APPLICATIONS of TISSUE CULTURE to the STUDY of the EPIDEMIOLOGY of RESPIRATORY VIRUSES

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by

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SUMMARY

The thesis is divided into two parts. The first section is concerned with tissue culture techniques and their use in the isolation and study of respiratory viruses. The second section describes the epidemiological results obtained from studies of respiratory illness.

Problems associated with the growth of certain tissue cultures, particularly human embryonic diploid cell strains were investigated. Attempts were made to derive cell strains from various organs of 36 human embryos and methods were found for improving the low temperature preservation of these cells. Cell strains of human embryonic kidney fibroblasts were shown to be more sensitive to rhinoviruses than the widely used human embryonic lung cell strains. Detailed studies on eight cell strains also revealed differences in sensitivity to rhinoviruses between various lung cell strains and between various kidney cell strains. Three rhinovirus serotypes were discovered which appear to grow much more readily in kidney cells than in lung cells. Attempts to improve the sensitivity of tests for rhinovirus neutralizing antibodies were unsuccessful. Specific antisera were made against 15 rhinovirus strains and used in cross-neutralization studies. Twelve distinct serotypes were detected, 11 of which appear to be antigenically distinct from rhinoviruses so far described. Eighty-six rhinovirus strains were tested for haemagglutinating activity; no haemagglutination was detected. Factors affecting the

isolation of respiratory syncytial (RS) virus are described, including the repeated loss of sensitivity of 'Bristol' HeLa cells after continuous culture for long periods. Attempts to improve the sensitivity of tests for RS virus neutralizing antibodies revealed that the addition of unheated rabbit serum to the serum-virus mixtures increased antibody titres two- to eight-fold. This unheated serum was valuable both in detecting neutralizing antibody responses in young children and in revealing antigenic variation among RS virus strains. Methods for concentrating RS virus onto aluminium hydroxide and for preparing strain-specific antisera were found. Two antigenically distinct RS virus strains and seven possibly intermediate strains were detected.

A total of 302 viruses were isolated from respiratory secretions collected from 800 cases in Glasgow between January 1962 and April 1966. The viruses comprised 29 influenza viruses, 27 parainfluenza viruses, 83 RS viruses, 95 rhinoviruses, 26 herpes viruses, 24 adenoviruses and 18 enteroviruses. Most of the four strains of influenza A2 and 25 strains of influenza B were isolated from schoolchildren during three winter outbreaks. Parainfluenza viruses (7 type 1, one type 2, and 19 type 3) were found most commonly in children but showed no marked seasonal distribution. RS viruses were isolated only from young children during four winter outbreaks. Titration of RS neutralizing antibodies revealed that

RS virus isolation was associated with an absence of antibody in children over 6 months but not in younger children. Fifty-eight of the 95 rhinoviruses could be identified serologically and belonged to 24 different serotypes. Rhinoviruses were found most often in young adults but occurred in all populations and age-groups; they were isolated most frequently in spring and autumn. Herpes simplex virus occurred most commonly in children in hospital. Adenoviruses, belonging to five different serotypes, and enteroviruses, belonging to at least nine different serotypes, were isolated only from children. Most enteroviruses were isolated during the summer.

Three main population groups were studied: children and adults at home or at work, adults with chronic bronchitis and children in hospital. Ten per cent of 107 respiratory illnesses in children and 40% of 60 illnesses in adults, who were at home or at work, yielded rhinoviruses. Myxoviruses were found in 19% of these illnesses in children and in 10% of them in adults. The etiological significance of rhinoviruses in children at home could not be assessed because they were also found in 13% of symptomless contacts. Eighty-seven respiratory episodes in chronic bronchitics were investigated and rhinoviruses were isolated from 16% of them. Only one rhinovirus infection was not associated with an illness which affected the chest and 25% of bronchitic exacerbations were associated with rhinovirus infection. Virus isolation and serological results suggest that rhinoviruses can infect the lower respiratory tract

of chronic bronchitics. Among children in hospital RS virus appeared to be the most important single cause of severe respiratory illness and was isolated from 20% of 402 children. Other myxoviruses and rhinoviruses were isolated from 7% and 8% respectively of these children. However, rhinoviruses were also found in 9% of children in hospital with diarrhoeal illnesses; myxoviruses were found only rarely in this group of children. Herpes simplex virus, adenoviruses and enteroviruses were isolated from both the respiratory and diarrhoeal groups of children. Detailed study of 19 rhinovirus infections in children in hospital indicated that these infections could cause lower respiratory illnesses.

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ABBREVIATIONS

Used in text, tables and figures:

BH = Bristol HeLa

BM = basal medium

BSS = balanced salt solution

CF = complement fixation

DMS = dimethyl sulphoxide

HEK = human embryonic kidney

HEKF = human embryonic kidney fibroblasts

HEL = human embryonic lung

HI = haemagglutination inhibition

LH = lactalbumen Hanks'

MEM = minimum essential medium

PBS = phosphate buffered saline

PSS = penicillin and streptomycin solution

RMK = rhesus monkey kidney

RS = respiratory syncytial

TCD_{50} = 50% tissue culture infecting dose

TPB = tryptose phosphate broth

Used in tables and figures only:

AD = adenovirus

ENT = enterovirus

FL = influenza

HS = herpes simplex

PF = parainfluenza

RH = rhinovirus

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INTRODUCTION

Methods for the isolation of various new respiratory viruses had been reported by 1961. Consequently, there was a need to establish these techniques at the Regional Virus Laboratory, Ruchill Hospital to improve the study of various types of respiratory disease in Glasgow. This thesis is an account of my experiences in establishing these techniques and of the epidemiological results which were obtained.

The methods I first used were those which I observed at the Common Cold Research Unit, Salisbury. Certain modifications were made, among them the introduction of cell strains of human embryo kidney fibroblasts, which had not previously been used, for rhinovirus isolation. This gave a higher isolation rate than has previously been reported and led to the recovery of a number of strains with a particular "affinity" for these cells. Since completion of the work reported here, eleven of the rhinoviruses isolated have been further studied at the Common Cold Research Unit and submitted to Dr. Hamparian, Ohio State University, as possible new rhinovirus serotypes. The discovery that neutralizing antibody titres against RS virus were increased by the addition of unheated serum had applications both to the estimation of antibody in human sera and to the investigation of antigenic variation among RS virus strains.

The respiratory viruses were sought in three main types of respiratory disease: acute respiratory illnesses of adults and children at home or at

work; acute respiratory episodes in adults with chronic bronchitis, and acute respiratory illnesses in children in hospital. A large number of viruses were isolated from each of these groups. Although the etiological significance of these agents was not always clear, my results show that rhinovirus infections are more frequent than previous reports suggest.

Most of the epidemiological studies were necessarily collaborative. The early work on respiratory illness in children at home and the final survey of children in hospital were both part of nationwide surveys organised by the Medical Research Council. Dr. M.B. Eadie assessed the clinical state of the chronic bronchitics, collected all their specimens and made the final clinical diagnoses of their respiratory episodes. Dr. Eadie also collected the specimens from the matched groups of children in hospital. I depended on general practitioners, hospital clinicians or public health officers to collect specimens and make clinical diagnoses of the other patients in this work. Thus, although I sought the assistance and advice of clinical colleagues where this was necessary, I myself examined the specimens and analysed the results to be presented in this thesis.

Although these investigations are inter-related, they have for convenience been described in two parts. Part I is concerned with tissue culture techniques for the isolation and study of respiratory viruses and with their use for detailed studies of rhinoviruses and respiratory syncytial virus. Part II gives the results of applying these techniques to certain aspects of

the epidemiology of these agents. In summary, this work may be described as development and application of techniques for studying the epidemiology of respiratory viruses.

PART I

TISSUE CULTURE TECHNIQUES FOR THE ISOLATION AND STUDY OF RESPIRATORY VIRUSES

CHAPTER I

REVIEW OF THE LITERATURE.

The origins of tissue culture stretch back to the end of the last century. However this technique was not widely used in virology until 1950, after the introduction of antibiotics which simplified the manipulation of cultures and the discovery that viruses would grow *in vitro* in tissues other than those in which they grew *in vivo*.

Before 1950 the only group of respiratory viruses known were the influenza viruses. In the last 16 years adenoviruses, parainfluenza viruses, respiratory syncytial virus and rhinoviruses have all been isolated in tissue cultures. The recent introduction of organ cultures has already led to the detection of fastidious rhinoviruses not isolated in conventional tissue culture and at least one apparently new type of human respiratory virus.

In this review it is proposed to consider briefly the history of tissue culture and its use in work on respiratory viruses. A subsequent section will cover the types of cells and cultures currently used, the viruses which are isolated in them, the medium and conditions under which they are maintained, and their applications to the study of respiratory viruses.

History of Tissue Culture

The earliest recorded attempts at tissue culture were made at the end of the nineteenth century but the first unequivocal demonstration of the

continuation of function in vitro was made by Harrison in 1907 using frog embryo tissue.

Burrows (1910) introduced the plasma clot technique and Carrell (1912) discovered the growth-promoting effect of embryo extracts. The "traditional" technique of growing tissues in plasma clots supplemented with embryo extract was thus established by 1912. At about the same time vaccinia virus was grown in tissue cultures of rabbit and guinea pig corneas (Steinhardt, Israeli and Lambert, 1913). This was probably the first genuine cultivation of a virus in tissue culture. A different approach to the cultivation of tissues in vitro was initiated by Thomson in 1914 and later developed by Strangeways (1924) and Fell (1951). This organ culture technique was designed to maintain small fragments of tissue in a state as close as possible to their state in vivo.

. The main problem in performing tissue culture at this time was the risk of bacterial contamination. The elaborate surgical techniques which Carrell used engendered the belief that tissue culture was extremely difficult. It is probably for this reason that the early experiments on the growth of tissue culture and cultivation of viruses in them anticipated by 30 or 40 years the widespread use of these methods in virology.

By 1950 only 36 mammalian viruses had definitely been grown in tissue culture and another five possibly grown (Robbins and Enders, 1950). Of these 43 viruses, 25 were human pathogens. In the succeeding 15 years over

150 new human viruses have been recovered in tissue culture, many of them associated with respiratory disease. This rapid progress was largely due to the introduction of non-toxic antibiotics such as penicillin (Rose, Molloy and O'Neill, 1945) and streptomycin (Enders, Weller and Robbins, 1949) into the tissue cultures. Other important factors were the realization by Robbins, Enders and Weller (1950) that a cytopathic effect in tissue culture was an adequate criterion of virus presence and the widespread use of trypsin for the dispersal of cells.

The first viruses to be discovered as a direct result of the widespread use of tissue culture were members of the echovirus group (Robbins, Enders, Weller and Florentino, 1951; Melnick and Agren, 1952) which were isolated in cultures of human or monkey tissue.

In 1953 Rowe and co-workers reported the first isolations of adenoviruses from fragments of human adenoids grown as plasma clot cultures. After prolonged incubation, cytopathic changes were seen in epithelial-like cells of some of the cultures. The croup-associated virus later renamed parainfluenza type 2 was first recovered by Chanock (1956) in rolled cultures of cynomolgus monkey kidney cells; the virus produced large syncytia. In the same year Morris, Blount and Savage (1956) reported the first isolation of respiratory syncytial virus from chimpanzees. This virus produced islands of rounded cells seven days after inoculation into roller cultures of Chang liver cells, a continuous human epithelioid cell line. The following year Chanock, Roizman and Myers (1957) reported the isolation of this virus

from children by inoculation of throat swabs into KB cells, derived from a human epidermoid carcinoma, and noted its characteristic syncytial formation.

In 1957, Vogel and Shelokov described a technique for detecting influenza A viruses in monkey kidney cells. Washed guinea pig cells were found to adsorb to infected cells. This haemadsorption technique was later used in a study of respiratory illness in young children (Chanock et al. 1958) and led directly to the isolation of parainfluenza types 1 and 3 in monkey kidney tissue cultures. Strains of type 1 produced a questionable cytopathic effect on primary isolation, type 3 strains produced none. Parainfluenza type 4 was first isolated by Johnson, Chanock, Cook and Huebner (1960) in monkey kidney tissue cultures and detected by the haemadsorption technique.

In 1960, Tyrrell and Parsons described cytopathic effects produced in tissue cultures of human embryonic kidney and monkey kidney by common cold viruses. These effects were visible only if culture were maintained under carefully controlled conditions. However, some common cold viruses, such as the D.C. strain would not produce cytopathic effects even under these special conditions.

In 1961, Hayflick and Moorhead derived semi-continuous cell strains from various tissues of human embryos and showed that these cell strains were susceptible to rhinoviruses. Hamparian, Ketler and Hilleman (1961b) isolated a large number of rhinoviruses in WI-26 cells, one of Hayflick's

human embryonic lung cell strains. The D.C. virus was later shown to produce cytopathic effects in WI-26 and other human embryonic cell strains (Tyrrell, Bynoe, Buckland and Hayflick,1962).

Organ cultures of human embryonic ciliated respiratory epithelium were shown by Hoorn and Tyrrell (1965) to support the growth of several types of respiratory viruses. When nasal washings from patients with colds were inoculated into organ cultures a number of agents were recovered which had not been detected by normal tissue culture methods. Some of these agents were rhinoviruses and produced cytopathic effects when infective organ culture fluid was inoculated into human embryonic lung cell strains, others could only be detected by inoculating human volunteers (Tyrrell and Bynoe, 1966). Some of the agents which could not be adapted to tissue cultures appear to be fastidious rhinoviruses, such as the H.S. strain (Hoorn and Tyrrell, 1966) others, such as the B.814 strain, are ether-labile and may be myxoviruses or members of a new group of respiratory viruses (Tyrrell and Bynoe, 1965).

Types of Cell and Organ Culture

The types of cultures available may conveniently be grouped into (a) primary or secondary cell cultures, (b) semi-continuous cell strains, (c) continuous cell lines, and (d) organ cultures.

(a) Primary and Secondary Cultures

Rhesus monkey kidney cells or human embryonic kidney cells are the primary or secondary cultures which have been most widely used for the isolation and study of respiratory viruses.

Monkey Kidney. The original isolations of parainfluenza 2 were made in cynomolgus monkey kidney cells rolled in Eagle's medium containing calf serum at 37°C (Chanock, 1956). Parainfluenza types 1, 3 and 4 were first isolated in rhesus monkey kidney cultures rolled in serum-free medium at 37°C (Chanock et al. 1958; Johnson et al. 1960). Although the original isolations of influenza viruses were made in animals or eggs it has been shown (Report of W.H.O. Expert Committee, 1959) that influenza B may be isolated more frequently in rhesus monkey kidney cultures than in eggs. Early attempts to isolate influenza A2 viruses in monkey kidney cells gave conflicting results (Hamre, 1963) but Stern and Tippett (1963) produced evidence that influenza A viruses could be isolated as often in rhesus monkey tissue culture as in eggs provided the cultures were rolled at 33°C in medium 199. Influenza C may also be isolated in cultures under similar conditions (Mogabgab, 1962).

Some strains of influenza and most of the parainfluenza types fail to produce cytopathic effects in tissue cultures. Their presence may be detected by the haemadsorption test with guinea pig or human group '0' erythrocytes. Most infected cultures show strong haemadsorption after

incubation with erythrocytes at 4°C but cultures infected with parainfluenza type 4 require temperatures of 25 - 37°C to produce maximal patterns. Thus, most naturally occurring human myxoviruses may be isolated in monkey kidney cultures and detected by haemadsorption provided the cultures are maintained under the appropriate conditions. Myxoviruses, other than certain strains of influenza, grow as well at 33°C as at 37°C. Serum is omitted from the culture medium because it is likely to contain specific or non-specific inhibitors for myxoviruses. Rolling the cultures enhances the growth of parainfluenza 4 virus (Canchola et al. 1964). Hence, the optimal conditions for the isolation of most myxoviruses in monkey kidney tissue cultures are a temperature of 33°C, a medium free of serum and rotation of the cultures.

The species of monkey from which the kidney cells are derived may be important. Although parainfluenza types 1 - 3 are recovered equally often in kidney cells from rhesus and cercopithicus monkeys, the rhesus monkey cells are more sensitive for the recovery of parainfluenza 4 and influenza types A2 and B (Canchola et al. 1965). Primary or secondary monkey kidney cells are equally sensitive to parainfluenza types 1, 2 and 3 (Beem et al. 1965).

Tissue cultures have almost superceded eggs and animals for the isolation of influenza and parainfluenza viruses. Eggs and animals are cumbersome to use and are much less sensitive than monkey kidney cells to parainfluenza viruses. Monkey kidney cells have not been

compared adequately with other cell types but the available evidence suggests that they are the most sensitive culture system for influenza and parainfluenza virus isolation (Chanock et al. 1963; Holzel et al. 1965a).

Many other viruses may be recovered from respiratory specimens in monkey kidney tissue culture. RS virus is frequently isolated and some workers have reported that monkey kidney cells are more sensitive than HEp-2cells for recovery of this virus (Wulff, Kidd and Wenner, 1964b). Rhinovirus M types are isolated in monkey kidney cells but less frequently than in human embryonic cells; echovirus 28 causes degeneration more rapidly in secondary than in primary cultures (Mogabgab and Holmes, 1961). Adenoviruses are isolated in monkey kidney cells at 33°C in serum-free medium but less frequently than in continuous cell lines or primary human embryonic tissue (Holzel et al. 1965a; Pal, McQuillan and Gardner, 1963). Echoviruses and coxsackie group B viruses are recovered in monkey kidney cells more often than other cell types used in respiratory virus work (Pal et al. 1963) and it is possible that higher isolation rates might be achieved if cultures were held at 36°C rather than 33°C.

Many viruses are most conveniently identified in monkey kidney tissue cultures. Fluids from cultures infected with influenza viruses usually contain enough complement-fixing antigen and haemagglutinin for the virus type and strain to be determined by CF and HI tests with specific antiserum. Parainfluenza viruses do not usually produce high titres of haemagglutinin in

tissue cultures and are identified either by haemadsorption-inhibition which is quick but wasteful of antiserum or by neutralization which is slower but requires less antiserum. Most enteroviruses are identified in monkey kidney tissue cultures by neutralization tests with specific antisera.

Primary and secondary monkey kidney cells have other applications in the study of respiratory virus epidemiology. Parainfluenza infections are often diagnosed serologically by CF or HI tests using complement-fixing antigens and haemagglutinins prepared in monkey kidney cells. Neutralizing antibodies against influenza and parainfluenza viruses are most conveniently detected in monkey kidney tissue cultures. These cultures are also used to prepare parainfluenza antigens which are inoculated into animals to prepare specific hyperimmune antisera.

Primary and secondary monkey kidney cells have one main disadvantage. They frequently contain contaminating simian viruses. Some of these, such as foamy agent, cause premature degeneration of the cultures. Infection with others, SV-5 and SV-41 causes haemadsorption and renders the cultures useless for the detection of influenza and parainfluenza viruses. Non-specific, non-viral haemadsorption may also occur in kidney cultures (Dowdle and Robinson, 1966). Latent infections in the cultures may also interfere with viruses subsequently inoculated and vary their sensitivity for virus isolation.

Human Embryonic Kidney. Rhinoviruses of the H type were first shown to cause cytopathic effects in primary cultures of human embryonic kidney (Tyrrell and Parsons, 1960). These effects were only seen if cultures were rolled at 33°C in medium with a low bicarbonate concentration. These cells have since been widely used for the isolation of rhinoviruses. Hamre (1963) has shown that secondary human embryonic kidney cells are more sensitive than a strain of diploid human lung cells for the isolation of rhinoviruses but she subsequently found that WI-38 cells were more sensitive than secondary human embryonic kidney cells (Hamre, 1964). It appears that certain rhinoviruses can be isolated in human embryonic kidney cells but not in lung cells and others can be isolated only in lung cells (Tyrrell, et al. 1962; Higgins, 1966b).

Parainfluenza types 1, 2 and 3 can be isolated in primary human embryonic kidney cultures (Chanock et al. 1963). Adenoviruses are frequently isolated in these cultures (Vargosko et al. 1964, Bell et al. 1960). Human embryonic kidney cells are also susceptible to enteroviruses.

Neutralizing antibodies against rhinoviruses have been measured in human embryonic kidney cells by a microplaque-reduction test (Taylor-Robinson and Tyrrell, 1962) but the end-point method in human embryonic fibroblasts is now more generally used (Taylor-Robinson et al. 1963).

Primary and secondary embryonic kidney cells have several disadvantages. It is usually difficult to obtain adequate and regular supplies of tissue. Tissue from different embryos may vary considerably in rhinovirus sensitivity (Johnson et al. 1962a). Human embryonic tissues may be chronically infected with viruses particularly rubella virus (Selzer, 1963; Kay et al. 1964; Goffe, 1965). The cytopathic effect of viruses is often transitory in primary and secondary human embryonic kidney cells; this has been observed with rhinoviruses (Tyrrell and Parsons, 1960) and enteroviruses (Likar, 1961).

(b) Semi-Continuous Cell Strains

A cell strain is defined by Hayflick and Moorhead (1961) as "a population of cells derived from animal tissue, subcultivated more than once in vitro, and lacking the property of indefinite serial passage while preserving the chromosomal karyotype characterizing the tissue of origin". This definition does not distinguish between secondary cultures and cell strains, although most cell strains may be carried through 25 - 50 passages and are therefore quite distinct from secondary cultures.

Human Embryonic Lung Fibroblasts. Numerous rhinoviruses were recovered in the human embryonic lung cell strain, WI-26 by Hamparian and colleagues (1961b). This cell strain and its successor, WI-38 have since been used extensively. Rhinovirus D.C., which had consistently failed to grow in primary human embryonic kidney, was readily isolated in WI-38 cells (Tyrrell et al. 1962) thus confirming the original observation

of Andrewes and colleagues (1953) that the virus would grow in human embryonic lung cells. In comparative studies rhinoviruses are isolated more often in WI-38 cells than in primary or secondary human embryonic kidney cells (Hamre, 1964; Higgins, 1966b). The conditions under which the human embryonic lung cells are maintained must be carefully controlled and include rolling at 33°C in medium of pH 6.8 to 7.4, if rhinoviruses are to be isolated. Different lung cell strains vary in their sensitivity to poliovirus (Hayflick and Moorhead, 1961), influenza A virus (Kilbourne, Sigiura and Wong, 1964) and rhinoviruses (Tyrrell et al. 1962; Brown and Tyrrell, 1964). A cell strain may be resistant to rhinovirus infection if it is chronically infected with rubella virus (Goffe, 1965). Any cell strain should therefore be carefully tested for sensitivity before being used for virus isolation.

Human embryonic lung cell strains are sensitive to laboratory strains of a wide range of viruses (Hayflick and Moorhead, 1961). Primary isolation of rhinoviruses and herpes simplex virus is more frequent in WI-38 cells than in primary human embryonic kidney cells; but WI-38 cells are less efficient for the isolation of adenoviruses, enteroviruses and myxoviruses (Higgins, 1966b). There is one report indicating that RS virus may be isolated as frequently in WI-38 cells as in HEp-2 cells (Anderson and Beem, 1966). Human embryonic cell strains are also used for identifying respiratory viruses, titrating rhinoviruses and their neutralizing antibody and for

preparing rhinovirus antigens. Rhinoviruses are identified by neutralization tests with specific antisera or antiserum pools either in tubes or in modified microtitre plates (Gwaltney, 1966). Respiratory syncytial virus has been identified in human fetal diploid cells by an immunofluorescent staining method (Schieble, Lennette and Kase, 1965). Rhinovirus neutralizing antibody is estimated by the microplaque-reduction test or tube neutralization test; the latter is almost as sensitive if small quantities of virus are used (Taylor-Robinson et al. 1963). Macroplaques are produced by rhinoviruses in human embryonic lung cells (Porterfield, 1962; Kisch, Webb and Johnson, 1964; Haff, Wohlsen, Force and Stewart, 1966) but this technique has not been widely used. Hyperimmune rhinovirus antisera have been obtained from animals immunized with antigens prepared in WI-38 cells (Fenters, Gillum, Holper and Marquis, 1966). In some cases lung fibroblasts were grown as a monolayer around the entire inner circumference of roller bottles (Hamre, Connelly and Procknow, 1964).

