratory disease into five clinical categories: upper respiratory tract infection; croup; acute bronchitis; acute bronchiolitis; and pneumonia.

Upper Respiratory Tract Infection

This includes a number of different upper respiratory infections.

Colds are illnesses whose main feature is a watery mucoid or purulent nasal discharge; a severe cold may be associated with a moderate pyrexia.

Pharyngitis is defined as a generalised erythema of the pharynx without localisation on the tonsils and without signs of a cold. The illness is usually associated with fever or malaise.

Tonsillitis is a local infection of the tonsils, which are red, swollen and often show an exudate; fever is frequently present.

Otitis media is characterised by redness of the drum which shows swelling and often a purulent discharge. Earache and fever are associated with this illness which may be primary, but more often is secondary to other respiratory symptoms.

Croup or Laryngotracheobronchitis

This illness is often preceded by a cold. The main symptoms are pyrexia, hoarseness, a croaking cough with stridor and restlessness; pallor and cyanosis may be present in the most severe forms indicating anoxia.

Acute bronchitis

This is a febrile illness whose main features are cough, frequently associated with wheezing and widespread rhonchi; râles are often present.

Acute bronchiolitis

This affects infants and young children, usually in epidemics. Main features are coryzal symptoms which precede the major illness by about 24 hours to several days. These are quickly followed by rapid respiration and fever which is variable. There is a frequent harsh exhausting cough, respiratory wheeze, and in most cases, fine râles are widespread throughout the lungs. The main physical sign is pulmonary overdistension with costal recession; chest radiographs are used to confirm the pulmonary overdistension and in a few cases there may be linear areas of collapse.

Pneumonia

In young children, this presents as an acute respiratory disease associated with pyrexia, cyanosis, restlessness and prostration. A cough is often present and the respirations are rapid. Clinical evidence of consolidation is only present in a small number of those affected, the main signs in the lung being diminished air entry and the presence of râles. Radiographs show shadows which may represent lobular, segmental or lobar consolidation. In older children, the disease is more insidious in onset and signs may persist for several weeks.

It has been found that these clinical definitions can be applied in practice. By correlating clinical categories with the type of virus isolated, we can gain a better insight into the causation of these illnesses. Furthermore, a start can be made towards prophylaxis and treatment when we know which are the important agents responsible. It was by careful studies, for example, that the great importance of R.S. virus as a major cause of respiratory illness in children was established and efforts are now being channelled towards preventive measures.

CONVENTIONAL LABORATORY TECHNIQUES

The ordinary routine isolation of the virus may take up to 28 days, but for the busy general practitioner and even the hospital consultant, reports which come in when the patient is better can only satisfy their curiosity in retrospect. Nevertheless, this function of defining the agents causing disease has played an important part in the prevention of infection. Unless we know the pathogenic potential of each virus and the frequency of its occurrence, prophylactic measures cannot even be considered. In the near future, antiviral drugs effective against certain viruses may become available. The virus laboratory must then be in a position to identify the virus causing the illness at a very early stage.

Tissue Culture Techniques

Specimens may be taken at the bedside, usually cough and nasal swabs. The swabs are broken off into a transport medium of a basic buffered salt solution containing a small quantity of bovine albumin (0·2 per cent). They are quickly taken to the laboratory on ordinary ice (4°C), because of the instability of most respiratory viruses, and inoculated on to tissue culture cells as soon as possible.

Certain basic types of tissue culture cells are employed in the virus laboratory. There are two widely used cell lines of human origin: HeLa cells, derived originally from carcinoma of a cervix uteri, and another malignant cell line known as HEp 2. These two cell lines are examples of continuous cell lines, which means they can be passaged from generation to generation in the laboratory. They are useful for growing viruses such as R.S. virus, adenoviruses, herpesvirus hominis and some of the picornaviruses.

All virus laboratories should also use rhesus monkey kidney cells. These are primary cell lines and each time these tissues are used they come from the kidneys of a new monkey; these cells cannot be passaged. They are used for the identification of influenza and parainfluenza viruses. Although these viruses do not produce any gross changes in the cell sheet, their presence may be noted by using the haemadsorption technique based on the original method of Vogel and Shelokov (1957). In this, guinea-pig red blood cells are added to the tissue cultures and if one of these viruses is present, then the red cells will adsorb to the monkey kidney cell sheet.

Monkey kidney cells are also useful for growing certain strains of rhinovirus, as well as Coxsackie and echoviruses which are occasionally associated with respiratory illnesses. These viruses will produce a cytopathic effect on monkey kidney tissue culture.

Another popular culture in use is the human embryo lung diploid cell, a semi-continuous line which means that it can be passaged only for a limited number of generations, usually about 40. The best known strain of these cells is the W.I.38. Monkey kidney cells and human diploid cells are normally incubated at 33°C on a roller drum. This is because they are used for cultivating rhinoviruses which will only grow under these conditions.

Organ cultures of human embryo trachea have been used for growing rhinoviruses and many which will not grow on monkey kidney and W.I.38 cell cultures will grow on organ cultures (Tyrrell, Bynoe and Hoorn, 1968).