Human embryonic kidney Fibroblasts. Hayflick and Moorhead (1961) reported that rhinoviruses would grow in human embryonic kidney cell strains; but they have not been used for rhinovirus isolation. They are slightly less sensitive than primary or secondary monkey kidney cells for the isolation of enteroviruses but more sensitive to adenoviruses (Schmidt, Ho and Lennette, 1965).

Human embryonic kidney strains are not as easy to derive from the primary cells as lung strains because after 6 - 9 passages the morphological appearance of the cell monolayer changes from epthelioid to fibroblastic. Cell strains may not always survive this critical stage.

Human Aorta Cells. Four cell strains have been derived from human atheromatous lesions and carried through 30 passages (Behbehani, Melnick and DeBakey, 1965). One of these, A-39, has been compared with WI-38 and human embryonic kidney cells for the isolation of rhinoviruses (Phillips, Melnick and Grim, 1965a). Rhinoviruses were isolated from similar numbers of specimens in each of the cell types but not always from the same specimens. More rhinoviruses were isolated in WI-38 and A-39 cells than in human embryonic kidney cells during two periods but during a third period more were isolated in kidney cells. The human aorta cells also appear to be sensitive to myxoviruses, adenoviruses and enteroviruses.

Human embryonic cell strains retain the wide range of virus susceptibility of primary and secondary cells. They are usually capable of 30 - 50 serial passages. Thus, vast quantities of cells may be obtained from a single embryo. Early passages of a cell strain may be frozen and its virus sensitivity thoroughly tested before use. Cell strains, therefore, overcome the problems of shortage of fetal material and variation in virus sensitivity between embryos and yet retain most of the range of virus susceptibility of primary cells.

(c) Continuous Cell Lines

A cell line is defined by Hayflick and Moorhead (1961) as "a population of cells derived from animal tissue and grown in vitro by serial subcultivations for indefinite periods of time with a departure from the chromosome number characterizing its source". A recent publication of the Cell Culture Collection Committee (1965) lists over 40 such cell lines or clones of cell lines and there are probably many more not yet properly characterised. However, only two cell lines have been widely used in respiratory virus work: HeLa cells, originally derived by Gey, Coffman and Kubicek (1952) from a carcinoma of the human cervix and HEp-2 cells, originally derived from a carcinoma of the human larynx (Moore, Sabachewsky and Toolan, 1955).

HeLa Cells Respiratory syncytial virus has been isolated in HeLa cells which were grown and maintained in medium containing horse serum (Hamparian et al. 1961a) and in HeLa cells grown and maintained in medium containing rabbit serum (Peacock and Clarke, 1961). The latter cells have become known as 'Bristol' HeLa cells and been widely used in Britain.

The isolation of respiratory syncytial virus does not appear to require rigidly controlled conditions of culture. The virus has been isolated in either stationary or rolled cultures maintained at 33°C or 37°C. Some reports suggest that freezing specimens before inoculation into tissue cultures inactivates respiratory syncytial virus but the virus has been recovered from a high proportion of selected specimens which had been frozen in dry ice in

glass sealed ampoules and thawed twice (Hamparian et al. 1961a). Cytopathic changes may not develop if the medium of inoculated cultures is not changed every 3 - 4 days (Yabrov, Golubev and Smorodintsev, 1964). Parainfluenza viruses, herpes virus, adenoviruses and enteroviruses may also be isolated in HeLa cells. Parainfluenza viruses and most enteroviruses are less frequently isolated in HeLa than in primary or secondary monkey kidney cells (Holzel et al. 1965a; Kelly and Sanderson, 1962; Pal et al. 1963). Adenoviruses and herpes simplex virus are isolated more often in HeLa than in monkey kidney cells (Pal et al. 1963: Holzel et al. 1965a).

HeLa cells are used in other aspects of respiratory virus work. Respiratory syncytial virus is identified either by complement fixation using antigen grown in HeLa cells or by neutralization in HeLa cells. Adenoviruses grown in HeLa cells may be identified by complement fixation and then by neutralization in HeLa cells or by HI. Respiratory syncytial virus and adenovirus infections are often diagnosed serologically by complement fixation tests using antigens prepared in HeLa cells. Neutralizing antibodies against RS virus and adenoviruses are measured in HeLa cell cultures. Antisera against RS virus, adenoviruses and rhinoviruses have been prepared in animals immunized with virus grown in HeLa cells (Doggett and Taylor-Robinson, 1965; Rosen, 1960; Ketler, Hamparian and Hilleman, 1962). Rhinoviruses form macroplaques under agar provided a sensitive line of HeLa cells and appropriate conditions are used (Fiala and Kenny, 1966).

HEp-2 Cells Most of the viruses isolated and grown in HeLa cells may also be isolated and grown in HEp-2 cells. In the USA, RS virus is most commonly isolated in HEp-2 cells. In one comparative study, adenoviruses were recovered equally often in HEp-2 and HeLa cells (Pal et al. 1963).

Other Continous Cell Lines. KB cells, derived from human epidermoid carcinoma of the mouth (Eagle, 1955) have been used to isolate RS virus (Chanock et al. 1957) and to prepare high titred pools of rhinoviruses (Johnson and Rosen, 1963).

The original isolations of RS virus were made in Chang liver cells (Morris et al. 1956).

Cells of continuous lines multiply rapidly and, therefore, may be produced in large quantities. However, HeLa cells do not all have the same virus sensitivity; some are sensitive for rhinovirus plaque production others are not (Fiala and Kenny, 1966). "Bristol" HeLa cells become less susceptible to RS virus if they are grown in calf serum (Tyrrell, 1963). HeLa cells resistant to echoviruses have also been reported (Nakano, 1959). HeLa cells grown in serum containing poliovirus inhibitors are less sensitive to poliovirus even after serum has been removed by repeated washing (Tomlinson, Davies, and Madigan, 1964).

(d) Organ Cultures

"The principle object of organ cultures is to retain the various constituents of a tissue in normal relationship to each other and functioning in a normal way, while they are outside the body in an artificial culture medium" (Hoorn and Tyrrell, 1965). This technique has been applied to the study of respiratory diseases and has recently led to the discovery of new viral agents.

Rhinoviruses may be isolated in organ cultures of human embryonic trachea, from specimens which yield no virus in tissue cultures of semi-continuous human embryonic lung cell strains or primary human embryonic kidney cells (Tyrrell and Bynoe, 1966; Higgins, 1966c). A new type of virus, B814, has been isolated in organ cultures from nasal washings obtained from a boy with a common cold (Tyrrell and Bynoe, 1965). This virus is ether-labile and morphologically similar to a virus isolated from human respiratory disease in Chicago (Hamre and Procknow, 1966) and to avian infectious bronchitis virus (Almeida and Tyrrell, 1967).

Organ culture appears to be a highly sensitive system for the isolation of respiratory viruses. This technique may also be useful to study the pathogenesis of respiratory viruses since it supplies conditions similar to those found in vivo. However, fetal tissue is not always readily available and the growth of some viruses in organ cultures is only detected by inoculation of adult human volunteers. These two factors are limitations

on the use of the technique in diagnostic virology.

Tissue culture is now the essential foundation upon which all study of the epidemiology of respiratory viruses is built. The introduction of new types of cells, cultural conditions and techniques has led to the discovery of new types of respiratory viruses. The importance of tissue culture to the understanding of respiratory disease is emphasized by the rapid progress which has been made since the introduction of this technique. In 1951 the causes of severe respiratory illness in children were virtually unknown; it is now possible to diagnose a causal agent in over half of these cases. Fifteen years ago the aetiology of the common cold could only be studied in human volunteers; it is now possible to culture viruses in vitro from 75% of such illnesses. The isolation and in vitro cultivation of causal agents is the first step towards an understanding of the complex epidemiology of respiratory disease.

CHAPTER 2

MATERIALS AND METHODS

Most of the materials used in this work were obtained from commercial sources and are similar to those used in many virus laboratories using tissue cultures. I shall not therefore describe their preparation in detail but will indicate their composition and important aspects of their preparation and storage.

The methods I have used for the preparation of monkey kidney and HeLa tissue cultures are those in use at the Regional Virus Laboratory, Ruchill Hospital, Glasgow. The cultivation and handling of human embryonic tissue cultures are not described here as they will be considered in detail in Chapter 3. Techniques for the collection, preparation and testing of clinical specimens for virus isolation were initially similar to those seen at the Common Cold Research Unit, Salisbury in 1961. They were, however, modified in the light of experience and experiments which I shall describe in this Chapter and in Chapters 4 and 6. Methods used for the identification and titration of viruses and for neutralization, haemagglutination and haemagglutination-inhibition tests will be described in this Chapter. Certain aspects of identification and neutralization tests which relate specifically to rhinoviruses and RS virus will be described in Chapters 4 and 6 respectively.

Materials

The composition of the various balanced salt solutions and tissue culture media are shown in Tables 2.1, 2.2 and 2.3.

Phosphate Buffered Saline (PBS)

Obtained from Oxoid Ltd. and prepared according to manufacturer's instructions. 5 ml of 0.4% phenol red solution added per litre. Autoclaved at 10 lbs/in^2 for 15 min and stored at room temperature.

Hanks' Balanced Salt Solution (BSS)

Obtained from Oxoid Ltd. and prepared according to manufacturer's instructions. Autoclaved at 10 lbs/in^2 for 15 min and stored at 4^{o}C. Before use pH adjusted as required with 4.4% sodium bicarbonate solution.

Lactalbumen Hanks' Solution (LH)

Prepared as BSS, with lactalbumen hydrolysate (5 g/litre) added before sterilization.

Tryptose Phosphate Broth (TPB)

Obtained from Difco Laboratories and prepared according to manufacturer's instructions. Autoclaved at 10 lbs/in^2 for 15 min and stored at 4^{o}C.

Eagle's Minimum Essential Medium (MEM)

Obtained as 10 times concentrate without sodium bicarbonate from Burroughs Wellcome Ltd. Before use pH adjusted as required with 4.4% sodium bicarbonate solution.

Eagle's Basal Medium (BM)

Obtained from Flow Laboratories Ltd. as a series of stock concentrates and made up according to manufacturer's instructions. Glutamine added immediately before use, and pH adjusted as required with 4.4% sodium bicarbonate solution.

Parker's Medium 199

Obtained from Glaxo Laboratories Ltd. as a 10 times concentrate containing sodium bicarbonate (8.8 g/litre) and antibiotics (2000 units/ml penicillin, 1000 µg/ml streptomycin).

Sodium Bicarbonate Solution

Sodium bicarbonate		4.4 g
Phenol red solution (0.4%)		0.5 ml
Deionized water	to	100 ml

Saturated with carbon dioxide until orange. Containers tightly sealed and autoclaved at 10 lbs/in^2 for 15 min. Stored at 4°C.

Sodium bicarbonate solution (4.4%) obtained from Burroughs Wellcome Ltd. was used to prepare media for human embryonic cultures.

Penicillin and Streptomycin Solution (PSS)

'Crystamycin' (Glaxo Laboratories Ltd.)		0.5 g
Hanks' BSS	to	50 ml

Prepared aseptically. Stock solution, containing 10,000 units/ml penicillin and 10,000 µg/ml streptomycin, stored at -20°C.

Mycostatin Solution

'Nystatin' (E.R. Squibb)	500,000 units
Hanks' BSS	to 200 ml

Prepared aseptically and stored at -20°C.

Aureomycin Solution

Aureomycin	0.5 g
Sterile Deionized water	to 100 ml

Filtered through asbestos pads previously washed in the solution (1 ml/4 cm^2). Stored at -20°C.

Calf Serum

Pooled blood from several calves allowed to clot overnight at room temperature. Serum separated by centrifugation at 1,500 rev/min for 10 min. Filtered through asbestos pads (Ford's SB pads) previously washed with serum (1 ml/4 cm^2 of filter pad). Stored at -20°C. Incubated at 56°C for 30 min before use in maintenance media.

Sterile calf serum also obtained from Burroughs Wellcome Ltd. and Flow Laboratories Ltd.

Newborn Calf Serum

Blood obtained from individual newborn unsuckled calves. Not pooled. Prepared and stored as for ordinary calf serum.

Rabbit Serum

Initially obtained from individual rabbits by cardiac puncture. Not pooled. Prepared and stored as for calf serum. Incubated at 56°C for

30 min before use. From July 1964, pooled inactivated rabbit serum obtained commercially (Burroughs Wellcome Ltd.)

Fresh Unheated Rabbit Serum

Blood obtained from individual rabbits by cardiac puncture. Not pooled. Allowed to clot for 4 hours at room temperature. Serum separated by centrifugation at 1,500 rev/min for 10 min and stored immediately at -20°C.

Fowl Serum

Blood obtained from individual fowls by cardiac puncture. Not pooled. Serum prepared and stored as for calf serum. Heavy precipitate often developed on thawing the serum for use. Precipitate removed by centrifugation and serum incubated at 56°C for 30 min before addition to medium.

Bovine Plasma Albumen Solution

Bovine plasma albumen (Armour Pharmaceuticals Ltd.)		10 g
Hanks' BSS	to	100 ml

Filtered through asbestos pads previously washed in solution (1 ml/4 cm^2 of filter pad). Stored at 4°C.

Trypsin Solution

Trypsin (Difco 1:250)		1 g
Sodium bicarbonate solution		10 ml
PBS	to	400 ml

Stirred at 37°C for 30 min. Filtered through asbestos pads previously washed with solution (1 ml/4 cm^2 of filter pad). Stored at -20°C.

Trypan Blue Solution

Trypan blue		0.1 g
PBS	to	100 ml

Stored at room temperature.

Neutral Red Solution

Neutral red		0.1 g
Deionized water	to	100 ml

Autoclaved at 10 lbs/in^2 for 15 min. Stored at room temperature.

Diluted 1/80 in tissue culture medium for use.

Buffer Solutions

Phosphate Buffer, 0.5M, pH 7.2

KH_2PO_4		1.94 g
$Na_2HPO_4.2H_2O$		6.20 g
Deionized water	to	100 ml

Phosphate Buffer, 0.1M, pH 7.0

KH_2PO_4		0.53 g
$Na_2HPO_4.2H_2O$		1.05 g
Deionized water	to	100 ml

Sodium Citrate - Citric Acid Buffer, 0.1 M, pH 4.0

Disodium citrate solution

Citric acid (H_2O)		2.1 g
1N NaOH		20 ml
Deionized water	to	100 ml

Buffer prepared by mixing:

Disodium citrate Solution	55.8 ml
O.1N HCl	44.2 ml

Each buffer solution autoclaved at 10 lbs/in^2 for 15 min. pH measured on Radiometer pH 4 meter. Acceptable if within 0.1 of desired pH. Stored at room temperature.

Transport Medium

Bovine Plasma Albumen solution	20 ml
$NaHCO_3$ solution	2 ml
PSS	1 ml
Mycostatin solution	1 ml
Hanks' BSS	76 ml

Prepared aseptically each month. Stored at 4°C.

Virus Stock Strains

RS virus. Randall strain

CH 18537 strain

Parainfluenza type 1 Copenhagen 222 strain

Parainfluenza type 2 ALTB strain

Parainfluenza type 3 Bovine strain

Echovirus 28 JH strain

Rhinovirus HGP

Rhinovirus FEB

Specific Neutralizing Antisera

Antisera against influenza and parainfluenza viruses, RS virus, herpes simplex virus, adenoviruses and echovirus 28, were obtained from Dr. P. Bradstreet, Standards Laboratory, Colindale.

Rhinovirus antisera NIH 1200, NIH 33342, Chicago 164-A and Chicago 137-3 were obtained from Reference Reagents Branch of National Institutes of Health. Dr. C.A. Phillips of Houston supplied Baylor type 3 antisera. All the remaining rhinovirus antisera except those made against Ruchill Strains were obtained from Dr. P.J. Chapple of the Common Cold Research Unit, Salisbury.

Methods

Preparation and Maintenance of Tissue Cultures

Rhesus Monkey Kidney (RMK)

Media	Growth	Maintenance
Eagle's MEM	93 ml	95 ml
Calf serum	5 ml	1 ml
$NaHCO_3$ solution	1 ml	3 ml
PSS	1 ml	1 ml

Primary. A single kidney was aseptically removed from a rhesus monkey. The capsule and medulla were discarded and the cortex finely chopped with scissors. The minced tissue was washed with PBS and then added to 100 ml of trypsin solution. This was agitated by a magnetic

stirrer at 36°C for 30 min. The fluid was then decanted off the minced tissue and replaced by a fresh 100 ml of trypsin solution. This was agitated until the pieces of tissue had disintegrated (about 2 - 3 hours). The kidney cells were centrifuged out of suspension and resuspended in growth medium. This cell suspension was diluted 1/10 in trypan blue solution and the unstained cells were counted in a haemocytometer. The cell suspension was diluted to contain 1.5×10^5 viable cells/ml and dispensed in 0.8 ml volumes into 4 in x ½ in Pyrex test tubes or in 60 ml volumes into Pyrex Roux flasks. A monolayer of cells usually formed in 5 - 7 days. The growth medium was then replaced with maintenance medium.

Secondary. The medium was discarded from a Roux flask of primary RMK and the cell layer covered with 5 ml of 0.25% trypsin solution. After 2 min the trypsin was poured off and the bottle incubated at 36°C until the cells were free from the glass (about 30 min). The cells were resuspended in 5 ml growth medium and this cell suspension diluted 1/10 in trypan blue solution. Unstained cells were counted in a haemocytometer. The viable cell concentration was adjusted to 8×10^4 cells/ml with growth medium and dispensed into tubes. Cells formed a confluent sheet in 2 - 3 days. The cultures were then changed to maintenance medium.

"Bristol" HeLa

Media	Growth	Maintenance
Eagle's MEM	92 ml	93 ml
Rabbit serum	5 ml	-
Fowl serum	-	2 ml
$NaHCO_3$ solution	2 ml	4 ml
PSS	1 ml	1 ml

Two lines of these cells were continuously cultured in 4 oz medical flat bottles or Roux flasks. Both cell lines were reseeded weekly but never on the same day. The medium was replaced by 2 ml of 0.25% trypsin solution. After 2 min on the cell layer the trypsin was decanted and the bottle incubated at $36^{o}C$ until the cells detached from the glass (about 5 - 10 min). Cells were then aspirated in 5 ml of growth medium. The resulting suspension was diluted 1/10 in trypan blue solution. Unstained cells were counted in a haemocytometer. The cell suspension was then adjusted to 8×10^4 cells per ml and seeded into tubes or further bottles as required.

After two days the tubes were changed to maintenance medium.

Collection and Treatment of Specimens

At the beginning of the study in January 1962 nasal and throat specimens were collected separately. Nasal washings were obtained by dropping 2 ml of transport medium up each nostril of the patient who then tilted the head

forward and expelled the fluid into a sterile glass jar. If the patient was too young to co-operate, nasal specimens were obtained by placing a pledgelet of cotton wool up each nostril in turn and placing this in 2 ml of transport medium. The throat was swabbed with cotton wool on the end of a wooden stick which was immediately broken into 2 ml of transport medium. Cough sputum was collected from chronic bronchitics where possible.

Most specimens were transported to the laboratory within 2 hours but some specimens from general practitioners were held overnight at 4°C. On arrival at the laboratory specimens were immediately treated. The throat swab was squeezed with forceps to express the fluid. The nasal pledgelet was squeezed in a 2 ml disposable syringe. The sputum was diluted approximately 1 in 4 with transport medium and shaken for 5 min with sterile glass beads. The resulting fluids were then centrifued at 3,000 rev/min for 10 min and the supernate constituted the inoculum.

Nasal specimens and sputa were inoculated into primary or secondary RMK cells and, when available, primary HEK cells (Table 2.4). The medium of these cultures was changed every 2 - 3 days to keep the pH between 7.0 and 7.4. Cultures were examined for minimal cytopathic changes every two days and if no such changes occurred within 14 days they were considered negative and discarded.

Throat swab and sputum material was inoculated into primary or secondary RMK which had been washed twice with PBS before changing to Parker's 199 medium (Table 2.4). Cultures were examined microscopically for cytopathic changes every 3 - 4 days. Every 7 days they were tested for haemadsorption: medium was replaced by 0.2 ml of 0.5% human group '0' erthrocytes in 0.85% saline. Cultures were incubated at 4°C for 20 min in the sloped position and examined microscopically. Cells were then washed with PBS, fed with Parker's medium 199 and further incubated at 36°C. RMK cultures were considered negative and discarded if no cytopathic effect or haemadsorption was seen within 3 weeks. Throat swabs and sputa were also inoculated into BH cultures (Table 2.4). Every 2 - 3 days these were examined microscopically and changed to fresh medium to keep the pH above 7.4. If no cytopathic effects were seen within 3 weeks, the culture medium was replaced by fresh medium containing 1/80,000 neutral red. After incubation at 36°C overnight cultures were stained and examined for syncytia which might have been overlooked. Cultures were considered negative and discarded if no syncytia were present.

Each specimen was inoculated into two tubes (0.2 ml per tube) of each type of tissue culture and for each specimen one tube of each tissue was inoculated with transport medium (0.2 ml per tube) as control. Test and control cultures were treated in the same way throughout. Excess specimen material was stored at -70°C in a glass sealed ampoule.

If cultures degenerated through age or toxicity before the observation

period was complete, the cells were scraped off the tube with a pasteur pipette and approximately 0.2 ml of cells and tissue culture fluid transferred to fresh cultures without prior freezing. Cultures showing cytopathic effect or haemadsorption were passed to additional cultures to provide a virus pool which was supplemented with 1% bovine plasma albumin if in serum-free medium. Virus pools were divided into aliquots of about 0.5 ml in glass sealed ampoules and stored at -70°C until required for identification.

Modifications to Collection and Treatment of Specimens

At the beginning of August 1962 a cell strain, RHEL-7 was derived from human embryonic lung (Chapter 3). Since primary HEK cells were so scarce that less than 12% of specimens had been inoculated into them, they were replaced by RHEL-7 cells from the end of August 1962. RHEL-7 cells were replaced by WI-38 cells from March to June 1963, by RHEKF-13 from July 1963 to August 1964 and by RHEKF-30 or RHEKF-35 from September 1964. The reasons for these changes will be discussed in Chapters 3 and 4.

From September 1963, throat, nose and sputum specimens were each inoculated into three different tissues under appropriate conditions (Table 2.5).

Although throat, nose and sputum specimens were tested separately, different viruses were never isolated from different specimens from the same individual. Therefore, from May 1964 throat and nose specimens were placed in the same bottle of transport medium and examined as one

specimen, except during studies on chronic bronchtics when it was desirable to know which part of the respiratory tract yielded the virus.

After April 1964, specimens were not centrifuged but 100 units penicillin, 100 μg streptomycin and 50 units mycostatin were added to each tube inoculated with clinical material for reasons described in Chapter 6.

From January 1965, adults with upper respiratory tract infection were asked to 'blow' nasal secretions into a small polythene bag. This specimen replaced nasal washings except where there was little or no rhinitis. Glass beads and medium from the throat swab were added to the polythene bag and shaken to provide the tissue culture inoculum.

Identification of Viruses

Viruses isolated from clinical material could often be partially identified by the cytopathic effects they caused and the type of tissue cultures in which these effects were seen. The identity of such viruses was confirmed by neutralization or complement fixation tests with specific antisera. This was not always possible with rhinoviruses and a virus was classified as a member of this group if it produced an enterovirus-like cytopathic effect in human embryonic cultures rolled at 33°C. in medium of low pH, was chloroform-stable and acid-labile.

Cytopathic Effects

Rhesus Monkey Kidney Cultures. The appearance of small areas of shrunken rounded cells which rapidly spread to involve the whole cell layer suggested

enterovirus or, rarely, rhinovirus infection. Swelling and clumping of the cells was characteristic of adenovirus infection. Influenza viruses caused cells to round off but often remain attached to each other by thin cytoplasmic strands; this effect was usually seen within a week of inoculation and such cultures strongly haemadsorbed human group "0" erythrocytes. Parainfluenza viruses caused no cytopathic effect on first passage but infected cultures showed haemadsorption. RMK cultures infected with RS virus gave a stringy appearance which developed slowly from the edges of the cell layer to involve the whole culture after about two weeks.

Bristol HeLa Cultures. Enteroviruses and herpes simplex virus caused shrinkage and rounding of BH cells, but the herpes virus grew more readily in human embryonic tissue in which it could be distinguished from enteroviruses. The appearance of large giant cells usually indicated the presence of RS virus, and cell clumping and rapid detachment from the glass was characteristic of adenoviruses.

Human Embryonic Fibroblast Cultures. Rhinoviruses and enteroviruses caused human embryonic fibroblasts to round up. Herpes simplex caused swelling and separation of the cells which remained joined only by thin cytoplasmic threads. Englargement of the cells accompanied by clumping indicated adenovirus infection. The appearance of giant cells, usually at the edges of the layer, was characteristic of RS virus infection.

Neutralization tests

SErum and virus in 0.1 ml volumes were mixed in WHO agglutination trays and held at room temperature for 1½ hours before inoculation into tissue cultures. Antiserum was used at the recommended dilution if obtained from other centres and diluted to contain 20 antibody units/0.1 ml (Committee on the Enteroviruses, 1957) if prepared at Ruchill. Virus was used at two dilutions estimated to contain 10 and 100 TCD_{50}/0.1 ml, except for rhinoviruses which were used at one dilution and the test read when a parallel virus titration showed that 32 - 100 TCD_{50} were present.

Viruses which produced characteristic giant cells in BH cells were tested for neutralization by RS virus antiserum.

Haemadsorbing viruses were tested for neutralization with antisera against parainfluenza types 1-3 and simian virus type 5. If cytopathic effects were present, antisera against influenza A and B were also included in the test. Some strains of influenza gave equivocal results in such tests and were identified by Dr. C.A.C. Ross in complement fixation tests against human convalescent sera.

Viruses producing cytopathic effects characteristic of herpes were tested for neutralization by specific antiserum.

Agents suspected of being adenoviruses were tested for neutralization by antisera against adenovirus types 1-7, 15 and 21.

Enteroviruses were tested against antisera to polioviruses 1-3, coxsackievirus A9, coxsackieviruses B1-6, echoviruses 1-9, and 11-27.

Rhinoviruses were tested against antisera to:

echovirus 28

Salisbury strains:	HGP, B632, FEB, Norman, Thompson, 16/60.
West Point strains:	1, 68, 181, 204, 5986, MRH.
NIH strains:	353, 363, 1059, 1734, 11757, 33342, 1200.
Chicago strains:	106F, 140F, 179E, 127-1, 164A, 137-3.
Baylor strain:	Baylor 3.
Ruchill strains:	409-62, 1321-62, 4574-62, 4704-62, 130-63, 1647-63, 1833-63, 4462-63, 1376-64, 1192-65, 1767-65, 2030-65, 4411-65.

Chloroform Sensitivity

The method used was similar to that described by Feldman and Wang (1961). 1 ml of virus was shaken for 5 min with 0.1 ml of chloroform and then centrifuged at 1000 rev/min for 5 min. A control sample of virus was treated similarly except that chloroform was replaced by PBS. The supernates after centrifugation were then inoculated into tissue cultures which were observed for the appearance of cytopathic effects. When testing viruses in this way a known chloroform-sensitive virus (herpes simplex) was always included as a positive control. If cytopathic effects appeared at the same time in cultures inoculated with control and chloroform-treated material the virus was considered to be stable to chloroform. Herpes simplex virus was always completely inactivated by chloroform.

Acid Sensitivity

The method used was based on that described by Tyrrell and Chanock (1963). 0.2 ml of virus was added to (a) 0.2 ml of 0.1 M citrate buffer, pH 4, and (b) 0.2 ml of 0.1 M phosphate buffer, pH 7, both in ¼ oz. bottles. After shaking, the bottles were incubated at 36°C for 1 hour and then 0.4 ml of 0.5 M phosphate buffer, pH 7.2, and 3.2 ml of tissue culture medium were added to each bottle. The contents of each bottle were then titrated in tissue cultures using tenfold dilutions and two tubes per dilution. If incubation at pH 4 caused a drop in titre of an hundredfold or more the virus was considered to be acid-labile. When testing for this property a known acid-labile virus (rhinovirus FEB) and a known acid-stable virus (echovirus 7) were always included as controls.

Virus Titrations

Tenfold dilutions of virus were made in PBS or maintenance medium and 0.1 ml inoculated per tissue culture tube. Three to six tubes were used per dilution except in titrations for acid lability of rhinoviruses where only two tubes were used. Titrations were read after 7 days and the TCD_{50} calculated by the method of Kärber.

RS virus titrations were changed to medium containing 1/80,000 neutral red after 6 days.

Rhinoviruses were sometimes estimated by microplaque counts made 60 - 65 hours after inoculation. A microplaque was defined as a discreet

lesion containing at least six rounded cells. Counts from a number of tubes were averaged to give the number of microplaque-forming units (mfu) per 0.1 ml.

Neutralizing Antibody Titrations

Twofold (for rhinovirus and early RS virus antibody titrations) or fourfold (for later RS virus antibody titrations) dilutions of sera were made in 0.25 ml amounts in PBS in WHO agglutination trays. 0.25 ml of virus diluted to contain between 10 and 68 TCD_{50}/0.1 ml (for rhinovirus) or 32 and 100 TCD_{50}/0.1 ml (for RS virus) were added to each serum dilution. After 1½ hours at room temperature 0.2 ml of each serum-virus mixture was inoculated into each of two tubes of tissue culture. The dilution of virus used was diluted tenfold and hundredfold and 0.1 ml of each dilution inoculated into three tissue cultures to estimate the amount of virus used.

The BH cultures inoculated with RS virus antibody titrations were changed to fresh medium containing 1/80,000 neutral red after 3 days and examined the following day.

Human embryonic cells inoculated with rhinovirus titrations were examined when the virus titration showed 10-32 TCD_{50} of virus had been used, usually after 3 days.

End-points were calculated by the method of Kärber and neutralizing antibody titres are expressed as the reciprocal of the dilution causing complete neutralization in 50% of the tubes inoculated.

Haemagglutination Tests

Haemagglutination tests for rhinoviruses were done in WHO agglutination trays using 0.3 ml volumes of tissue culture fluids and 1% suspensions of erythrocytes. The blood cells were collected in acid-citrate dextrose. They were washed 3 times and resuspended in isotonic (0.85%) saline not more than 24 hours before use. Tests were read by the pattern method after incubation for 1 hour.

Haemagglutination-Inhibition Tests

The microtitre system was used for haemagglutination-inhibition tests against parainfluenza viruses.

Haemagglutinin was prepared in tissue cultures of secondary RMK rolled at 33°C in Parker's medium 199. The tissue culture fluid was harvested when the haemagglutinin titre had reached the maximum, centrifuged at 3,000 rev/min and stored in 1 ml amounts at -70°C.

Preliminary titration of haemagglutinin against human group '0', guinea pig and fowl erythrocytes at 4°C, 18°C and 36°C (Table 2.6) indicated that guinea pig cells were the most suitable and that tests for parainfluenza types 1 and 2 could be done at room temperature but for type 3 higher titres were obtained at 36°C. Haemagglutination-inhibition tests were therefore done using guinea pig cells at room temperature for types 1 and 2 and at 36°C for type 3.

Haemagglutinins were titrated on the day of each test and diluted in isotonic saline (0.85%) to contain 4 haemagglutinating units. Sera were treated with receptor-destroying enzyme (Burroughs Wellcome Ltd.) according to manufacturers instructions: 0.1 ml of serum was added to 0.4 ml of enzyme and incubated at 36°C for 16 hours; the mixture was then heated at 56°C for 1 hour. Twofold dilutions of enzyme-treated sera were made from 1/10 to 1/640 in isotonic saline for each virus and 1/10 dilutions for controls. Haemagglutinin was then added to each serum dilution and saline to the serum controls. Haemagglutinin was again titrated to confirm that 4 units had been used in the test. After 1 hour at room temperature 1% guinea pig erythrocytes were added and the parainfluenza type 3 tests incubated at 37°C. After 2 hours the tests were read by the pattern method and the titre taken as the highest dilution of serum which caused at least 50% inhibition of haemagglutination.

Sera which caused non-specific haemagglutination in the serum controls were absorbed with guinea pig erythrocytes: 0.4 ml of enzyme-treated serum was added to 0.1 ml of packed erythrocytes and held at 4°C for 2 hours. After centrifugation the serum was removed and retested.

CHAPTER 3

PREPARATION AND PRESERVATION OF HUMAN EMBRYONIC TISSUE CULTURES

Successful isolation of respiratory viruses requires the use of three types of tissue cultures: monkey kidney cells, HEp-2 or HeLa cells and human embryonic cells. When I began this work, the former two types of cultures were routinely used in the laboratory but there was no method in use at Ruchill Hospital for the preparation of human embryonic cell cultures.

Initially, I used primary human embryonic kidney cells prepared according to a method I had observed at the Common Cold Research Unit. However, this tissue had serious disadvantages: the local supply of human tissue was insufficient; cells multiplied so rapidly that, even if inoculated when semi-confluent, cultures were virtually unreadable within 5-8 days due to the formation of large aggregates of cells; the cytopathic effect of rhinoviruses could easily be missed if cultures were not carefully examined each day; kidney cells from different embryos varied in their sensitivity to rhinoviruses (Johnson et al. 1962a). I therefore attempted to derive semi-continuous cell strains from human embryonic kidney and lung tissue by the methods of Hayflick and Moorhead (1961). Considerable difficulties were encountered because these cell strains, particularly those derived from the kidney, appeared to be fastidious in their growth requirements. At various times toxicity in calf serum, medium and glassware were thought to be responsible

for inhibition of cell growth. The preservation of cells from early passages was essential if a semi-continuous cell strain was to be used over a long period. I found that certain modifications of the published method (Hayflick and Moorhead, 1961) improved cell viability.

Preparation of Primary Human Embryonic Tissue Cultures

Human foetuses from therapeutic abortions performed between the 8th and 24th week of gestation were collected in Hanks' BSS and immediately transported to the laboratory. Kidneys and lungs were removed aseptically, washed twice in PBS and chopped finely with scissors. Material from Inverness was received 24-48 hours after removal, as chopped tissue in LH containing 20% human serum. Chopped tissue from each organ was repeatedly washed in PBS until the supernate was clear. Tissue was incubated with ten times its volume of trypsin solution at 36°C with occasional shaking until tissue fragments had been dispersed (1-3 hours). Foetal material was incubated with trypsin solution at 4°C overnight if received late at night since this method had been shown to give satisfactory results (Tyrrell, 1961). The resulting cell suspension was filtered through sterile gauze, centrifuged in a graduated conical centrifuge tube at 1,000 rev/min for 10 min and the deposit resuspended in 100 times its volume of growth medium. At various times the following growth media were used:-

1.	LH	87 ml
	Calf serum	10 ml
	$NaHCO_3$ solution	2 ml
	PSS	1 ml
2.	Eagle's MEM	87 ml
	Calf serum	10 ml
	$NaHCO_3$ solution	2 ml
	PSS	1 ml
3.	Parker's medium 199	90 ml
	Inactivated calf serum	10 ml
4.	Eagle's MEM	92 ml
	Newborm calf serum	5 ml
	$NaHCO_3$ solution	2 ml
	PSS	1 ml

The cell suspension was seeded into 4 oz medical flat bottles (10 ml per bottle) or Roux flasks (60 ml per flask) and, in the case of kidney, into 4 in x ½ in tubes (0.8 ml per tube). When kidney cells formed a semi-confluent monolayer tube cultures were changed to maintenance medium:

Eagle's MEM	96 ml
Calf serum	1 ml
$NaHCO_3$ solution	2 ml
PSS	1 ml

Kidney and lung cells normally formed confluent monolayers in bottles in

3 - 10 days and were then reseeded to initiate semi-continuous cell strains.

Cultivation of Semi-Continuous Cell Strains

The medium was decanted from a bottle containing a monolayer of primary kidney or lung cells. The cells were covered with prewarmed trypsin solution (2 ml per 4 oz bottle, 5 ml per Roux flask). After 2 min the trypsin solution was discarded and the bottle incubated at 36°C until the cells were free from the glass (5 - 20 min). The dislodged cells were resuspended in 10 ml of growth medium (as used for primary cultures). Half of the cell suspension was transferred to a second similar bottle. Growth medium was then added to the two bottles (5 ml per 4 oz bottle, 55 ml per Roux flask). The cells from three 4 oz bottles were sufficient to seed a Roux flask. Such 1:2 divisions were repeated at alternate 3 - and 4 - day intervals throughout the life of a cell strain.

Lung cell strains retained a fibroblastic appearance throughout culture. Kidney strains appeared epithelioid in the initial passages. After four to six passages patches of fibroblasts appeared and overgrew the degenerating epithelioid cells. During this change the cells formed monolayers more slowly and it was usually necessary to allow more than 3 or 4 days between passages. After six to nine passages fibroblasts formed the entire monolayers and thereafter the cell strains could be passaged regularly twice a week.

When preparing tubes, 0.8 ml of cell suspension containing 5×10^4 cells per ml was seeded into each tube. After December 1965 consistent growth of embryonic cell strains was achieved and it was possible to seed tubes with cell suspensions obtained from 1 : 2 divisions of bottles without counting the cells. When the cells had formed confluent monolayers (3 - 4 days), tubes were changed to the maintenance medium used for primary embryonic cultures.

Results of Cultivation of Tissue from 36 embryos

The first 12 embryos were used to compare Growth Media 1, 2 and 3. Medium 1 was adequate for the preparation of primary cultures but none of the tissues from Embryos 1 - 5 (Table 3.1) could be passaged more than 4 times in this medium. Therefore, Media 2 and 3 only were used for subsequent embryos. Cell strains could be passaged an equal number of times in either of these media.

Several other observations were made on the serial passage of Embryos 1 - 12. Embryos 8 and 9 which had been stored at $4^{\circ}C$ before arrival at the laboratory yielded primary cultures which were heavily contaminated. Tissue from Embryos 2 and 3 failed to yield viable primary cultures. Only two (Embryos 6 and 7) of the remaining eight embryos yielded cells which could be subcultured more than 13 times. The tissue from both these embryos was grown in medium containing the same batch of calf serum which was used only for cells from Embryos 6, 7, 8 and 9. This suggested

that the quality of calf serum might be critical for successful serial subcultivation. Efforts were therefore made to obtain more satisfactory and consistent supplies of serum. While passaging cells from Embryos 6 and 7, it was noticed that cells formed monolayers more rapidly in the "parent" bottles than in the new bottles. Cell growth in new bottles was improved by soaking bottles in growth medium at 36°C for 30 min before use. This practice was continued throughout further work.

At the beginning of 1963, I was able to obtain supplies of newborn calf serum. This serum in Eagle's MEM (Medium 4) was used for Embryos 13 to 29. Tissues from two embryos (18 and 19) of this group failed to produce primary cultures; one of these had been stored at 4°C overnight before arrival at the laboratory. Cell strains were derived successfully from the lungs of all the remaining 13 embryos from which these organs were obtained. The lung cell strain of Embryo 14 was passaged 56 times before it degenerated. Only two (Embryos 22 and 28) of 13 cell strains derived from kidney tissue degenerated before becoming fibroblastic. Kidney cell strains 13 and 29 were cultured until degeneration occurred after 40 and 36 passages respectively. All cell strains which were frozen were multiplying rapidly and the cells appeared much less "granular" than those from embryos grown in Media 1, 2 or 3. Thus, the use of newborn calf serum resulted in much more satisfactory growth of embryonic cell strains.

Unfortunately, at the end of 1963 the supplies of newborn calf serum stopped abruptly and despite several efforts it was never again possible to obtain adequate quantities.

Before stocks of newborn calf serum were exhausted, batches of calf serum from commercial sources were tested. A batch of unheated serum from Burroughs Wellcome Ltd appeared to give satisfactory results. The entire stock of this batch was reserved and used in Medium 2 for the later passages of Embryo 30 and entirely for Embryos 31 - 36. Cell strains were successfully derived from all the tissues used. One of the two lung and three of the six kidney cell strains kept in continuous culture, degenerated before the 21st passage, suggesting that this medium was deficient and probably inferior to Medium 4.

At the end of 1965, the satisfactory batch of Burroughs Wellcome calf serum was virtually exhausted and none of the subsequent batches tested was satisfactory. Medium and calf serum from the United States (Flow Laboratories) were then used as Medium 5:

Eagle's BM	87 ml
Calf serum	10 ml
$NaHCO_3$ solution	2 ml
PSS	1 ml

This medium appeared to be superior to the Medium 2 which had been used for Embryos 31 - 36, When cells from certain of these embryos

were recovered from frozen stock they could be passaged further in Medium 5 than had been possible in Medium 2.

Cell strains were derived from the kidneys of a total of 20 human embryos. Fibroblasts first formed the entire monolayer in the 6th to 9th passage between 23 and 46 (mean 32) days after the start of the culture and between 3 and 21 (mean 6) days after the previous subculture.

The conditions under which the primary tissue was trypsinized did not appear to affect cell viability. Successful cell lines were derived with similar frequency from embryos 13 - 36 whether tissue was incubated at 36°C for 1 - 3 hours or at 4°C overnight.

Organs which were dissected from the embryos, chopped and suspended in medium within a few hours of the embryo's removal from the mother, were viable at room temperature for up to 3 days. Most of the material from Inverness had been treated in this way. In contrast, organs which were left in the intact embryo at 4°C overnight were either microbially contaminated (Embryos 8 and 9) or produced no viable primary cultures (Embryo 18).

For convenience hereafter cell strains derived at Ruchill Hospital will be prefixed by the letter 'R' and followed by the number of the embryo from which they were derived. Thus, RHEKF-30 is the Ruchill strain of human embryonic kidney fibroblasts derived from Embryo 30.

Preservation of Human Embryonic Cells

Hayflick and Moorhead (1961) preserved cells at -70°C in growth medium containing 10% glycerol. Trypsinized cells from mature cultures were resuspended in a few ml of growth medium and the concentration adjusted to $1.5 - 2.0 \times 10^6$ cells per ml. Sterile glycerol was added to give a final concentration of 10% and the cell suspension dispensed in 2 ml amounts into 5 ml ampoules. The ampoules were sealed, held at 5°C overnight and transferred directly to -70°C the next day. Ampoules were thawed rapidly in a water bath at 37°C. The cell suspension was then transferred to a bottle (40 cm^2 surface area) and sufficient growth medium added to cover the surface of the bottle. After incubation at 37°C for 1 day the medium was changed. The culture was fed periodically until the cell sheet was confluent. I found the rate of cell recovery was poor using this method and I therefore attempted to improve it.

Results of Experiments on Cell Preservation

Rate of Freezing

In view of the discrepancies between reports on the optimal rate of temperature drop when freezing cells (Scherer and Hoogasian, 1954; Swim, Haff and Parker, 1958; Stulberg, Rightsel, Page and Berman, 1959) HEL cells were frozen by three methods;

1. Ampoules were held at 4°C overnight and transferred directly to -70°C the following day. The temperature as measured by an alcohol thermometer fell from 4°C to -20°C in 5 min.
2. Ampoules were held at 4°C for 30 min, then -40°C for 60 min before storage at -70°C. The temperature, as measured by a thermometer whose bulb was placed in medium in an ampoule, dropped at an overall rate of about 1°C per min but there were rises of temperature (2 - 5°C) at each transfer.
3. Ampoules were placed in a polystyrene box having walls about ½" thick. After 30 min at 4°C the box was transferred to -70°C. The temperature inside the box fell from 4°C to -15°C in 10 min.

After rapid thawing at 36°C, a portion of the cell suspension was diluted in trypan blue solution and the percentage of viable cells was determined. The remainder of the cell suspension was diluted 1/10 in growth medium. Further two-fold dilutions were made and four tubes inoculated with each dilution of cell suspension. The growth medium was changed after 24 hours and the tubes examined after 10 days to determine the highest dilution which had formed a complete monolayer. The results of two experiments (Table 3.2) indicated that Method 1 was unsatisfactory. The cells had a low percentage viability and formed a monolayer at 1/10 dilution only once. Cells frozen by Method 3 had higher percentage viability than those frozen by Method 2, but both types of cells formed monolayers at similar dilutions. Method 3 was more convenient than

Method 2 and therefore adopted as the standard procedure.

Freezing Medium

In 1959, Lovelock and Bishop described the use of dimethyl sulphoxide (DMS) for the prevention of freezing damage to cells. Porterfield and Ashwood-Smith (1962) produced evidence that DMS gave superior results when compared with glycerol for the preservation of chick fibroblasts and HEL cells. Glycerol and DMS were compared for the preservation of HEL cells in experiments similar to those used in the comparison of freezing methods. The results (Table 3.3) indicated that the percentage viability of cells frozen in DMS was higher than that of cells frozen in glycerol but the two types of cells formed monolayers at similar dilutions. DMS was convenient to use because it did not require sterilisation and was less viscous than glycerol. Growth medium containing 10% DMS was therefore adopted as the standard freezing medium.

Duration of Storage

Cells stored at -70°C appeared to deteriorate after periods of 12 months or more. Experiment 1 of Table 3.2 is an example. Cells from ampoules thawed 1 and 2 months after freezing formed monolayers at 1/20 but cells from ampoules thawed after 16 months failed to form monolayers at 1/10. Similar observations were made with several batches of frozen cells. Most of the frozen cells from earlier embryos were lost because of this deterioration.

CHAPTER 4

STUDIES WITH RHINOVIRUSES
1. ISOLATION, ESTIMATION OF NEUTRALISING ANTIBODIES, PREPARATION OF ANTISERA AND IDENTIFICATION OF SEROTYPES.

My earlyattempts to isolate rhinoviruses were disappointing. The original techniques were therefore modified by altering the tissue culture cells and their maintenance medium. The rhinovirus isolation rate increased considerably after the introduction of human embryonic kidney fibroblast cell strains, and comparative studies showed that more rhinoviruses were isolated in cell strains derived from kidneys than those derived from lungs.

The detection of neutralizing antibodies in human sera proved difficult. Efforts were therefore made to increase the sensitivity of the tests by using minimal amounts of virus, by varying the conditions of incubation of the serum-virus mixtures and by adding unheated rabbit serum to the serum-virus mixtures.

Despite generous gifts of rhinovirus antisera, most of the rhinoviruses isolated in Glasgow were not neutralized by the available antisera. Antisera were therefore prepared against 15 Glasgow rhinoviruses by a simple but effective method. These antisera were used in cross-neutralization studies and to identify other rhinoviruses isolated in Glasgow.

Since rhinoviruses have many of the properties of enteroviruses it seemed possible that some serotypes might possess haemagglutinating activity. Rhinovirus haemagglutinins were therefore sought using several species of

erythrocytes under various conditions.