This brief outline of tissue culture techniques shows that a great deal of the resources of the laboratory must be devoted to growing cells. The viruses isolated on tissue culture are identified by various means according to their properties. It has been seen that some viruses can be identified by the haemadsorption test, but this merely shows that a virus of this group is present in the culture and its further identification by a haemadsorption neutralisation test is required. The suspected virus agent and a range of antisera react together for an established period of time at a fixed temperature to allow neutralisation to take place. The mixtures are then inoculated on to tissue culture which are tested several days later for haemadsorption. Other viruses, such as R.S. virus, adenoviruses and picornaviruses, produce characteristic cytopathic effects on tissue culture but for confirmation of the individual types, a neutralisation test must also be performed; the specific antiserum for the virus type neutralises that virus and prevents degeneration from occurring.

Serological Techniques

The serum of patients can also be investigated in the laboratory by serological tests. The basic serological test is the complement-fixation test and two difficulties may be encountered. If the patients are young, there may be difficulty in obtaining suitable quantities of blood. Most of the children examined are bled by heel stab; if this does not provide enough blood for the ordinary test, a microtechnique is used. The second difficulty is that it is important to obtain paired sera, the first one taken at the onset of illness, and the second one 10–14 days after the first. The two samples of blood are then compared with a range of respiratory virus agents to detect a change in antibody titre. A four-fold rise in antibody titre is considered diagnostic.

Other serological tests which may be performed are serum neutralisation tests and the haemagglutination-inhibition test, but these are seldom used in routine diagnosis. Routine procedures for virus identification and serological diagnosis are discussed in detail in Chapter 8.

The Rapid Diagnosis of Respiratory Virus Infection

The delays inherent in conventional methods of diagnosing virus disease led workers to seek more speedy methods. Some have favoured electron microscopy, but difficulties are encountered in differentiating the various myxoviruses and paramyxoviruses (Doane *et al.*, 1967; Joncas *et al.*, 1969). Here as elsewhere, fluorescent antibody techniques

have been used (Gardner and McQuillin, 1968; McQuillin and Gardner, 1968; Hers, Kuip and Masurel, 1968). There are two common methods for diagnosing virus diseases by fluorescent antibody techniques, the direct and the indirect.

The Direct Technique

In this method, a specific virus antiserum is conjugated directly with a dye, such as fluorescein isothiocyanate. If the suspected virus antigen, or cells infected with the virus, are treated with this conjugated antiserum, fluorescence will be seen under the ultraviolet (UV) light microscope if the appropriate antigen is present.

The Indirect Technique

Infected cells from the patient or infected tissue culture cells may be taken as a source of virus antigen. They are fixed on to slides and treated with a specific animal antiserum, e.g. rabbit. Both for this and the direct test, the antisera must be highly specific and show no cross-reaction with other viruses or human tissue. If a reaction has taken place between virus and antiserum, when the preparation is washed, rabbit antibody will remain attached to infected cells at the sites where virus antigen is present. If the preparation is now treated with an anti-rabbit globulin (made in another animal, for example, a goat or dog) and labelled with a fluorescent dye, it will react with any rabbit antibody remaining on the preparation. The sites where virus antigen is present will then appear brightly fluorescent when examined under the UV microscope.

The indirect fluorescent antibody technique was chosen because it may be as much as ten-fold more sensitive than the direct method (Coons, 1956). Of even greater importance is the fact that one fluorescent conjugate can be used for the diagnosis of a number of different virus infections and there is no need to label each individual virus antiserum with a fluorescent dye.

The Indication for Rapid Diagnosis

Virology must make progress in the clinical field and, as antiviral agents appear, then accurate and speedy diagnosis becomes essential. The antiviral drugs which are available at the moment seem to be specific for particular groups of viruses and they must be given as early as possible in the disease, the best results being obtained if the drug is used prophylactically.

Examples of antiviral drugs are:

amantadine hydrochloride for the influenza virus group;
5-iodo-2-deoxyanidine (idoxuridine) for the herpesvirus group;
N-methylisatin°beta thiosemicarbazone (Methisazone) for the pox group.

Two more recent drugs, isoquinolines, (UK 2054 and UK 2371) are reported to be active against a number of respiratory viruses, when given before clinical symptoms appear. If these drugs are to be ultimately used clinically, then rapid diagnosis is essential.

The clinician too needs a rapid diagnosis in order to evaluate the treatment. The child with bronchiolitis from whom R.S. virus has been isolated with no potential pathogenic bacteria present needs oxygen therapy, humidity and correction of biochemical imbalance. Antibiotics will only be helpful in the event of secondary bacterial invasion; this is a very rare occurrence in bronchiolitis caused by R.S. virus. The excessive and unnecessary use of antibiotics in this condition, when the involvement of pathogenic bacteria remains unproved, is not only uneconomic but harmful since it encourages the development of resistant strains of bacteria.

To be justified, the fluorescent antibody technique for rapid diagnosis in virus infections must fulfil some of the following conditions:

1. By definition, it must be more rapid than the conventional techniques in current use.
2. It should be at least as sensitive as conventional methods, preferably more so.
3. The rapid diagnosis of the virus infection should benefit the clinician in his management and treatment of the case—if not immediately, at any rate in the future.
4. The agent investigated must be sufficiently prevalent to make its rapid diagnosis an economic proposition.
5. Isolation techniques are limited in respiratory virus infections to specimens which must be collected and rapidly transported to the laboratory for immediate inoculation into tissue culture. This restricts investigations mainly to hospital patients. If specimens could be taken and simple methods devised to prepare them for staining by the fluorescent antibody technique, diagnosis of virus infections in areas distant from virus laboratories would be simplified. This could provide a service for general practitioners.
6. The technique used might replace more tedious laboratory

procedures; for example, the use of this method instead of a neutralisation test for the identification of R.S. virus provides a result in three hours instead of four to seven days.

Rapid diagnostic techniques have been devised in this Department for the investigation of R.S. virus, influenza A and parainfluenza viruses 1, 2 and 3. Hers had previously shown the value of immunofluorescence for the diagnosis of influenza infections (Hers, 1963; Hers, Kuip and Masurel, 1968).

Methods of Collecting Specimens

Respiratory virus infection of childhood in hospital practice can be investigated by aspirating respiratory secretions from children mainly

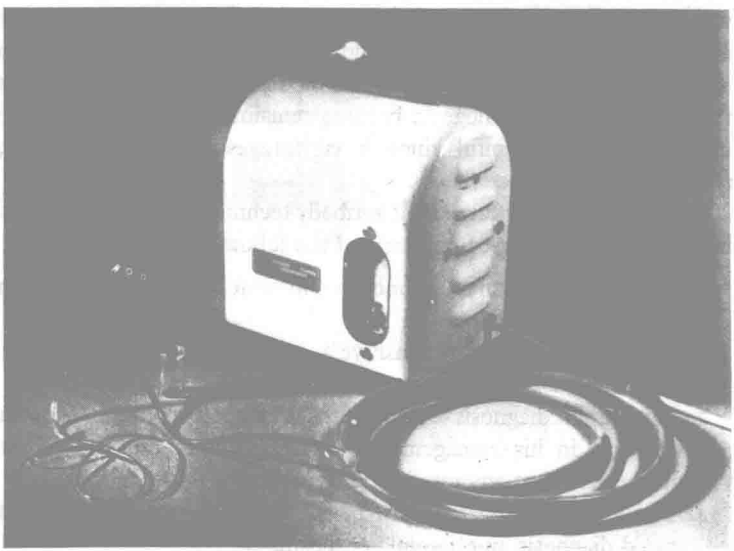

Figure 3. Suction apparatus used for the aspiration of infants with respiratory infection. Catheters and mucus extractor are attached.

under the age of one, but often up to the age of two. The apparatus shown in Figure 3 is used.

The intranasal catheter is passed into the region of the nasopharynx and secretions are aspirated and collected in the mucus extractor. This material contains masses of cells which are washed, deposited on slides and air-dried, then fixed and stained, as described above. The apparatus must not be used at more than 26 lb negative pressure. Many of these children are aspirated in any case, in the normal course of treatment

for their respiratory disease. A method has recently been devised for taking cough swabs from children, eluting cells from them, which are then concentrated, washed and fixed (McQuillin, Gardner and Sturdy, 1970). This method of using cough swab preparations has several advantages; it can be used for children of all ages in hospital and in general practice, and it can also be used for adults. The method has the disadvantage of containing fewer cells for examination and is therefore less sensitive; such preparations require a much longer microscopic examination than nasopharyngeal secretion. In a recent series of 29 children, a diagnosis of R.S. virus infection was made on preparations of nasopharyngeal secretions; the diagnosis was also made on cells taken on cough swabs in 27 of these children, but the time taken to study the preparations of nasopharyngeal secretions was only a few minutes per slide, while in many cases, 20 minutes or more was needed for the examination of cough swab preparations. An ordinary throat swab cannot be smeared on to a slide and examined in this way, since very few cells are transferred to the slide and those which are, are damaged. It is impossible to diagnose a virus infected cell by a fluorescent antibody technique unless the cell is intact.

Preparations of nasopharyngeal secretions are not only suitable for rapid diagnosis but provide material from which isolation of virus can easily be made. Consequently they have proved to be a better source of virus than material on cough swabs. Therefore, whenever possible, collection of nasopharyngeal secretion should be attempted (Sturdy, McQuillin and Gardner, 1969). Nasopharyngeal secretions have also been employed to study the role of local antibody in R.S. virus infections (Scott and Gardner, 1970).

The Significance of Viruses Isolated from the Respiratory Tract

The significance of a virus isolated from the respiratory tract presents a problem of interpretation in diagnostic virology. To survive, respiratory viruses must be intracellular and the concept has developed that if a virus occurs in a patient, it must be of pathogenic significance; this is not necessarily true.

Various methods have been used to show possible significance. The first is to compare the number and types of viruses isolated from children with respiratory illness with those viruses isolated from a control population of normal children. 'Normal' children in this context are those not suffering from a respiratory disease—selection of 'controls'