Cells for Virus Isolation

The 52 months during which rhinoviruses were isolated from clinical material, may be divided into three periods according to the types of embryonic cells used (Table 4.1):

1. Between January 1962 and the end of March 1963, specimens were inoculated into primary HEK, RHEL-1 or RHEL-7 cells.

2. At the beginning of 1963 I was able to obtain WI-38 cells and to compare them with some of the Ruchill cell strains. Rhinovirus FEB was titrated in six cell strains (Table 4.2). The highest titres were obtained in RHEKF-13 and WI-38 cells and were at least tenfold higher than the titre in RHEL-7. At the end of March 1963, RHEL-7 cell strain was replaced by RHEKF-13, or occasionally WI-38 cells, for the routine isolation of rhinoviruses. These cells were used, with few exceptions, until the end of September 1964.

3. When stocks of newborn calf serum were almost exhausted and it was found that RHEKF-13 cells would not grow in ordinary calf serum, 11 cell strains (six HEKF and five HEL) grown in ordinary calf serum were tested for their sensitivity to rhinovirus FEB and compared with WI-38 cells. A frozen pool of rhinovirus FEB was titrated at various times in these different cell strains. The geometric means of the titres from three titrations in each tissue were not significantly different: the highest mean titre (expressed

as log TCD_{50}/0.1 ml) was 5.0 $\pm$ 0.2 and the lowest 4.3 $\pm$ 0.4. The geometric mean of the 18 values in the six HEKF strains was 4.87 $\pm$ 0.07 and of the 18 values in the six HEL cell strains was 4.65 $\pm$ 0.09; these were not significantly different. A detailed investigation of the rhinovirus sensitivity of eight of these cell strains was therefore initiated and will be described in Chapter 5. Meanwhile, since the highest geometric mean titres had been obtained in RHEKF-30 and RHEKF-35 cells, from October 1964, all specimens were examined in one of these two cell strains. Between December 1964 and April 1965, specimens from children in hospital were also examined in BWHEL-7 cells (a cell strain supplied by Burroughs Wellcome) and between April 1965 and April 1966, specimens from children in hospital and adults with colds were also tested in WI-38 cells.

The rates of isolation of rhinoviruses during the three periods (Table 4.1) varied from 3.4% to 13.0%. Although some of this difference might have been due to the different groups of patients studied, it seemed likely that the cells used during the first period was insensitive. I, therefore, decided to re-examine as many as possible of the specimens which had not been inoculated into HEKF. The specimens, which had been stored at -70°C for 2 - 3 years, were thawed and inoculated into RHEKF-30 cells and sometimes into WI-38 cells as well. Re-testing specimens from 131 patients (Table 4.3)

revealed 22 rhinoviruses, 17 of which had not been detected when the specimens were originally inoculated into human embryonic cells. Two of the 22 additional rhinovirus-positive patients were studied during the second period and the remainder during the first period. Thus, the final isolation rate for the first period was 24/132 (18.2%), for the second period was 16/206 (7.7%) and for the third period was 55/423 (13.0%). These results indicated that RHEKF-30 cells were more sensitive to rhinoviruses than the HEL cell strains originally used.

When specimens were examined in parallel in HEKF and HEL cell strains more rhinoviruses were isolated in HEKF than in HEL cells. Between December 1964 and April 1965, 110 specimens were inoculated simultaneously into RHEKF-30 and BWHEL-7 cells. Rhinoviruses were isolated from seven specimens in RHEKF-30 cells but from only two in BWHEL-7 cells. Between April 1965 and April 1966, 298 specimens were examined under identical conditions in WI-38 cells and RHEKF-30 or RHEKF-35 (Table 4.4). Of the 43 rhinoviruses isolated, 40 (93%) were detected in HEKF but only 29 (67%) were detected in WI-38 cells. These results suggested that HEKF may be superior to the widely used WI-38 cells for the isolation of rhinoviruses.

A further advantage of HEKF strains over WI-38 is the earlier appearance of cytopathic changes. For 17 specimens which were positive in both RHEKF-30 and WI-38 the average number of days between inoculation and the first definite cytopathic effect was 5 in RHEKF-30 and 7 in WI-38; the intervals for the nine specimens yielding rhinoviruses in RHEKF-35 and

WI-38 were 4 days in RHEKF-35 and 6 days in WI-38. Thus, on average, virus was detected two days earlier in RHEKF strains than in WI-38 cells.

Medium for Virus Isolation

When human embryonic cell strains were introduced for rhinovirus isolation the maintenance medium which had been used for HEK cells had to be improved. Cells in LH medium began to degenerate after a week but cells in Eagle's MEM remained healthy for periods of up to four weeks. Embryonic cell strains were also particularly sensitive to toxic substances in clinical material, but the addition of 5% TPB to the medium reduced the effect of toxic specimens. The effect of TPB on rhinoviruses was investigated by titrating strain FEB in HEL cells maintained in medium with and without TPB. In each of three experiments rhinovirus microplaque counts were more than tenfold higher in the presence of TPB although the final end-point after seven days was unaffected. The counts of microplaques at daily intervals in a typical experiment are shown in Table 4.5. As a result of these observations, the following medium was used for all rhinovirus work in human embryonic cell strains:-

Eagle's MEM	91.5 ml
Calf serum	1 ml
TPB	5 ml
$NaHCO_3$ solution	1.5 ml
PSS	1 ml

Estimation of Neutralizing antibodies

Antibody responses in patients infected with rhinoviruses were often only detected if small amounts of virus were used in the serum-virus mixtures. Five pairs of sera were tested on two occasions against virus at two dilutions tenfold apart (Table 4.6). In each case the antibody titre in the convalescent serum was fourfold or nearly fourfold higher against the low virus dose than against the high dose. Therefore, virus was used at a dilution which gave a titre of 10-68 TCD_{50} when the tests were read after three days.

Twofold dilutions of an acute and a convalescent serum from an adult were incubated with virus in three different ways: 1½ hours at room temperature, 1½ hours at 36°C and 3 hours at room temperature. There was no significant difference between the neutralization end-points of the sera. All rhinovirus neutralizing antibody titrations were therefore incubated with virus for 1½ hours at room temperature.

Five pairs of sera were titrated against their homologous rhinoviruses diluted in PBS and in PBS containing 25% fresh unheated rabbit serum (Table 4.7). The neutralization end-points were not significantly different even with sera from young children. Rhinoviruses used in neutralizing antibody titrations were therefore diluted in PBS.

Preparation of Antisera

Fifteen rhinoviruses which were not neutralized by any of the 27 available prototype antisera were used to prepare specific antisera. These

viruses included 11 isolated from chronic bronchitics between 1962 and 1964 and 4 isolated from children in hospital in 1965.

Rhinovirus antigen was prepared in roller bottles. Five million cells of RHEKF-30 or RHEKF-35 in 10 ml of growth medium were placed in 250 ml round centrifuge bottles which were rotated at 12 rev/min at 36°C until a cell monolayer formed over their entire inner circumference. The growth medium was replaced by 5 ml of rhinovirus maintenance medium and 5 ml of fluid from rhinovirus infected tissue cultures. Bottles were then rolled at 33°C and the medium changed daily. When 75 - 100% of the cell layer was destroyed the bottles were frozen at -70°C and thawed. Cell debris was removed by centrifugation at 3,000 rev/min for 20 min and the supernate distributed into ampoules and stored at -70°C.

Concentration of antigen was attempted by the use of aluminium phosphate gel according to a method used at the Common Cold Research Unit (Doggett, 1963).

The $AlPO_4$ gel was prepared by the method of Fantes (1962): 20 ml of 16% $Na_3PO_4.12\ H_2O$ were diluted to about 1 litre in deionized water and mixed with 20 ml of 10% $AlCl_3.6H_2O$. After allowing the precipitate to settle, the supernate was discarded. The precipitate was then washed three times in deionized water and resuspended in O.1M phosphate buffer, pH 6.8 to give a concentration of 10 - 15 mg/ml. The suspension was sterilized by autoclaving at 20 lbs/in^2 for 1 hour and stored at 4°C.

$AlPO_4$ suspension was added to clarified virus suspension to give a final

concentration of 1 mg/ml and the pH adjusted to 7 with 0.1M phosphate buffer. This suspension was stirred for 1½ hours at 4°C and then centrifuged at 2,500 rev/min for 10 min. The supernate was discarded and the deposited $AlPO_4$ resuspended in 0.5M phosphate buffer, pH 8 (one-tenth the volume of the original virus suspension). The suspension was left at 4°C for 30 min to allow elution of virus then centrifuged to remove $AlPO_4$ and the supernate stored at -70°C.

Antisera were prepared in guinea pigs weighing between 800 and 1,200g. Blood (5 ml) was removed from each pig by cardiac puncture before immunization. Three schedules of immunization were used in an attempt to find the simplest means of obtaining satisfactory antisera:

1. Animals were given two intramuscular injections of 1 ml virus, concentrated by $AlPO_4$, two weeks apart and exsanguinated five days after the second injection.
2. Two injections were given as in 1. but using virus which had not been concentrated.
3. Two intramuscular injections of 1 ml of a 50/50 emulsion of virus and Freund's incomplete adjuvant (Difco) were given two weeks apart. After a further 3 weeks, 1 ml of virus alone was injected intramuscularly and the animals exsanguinated 5 days later.

The results of using these three methods with three different viruses are shown in Table 4.8. The method used for the concentration of rhinoviruses was not satisfactory. Infectivity titres were not increased and the antigenicity of virus concentrated by $AlPO_4$ was not high since method 1 produced only low or undetected antibody responses. Method 2 induced a good antibody response against 4574-62 (an M strain) but not against the other two rhinoviruses (H strains). Method 3 was completed with two rhinoviruses (H strains) and in both cases produced antibody levels more than tenfold higher than those produced by the other methods. Method 3 was therefore used in the preparation of all further rhinovirus antisera.

Antisera were prepared against a further 12 rhinovirus strains, using two guinea pigs for each virus. The titres of these antisera are shown in Table 4.9. Only two of the 24 guinea pigs had antibody titres below 256 after immunization. None of the animals had antibody in their pre-immune sera. One serum against each strain was selected for use as typing antiserum either because it had the higher titre or because it was available in larger quantities. These typing antisera were used at dilutions calculated to contain at least 20 neutralizing antibody units.

Cross-Neutralization Tests

Cross-neutralization studies with the 15 rhinovirus strains and their typing antisera revealed four cross-reactions (Table 4.10).

Two strong reciprocal cross-reactions were found. These indicated that strains 409-62 and 1746-62 belonged to the same serotype and strains

130-63 and 992-63 belonged to the same serotype. In future chapters, strains neutralized by 409-62 and 1746-62 antisera are called 409-62 serotype and strains neutralized by 130-63 and 992-63 antisera are called 130-63 serotypes.

A weaker reciprocal cross-reaction was observed between 4574-62 and 1767-65 strains. The titre of 1767-65 antiserum showed a fourfold difference against the two virus strains. There was a 32-fold difference between the titres of 4574-62 antiserum against homologous and heterologous viruses. The large difference between the 4574-62 antiserum titres might have occurred because this antiserum was prepared by method 2 and therefore more strain-specific. There was a further difference between the two strains; 4574-62 was an M strain and 1767-65 an H strain. Because of these differences in future work M strains neutralized by 4574-62 antiserum were called 4574-62 serotypes and H strains neutralized by 1767-65 antiserum were called 1767-65 serotypes. They are, however, related serologically.

The fourth cross-reaction was a strong one-way cross-reaction between 1321-62 and 4411-65. The 4411-65 antiserum neutralized both viruses but the 1321-62 antiserum neutralized only the homologous virus. There were two possible explanations of this finding: 4411-65 virus was a "prime" strain with a broader antigenic composition than 1321-62 virus, or 4411-65 virus was a mixture of 1321-62 virus and another virus. The cross-reaction was therefore further investigated. The 4411-65 virus was purified twice by

terminal dilution in the presence of 1321-62 antiserum at 1/8. The pure virus was then inoculated into a guinea pig using Method 3. The resulting antiserum had a titre of 128 against purified 4411-65 virus but did not neutralize the original 4411-65 virus or 1321-62 virus. It was therefore concluded that the original 4411-65 was a mixture of two viruses. Only those rhinoviruses which were neutralized by the antiserum made against purified 4411-65 virus were called 4411-65 serotypes. It was not possible to re-isolate virus from the original 4411-65 specimen and it is not known whether two viruses were present in the original specimen. However, since the paired sera from the child from whom this specimen was collected showed a four-fold titre rise against the unpurified 4411-65 virus it is possible that the patient was infected with two rhinoviruses.

Tests for Haemagglutinin

Tissue cultures which had been completely destroyed by rhinoviruses were frozen and thawed and the resulting fluids tested for haemagglutinin. Human group '0', guinea pig, rhesus monkey and fowl (vaccinia-agglutinable) erythrocytes were used against 86 of the 95 rhinoviruses isolated in Glasgow. The tests were done at $4^{\circ}C$, room temperature and $36^{\circ}C$ by the methods described in Chapter 2. No evidence of haemagglutination was found.

CHAPTER 5

STUDIES WITH RHINOVIRUSES

2. RHINOVIRUS SENSITIVITY OF EIGHT CELL STRAINS

The results reported in Chapter 4 suggested that HEKF strains might be superior to HEL cell strains for isolating rhinoviruses and that there might be considerable variation between HEL cell strains. These variations would greatly influence the isolation rate of rhinoviruses. I, therefore, decided to investigate the rhinovirus sensitivity of three HEKF strains and five HEL cell strains.

In this chapter I shall describe the characteristics of the eight cell strains, the cytopathic effects of a rhinovirus in them and the results of inoculating them with specimens and with pools of virus grown in tissue culture.

Characteristics of Cell Strains

The cell strains used and their characteristics are shown in Table 5.1. Ruchill strains were derived from both the kidneys and lungs of three embryos and from the lung only of another embryo by the methods described in Chapter 3. These embryos were all male and between 14 and 20 weeks of gestation. They were obtained from pregnancies which were terminated for reasons other than suspected fetal abnormality and during which there was no history of infection. The gross appearance of each of the fetuses was

normal. WI-38 cells were obtained every three weeks from Mr. J.P. Jacobs.

After recovery of Ruchill strains from frozen stock or receipt of the WI-38 strain from Mr. Jacobs, all cells were grown and maintained in the same batch of medium.

Cell strains were examined for mycoplasma contamination at two different passage levels (Table 5.1) by Mr. W. House. Three methods were used (House and Waddell, 1967): a) Chanock's medium (Chanock, Hayflick and Barile, 1962), b) a feeder cell layer with Chanock's medium, c) the medium described by Herderschee (1963). All cultures were incubated at 37°C in an atmosphere of 5% carbon dioxide in nitrogen. At no time were mycoplasmas detected in any of the cell strains.

Virus sensitivity tests were made in cells from passages before the cell strains showed signs of "senescence".

Cytopathic Effects of Rhinoviruses

Early cytopathic effects of rhinovirus FEB were similar in each of the eight cell strains (Figs. 5.1 to 5.8). Elongated fibroblasts contracted and became rounded in discrete areas. These foci were more prominent and spread more rapidly in HEKF than in HEL cells. Different serotypes of rhinoviruses gave similar results.

Examination of Specimens

Specimens were inoculated in 0.1 ml or 0.2 ml volumes into one tube of each cell type. Most specimens were undiluted but some were diluted

1 in 2. The same volume was inoculated into each cell strain for any one specimen.

Thirty-six previously positive specimens which had been stored at -70°C for periods up to four years and 24 specimens similarly stored, but which were negative when initially tested in HEL cells were inoculated into the eight cell strains (Table 5.2). Five of the rhinovirus-positive and 17 of the rhinovirus-negative specimens failed to yield virus in any of the eight cell strains. Most (79%) of the 38 specimens which contained virus detectable in at least one cell strain, were positive in RHEKF-30. The other two HEKF cell strains detected 63% and 68% respectively. WI-38 cells were more sensitive than any of the other four HEL cell strains; RHEL-35 was apparently least sensitive, detecting only 18% of the positive specimens.

Six specimens were positive in all eight cell strains; they all caused obvious cytopathic effects within 3 days of inoculation. The viruses isolated belonged to five different serotypes; two were M strains and the remainder H strains.

Fourteen specimens were positive in three or more of the HEL cell strains; of these one was positive in two HEKF strains and the remainder were positive in all three HEKF strains. The viruses isolated from these 14 specimens belonged to ten different serotypes; four were M strains and the remainder H strains.

Twenty-seven specimens were positive in two or three HEKF strains; of these, 14 were positive in more than two HEL strains, three in two, five

in one and five in none of the HEL strains. Four of the latter five belonged to Glasgow serotype 2030-65 and one to Glasgow serotype 1647-63. Examination of records showed that although two of four 1647-63 strains had been isolated in WI-38 cells, all six strains of 2030-65 had been isolated in HEKF cells only. Thirty-six of the rhinoviruses isolated from the 38 positive specimens belonged to 22 serotypes and two were untyped.

Three specimens were titrated in the eight cell strains (Fig. 5.9; Table 5.3). Virus titres are expressed as log TCD_{50}/0.1 ml throughout this Chapter and mean titres refer to geometric mean titres. The mean titre of the three specimens in the three HEKF strains was 2.52 ± 0.26 and in the five HEL strains was 1.35 ± 0.29. The difference between these two mean titres was 1.17 ± 0.27 which is statistically significant ($t = 4.88$; $0.02 < p < 0.05$).

These experiments with clinical material indicate that in general HEKF strains are more sensitive to rhinoviruses than HEL strains and suggest that this difference may be greater with certain serotypes. There is also evidence indicating that different HEL strains vary considerably in their sensitivity.

Titrations of Virus Pools

Further experiments were designed to confirm whether or not certain serotypes produce cytopathic effects more readily in HEKF than in HEL strains and whether differences in sensitivity between various cell strains could be observed after rhinoviruses had been cultivated in vitro. Therefore, twenty-one titrations of virus pools representing 17 different serotypes were

made in the eight cell strains (Tables 5.4, 5.5 and 5.6). Seventeen of the virus pools were prepared in HEKF and four in HEL cells.

The mean titre in four of the HEL strains (RHEL-33 was excluded because four titrations could not be made owing to bacterial contamination) was subtracted from the mean titre in the three HEKF strains for each virus titration. The results (Fig. 5.10) fell into two groups: a group in which the difference between the mean titres in HEKF and HEL cells was small (0.6 or less) and which included 16 of the 21 titrations and 14 of the 17 serotypes represented (Tables 5.4 and 5.5); a group in which the difference was large (1.4 or higher) and which included five titrations representing three different serotypes, Glasgow serotypes 1647-63, 1376-64 and 2030-65 (Table 5.6). For convenience viruses in the first group will be called N (normal) viruses, and those in the second group K (kidney) viruses.

The difference between mean titres in HEKF and HEL cells for N viruses was 0.16 ± 0.06 which is significant ($t = 2.5$; $0.02 < p < 0.05$). The difference for K viruses was 1.66 ± 0.07 which is highly significant ($t = 22.1$; $p < 0.005$).

The means of 14 of the 16 titrations of N viruses (two were excluded because of contamination in RHEL-33) in each of the eight cell strains (Table 5.7 and Fig. 5.11) show no significant differences between the cell strains. Similar results from the five titrations of K viruses in seven cell strains (RHEL-33 was excluded because of bacterial contamination in two titrations) show more striking differences (Table 5.7 and Fig. 5.12). The mean titre in RHEKF-30 cells is significantly higher than that in any other

cells except RHEKF-35. Mean titres in the HEL cells, except WI-38 cells, are significantly lower than those in the three HEKF cell strains. The mean titre in WI-38 cells is significantly higher than that in RHEL-36.

These results confirm the existence of certain rhinovirus serotypes to which HEKF are much more sensitive than HEL cells. Titration of N viruses after growth in vitro did not show any significant differences in sensitivity between the eight cell strains. Certain differences were evident when K viruses were titrated.

CHAPTER 6

STUDIES WITH RESPIRATORY SYNCYTIAL VIRUS

Respiratory syncytial virus had been described as a labile virus (Beem et al. 1960). Modifications of the usual methods for virus isolation were therefore made in an attempt to improve the isolation rate of this virus. During studies with RS virus the sensitivity of BH cells was found to be influenced by the serum in which they were maintained and the length of time they had been in continuous culture.

Early attempts to detect RS virus neutralizing antibodies in sera from infected children were disappointing. After modifying the method in various ways I found that the addition of unheated rabbit serum to the serum-virus mixtures significantly increased neutralizing antibody titres.

Unheated rabbit serum was also valuable in the study of antigenic variation among RS virus strains. Antisera were prepared by various methods against two strains of RS virus. When these sera were titrated against nine RS virus strains they revealed two antigenically distinct strains; the other seven strains were possibly intermediate.

Virus Isolation

During a small study of children in hospital with respiratory illness from December 1962 to May 1963, throat swabs were inoculated directly into tubes of BH cells in the wards. The cultures were collected each day and subsequently

incubated and treated as usual. During December, January and February, RS virus was isolated from eight (26%) of 31 children. This isolation rate was no higher than that found by other workers using the more conventional technique of inoculating tissue cultures with transport medium. The method had two disadvantages: only one type of tissue culture could be inoculated unless more swabs were collected; twenty (34%) of 59 specimens collected were microbially contaminated. Direct inoculation of tissue cultures was therefore discontinued.

When studies on children in hospital were continued in October 1963, I compared increased antibiotic concentrations with centrifugation for the control of microbial contamination. When throat or nose specimens had been centrifuged the supernate was inoculated into tissue cultures as usual and the deposit was resuspended in a portion of the supernate and 0.1 ml inoculated into one tube of each type of tissue culture. To the tubes inoculated with the deposit material from alternate specimens additional antibiotics (50 units nystatin, 100 units penicillin and 100 μg streptomycin per tube) were added. The results of the examination of 80 specimens (Table 6.1) show that, of tubes inoculated without extra antibiotics, those inoculated with deposit material were contaminated more often than those inoculated with supernate but there was no difference in the group where extra antibiotics were added with the deposits. Centrifugation also appeared to affect the isolation of RS virus. Of 17 specimens yielding RS virus, the virus was isolated from both supernate

and deposit of 11, supernate only of one and deposit only of five. Since centrifugation was time consuming and might remove RS virus from specimens it was discontinued after April 1964 and microbial contamination was controlled by the addition of extra antibiotics to cultures inoculated with specimens.

In February 1964, batches of fowl serum were obtained which were toxic to BH cells and inhibited RS virus. An alternative serum was therefore sought for the maintenance of BH cells. Pools of calf, rabbit and fowl sera were tested for RS virus inhibitors. One of three pools of fowl serum and two pools of calf serum inhibited the growth of 68 TCD_{50} of RS virus at dilutions of 1/20 to 1/40 in tissue culture medium. No inhibition was found in two batches of newborn calf serum or in four pools of rabbit serum. Rabbit serum was therefore substituted for fowl serum in BH maintenance medium after March 1964.

It was repeatedly found that, after about a year in continuous culture, BH cells tended to aggregate into clumps. Fresh cells were therefore thawed at the end of each summer from a stock frozen at $-70^{\circ}C$. Titrations of RS virus in cells which had recently been thawed and in cells which had been in culture over twelve months (Table 6.2) showed that the virus gave a higher titre in recently thawed cells. The BH cells had been shown by Mr. W. House to be infected with a mycoplasma. Cells were therefore cultured in the presence and absence of 50 µg/ml of aureomycin in an attempt to

eliminate the mycoplasma and investigate its effect on the RS virus sensitivity of the cells. Mycoplasma were not isolated from cells which had been grown in the presence of aureomycin for six weeks or more. However, the titres of RS virus were similar whether cells had been grown in the presence of aureomycin or not (Table 6.2 Experiment 2). The geometric mean of the titres in cells which had been in culture four months or longer was 2.85 $\pm$ 0.26 (log TCD_{50}/0.1 ml) and in cells which had been in culture six weeks or less was 4.15 $\pm$ 0.27 (log TCD_{50}/0.1 ml). This indicated a significant difference. Thus BH cells were shown to lose sensitivity for RS virus but this could not be associated with mycoplasma infection.

During the study, respiratory specimens from 476 children in hospital were tested in parallel in RMK, BH and HEKF cells (Table 6.3). RS virus was isolated in BH cells from 65 (88%) of the 74 cases which yielded this virus, from 38 (51%) in RMK and from 13 (18%) in HEKF. The results indicate the superior sensitivity of BH cells during each of the three winters of the study.

Estimation of Neutralizing Antibody

Various conditions were investigated to establish a sensitive method for detecting RS virus neutralizing antibodies: the amount and strain of the virus used, the temperature and duration of incubation of serum-virus mixtures; the addition of unheated serum to the reaction mixtures and the duration of incubation before tests were read.

Acute and convalescent sera from four patients from whom RS virus was isolated were titrated against tenfolded dilutions of RS virus and the tissue cultures read four and seven days after inoculation (Table 6.4). When more than 100 TCD_{50} of virus were used, a fourfold or greater antibody titre rise was found once in readings at 4 days and not at all at 7 days. When 32 TCD_{50} of virus were used fourfold or greater titre rises were found in two of the four patients at 4 days and in one at 7 days. In most cases, the antibody titres were higher if readings were taken at 4 days. In all further tests, therefore, the virus dose was less than 100 TCD_{50} and cultures were changed to fresh medium (containing 1/80,000 neutral red) after 3 days and read the following day.

Acute and convalescent sera from a child infected with RS virus were titrated and the serum-virus mixtures held under three sets of conditions: a) 1½ hours at room temperature, b) 1½ hours at 36°C, c) 6 hours at room temperature. No difference in antibody titres was found. In the standard method serum-virus mixtures were incubated for 1½ hours at room temperature.

Sera collected from children aged between 5 and 16 months in hospital with acute respiratory illness during the 1962-63 winter were tested for neutralizing antibodies against two strains of RS virus, using the above standard methods. The neutralizing antibody titres against the

Randall strain were low, particularly compared with the CF antibody titres (Table 6.5). Only four of seven cases showed a fourfold or greater titre rise. The same sera were tested against a similar dose of a strain of RS virus isolated at the beginning of February 1963 (strain 329-63). Neutralizing antibody titres in convalescent sera were higher against 329-63 than against Randall and all seven cases showed fourfold or greater titre rises against 329-63.

Unheated rabbit and guinea pig sera were incorporated into the serum-virus mixtures in an attempt to increase the sensitivity of the neutralization test. Acute and convalescent sera were titrated against the same dilution or RS virus made in PBS alone, PBS containing 1% unheated guinea pig serum and PBS containing 25% unheated rabbit serum (Table 6.6). Both guinea pig and rabbit sera considerably enhanced the antibody titre in the convalescent serum without affecting the virus dose. Rabbit serum had no effect if it was heated at 56°C for 30 min before addition to the serum-virus mixture (Table 6.7).

During the two following winters (1963-64 and 1964-65) the effects of different virus strains and unheated rabbit serum on neutralization tests was investigated further. Paired sera from some of the patients from whom RS virus was isolated were tested in the presence and absence of fresh rabbit serum against both the Randall strain and a strain isolated during each of the two winters (121-64 and 143-65). The value of unheated rabbit serum was confirmed since in all cases it increased the antibody

titres, in three instances more than eightfold (Table 6.8). Unheated serum increased the number of fourfold or greater titre rises detected from two to five both against epidemic and against Randall strains of RS virus. No significant differences were detected when results obtained using the Randall and epidemic strains of virus were compared. Titrations of four pairs of sera from the 1963-64 and 1964-65 winters were repeated against the Randall and 329-63 strains (Table 6.9). The antibody titres were similar against both strains.

Antigenic Variation

In view of the different levels of neutralizing antibody found in 1962-63 human sera against Randall and 329-63 strains of RS virus, I decided to determine if these two strains differed antigenically.

Antisera against both the Randall and 329-63 strains were prepared by intranasal inoculation of guinea pigs (weighing 800 - 1100 g) with 0.2 ml per pig of unfrozen tissue culture fluid (containing 10^5 to 10^6 TCD_{50}/ml). Further intraperitoneal inoculations of 2 ml per pig of virus (10^4 to 10^5 TCD_{50}/ml) were given 3 and 6 weeks later. The animals were exsanguinated 1 week after the final inoculation. Titrations of these antisera (Table 6.10) showed that all the animals had a greater than fourfold rise in antibodies against both strains of virus, irrespective of whether unheated rabbit serum was used or not. The degree of the antibody response was not always the same against the two virus strains. Post-

immune Randall antiserum 1 showed a 32- to 45- fold difference in titre against homologous and heterologous virus strains. The difference with Randall antiserum 2 was similar but much less. The only significant difference (fourfold or greater) with the two 329-63 post-immune antisera was antiserum 1 which, in the absence of unheated serum, showed a fourfold higher titre against the Randall strain than against the homologous strain.

The neutralization indices of post-immune antisera, Randall/1 and 329-63/2, were also estimated. Equal volumes of tenfold dilutions of the two virus strains and 1/10 dilutions of the antisera and normal guinea pig serum were incubated for 1½ hours at room temperature both in the presence and absence of unheated rabbit serum. The mixtures were inoculated into BH cells (0.2 ml per tube; 5 tubes per mixture) and the titres of virus estimated after 7 days. The neutralization index (NI) for each virus against each antiserum was calculated by subtracting log TCD_{50} titre of virus with antiserum from log TCD_{50} titre with normal serum. The results (Table 6.11) confirm those shown in Table 6.10. Randall antiserum 1 differentiates between the two virus strains both with unheated serum (Randall NI >4.2, 329-63 NI = 2.4; difference>1.8) and without it (Randall NI>3.8, 329-63 NI = 0.6; difference >3.2) whereas 329-63 antiserum 2 does not (NI differences <0.2 and 0.2).

Attempts were made to produce more narrowly specific antisera by injecting guinea pigs intramuscularly with 1 ml of virus ($10^{4.7}$ to $10^{5.3}$ TCD_{50}/ml) and exsanguinating them 7 days later. This method was

successfully used by Goffe and Bell (1963) for the production of highly specific poliovirus antisera. However, no antibody response against RS virus could be detected.

Efforts were then made to increase antibody response by concentrating the virus and by increasing the number of injections. Attempts to produce high-titred virus by growing it in roller bottles containing a monolayer of cells around the inner circumference failed because BH cells formed large aggregates, not monolayers, under these conditions.

Concentration of antigen was then attempted by the use of aluminium salts. Two methods were used:

(a) The $AlPO_4$ gel was prepared by the method of Fantes (1962) and used as described in Chapter 4 for concentrating rhinoviruses.

(b) The method described by Midulla, Wallis and Melnick (1965) was used. 1 ml of 0.25 M $AlCl_3$ and 1 ml of 0.5M Na_2CO_3 were added to 8 ml of cell-free virus suspension, the mixture shaken and incubated at 36°C for 15 min. After centrifugation at 2,000 rev/min for 10 min the supernate and a portion of the precipitate were removed for titration, and the remainder of the precipitate distributed into ampoules and stored at -70°C.

No increase in titre of the virus was detected using Method (a) in either of two experiments. Method (b) resulted in a 5- to 30- fold increase in the virus titre in the precipitate (Table 6.12).

Randall and 329-63 antigens were prepared both as normal tissue culture fluid and as precipitate after the addition of $AlCl_3$ and Na_2CO_3. Each of the four preparations was inoculated into two guinea pigs. Two intramuscular injections of 1 ml were given 3 weeks apart and the animals exsanguinated 5-6 days after the final injection.

Concentration of the virus antigen induced higher antibody levels with 329-63 strain but not with Randall strain with which there was less difference between the infectivity titres of normal and concentrated virus (Table 6.13).

The antibody responses of the guinea pigs were low when sera were tested without unheated rabbit serum (Table 6.13). In the presence of unheated serum, four post-immunization sera (Randall/5, 329-63/5, 6 and 7) had homologous titres of 128 or greater. When these four sera were titrated against the heterologous strain the titres were more than eightfold lower. These results indicate a reciprocal antigenic difference between Randall and 329-63 strains.

The distribution of these two antigenic types amongst strains isolated at Ruchill and in the U.S.A. was next investigated. Four antisera, Randall/1 and 5 and 329-63/2 and 5 were titrated against six other strains of RS virus isolated from three epidemics at Ruchill and a strain obtained from Dr. R.M. Chanock (CH 18357). The results (Table 6.14) indicate that these virus strains may be divided into four groups:

1) Strains which are neutralized strongly by all four antisera; e.g. the U.S. strain (CH 18357) and two strains isolated at Ruchill in Autumn 1965 (3892-65 and 4410-65).

2) Strains which are neutralized strongly by all the antisera except 329/5; e.g. Randall strain.

3) Strains which are neutralized strongly by the two 329-63 antisera but weakly by the Randall antisera; e.g. 329-63 and a strain isolated in January 1965 (143-65).

4) Strains which are neutralized weakly by all antisera except 329-63/2; e.g. two strains isolated in Autumn 1965 (4414-65 and 4178-65) and a strain isolated in January 1964 (121-64).

The same grouping emerges (Fig. 6.1) when the log geometric mean of the titres of the two Randall antisera is plotted against that of the two 329-63 antisera. Group 1 strains give titres within fourfold of the homologous titres of both Randall and 329-63 strains. The Group 2 strain gives a titre within fourfold of the Randall homologous titre but more than fourfold less than the 329-63 homologous titre. Group 3 strains give titres within fourfold of homologous 329-63 titre but more than fourfold less than homologous Randall titre. Group 4 strains give titres more than fourfold lower than either 329-63 or Randall homologous titres.

The relationship of the six Ruchill and one U.S. strains to Randall and 329-63 strains may be deduced by considering the fold difference between the geometric mean titre of the Randall antisera and that of the

329-63 antisera for each strain (Table 6.15). No strains were identical with either Randall or 329-63 strains. The fold differences of the seven other strains were between those of Randall and 329-63. Strain 143-65 was more like 329-63 than the others; strains 4414-65 and 4178-65 were more like Randall. The strains isolated at Ruchill did not show any pattern that could be related to the time at which they were isolated.

CHAPTER 7

DISCUSSION

The main purpose of the work described in Part I was to establish at Ruchill techniques for studying the epidemiology of respiratory viruses, particularly rhinoviruses and RS virus. Problems arose during the growth of cells and isolation of these viruses in them. The identification of viruses isolated and the estimations of neutralizing antibody against them also presented difficulties. Certain modifications of standard techniques improved results and led to some hitherto unpublished observations.

The difficulties I experienced in culturing human embryonic cell strains were shared by many workers and have been traced to reagents of inferior quality and to variations in the formulae of Eagle's media (Hayflick, Jacobs and Perkins, 1964). Newborn calf serum may have contained factors which compensated for the unsatisfactory Eagle's MEM and thus allowed satisfactory growth of cell strains. When satisfactory media were used I was able to obtain growth of cell strains similar to that described by Hayflick and Moorhead (1961). Although these workers reported that cell strains could be derived from material that had remained in the intact fetus up to 5 days after its removal from the uterus, I found that material from three fetuses which had remained intact at 4°C for 24 hours was either microbially contaminated or failed to yield viable cells. However, I found that, as stated by Hayflick and Moorhead (1961), chopped tissue placed in medium

remained viable for at least 3 days.

The original method for preserving human diploid cell strains (Hayflick and Moorhead, 1961) could be improved by replacing glycerol with DMS in the freezing medium. This observation is in agreement with those of other workers using primary human embryonic lung cells (Porterfield and Ashwood-Smith, 1962) and human diploid cell strains (Wallace, 1964). Cell recovery was further improved by freezing the cells more slowly than Hayflick and Moorhead (1961). This finding agrees with work on many cell types (Hauschka, Mitchell and Niederpruem, 1959) although Wallace (1964) found the freezing rate was not critical for human diploid cell strains. The deterioration of cells stored at -70°C over long periods which I observed has also been noted by other workers (Takano, Yamada and Hirokawa, 1961; Wallace, 1964). Acceptable upper temperatures have not been established but Merryman (1962) has stated that storage of biological material should be below -100°C. At this temperature crystal growth and biochemical degradation are believed to cease.

My studies on the handling and storage of human embryonic diploid cell strains indicates that such cells may be readily grown provided high quality medium of the correct type is used. Storage of these cells should be in medium containing DMS and at temperatures below -100°C.

Human embryonic lung cell strains have been widely used for the isolation and growth of rhinoviruses but few workers have compared them with other cell types and no one has reported the isolation of rhinoviruses

in human embryonic kidney cell strains. Comparative studies have shown that more rhinoviruses are isolated in WI-38 cells than in primary embryonic kidney cells and more in primary embryonic kidney cells than in another embryonic lung cell strain (Hamre, 1963, 1964). The work reported here indicates that HEKF cell strains are more sensitive than WI-38 cells for primary isolation of rhinoviruses. Higgins (1966b) has recently shown that some rhinoviruses were repeatedly isolated in WI-38 cells but not in primary embryonic kidney cells and others were isolated in kidney cells but not in WI-38 cells. The discovery in this work of three rhinovirus serotypes (K strains) which grow more readily in HEKF than in HEL cells proves the existence of rhinoviruses with different tissue tropism and may be the explanation of Higgins' findings. I did not, however, find evidence of rhinoviruses which grew better in HEL than in HEKF cells. The HEKF cell strains may possibly be sensitive to both the types of rhinoviruses described by Higgins and therefore combine the advantages of WI-38 cells and primary embryonic kidney cells.

Cells from different embryos vary in their sensitivity to rhinoviruses. This has been shown in both primary cells (Johnson et al. 1962a) and semi-continuous lung cell strains (Brown and Tyrrell, 1964). My observations confirm those of Brown and Tyrrell (1964) and indicate that similar variation occurs among HEKF strains, though to a lesser extent in the three strains I tested. The reasons for this variation are not understood. The rhinovirus sensitivity of a cell strain does not appear to be entirely

genetically controlled since I derived a sensitive HEKF strain and comparatively resistant HEL strain from the same fetus. Infection of cell strains with viruses, mycoplasmas or protozoa which may produce interferon and thus inhibit rhinovirus growth is a more likely explanation of resistance to rhinovirus infection. Goffe (1965) described a HEL strain which was resistant to rhinoviruses and found to be chronically infected with rubella virus. No mycoplasmas were isolated from the cell strains I used and no cytopathic or haemadsorbing agent was found when cell strains were inoculated into BH or RMK cells but it is difficult to prove the absence of infectious agents from a cell strain.

Haemagglutination by rhinoviruses has been sought on a limited scale by several workers but never detected (Hamparian et al. 1961b; Johnson and Rosen, 1963; Mufson et al. 1965). Since rhinoviruses are biologically and morphologically similar to enteroviruses it seemed possible that certain types under appropriate conditions might agglutinate erythrocytes. I was unable to detect any rhinovirus haemagglutinin although a large number of strains were tested against erythrocytes of several species at three different temperatures.

The method I used for estimating rhinovirus neutralizing antibody is similar to that described by other workers and is only slightly less sensitive than the microplaque-reduction technique (Taylor-Robinson et al. 1963). Attempts to enhance rhinovirus neutralization have not previously been reported but I was unable to increase neutralization titres by varying

temperature or time or by adding unheated serum.

Specific antisera should ideally be prepared using viruses which have been purified either by picking single plaques or by terminal dilution passage (Tyrrell and Chanock, 1963). However, in a laboratory where rhinoviruses are being continually isolated there is a risk of cross-contamination. I therefore passed viruses in tissue culture as little as possible before storing. The viruses I used for antiserum preparation were not purified as I believed that, in these circumstances, triple terminal dilution passage might result in cross-contamination rather than purification. Rhinovirus HGP has been successfully concentrated by adsorption onto aluminium phosphate (Chapple and Harris, 1966) but it is not clear what degree of concentration was achieved. I was not able to concentrate three rhinoviruses in this way, possibly because too much cellular protein or serum was present in the virus suspension I used, or less likely, because not all rhinoviruses are adsorbed equally well. Rhinovirus antisera have been prepared in guinea pigs by several workers (Ketler et al. 1962; Johnson and Rosen, 1963; Hamre et al. 1964). Although the methods I used were more simple, they gave satisfactory results.

Cross-neutralization studies with rhinoviruses isolated at Ruchill Hospital and guinea pig antisera prepared against them revealed two pairs of serologically identical viruses, a pair closely related and a pair showing a strong one-way cross-reaction. This one-way cross-reaction was shown

to be due to a mixture of viruses and not to antigenic differences. The absence of true one-way cross-reactions agrees with the results of most other workers who have prepared antisera in guinea pigs. However, one-way cross-reactions have been demonstrated using antisera prepared in guinea pigs between rhinovirus B632 and strains of echovirus 28 and, to a lesser extent, between strain NIH 353 and certain Chicago rhinoviruses (Monto and Johnson, 1966; Hamre et al. 1964). Low level heterotypic responses have frequently been found in antisera prepared in cattle (Fenters et al. 1966). Fenters and his colleagues believe that serologically distinct rhinoviruses may be grouped by these low-level cross-reactions. It is however possible that cattle are naturally infected with rhinoviruses serologically related to the human viruses and that heterotypic responses are due to the 'recall' phenomenon similar to that found in coxsackievirus type B infections in man. The reciprocal cross-reaction between strains 4574-62 and 1767-65 suggests that they belong to the same serotype, although one was an M strain and the other an H strain. The isolation of M and H strains of the same serotype has been reported for two serotypes (Phillips, Riggs, Melnick and Grim, 1965b). Of the viruses isolated in Glasgow, two M strains and four H strains were neutralized by both 4574-62 and 1767-65 antisera and one M strain and three H strains were neutralized by 1321-62 antiserum. These findings indicate that the grouping of rhinoviruses into M and H strains may be a guide to their serotype but is not necessarily so. Recently Douglas, Cate and Couch

(1966) have shown that five H rhinoviruses would produce cytopathic effects in monkey kidney cells if enough virus was inoculated, but there is evidence that one of these viruses, strain NIH 1059, failed to grow in monkey kidney cultures because it was not absorbed by the cells to any appreciable extent (Haff et al. 1966). There are also differences between M and H strain viruses both in their ability to stimulate antibody production (Taylor-Robinson et al. 1963) and in their seasonal pattern of infection (Bloom, Forsyth, Johnson and Chanock, 1963). Thus, an important distinction may yet exist between rhinoviruses which can be isolated and grown in monkey kidney cells and those which cannot.

The isolation of RS virus was influenced by the treatment of specimens and the sensitivity of cells into which they were inoculated. The removal of RS virus from specimens by centrifugation has not previously been reported and indicates that virus is probably attached to cells, cell debris or other particulate matter.

BH cells grown in calf serum lose some of their sensitivity to RS virus (Tyrrell, 1963). This might be due to the RS virus inhibitors in calf serum reported by Taylor-Robinson and Doggett (1963) and also found in this study. A HeLa cell line grown in human serum containing poliovirus inhibitors was considerably less sensitive to polioviruses than the same cell line after growth in other sera (Tomlinson et al. 1964). The sensitivity of the cells grown in human serum could be partly restored

by mild treatment with trypsin but not by washing the cell monolayer before infection. Loss of sensitivity by BH cells grown in rabbit serum has not previously been reported. Since rabbit serum does not appear to contain RS virus inhibitors it is unlikely to have lowered the sensitivity of BH cells. Contamination by mycoplasmas does not appear to be relevant since the phenomenon occurred in cells from which mycoplasmas were isolated and in cells from which they were not. Chance contamination by other infectious agents is unlikely to be responsible since the phenomenon was repeatable. Parental and clonal strains of HeLa cells have been shown to vary in their sensitivity to poliovirus (Darnell and Sawyer, 1959). Hence, overgrowth of the BH cell strain by a clone of cells resistant to RS virus may explain the loss of sensitivity of BH cells to this virus.

The BH cells were consistently more sensitive for RS virus isolation than RMK, HEKF or WI-38 cells. This finding appears to conflict with that of Wulff, Kidd and Wenner (1964b) who isolated RS virus more frequently in RMK than in HEp-2 cells and that of Anderson and Beem (1966) who found WI-38 cells as sensitive as HEp-2 cells. Other workers (Holzel et al. 1965a) who have compared BH cells with RMK, isolated RS virus more often in BH than in RMK. It is possible that the HEp-2 cells used in the American studies were less sensitive than BH cells. There may also be differences between virus strains which are reflected in the cells in which they may be isolated. In this context

it is interesting that one of the strains isolated in RMK by Wulff and colleagues (1964b) differs antigenically from the prototype Long strain (Wulff, Kidd and Wenner 1964a).

Unheated rabbit serum increases the rubella neutralizing antibody titre of human sera (Neva and Weller, 1964; Plotkin, 1964; Parkman, Mundon, McCown and Buescher, 1964). Unheated serum has a similar effect on herpes simplex neutralizing antibody (Yoshino and Taniguchi, 1964). The finding that unheated rabbit serum increases the titre of RS virus neutralizing antibody has not previously been reported. The increase of herpes simplex neutralising antibody titres by unheated serum appears to be due to complement and is most marked in sera from recently infected patients (Yoshino and Taniguchi, 1964, 1966). Although I did not study the effect of unheated rabbit serum on RS neutralizing antibodies in detail, it is probable that its mode of action is similar. Yoshino and Taniguchi (1966) found that guinea pigs immunized with herpes simplex virus produced detectable complement-requiring neutralizing antibodies after 7 days. Non-complement requiring neutralizing antibodies were not detected until 28 days after immunization was started, when the complement-requiring neutralizing antibody titres were 320. Comparable results for the immunization of guinea pigs with RS virus are not available but the following results suggest that the formation of RS antibody may be similar: sera from guinea pigs which had been immunized for less than 4 weeks showed high homologous antibody titres

only in the presence of unheated serum (Table 6.13); therefore, high levels of complement-requiring antibody were present but little or no non-complement-requiring antibody. Sera from guinea pigs which had been immunized for 7 weeks showed high homologous antibody titres both in the presence and in the absence of unheated serum (Table 6.10); therefore high levels of non-complement-requiring antibody were present. This effect is more striking with Randall strain than with 329-63 strain.

In rabbits immunized with a high dose of poliovirus, IgM immunoglobulins appeared first and were later replaced by IgG immunoglobulins (Svehag and Mandel, 1962). It is possible that with herpes simplex and RS virus the complement-requiring antibodies are IgM and the non-complement-requiring antibodies are IgG. This is probably an over-simplification of the situation for it is clear that not all viruses induce the production of complement-requiring neutralizing antibody. For example, unheated rabbit serum did not affect rhinovirus neutralizing antibody titres.

Unheated serum was valuable in studying certain aspects of the epidemiology of RS virus. Maternal antibody and significant antibody titre rises in young children could often be detected only if unheated rabbit serum was present in the serum-virus mixtures. Unheated serum was also valuable during an investigation of antigenic variation among RS virus strains. The most strain-specific antisera often had little or no antibody if titrated without unheated serum but had high titres ($\geq$ 512) in the presence of unheated serum.

Antigenic differences between strains of RS virus have been reported (Coates, Kendrick and Chanock, 1963; Wulff et al. 1964a, Doggett and Taylor-Robinson, 1965; Suto et al. 1965; Coates, Alling and Chanock, 1966) but since there has been little comparison of strains between these workers it is not possible to say how many different strains there may be. However, it has never previously been possible to demonstrate antigenic differences using human sera. My finding with the 329-63 strain and sera collected from children during the 1962-63 winter (Table 6.5) is therefore surprising. I was not able to demonstrate such a difference with sera from subsequent years (Table 6.9) and it is possible that the original observations were the result of differences in the antigen/infectivity ratio of the virus pools used, although their preparation was similar.

Most work on the antigenic variation of RS virus has been done using ferret sera (Doggett and Taylor-Robinson, 1965; Coates et al. 1966) but rabbit and guinea pig sera have also been used and appear to be more strain specific (Coates and Chanock, 1962). The guinea pig sera I initially prepared against the Randall and 329-63 strains showed a one-way cross-reaction (Table 6.10), but some of the sera prepared by only two intra-muscular injections showed a reciprocal antigenic difference (Table 6.13). This discrepancy could be explained by the method of preparation of the sera. The sera Randall/1 and 329-63/2 were prepared by giving guinea pigs an intranasal inoculation followed by two intramuscular injections. It is possible that intranasal inoculation failed to infect the pig yielding

Randall/1 serum (Hambling, 1966) hence its antibody response was against the two intramuscular injections only and therefore more specific.

The results of cross-neutralization tests with Randall and 329-63 strains indicated reciprocal antigenic differences which could not be explained by differences in avidity for antibody. Seven other strains were neutralized by Randall and 329-63 antisera. One of these (143-65) appeared to be closely related to 329-63. Three strains were poorly neutralized by both 329-63 and Randall antisera and three other strains (including CH 18537) were strongly neutralized. The neutralization of CH 18537 by Randall antisera is surprising since hyperimmune rabbit serum against the Long strain had little or no neutralizing activity against CH 18537 (Coates et al. 1963) and the Long and Randall strains have been shown to be antigenically similar (Bennett and Hamre, 1962; Doggett and Taylor-Robinson 1965). This discrepancy might be due to the different species of animals used. Differences between poorly-neutralized and well-neutralized strains are not due to tissue culture passage history since strains which had been passaged similarly fell into both groups. Differences in avidity for antibody could be responsible but antigenic variation seems the most likely explanation since the results are similar to those of Coates and colleagues (1966). They tested 12 strains isolated during one year against Long and CH 18537 anti-sera: nine strains were neutralized by Long but not by CH 18537, two were neutralized by both sera, one was neutralized only by the CH 18537 serum. Of the five 1965 strains I tested, one was similar

to 329-63, two were neutralized by both 329-63 and Randall sera and two were only poorly neutralized by the two sera. Thus, it seems that different antigenic types circulate at the same time and that new 'antigenic subtypes' do not replace previous ones.

Although strains can be distinguished in neutralization tests they are all quite closely related and RS virus has been compared to the A, or A2 families of influenza A virus in the magnitude of its antigenic heterogenicity (Coates et al. 1966). Available evidence does not suggest that antigenic variation of RS virus is important in human disease. Although reinfection of children and adults with RS virus occurs despite the presence of circulating antibody (Kapikian et al. 196la; Johnson et al. 1962c) the strains isolated from the same individual during different episodes are antigenically indistinguishable (Beem, 1965). Thus, there is little selective pressure for new serotypes to emerge and the role of antigenic variation among RS strains remains obscure.

PART II

THE EPIDEMIOLOGY OF HUMAN RESPIRATORY VIRUSES ISOLATED IN GLASGOW, 1962 - 1966.

CHAPTER 8

REVIEW OF THE LITERATURE

The literature on the epidemiology of human respiratory viruses is now vast and the object of this review is to summarize briefly the epidemiological characteristics of the most important viral agents. The viruses or virus groups to be considered are influenza viruses, adenoviruses, enteroviruses, reoviruses, parainfluenza viruses, respiratory syncytial virus and rhinoviruses. The history of their isolation, their geographical and age distributions, their behaviour in different population groups, their association with disease and their seasonal occurrence will be outlined. Where appropriate, there are comments on the relative importance or special characteristics of particular serotypes within a group.

Influenza Viruses

Early information about the prevalence of influenza in England and Wales may be obtained from the Registrar-General's figures for deaths from influenza. In the seventeenth and eighteenth centuries there were many epidemics of this disease but in 1850 the disease virtually disappeared until the pandemic of 1890 and thereafter the death rates per million of the population remained at a higher level than ever before. The highest mortality of the past hundred years occurred during the 1918 pandemic and since then years with major epidemics have alternated with years of minor prevalence

(Stuart-Harris, 1965).

In 1932 the first influenza virus, later called the W.S. strain of influenza A virus, was recovered (Smith, Andrewes and Laidlaw 1933) and until 1946 all influenza A viruses recovered resembled W.S. or the related PR 8 strain recovered in America in 1934 (Francis, 1934). In 1946 a serologically distinct A virus of the A1 family, the CAM virus, was recovered in Australia (Anderson and Burnet, 1947; Anderson, 1947) and similar viruses caused outbreaks in Britain and the U.S.A. in 1947. Viruses of the A1 family were thereafter recovered from outbreaks all over the world (Mulder, Braus and Masurel, 1956) until 1957. Strains of a third distinct family of influenza A viruses, the A2 family, were first isolated in February, 1957 in South-east China (Tang and Liang, 1957). Within a year A2 viruses spread as a pandemic and were isolated from widely scattered areas of the world (Payne, 1958).

In 1940, an influenza virus with no serological overlap with any earlier virus was discovered independently by Francis (1940) and Magill (1940) and named influenza B. This virus resembled influenza A epidemiologically but outbreaks of influenza B were usually localized and seldom involved an entire country, in addition its antigenic variation seemed to be less than that of influenza A.

In 1947 a third type, influenza C, was recovered by Taylor (1949, 1951) and has since been isolated from a few cases with respiratory disease and rare outbreaks in isolated communities (Francis, Quilligan and Minuse, 1950;

Gerber, Woolridge, Seal and Ziegra, 1952). In Britain, this virus has occasionally been isolated from cases of influenza or common cold (Andrews and McDonald, 1955) and rising antibody titres have been detected during mild respiratory disease and during pneumonia (Grist, 1955). Antibodies are acquired during childhood and are found in a high proportion of adults in Britain and U.S.A. suggesting that the virus is widespread, although few isolations are made and little is known about its epidemiology.

The epidemiology of influenza A and B viruses is complex largely because of their ability to vary antigenically. Since influenza is a fairly well defined clinical disease much knowledge of the epidemiology of influenza has been based on mainly clinical evidence. However the epidemiology of the disease may not always accurately reflect the epidemiology of the virus since infection with influenza viruses can cause mild respiratory symptoms or even none at all. Sporadic infections with influenza viruses do occur but most infections are recognised during epidemics. The nature of each epidemic varies according to the antigenic composition of the causative agent and the previous experience of the population to it.

The geographical distribution of influenza viruses is worldwide as the influenza A2 pandemic illustrated. Antibodies again influenza A2 and B viruses were found in most of the sera collected between 1958 and 1962 from 16 widely scattered countries and three distinct isolated communities (Taylor-Robinson, 1965; Brown and Taylor-Robinson, 1966).

In a primary epidemic of a 'new' virus variant the highest incidence of disease is usually in the 5 - 14 year age group. There is a decline to about 25 years of age followed by a rise during 25 - 34 year old period. After 40 years there is always decreased incidence which tends to settle around 10 - 15 per cent of the population at risk. In contrast, the age distribution of later episodes associated with essentially the same virus may differ in that dominance of disease among the younger age groups disappears and incidence is more generally distributed (Fry, 1958, 1959 and 1961; Francis and Maasab, 1965). In two British surveys the isolation rate of influenza viruses from respiratory illnesses was the same (2 - 3%) in children aged 5 - 15 years as in adults, but less (1 - 2%) in children under 5 years-old (MRC, 1965b; Higgins, Ellis and Boston, 1966).

No population group appears to be particularly susceptible or resistant to influenza viruses. Surveys of respiratory disease indicate that these viruses may be isolated from 1 - 3% of illnesses in children or adults at home (MRC, 1965b; Higgins et al. 1966), children in hospital (Chanock and Parrott, 1965a; Holzel et al.1965a) or university students (Hamre, Connolly and Procknow, 1966). Serological evidence of influenza virus infection has been found in 3 - 4% of respiratory illnesses in industrial workers (Hilleman et al.1962; Gwaltney, Hendley, Simon and Jordan, 1966), 11% of illnesses in university students (Gwaltney and Jordan, 1966) and 2% of illnesses of children in hospital (Chanock and Parrott, 1965a). McDonald, Miller, Zuckerman and Pereira (1962) obtained

serological evidence that during two winters 26% of respiratory illnesses in military personnel were due to influenza viruses.

There is no doubt that influenza viruses cause respiratory disease. However, serological evidence of influenza A or B virus infection may be found as often in people without respiratory illness as in those with such illness (Hilleman et al. 1962; Gwaltney et al. 1966). Influenza viruses may also be isolated from children without respiratory symptoms (Chanock and Parrott, 1965a). The diseases associated with influenza A or B virus infections vary from a mild common cold to pneumonia (Parrott, Kim, Vargosko and Chanock, 1962a; Evans, Dick and Nystuen, 1963). Information about the relative frequency of different illnesses caused by influenza viruses is limited, partly because many infections, particularly in interepidemic periods are not recognised clinically (Hayslett et al. 1962). Most of the influenza infections detected in a recent British survey (MRC, 1965b) were associated with a clinical diagnosis of influenza but a quarter were associated with colds. In children in hospital influenza viruses have been isolated from between 0.8% and 2.4% of children with upper respiratory infections and about 1% of those with lower respiratory illnesses; serological evidence of influenza infection was most often found in children with croup (Chanock and Parrott, 1965a; Holzel et al. 1965a). Severe, often fatal, pneumonia associated with staphylococcal infection may occur during influenza epidemics (Stuart-Harris, 1965). Influenza viruses may also cause severe illness in

patients with chronic bronchitis (Stuart-Harris and Hanley, 1957). Between 5% and 10% of exacerbations of chronic bronchitis have been associated with influenza infections (Stark, Heath and Curwen, 1965; Ross et al. 1966). Fry (1961) found chest complications occurred in 20%, 12%, 10%, 3%, 10% and 14% of clinical cases of influenza in six successive epidemics in his practice; only a few of these cases had previous history of chronic bronchitis.

Seasonally, influenza epidemics occur in closely defined periods, usually in the early months of the year in Britain. Virus isolations from sporadic cases are also usually made at this time (Clarke et al. 1964; MRC, 1965b; Higgins et al. 1966). Influenza viruses may cause high morbidity and appreciable mortality during epidemics, but over a number of years their contribution to respiratory disease is comparatively small.

Adenoviruses

Despite early success in establishing the cause of major outbreaks of influenza, as recently as 1953, Stuart-Harris pointed out that the causes of the majority of human respiratory illnesses remained unknown. In the same year, 1953, the first adenoviruses were isolated from human adenoids (Rowe et al. 1953) and in the following year Hilleman and Werner (1954) reported the isolation of three adenoviruses from throat swabs of American soldiers suffering from acute respiratory diseases and suggested they might be responsible for some outbreaks of acute respiratory disease. However, by

1958 Huebner, Rowe and Chanock had shown that 13 adenovirus types could account for no more than 5% of the total problem and despite the increase of human adenovirus serotypes to 31 (Pereira, Pereira and Clarke, 1965) there is no evidence that the group contributes to more than a small fraction of respiratory illness in the general population.

The geographical distribution of adenovirus infections is probably world-wide, although most data have so far come from studies in North America and Europe.

Serological studies of the general population suggest that the initial adenovirus infections which occur early in life are most frequently due to types 1 and 2 and less often to types 3, 5 and 7 (Jordan, Badger and Dingle 1958). A study in Holland indicates that by the age of 5 years practically all children have been infected with at least one type of adenovirus and about 50% of them with at least four types (van der Veen, 1963). In a civilian population 7% of children and 2% of adults showed serological evidence of infection during a winter season (Jordan, 1958). Age appears to affect the type of illness which occurs in adenovirus infected individuals. In civilian populations adenoviral pneumonia has been observed mainly in children under 4 years of age (Chany et al. 1958; Parker, Wilt and Stakiw, 1961; Clarke et al. 1964). In Chany's study however overcrowding, retarded development and malnutrition were also thought to have contributed to the severity of the illness. Pharyngo-conjunctival fever seems to be most commonly found in school children (Kendall et al. 1957; Fukumi et al. 1958;

van der Veen and van der Ploeg, 1958). Amongst adults in the general population adenovirus infection seems to be infrequent but sporadic cases with upper respiratory illness do occur (Higgins et al. 1966).

The frequency of adenovirus infections has been studied in several population groups including families living at home, hospital outpatient and in-patient groups and military recruits. In family studies adenovirus infections have been found in about 2 - 3% of children with respiratory illness (Jordan et al. 1956a; Kendall, Cook and Stone, 1960) and less than 1% of adults (Hilleman et al. 1962; Robinson et al. 1962). Adenoviruses were isolated from 1% of all the respiratory specimens collected during four years of continuous fortnightly surveillance of New York families (Spigland et al. 1966); 30% of the adenovirus infections detected in the survey were associated with respiratory illness (Elveback et al. 1966). In outpatient groups with respiratory illness the adenovirus infection rates appear to be slightly higher, 5% for children (Hilleman et al. 1962) and 2% for adults (Hamre et al. 1961). A study of infants attending a well-baby clinic (Moffet and Cramblett, 1962) revealed adenovirus infection in 33 of 524 visits; 42% of these infections were associated with respiratory illness. Adenoviruses were isolated from infants aged 1 - 6 months ten times more often than from newborn infants, children aged 5 - 18 years or their mothers aged 15 - 38 years. Estimates of adenovirus infection rates in respiratory illness in hospitals vary from 4 - 25% for children and 1 - 4% for adults (van der Veen, 1963), although in

one survey of selected hospital patients (nearly all adult) 18% had evidence of adenovirus infection (Jordan et al. 1956a). Two recent reports of studies of large numbersof children in hospital over several years reveal adenovirus isolation rates of 8.5% and 4% from patients with respiratory illness and 3.9% and 1.2% from patients with no respiratory illness (Chanock and Parrott, 1965a; Holzel et al. 1965a). The highest infection rates of adenoviruses, 30 - 50% have been found in military recruits with acute respiratory illness (van der Veen, 1963). A more recent report (Bloom et al. 1964) shows that during a particular 4-month period adenovirus infection rate in men with respiratory disease was 68% but during the same period 33% of men without respiratory disease also had evidence of infection.

It is often difficult to prove that adenovirus infections cause clinical symptoms because adenoviruses are found in apparently normal tonsils and adenoids and may be shed intermittently for long periods after infection, particularly by children, and especially types 1, 2 and 5. However, a survey in an American orphanage demonstrated a causal relationship between illness and infection by types 1, 3 and 5; infection by type 2 did not show such a relationship. The same survey suggested that a child is very likely to become ill when first infected with types 1, 3 and 5 but, upon re-infection (or latent shedding) is much less likely to have an illness (Bell, Rowe and Rosen, 1962). Pharyngitis and pharyngoconjunctival fever associated with adenovirus infection occur sporadically in both civilian and military

populations but epidemics due to type 3 and less often type 7 are found in children. Epidemics of acute respiratory disease in military recruits have been associated with types4, 7 and, more recently, 21 (Bloom et al. 1964; van der Veen and Dijkman, 1962). In these epidemics 20 - 40% of recruits required hospitalization. Only two similar outbreaks in civilian populations have been reported (Tyrrell, Balducci and Zaiman, 1956; Ormsby, Fowle and Doane, 1957). Severe and fatal cases of pneumonia due to adenovirus types 3 and 7 have been reported (Stuart-Harris, 1965) and recently Clarke and colleagues (1964) recovered adenovirus 21 from six children with severe broncho-pneumonia, one of whom died.

Marked seasonal variation of adenovirus epidemics is well recognized. Most large outbreaks of pharyngo-conjunctival fever have been observed in the summer months (van der Veen, 1963). In contrast, adenoviral epidemics in military recruits occur almost exclusively in the winter (van der Veen and Dijkman, 1962; Bloom et al. 1964), as does adenoviral pneumonia of children (Chany et al. 1958; Clarke et al. 1964). Athough van der Veen (1963) suggests that sporadic cases of upper respiratory illness due to adenoviruses also tend to occur predominantly during the winter and spring, recent studies of patients in general practice (Higgins et al. 1966) and children in hospital (Holzel et al. 1965a) show no definite seasonal variation in adenovirus infections. The family survey of Spigland and colleagues (1966) again reveals no definite seasonal variation in adenovirus excretion but suggests initial onset of

infection tends to occur in winter or early spring.

Enteroviruses

The introduction of the suckling mouse and widespread use of cell cultures resulted in the discovery of large numbers of small ether-resistant viruses in the 1950's. The enteroviruses now comprise over 60 serotypes and much is known about their epidemiology and ability to cause such diverse human syndromes as aseptic meningitis, epidemic pleurodynia, acute pericarditis, febrile exanthem and encephalomyocarditis of the newborn. However, the role of enteroviruses in respiratory disease is, far from clear, partly because many diseases such as poliomyelitis or aseptic meningitis may begin with symptoms suggestive of a respiratory illness, and partly because the ratio of clinically apparent to inapparent enteroviral infections may be quite low. There are nevertheless a number of enteroviruses which appear to be associated with respiratory disease.

The coxsackieviruses of group A were first isolated by Dalldorf and Sickles (1948) in suckling mice. In 1951, Huebner and co-workers showed that types A2, 4, 5, 6, 8 and 10 could be isolated significantly more often from cases of herpangina than from matched healthy control subjects. There is also an unconfirmed report (Lerner, Klein, Levin and Finland, 1960) of the isolation of coxsackievirus A 9 from the lung of a 16 month old girl who died of pneumonia. The Pett strain of coxsackievirus A 24 was recovered from the faeces of 8 out

of 10 patients in an outbreak of respiratory illness in Washington (Kasel, Cramblett and Utz, 1958). One of these strains however produced no illness when given to volunteers (Kasel and Knight, 1963).

Coxsackievirus A 21 (Coe virus) is different from others in the group. It is definitely associated with respiratory illness, particularly in military personnel, and its epidemiology is similar to that of adenovirus types 4 and 7. Although the prototype strain of coxsackievirus A 21 was recovered from faeces (Sickles, Mutterer and Plager, 1959) most isolations have been made from throat specimens. Lennette, Fox, Schmidt and Culver (1958) first reported the isolation of this virus from military personnel with upper respiratory diseases; but as their virus was of low pathogencity for suckling mice it was not thought to be a group A coxsackievirus until Schmidt, Fox and Lennette (1961) showed it to be identical with coxsackievirus A 21.

This virus has since been isolated in Britain (Pereira and Pereira, 1959), Japan (Fukumi, Nishikawa, Sonoguchi and Shimizu, 1961) and Holland (van der Veen, Dei and Prins, 1960).

Coe virus antibodies are rarely found in children but become more prevalent with increasing age, like adenovirus type 4. Abraham (1962) found antibody in 2% of children, 5% of adults 20 - 30 years old and 12% of middle-aged adults in Pittsburgh. In the studies of Pereira and Pereira (1959) and Fukumi, Nishikawa, Sonoguchi and Shimizu (1962) antibody was present in

higher proportions of civilian adults (over 25% of the 20 - 30 age group). There is also a sex difference in that males appear to acquire antibody at an earlier age than females but this may be related to the military associations of this virus.

All reported isolations of Coe virus from natural illness appear to have been made from military populations although sero-conversion occurs in the civilian population during the winter (Fukumi et al. 1962). Recent reports of large surveys of children and adults at home and in hospital with respiratory disease do not include one isolation of Coe virus (Chanock & Parrott, 1965a; Holzel et al. 1965a; MRC, 1965b; Higgins et al. 1966).

The natural infections so far reported have been associated with colds and pharyngitis. Johnson, Bloom, Mufson and Chanock, (1962b) showed a significant association between virus isolation and mild respiratory disease during the first 6 weeks of an outbreak but during the last 4 weeks virus isolation was not correlated with disease. This was thought to be due to the unusually long persistance of virus in the throat. The ability of the virus to cause colds has been confirmed by inoculation of human volunteers (Parsons, Bynoe, Pereira and Tyrrell, 1960; Spickard, Evans, Knight and Johnson, 1963). The work of Spickard and colleagues (1963) has also shown that circulating antibody will prevent inoculated volunteers becoming ill. Two volunteers given large doses of virus developed severe illness (Spickard et al. 1963)

and two of 71 volunteers inoculated with virus in a fine spray developed pneumonia (Couch et al. 1964). Severe illnesses of these kinds have not been found in natural infections. Coe virus is more frequently isolated from the throat than the faeces (Johnson et al. 1962b), and infection of the gut through tube or capsule produced no illness in volunteers (Spickard et al. 1963). These findings suggest that the natural habitat of Coe virus in man is the respiratory tract rather than the enteric tract.

Reported outbreaks of Coe virus infection in military populations have occurred in autumn or winter (Bloom, Johnson, Mufson and Chanock, 1962; McDonald et al. 1962). Bloom and colleagues (1962) comment that the end of the outbreak was not due to a lack of susceptibles and suggest that climatic conditions may have been responsible. Similar observations were made in studies of adenovirus epidemics in military camps (Forsyth, Bloom, Johnson and Chanock, 1964).

Coxsackieviruses of the B group were first isolated in suckling mice by Melnick, Shaw and Curnen (1949) and Dalldorf, Sickles, Plager and Gifford (1949) from cases of aseptic meningitis. These viruses have since been associated with pleurodynia, myocarditis, pericarditis and occasionally paralysis (Dalldorf and Melnick, 1965). Coxsackieviruses of the B group have been found in all parts of the world (Dalldorf and Melnick, 1965).

Coxsackievirus group B infections appear to be most common in young children and to become less common with increasing age.

Infection with group B coxsackievirus was first shown to be associated with respiratory illness by Jordan, Stevens, Katz and Dingle (1956b). Heggie and associates (1960) recovered coxsackievirus B 2 significantly more often from ill persons than from subjects without illness during an outbreak of mild febrile disease. Virus was recovered more often from throat than from rectal swabs of ill persons. During a twelve-week period in summer, coxsackievirus B 3 was isolated from 19 of 39 children with respiratory illness but from none of 22 children without febrile respiratory symptoms (Kendall et al. 1960). Coxsackievirus B 5 has been isolated from 20% of 126 children with mild or febrile upper respiratory illness seen in an outpatient department; the agent was also isolated from 5% of 118 children with lower respiratory illness and 5% of 212 children without respiratory illness (Vargosko, Kim, Parrott and Chanock, 1962). These results suggest a causal relationship of coxsackievirus B 5 with upper respiratory illness but probably not with lower respiratory tract disease.

Coxsackievirus B 1 has been isolated from the lung of a 21-year old woman who died of rheumatic heart disease and broncho-pneumonia (Jahn, Felton and Cherry, 1964). Large studies of children in hospital (Chanock and Parrott, 1965a; Holzel et al. 1965a) both showed coxsackieviruses of the B group to be associated with 1.9% of cases of respiratory illness but these viruses were also found in 0.8% to 1.6% of children without respiratory illness. Paffenberger and associates (1959) studying boys in a summer

camp found respiratory symptoms in a significantly higher proportion of boys excreting coxsackievirus B 4 than in those who were not. Two studies in childrens' institutions have revealed an association of coxsackievirus B 3 with outbreaks of upper respiratory illness (Bell et al. 1961; Roberts, Rendtorff and Hale 1965). Cramblett et al. (1964) in a survey of 114 coxsackievirus group B infections in North Carolina, found 35% associated with respiratory illness, 45% with neurological, enteric or undifferentiated febrile illness and the remainder associated with unrelated or no disease.

Almost all the reported respiratory illnesses caused by coxsackieviruses of Group B have occurred in children during the summer and early autumn. The types most commonly involved appear to be 2, 3, 4 and 5.

Echoviruses were first isolated in 1951 (Robbins et al. 1951) but for many years their relationship to human disease was unknown. It is now known that echoviruses may be associated with many clinical diseases including aseptic meningitis, paralysis, myocarditis, pericarditis, encephalitis, rashes and diarrhoea (Melnick, 1965). There are also many reports of several echovirus serotypes being associated with respiratory disease. Types 1 and 6 were associated with outbreaks of febrile respiratory disease in Japan (Matumoto, 1963). Type 3 has been associated with mild febrile respiratory illness in infants and nursery children (Rosen, Kern and Bell, 1964). Of the higher serotypes, types 13, 19, 22 and 25 have been reported in association with respiratory illness (Cramblett et al. 1962; Wigand and Sabin, 1961;

Hooft, Nihoul, Lambert and Valcke, 1963; Reilly, Stokes, Hamparian and Hilleman, 1963). Echovirus 25 has produced respiratory illness when inoculated into adult volunteers (Kasel, Rosen, Loda and Fleet, 1965). Philipson and Wesslén (1958) reported the isolation of a virus later shown to be echovirus 11 from 32% of 53 children with croup and from 11% of 63 children without such illness; however the healthy children were selected from a day nursery and not hospital and therefore were not strictly comparable. Such results have not been repeated despite the testing of very large numbers of specimens from cases of respiratory disease. Adult volunteers inoculated with a strain isolated from the outbreak developed symptoms which suggested that the virus might cause a feverish sore throat (Philipson, 1958) but no respiratory symptoms were observed in a second group of volunteers inoculated with the same pool of virus (Buckland , Bynoe, Philipson and Tyrrell, 1959). Echovirus 20 was first recovered from orphanage children, some of whom were well and some of whom had respiratory illness (Rosen, Johnson, Huebner and Bell 1958; Cramblett et al. 1958). Adult volunteers inoculated with echovirus 20 developed febrile pharyngitis in most cases and a common cold in two (Buckland, Bynoe, Rosen and Tyrrell, 1961).

Most respiratory illnesses associated with natural echovirus infection have occurred in children, usually in the summer months.

Reoviruses

Reoviruses have been recovered from a wide range of human diseases

but in no case has their aetiological role been established with certainty. Rosen and co-workers (1960b) recovered 22 strains of reovirus type 1 from orphanage children between January and March 1957. The association of mild febrile respiratory illness with reovirus infection was of borderline statistical significance. In two other outbreaks in the same orphanage reovirus type 3 was isolated (Rosen et al. 1960a); although diarrhoea and coryza were associated with virus isolations there was insufficient evidence to prove a causal relationship.

Jackson, Muldoon and Cooper (1961) inoculated volunteers with nasal secretions collected from a subject with a common cold and known to contain reovirus type 1. Interpretation of the results was complicated by the isolation of echovirus 28 from the same secretion (Jackson, Muldoon, Johnson and Dowling, 1963); nevertheless suggestive evidence was obtained that reovirus 1 in the secretion produced common colds. Rosen, Evans and Spickard (1963) inoculated human volunteers with tissue culture grown strains of reovirus types 1, 2, and 3 but produced no illness which could be definitely attributed to the inoculum although there was evidence of infection in most of the volunteers.

A number of surveys of respiratory illness in children and adults do not suggest that reoviruses are an important cause. A serological survey by Hilleman and colleagues (1962) revealed one reovirus infection in 667 out-

patient children with upper and lower respiratory illnesses, another infection in 153 control children, no infections in 155 adults with common colds and one in 54 adult controls. Wulff, Kidd and Wenner (1964c) isolated 5 reoviruses from 114 hospital children with respiratory illness but did not investigate children without respiratory illness. The large survey of Spigland and colleagues (1966) revealed 6 isolations of reovirus and herpes virus (the exact number of each is not specified) from over 31,000 specimens taken between 1961 and 1965. Other surveys of respiratory diseases (Clarke et al. 1964; Chanock & Parrott, 1965a; Holzel et al. 1965a; MRC 1965b; Higgins et al. 1966) do not mention reoviruses at all.

In one study reoviruses were isolated during all seasons of the year but most often in winter unlike the enteroviruses which occur most frequently in the summer (Gelfand, 1959). The three outbreaks of reoviruses infections in an American orphanage were detected in the first, third and fourth quarters of the year (Bell et al. 1961). Whenever virus isolation attempts have been made on both rectal and throat specimens, the isolation rates have always been higher from the rectal specimens suggesting that the natural human habitat of reoviruses is more likely to be the gut than the respiratory tract.

Despite many reports of respiratory symptoms associated with entero-virus and reovirus infections and numerous experiments with these viruses in volunteers it seems unlikely that they contribute greatly to respiratory

illnesses in the general population.

Parainfluenza Viruses

The first of the parainfluenza viruses to be isolated, the Sendai or HVJ strain of parainfluenza 1, was found in mice inoculated with lung tissue from children who died of pneumonia (Kuroya, Ishida and Shiratori, 1953). Later studies have shown that Sendai virus is commonly found in mice and doubt has been expressed about its human origin (Fukumi et al. 1959). In 1955 the croup-associated or CA virus (later called parainfluenza 2) was isolated in tissue culture from infants with croup (Chanock, 1956) and in 1958 the isolation of parainfluenza types 1 and 3 from children with respiratory disease was reported (Chanock et al. 1958). More recently, Johnson and colleagues (1960) reported the isolation of a parainfluenza 4 virus. Hsiung, Isacson and McCollum (1962) have suggested that a virus which they isolated from human blood and which is antigenically similar to SV-5 should be classified as parainfluenza 5. However, there is no evidence that parainfluenza 5 causes disease in man.

Since their original isolation from man in the U.S.A. parainfluenza viruses have been found in most parts of the world where they have been sought, including Britain (Sutton, Clarke and Tyrrell, 1959), Russia (Bukrinskaya & Paktoris, 1960), Canada (McLean et al. 1961) and Australia (Lewis, Lehmann and Ferris, 1961a; serological studies have also revealed evidence

of parainfluenza infections in Holland (van der Veen and Smeur, 1961) Italy and India (La Placa & Moscovici, 1962) and 13 other countries (Taylor-Robinson, 1965) as well as three isolated communities (Brown and Taylor-Robinson, 1966). Recovery of parainfluenza 4 has not yet been reported outside the United States.

Infants and young children appear to be most susceptible to and severely affected by parainfluenza infection. Most infants possess type 3 neutralizing antibody by 2 years of age. Such antibody against types 1 and 2 is acquired more slowly but most children have it by the fifth year (Parrott et al. 1962b). Essentially the same conclusions may be drawn from studies of haemagglutination-inhibiting antibody (Stark, Heath and Peto, 1964). Although primary infectionsoccur early in life, reinfections have been detected (Parrott et al. 1962b). Serological studies of pneumonia in various countries have revealed parainfluenza infection rates of 6 - 17% (with one estimate of 71%) in infants and children and 3 - 5.5% in adults (Chanock et al. 1963). A study of acute respiratory illnesses in general practice (Higgins et al. 1966) has shown that parainfluenza viruses may be isolated from 6.3% of children under 5 years and 2.3% of children between 5 and 15 years and of adults over 16 years. A large virus survey of New York families suggests that there is little difference in parainfluenza isolation rate with age (Elvebach et al. 1966; Fox et al. 1966). However, parainfluenza viruses may be more difficult to isolate from adults than from children and parainfluenza infections

may not be detected by virus isolation if tissue cultures are retained for only three weeks (Bloom, Johnson, Jacobsen and Chanock, 1961; Banatvala, Anderson and Reiss, 1964).

In the normal population living at home parainfluenza infection does not appear to be common. In one study only 21 parainfluenza viruses were detected in over 12,000 respiratory specimens collected during over 10,000 person-months of observation (Fox et al. 1966; Spigland et al. 1966). Surveys of mild respiratory disease (Chanock et al. 1963; Hamre et al. 1966; Higgins et al. 1966) have revealed parainfluenza infection rates of 1.9% to 9.1% in adults and 6 to 16% in infants and children.

Children in hospital are the group in which parainfluenza viruses are most commonly found. In two recent reports of large studies in Britain and America parainfluenza viruses were isolated from 5.4% and 6.5% respectively of children with respiratory disease; but from less than 1% of those without such symptoms. The use of serological diagnoses in one of these studies revealed 16% parainfluenza infections in respiratory illness of children and 6% in children with no respiratory illness. In both studies parainfluenza type 3 was most frequently isolated, then type 1 and finally type 2 least frequently (Chanock & Parrott, 1965a; Holzel et al. 1965a).

Parainfluenza viruses have been isolated from military personnel with a similar frequency to that found in civilian adults. Bloom and colleagues (1961) isolated 11 strains from 230 (5%) cases of respiratory illness and 3 from

74 (4%) controls without such illness. McDonald et al. (1962) found serological evidence of parainfluenza virus infection in 2 - 3% of respiratory illness in Air Force recuits but in less than 0.4% of similar illnesses in men at operational stations.

In institutions, particularly for children, there have been reports of sharp outbreaks of parainfluenza infections. There were eight such outbreaks during three years of study in a Washington Welfare Nursery (Chanock et al. 1963). Five were due to type 3, 2 to type 1 and 1 to type 2. The duration of each of the outbreaks was about 4 - 6 weeks. In an outbreak of type 3 infection in a residential nursery in Sheffield (Sutton, 1962) all the 16 children in the nursery at the beginning of the outbreak developed illnesses similar to that of the index case. A similar high infection rate was found by Chanock and colleagues (1963).

A significant difference between the isolation rates of parainfluenza viruses from children with respiratory disease and from those without has been demonstated in both orphanage and hospital populations (Bell et al. 1961; Chanock & Parrott 1965a). In a survey of parainfluenza virus infections in general practice croup was the commonest clinical manifestation of infection in children (Banatvala et al. 1964). Surveys in various countries have shown that parinfluenza viruses (mainly type 1) may be isolated from 26 - 51% of cases of croup (Chanock et al. 1963). Types 1, 2 and 3 viruses may be isolated from 3 - 4% of lower respiratory illness and 6 - 9% of upper respiratory illness of

children in hospital (Chanock & Parrott, 1965a; Holzel et al. 1965a). Fatal cases of parainfluenza infection have been reported: type 2 virus was isolated from the lungs of two infants who died (Von Euler, Kantor and Hsiung, 1963). During a series of parainfluenza virus outbreaks in a Welfare nursery it was found that approximately one third of primary type 3 infections and one quarter of primary type 1 infections were associated with lower respiratory signs. However, croup developed in only 1 of 45 type 1 infections and 1 of 31 type 2 infections. Neutralizing antibody conferred some protection against such illness. Reinfection with parainfluenza type 3 was proved by isolation during 3 outbreaks within 9 months. Seventeen per cent of children at risk who were infected during the first or second outbreaks were reinfected during the second or third outbreak. Triple infections were not observed. Serological findings of significant neutralizing antibody rises in 10 of 15 children during reinfection support the interpretation of reinfection rather than persistent infection with intermittent shedding. Most of the second infections were symptomless. Of 16 reinfections 8 occurred in children who possessed neutralizing antibody before the first infection, suggesting that reinfection can occur more than once (Chanock et al. 1963). Illness produced by type 4 virus in children appears to be mild, and limited to the upper respiratory tract (Canchola et al. 1964).

The significance of parainfluenza virus infection in adults with upper respiratory disease is less clear. There are several reports of such infections with symptoms of either influenza (Banatvala et al. 1964) or febrile sore throat

(Meenan, Clarke and Tyrrell, 1959; Bloom et al. 1961; Dick, Mogabgab and Holmes, 1961). Only the study of Bloom and colleagues (1961) included symptomless controls and it did not show a statistically significant association of infection with respiratory disease. In a serological survey of adults with chronic bronchitis there was evidence that parainfluenza viruses were associated with a small number of exacerbations (Stark et al. 1965). The only reported type 4 infection of an adult was in a student with pharyngitis (Johnson et al. 1960).

Adult volunteers have been inoculated with each of types 1, 2 and 3 (Reichelderfer et al. 1958; Tyrrell et al. 1959; Kapikian et al. 1961b; Taylor-Robinson & Bynoe, 1963). The results of these studies suggest that types 1 and 2 cause common colds and influenza-like illnesses and type 3 common colds, in adults who may have high levels of circulating antibody.

The seasonal distribution of parainfluenza virus infections appears to vary. Chanock & Parrott (1965b) reported the isolation of types 1 and 3 all the year round with peak prevalence in the colder months and of type 2 only in the colder months. In British studies (Higgins et al. 1966; Holzel et al. 1965a) type 3 also appears to be endemic but type 1 has occurred mainly in outbreaks. Occasionally parainfluenza viruses occur in an epidemic pattern. Chanock & Parrott (1965b) have reported one such epidemic in Washington during the 1962-63 winter when type 1 was recovered from up to 25% of children entering hospital. It is of considerable interest that during the same winter type 1 was prevalent in New Haven (Horstmann & Hsiung, 1963), in Toronto, Canada

(McLean, Bach, Lark and McNaughton, 1963) and in Cambridge, England (Banatvala et al. 1964). All the type 1 isolations reported in two other English surveys (Holzel et al. 1965a; Clarke et al. 1964) and 8 of the 10 reported by Higgins and co-workers (1966) occurred during the same winter. These facts suggest that occasionally this virus may assume an epidemic pattern over a large geographical area. Epidemic behaviour of types 2 and 3 has also been reported (Chanock & Parrott 1965b).

Antigenic variation among parainfluenza viruses appears to be uncommon. There is an unconfirmed report of variant strains of type 1 found in Moscow (Bukrinskaya, Yun-De and Gorbunova, 1962). Two subtypes, A and B, of type 4 have been reported (Canchola et al. 1964); both A and B types were recovered in Washington in 1962, but only B types were recovered in 1963. No such variations have been reported for types 2 and 3.

Respiratory Syncytial Virus

Respiratory Syncytial (RS) virus was first recovered from chimpanzees with common cold-like illnesses and from a laboratory worker who had been in contact with them; it originally was named chimpanzee coryza agent (Morris et al. 1956). Chanock and colleagues (1957) isolated RS virus from children with lower respiratory disease and noted the characteristic syncytial appearance of the virus in tissue culture. The virus was then renamed respiratory syncytial virus (Chanock and Finberg, 1957). Many studies in various parts of the world have since established that RS virus is the most

important viral respiratory pathogen of infancy.

After the original American reports RS virus was isolated in Britain (Peacock and Clarke, 1961), Australia (Lewis, Rae, Lehmann and Ferris, 1961b), Norway (Ulstrup, 1964) and Finland (Berglund, Vihma and Wickström, 1965). Serological evidence of RS virus infection has been found in sera from 14 different countries (Doggett, 1965).

Primary infection with RS virus occurs early in life. One third of infants develop neutralizing antibody by one year of life and 80 - 90% by 4 years (Chanock et al. 1957; Moss, Adams and Tobin, 1963; Beem, Egerer and Anderson, 1964; Hambling, 1964). In studies of respiratory illness of children in hospital RS virus isolation rate decreases sharply with increasing age from about 30% in children under 1 year (mostly under 6 months) to about 10% in children between 1 and 4 years old and even less in children over 4 years (Chanock et al. 1961; Chanock and Parrott, 1965a; Holzel et al. 1965a). In a study of respiratory illness in general practice RS virus was isolated from 7% of 381 cases under 4 years, 0.8% of 353 cases between 5 and 15 years and 0.1% of 798 cases over 16 years (Higgins et al. 1966). Similar results were found among the general practice cases of a collaborative study (MRC, 1965b). The marked predeliction of RS virus for causing respiratory diseases in the first half year of life is not found with other respiratory viruses (Chanock and Parrott, 1965a).

In surveys of respiratory illnesses in non-hospitalized populations RS

virus has been isolated from 7% of children under 4 years or 4% of children under 5 years but from less than 1% of older children or adults (Higgins et al. 1966; MRC, 1965b). One large survey (Spigland et al. 1966) failed to detect a single RS virus infection after examining over 12,000 respiratory specimens during about 4 years, although tests indicated that the techniques used were sensitive to RS virus. During a four-year study of respiratory illness in University students (Hamre et al. 1966) RS virus was isolated from 15 (1.7%) of 894 specimens collected during the acute stage of illness, none of 808 convalescent specimens and 1 of 2,130 specimens collected on a routine schedule unrelated to illness. Eleven of the 16 RS virus isolations were made during January and February 1961, indicating that RS virus may cause sharp outbreaks of respiratory illness in adults (Hamre and Procknow, 1961).

RS virus infections have been reported most frequently among children in hospital, where they appear to account for 9.5% to 14% of total childhood respiratory illnesses (Chanock and Parrott, 1965a; Holzel et al. 1965a) and almost 30% of all bronchiolitis. In one study (Chanock et al. 1961) a marked disparity was found between the frequency of virus isolations (1%) and of CF antibody development (15%) in the control group of children without respiratory disease. Such a disparity did not exist for patients with lower respiratory disease, suggesting that infection in the control group was acquired by cross-infection while in hospital.

Twenty-two RS virus infections occurred in a military population between

January and March 1961 (Johnson, Bloom, Mufson and Chanock, 1962c). This is further evidence that RS virus can cause sharp outbreaks of respiratory illness in adults. Fifteen of the 22 infections were associated with mild respiratory illness but 7 were not associated with any illness.

During an outbreak of RS virus infections in a childrens' institution, serological studies showed that 91% of the study population were infected. Pneumonia occurred in 40% of the 90 children in the institution, and its occurrence was not significantly influenced by the presence or absence of pre-outbreak neutralizing antibody (Kapikian et al. 1961a).

The symptoms associated with RS virus infection are closely related to the age of the infected individual. In studies of children in hospital, 74% to 100% of children under 1 year with virologically confirmed RS virus infection have lower respiratory tract symptoms, whereas only 20% to 58% of similar children over 1 year have such symptoms (Chanock et al. 1961; Holzel et al. 1965a; Berglund et al. 1965). Chanock and colleagues (1961) in an addendum mention three fatal cases associated with RS virus infections in children under 7 months and Beem and co-workers (1960) cite two fatal cases aged 3 months and 1 year respectively. Studies in both hospital and general practice populations have shown that RS virus is rarely isolated from children without respiratory illness and there is ample evidence that RS virus is a cause of respiratory illness in children (Chanock and Parrott, 1965a; Holzel et al. 1965a; MRC, 1965b). RS virus infection of children occurs in the presence of serum neutralizing activity (Chanock et al. 1961; Hamparian et al. 1961a; Kapikian et al. 1961a; Suto et al.

1965). If this neutralizing activity is specific antibody, and the work of Beem and colleagues (1964) would suggest that it is, then RS virus infection can cause severe illness even in the presence of neutralizing antibody. Beem (1965) has provided proof that reinfections do occur by a report on ten children having two separate symptomatic virus-shedding infections in successive seasons of virus prevalence.

The two reports of outbreaks of natural RS virus infections in adults suggest that the virus may cause a common cold-like syndrome in adults (Johnson et al. 1962c; Hamre et al. 1966) but a number of such infections are symptomless. Inoculation of adult volunteers has also produced common cold-like symptoms even in the presence of circulating antibody (Johnson et al. 1961; Kravetz et al. 1961).

There is one report (Berkovich & Kibrick, 1964) of a 2-year-old child and a mother and her newborn infant all having rashes in association with RS virus infection but there has been no confirmation of this.

An association between RS virus infection and exacerbations of chronic bronchitis has been suggested by two reports. In one, 17.4% of acute respiratory illnesses of chronic bronchitics were associated with RS virus infection (Carilli, Gohd and Gordon, 1964). In the other serological evidence of RS virus infection was found in a high proportion of adults with respiratory illness and 50% of these infections were associated with acute exacerbations of chronic bronchitis; however, from the data given, it is not possible to tell how many

sera from chronic bronchitics were tested (Sommerville, 1963). The more detailed study of Ross and colleagues (1966) does not, however, confirm the importance of RS virus in this condition. Infection with RS virus was found in only 3.5% of acute exacerbations.

There is a marked seasonal distribution of RS virus outbreaks. Most reports indicate that the peak of outbreaks in both adults and children occurs in winter or early spring. The large studies of Chanock and Parrott (1965a) have shown that such outbreaks occur among infants and young children every year in Washington and other studies (Beem et al. 1962; Holzel et al. 1965a) suggest that this is the characteristic pattern for this virus in large urban communities. The only two reported outbreaks of RS virus infection in adults both occurred between January and March, 1961 (Johnson et al. 1962c; Hamre et al. 1966). Sporadic cases of RS virus infection do occur throughout the year but these are infrequent.

There are several reports of antigenic variation among RS virus strains Wulff et al. 1964a; Doggett & Taylor-Robinson, 1965; Coates et al. 1966; Suto et al. 1965) but differences have not been demonstrated using human sera and the epidemiological significance of these variants is not clear. Beem (1965) has isolated two RS virus strains from separate infections of the same individual in ten instances but was not able to show that the pairs of strains were different and concluded that reinfections could not be explained on the basis of strain variation.

Rhinoviruses

The in vitro growth of rhinovirus D.C. was first reported in 1953 (Andrewes et al. 1953) but could not be repeated until 1962 (Tyrrell et al. 1962). The first member of the rhinovirus group to be extensively studied was originally classified as echovirus 28 (Price, 1956; Pelon, Mogabgab, Phillips and Pierce, 1957). The discovery that several common cold viruses would produce a cytopathic effect in tissue cultures under appropriate conditions (Tyrrell and Parsons, 1960) has led to the isolation of many serotypes of the rhinovirus group.

Isolations of rhinoviruses have been reported from several centres in the U.S.A. and Britain (Hamparian, Ketler and Hilleman, 1961b; Hamre and Procknow, 1961; Johnson et al. 1962a; MRC, 1965b) and antibodies against M rhinoviruses have been found in sera collected from 15 different countries (Taylor-Robinson, 1965) suggesting a wide geographical distribution of these viruses.

Rhinovirus infections are more common in adults with respiratory disease than children with similar symptoms. These viruses have been isolated from 3 - 4% of children with respiratory illness and 6 - 14% of adults in the same surveys (Bloom et al. 1963; MRC, 1965b; Higgins et al. 1966). However, the infection rate in adults is probably higher (Hamparian, Leagus, Hilleman and Stokes, 1964; Hamre et al. 1966). Studies on the frequency of antibodies against rhinoviruses suggest that different serotypes may behave differently. Johnson and Rosen (1963) found neutralizing antibodies against rhinovirus 11757

in less than 15% of children under 15 years but the proportion increased with increasing age so that 80% of adults over 40 years possessed antibody. In contrast, rhinovirus 1734 antibodies were present in 30 - 60% of persons over 1 year old and showed little change with increasing age. Neutralizing antibody against rhinoviruses HGP and B632 was found more often with increasing age (Schild and Hobson, 1962).

Civilian populations living at home have been studied for rhinovirus infections but results vary. In early reports (Hobson and Schild, 1960; Tyrrell and Bynoe, 1961) rhinovirus-like agents were isolated from 30% and 16% of colds. In larger studies of industrial workers rhinoviruses were isolated from 11% and 19% of adults with acute respiratory illness and from 1% and 2% of symptomless controls (Hamparian et al. 1964; Gwaltney and Jordon, 1964). Slightly lower rhinovirus isolation rates were found in adults in two British studies (MRC, 1965b; Higgins et al. 1966). In children at home, rhinoviruses have been found in 3 - 5% of those with respiratory illness and 2 - 3% of those without (Hamparian et al. 1964; MRC, 1965b; Higgins et al. 1966).

Several studies on University students (Hamparian et al. 1964; Gwaltney and Jordan, 1966; Phillips et al. 1965b; Hamre et al. 1966) report isolations of rhinoviruses from 9 - 32% of acute respiratory illnesses and from only 1 - 3% of control cases.

Three reports (Chanock and Parrott, 1965a; MRC, 1965b; Portnoy, Eckert and Salvatore, 1965) suggest that rhinoviruses may be isolated from 3 - 4% of children in hospital with respiratory illness.

A study of R.A.F. recruits (Pereira, Hambling, McDonald and Zuckerman,

1963) revealed rhinovirus infection in 23% of those with colds. In the larger survey of Bloom and associates (1963) in marine corps personnel and their children, rhinoviruses were found in 20% of men with afebrile respiratory disease and 5% of those with no respiratory illness when the men were undergoing extensive training as a semiclosed population. Rhinoviruses were isolated less often from men living under conditions similar to civilian populations.

In a survey of respiratory illness at a boarding school Kendall, Bynoe and Tyrrell (1962) isolated 18 rhinoviruses from 59 boys.

The association of this group of viruses with common colds in adults is well established both from highly significant differences between isolation rates from persons with colds and those without (Bloom et al. 1963; Hamparian et al. 1964; Hamre et al. 1966) and also from volunteer experiments(Cate, Couch and Johnson, 1964; Taylor-Robinson and Bynoe, 1964). The volunteer experiments provided evidence that specific antibody protected against reinfection and illness but only partially. There is also evidence (Fleet, Couch, Cate and Knight, 1965) that rhinovirus infection induced a non-specific resistance to further heterologous infection lasting between 2 and 5 weeks. It has been shown that a rhinovirus can cause tracheobronchitis in adult volunteers when they are infected by inhalation of virus in a small particle aerosol (Cate et al. 1965); and there is our own evidence that, under natural conditions, rhinoviruses may infect the lower respiratory tract of adults with chronic

bronchitis (Eadie, Stott and Grist, 1966).

The importance of rhinoviruses in respiratory disease in children is less clear. There are significant differences in virus isolation rates between children with respiratory illness and those without (Bloom et al. 1963) but symptomless infections appear to be more common in children than adults. Although rhinoviruses are found in 3 - 4% of children in hospital with severe respiratory illness, Portnoy and colleagues (1965) have found serological evidence of simultaneous infection with either RS virus or parainfluenza viruses or both in 5 (38%) of 13 such cases. However, the hospital children with rhinovirus infections studied by Hamparian and associates (1964) had no evidence of infection by other agents. There is one report (Holzel, Smith and Tobin, 1965b) of 3 infants with meningo-encephalitis associated with rhinovirus infection, but the significance of this finding is not yet clear.

When isolating rhinoviruses from nasal washings, conventional tissue culture techniques may detect less than half the rhinoviruses which can be detected by the use of organ cultures of human embryo trachea (Tyrrell and Bynoe, 1966). Thus, the importance of rhinoviruses in human respiratory disease is probably greater than present estimates suggest.

Although rhinoviruses may be isolated throughout the year there is evidence that peak prevalence occurs in the spring (Spigland et al. 1966) or in the spring and autumn (Bloom et al. 1963; Hamre et al. 1966). Some serotypes, particularly M strains, may occur in sharp outbreaks and then disappear from

a community, other types occur sporadically over long periods of time (Hamre et al. 1966; Mufson, Bloom, Forsyth and Chanock, 1966). One report suggests that M strains are isolated more frequently in the autumn and H strains more often in the spring (Bloom et al. 1963).

At present there appear to be at least 80 different serotypes of human rhinoviruses (Hamparian, 1966) and it is not yet possible to decided whether some serotypes are more important than others. Controlled studies have shown that a number of individual serotypes are significantly associated with respiratory disease (Johnson, Bloom, Forsyth and Chanock, 1965; Mufson et al. 1966).

In conclusion, it may be said that whereas 12 years ago little was known of the cause of most respiratory disease other than influenza, it is now possible to isolate viruses from over 30% of such illnesses. Improvements in technique may permit the isolation of known viruses from a higher proportion of illnesses and there is evidence that there are other respiratory viruses only recently isolated or not yet isolated whose importance is unknown (Tyrrell, 1964; Tyrrell and Bynoe, 1965; Hamre and Procknow, 1966; Hoorn and Tyrrell, 1966). Despite the great advances made in recent years knowledge of the spidemiology of many respiratory virus diseases is still incomplete.

CHAPTER 9

ADULTS AND CHILDREN AT HOME AND AT WORK

The work described in this chapter falls into two main parts:

(a) a comparative study of children with acute respiratory illness and their symptomless contacts which was part of a collaborative survey organised by the Medical Research Council.

(b) a study of acute respiratory illness in children and adults.

All the throat and nasal specimens were collected and tested in tissue cultures of RMK, BH and HEKF as described in Chapters 2 and 3. Many specimens, however, had been preserved at -70°C for some months before they were tested in HEKF. No blood sera were collected.

Comparative Study of Children with Acute Respiratory Illness and their Symptomless Contacts

Throughout the period November 1962 to May 1963, 33 families, each with at least two children under 15 years, in the practices of five general practitioners were asked to notify their doctor of any acute respiratory illness in their children. The general practitioner then visited the family and took specimens from the sick child (case) and from the child in the same household nearest in age to the ill child, who who had not had any respiratory symptoms for at least one week(contact.) The specimens were collected within three days of

the onset of illness of the case and were transported to the laboratory as soon as possible (4 - 24 hours). The family received a second visit from the doctor several days later to assess the final diagnosis of the case and the health of the contact.

A total of 31 pairs of cases and contacts were investigated between November 1962 and April 1963. A summary of the age and sex composition of the group studied and the viruses isolated is shown in Table 9.1.

Ten of the 31 contacts became ill within seven days of the specimens being collected: four had a cough, five a common cold and one had measles. Rhinoviruses were isolated from two of the contacts who developed colds after four and five days respectively and from the contact who developed measles after seven days. The fourth contact from whom a rhinovirus was isolated had last had respiratory symptoms 12 days previously.

The illnesses of the 31 cases comprised 22 common colds, seven feverish colds and two influenza-like illnesses. Three rhinoviruses and the RS virus were isolated from common colds and one rhinovirus from a feverish cold.

The RS virus was isolated from a 3 year-old male, with a common cold. The two older sisters of this three-year old child had no respiratory symptoms but a brother of six months developed bronchitis two days later.

The ages of the eight children from whom rhinoviruses were isolated ranged from 2 to 13 years with an average of 5.1; three were male and five female.

The monthly distribution of the cases, contacts and viruses isolated is shown in Table 9.2.

There were two occasions on which rhinoviruses were isolated from both the case and its contact. On both occasions the viruses were identified serologically and both case and contact were found to have the same serotype: 179E in one pair, and 1767-65 in the other. None of the other 4 rhinoviruses isolated were neutralized by the 40 available antisera.

In conclusion, rhinoviruses were isolated from 13% of children between 2 and 13 years old, but no convincing evidence was found to indicate their causative role in acute respiratory disease since viruses were isolated with the same frequency from children with respiratory illness and from their contacts who were symptomless at the time of specimen collection.

Acute Respiratory Illness in Adults and Children

Most of the patients considered in this section were members of the laboratory staff or their families who had colds. A number of the children were patients visited by a general practitioner or public health department staff, during the investigation of two outbreaks of respiratory illness which occurred in February, 1962 and January 1966. Both outbreaks were due to influenza B virus.

A total of 56 episodes in adults and 61 in children under 16 years were investigated. Thirty viruses were isolated from adults and 24 from children.

Details of the virus isolations by age are shown in Table 9.3. The high

isolation rate (26%) of influenza B from children is largely due to the fact that half the episodes investigated occurred during the two periods when influenza B was prevalent. Rhinoviruses were isolated from six (10%) of the episodes in children and 24 (43%) of those in adults. The rhinovirus isolation rate was higher in adults between 16 and 30 years (53%) than in those between 31 and 45 years (33%).

The sex distribution of the virus isolations shows little difference between the sexes (Table 9.4).

In adults all the rhinoviruses were isolated from colds or sore throats and all the influenza viruses were isolated from influenza-like illnesses (Table 9.5). In children all the rhinoviruses were again isolated from colds, but influenza viruses were isolated almost as often from colds (26%) as from influenza-like illnesses (33%).

The monthly distribution of the virus isolations includes figures for over 4 years (Table 9.6). Influenza viruses were isolated only during January and February but rhinoviruses were isolated throughout the year except during January, July and August. The absence of rhinovirus isolations in January is noteworthy but the results for July and August could be due to the small number of episodes investigated.

The duration of illness between the first appearance of respiratory symptoms and collection of the first specimens was usually less than four days. The total virus isolation rate was almost the same whether the duration of illness was 1 - 3 or 4 - 7 days (Table 9.7). The rhinovirus isolation rate where duration of illness was 4 - 7 days (30%) was slightly higher than the rate when the duration was less (22%).

Further information about rhinovirus shedding was obtained from 13 rhinovirus-positive episodes from which serial specimens were obtained at 3 - 7 day intervals as long as coryza persisted. The results of the examination of 35 specimens from these 13 episodes indicate that rhinoviruses could be isolated from a consistently high proportion of specimens up to 14 days after onset of illness (Table 9.8). If the average number of days for a specimen to show earliest cytopathic effect is taken as a crude estimate of the quantity of virus in the specimen, there is a suggestion that the amount of virus shed decreased with longer duration of illness. Symptoms persisted for less than a week in two of the 13 episodes: in one, virus was isolated from two specimens taken at 1 and 5 days respectively and in the other, virus was isolated from a specimen taken at 3 days, but not from material collected at 1 day. Symptoms persisted for 7 - 14 days in seven episodes; rhinoviruses were isolated from five of eight specimens

taken 7 days or more after the onset of these episodes. The remaining four episodes lasted more than 2 weeks; specimens collected 14 days after onset from three of these episodes all yielded rhinoviruses.

Seventeen of the 30 rhinoviruses isolated were identified by neutralization tests using the 40 available antisera. The 17 strains belonged to 14 serotypes: two each of types 68, 16/60 and FEB and one each of types B632, 4574-62, 1059, 363, 409-62, 4704-62, 1647-63, 1833-63, 4462-63, 1192-65, 1767-65.

There were six adults from whom rhinoviruses were isolated during two or more separate episodes. (Table 9.9). Each episode was shown to be due to a different serotype, except for two episodes of adults I and 6 where the viruses were untyped and differences could not therefore be proved.

There were five instances among the laboratory workers and one in a family where the onset of a cold in one individual occurred during a cold in another individual who lived or worked in close proximity, and where rhinoviruses were isolated from both individuals. It was thought that there might be some evidence of virus spread in these instances; but among the laboratory workers the two viruses isolated were different in four instances and were both untyped in the other. In the family outbreak type 1833-63 was isolated from the father but untyped viruses were isolated from the mother and two-year-old son.

CHAPTER 10

ADULTS WITH CHRONIC BRONCHITIS

Virological studies were designed to investigate the role of respiratory viruses in the initiation or exacerbation of chronic bronchitis. These studies fall into three parts:

(a) a study of 15 chronic bronchitics and their families lasting $2\frac{1}{2}$ years,

(b) a study of rhinoviruses in 29 chronic bronchitics lasting 4 months,

(c) the examination of material from bronchial biopsies.

Fifteen Chronic Bronchitics and their families

Fifteen patients who were living at home and who had chronic bronchitis, as defined by Scadding (1959), were selected on the basis of two main criteria: (i) that they would be likely to co-operate effectively and (ii) that there would be other persons, preferably children, in the family who would increase the likelihood of virus infections being introduced into the household. In order to confirm diagnosis and to establish their initial condition, the patients were examined clinically by Dr. M.B. Eadie. An augmented form of the standard short questionnaire (MRC, 1960) was completed for each of the 15 patients and they were asked to notify the laboratory of any acute respiratory illness of themselves or of their family contacts.

Eight males and seven females aged from 29 to 76 years were accepted for the study. The duration of their bronchitis varied between 2 and 32 years and they have been distributed according to the MRC classification (MRC, 1965a) and questionnaire (MRC, 1960) in Tables 10.1 and 10.2. There were 41 family contacts, 16 less than 10 years of age.

The bronchitics were visited in their own homes every fortnight or more often when they gave notice of an acute respiratory illness in the household. Each respiratory episode was investigated virologically by the collection of a nasal washing, throat swab and, where possible, sputum. Blood sera were collected from the bronchitics only at initial enrolment, in pairs related to episodes of illness during the first 1½ years, and at the beginning (October) and end (April) of the 1963-64 winter. Specimens for virus isolation were examined in tissue cultures as described in Chapter 2, although several of the rhinoviruses were only isolated when specimens were re-examined in HEKF after freezing at -70°C. The paired blood sera were tested for complement fixing antibodies to influenza A, B and C, adenovirus group and RS virus by Dr. Ross.

During the period January 1962 to June 1964, 94 episodes of respiratory illness were investigated, 19 in family contacts and 75 in chronic bronchitics. Ten of the 15 bronchitics were observed for 2½ years, four for 1½ years and one for 1 year. The average number of illnesses per bronchitic per year was 2.5, ranging from 0 to 7.8.

The illnesses were described on clinical evidence, before any laboratory results were known, as follows:

(1) Cold. Upper respiratory tract infection with predominating rhinitis and pharyngitis. Such colds could be subdivided into:

(a) those which did not affect the chest or only caused slight increase in wheeze and cough and no incapacitation.

(b) those which were associated with an exacerbation of bronchitis.

(2) Influenza-like illness. Generalised aches, fever and headache with or without upper respiratory tract symptoms.

(3) Exacerbation. Marked increase in dyspnoea, wheezing and cough of at least 24 hours duration. This group included cases of acute exacerbation, subacute exacerbation and acute worsening of chronic deterioration. The latter subdivision included several older patients who showed an increase in signs or symptoms during a slow deterioration over the winter months, the time of 'onset' being difficult to determine.

The classification and virological results of the 94 episodes investigated are shown in Table 10.3. The types of specimens received for virus isolation from the 75 episodes in the bronchitics are shown in Table 10.4 and from the family contacts in Table 10.5.

Viruses were isolated from seven specimens from five of 19 episodes in the family contacts, all five were in children.

Of the 19 illnesses in family contacts only eight had a date of onset within two weeks of the start of an episode in the family bronchitic. In

two of these eight the same virus was recovered from both bronchitic and contact (one parainfluenza type 1 and one rhinovirus 1647-63).

Respiratory viruses considered relevant were isolated from 13 respiratory episodes of the bronchitics: from one of 26 exacerbations, from 11 of 42 colds and from one of seven influenza-like illnesses. Inclusion of three acceptable serological results (fourfold or greater titre rises) raised the proportion of identifiable virus infections to 16 (21%) of 75 illnesses in bronchitics. Four of these were attributable to myxoviruses and the rest to rhinoviruses. The latter were isolated from 11 of 47 (23%) illnesses affecting the chest from only one of 28 (4%) other illnesses. The rhinoviruses were isolated from seven of the 15 bronchitics: from three separate illnesses of one bronchitic, from two illnesses of three and from single illnesses of three patients. The seven bronchitics who had rhinovirus infections tended to be younger, have more family contacts under 10 years, and a lower M.R.C. phlegm production grade than the remaining eight bronchitics who had no rhinovirus infections.

The monthly distribution of illness and virus infections in the chronic bronchitics (Fig. 10.1) indicates that most exacerbations and rhinovirus infections occurred in the spring and autumn months.

Rhinoviruses were isolated from the sputum in five cases in two of which a throat swab collected 24 hours or immediately before the sputum yielded no virus. Titrations of virus in the sputum-transport medium

homogenate of three specimens were made in RHEKF-35 cells using five tubes per ten-fold dilution. Titres of virus in the original sputa were 2.1, 3.1 and 4.3 $\log_{10} TCD_{50}$/ml.

The antibody responses of the seven patients to their 12 rhinovirus infections are shown in Tables 10.6 to 10.12. In two cases (Tables 10.6 and 10.9) rhinovirus infection occurred in the presence of detectable homologous antibody; both episodes were associated with exacerabations. Fourfold or greater titres rises were found in all infections except two (Tables 10.8 and 10.10) but even these showed a measurable antibody response. One was associated with a cold which did not affect the chest and the other with an exacerbation with no upper respiratory symptoms. In all cases where there was a fourfold or greater antibody titre rise, neutralizing antibody was still present at the end of the study which in some cases was almost two years after the infection. In the two cases in which the highest antibody levels reached were greater than 256 there was no significant drop in titre after over 18 months (Table 10.6) or almost two years (Table 10.9). Five of the six infections in which the highest titres reached were between 32 and 256, and which were followed for more than six months, showed a four-to eightfold drop in titre.

In the four patients with more than one rhinovirus infection (Tables 10.6 to 10.9) there was no evidence that one rhinovirus caused a heterologous antibody response to the others.

Two of the twelve rhinoviruses were M strains (Table 10.6. Infection 2 and Table 10.9. Infection 2) and the remainder H strains. One of the M strains was neutralized by antiserum against Chicago 179-E but none of the other M or H strains could be neutralized by the prototype antisera available. Antisera were therefore made against the 11 unidentified viruses and the results of cross neutralization tests have been discussed (Chapter 4, Table 4.10). The 11 viruses belonged to nine different serotypes. No patient had two infections with the same serotype.

Rhinoviruses in 29 Chronic Bronchitics

The role of rhinoviruses in chronic bronchitis was further investigated by studying a larger number of patients. Unfortunately, the departure of Dr. M.B. Eadie at the end of April 1965 curtailed this work.

Forty-five chronic bronchitics, some taken from the original study and others suggested by Dr. A.W. Lees, were approached and 29 agreed to co-operate in the study. They were asked to notify the laboratory of any respiratory illness. They were then visited and blood, throat and nose, and where possible, sputum specimens were collected. When practical, patients with illnesses were visited every three to seven days and if symptoms were still present further throat swabs and sputa were collected. The specimens were inoculated only into cultures of human embryonic tissue for the isolation of rhinoviruses. A convalescent blood specimen was taken

three weeks after the first.

Thirty-eight specimens for virus isolation were collected from 12 episodes in the 29 chronic bronchitics between January and April 1965. Rhinoviruses were isolated from four specimens from two illnesses. The clinical classification and virus isolation results of the illnesses are shown in Table 10.13.

Details of the isolations and serological responses in the two rhinovirus infections are shown in Tables 10.14 and 10.15. In both cases there was a significant rise in neutralizing antibodies. In the second case (Table 10.15), where sputum was available, a specimen taken 9 days after the onset of the cold was found to contain rhinovirus, whereas the throat swab taken at the same time did not. The titres of virus in the two sputum samples containing rhinovirus were 3.9 and 2.4 $\log_{10}$ TCD_{50}/ml when assayed in RHEKF-35 cells.

One of the rhinoviruses (Table 10.14) was an M strain; the other of them could be identified using the 40 available antisera.

Examination of Bronchial Biopsy Material

The possibility that latent viruses might be present in the bronchi of chronic bronchitics was investigated by examining mucosal biopsies. The biopsies were collected by Dr. A.W. Lees and placed in 5 ml of PBS. This was immediately transported to the laboratory where the material was washed in PBS, chopped finely and washed twice more to remove blood,

bacteria and fungi.

The chopped tissue was then inoculated into tubes of the following tissue cultures:

(a) secondary RMK held stationary at 36°C in Parker's medium 199,

(b) RHEKF-30 or RHEKF-35 cells rolled at 33°C in Eagle's MEM containing 1% inactivated calf serum, 5% tryptose phosphate broth and 1% sodium bicarbonate solution.

(c) RHEKF-30 or RHEKF-35 cells held stationary at 36°C in Eagle's MEM containing 1% inactivated calf serum and 3% sodium bicarbonate solution.

(d) a 1 oz prescription bottle to which 3 ml of Eagle's MEM supplemented with 10% inactivated calf serum and 1% sodium bicarbonate solution was added.

These cultures were used between November and December 1964. From January 1965 the methods were slightly altered by:

(1) combining (c) and (d) so that tissue fragments were added to 1 oz prescription bottles containing a monolayer of RHEKF-30 or RHEKF-35 cells in Eagle's MEM supplemented with 10% inactivated calf serum and 1% sodium bicarbonate solution.

(2) inoculating tubes of BH cells held stationary at 36°C in Eagle's MEM supplemented with 2% inactivated rabbit serum and 4% sodium bicarbonate solution.

All tissue culture tubes were observed for 8 weeks, their medium changed when necessary and tissue culture fluid passed to fresh cultures when any cell degeneration was observed. The prescription bottles were also observed for 8 weeks and fed with fresh medium as required. Three and 6 weeks after inoculation fluid from the bottles was inoculated into tubes of secondary RMK, RHEKF-30 or RHEKF-35 and BH under the above conditions. These tubes were observed for 2 weeks. All secondary RMK cultures were tested weekly for haemadsorption with 0.5% human group '0' erythrocytes.

Mucosal biopsies were collected from 25 chronic bronchitics between November 1964, and April 1965. Five of the 25 specimens produced heavy growth of bacteria, yeasts or fungi in tissue culture and were discarded. Of the remaining 20, only one produced a transmissable cytopathic effect with complete destruction of BH cultures seven days after the original inoculation. No effect could be seen in cultures of RMK or RHEKF-30 inoculated with material from BH showing cytopathic effects. Cell degeneration did not occur in BH if the inoculum was treated with chloroform, nor if the cultures contained Kanamycin (100 µg/ml) or aureomycin (50 µg/ml). The agent was therefore thought to be a mycoplasma. Attempts to prove this were complicated by the discovery that uninoculated BH cultures were contaminated by a non-cytopathic mycoplasma. Attempts to prevent the cell degeneration by neutralizing the agent with serum taken from the patient

two months after the biopsy failed. Tissue fragments seeded into 1 oz bottles of HEKF produced some outgrowth or cell migration in most cases but this ceased within four weeks.

Thus, no evidence for the presence of latent viruses in the bronchi was obtained.

CHAPTER 11

CHILDREN IN HOSPITAL.

The studies of children in hospital fall into three main groups:

a) A pilot study of children entering hospital with respiratory disease between December, 1962 and May, 1963.

b) A comparative study of a group of children entering hospital with respiratory disease and a matched group of children with diarrhoeal illness, between October, 1963 and April, 1965.

c) A study of a larger proportion of the children entering hospital with respiratory disease between December, 1964 and April, 1966. This was part of a nationwide collaborative study organised by the Medical Research Council.

The purpose of these investigations was first to ensure that BH cultures were able to detect the labile RS virus and to see if direct tissue culture inoculation in the ward was advantageous. The expansion of the investigation to include isolation methods for all known respiratory viruses and a matched group of children without respiratory illness allowed analysis of the significance of the virus isolations and the relative importance of the various virus groups. This latter point was further studied in a larger group of children which also showed whether the matched group was a representative one or not.

Other sections in this chapter concern rhinovirus infections, serological studies and multiple infections in these children.

Pilot study of children with Respiratory Disease, December, 1962 to May, 1963

The children investigated during this period were those under 16 years entering hospital with respiratory disease. Throat swabs were collected within 24 hours of admission and each inoculated in the ward into two tubes of BH cells which were then transported to the laboratory.

Swabs were obtained from 59 children but 20 of these were heavily contaminated with bacteria or fungi and therefore discarded and excluded from the study.

The 39 children in this study comprised 21 males and 18 females aged between 1 month and 5 years with an average age of 11 months. RS virus was isolated from 3 males and 5 females. Adenovirus 1 and adenovirus 5 were each isolated from male children.

Over a quarter of the children under 1 year yielded RS virus and six of the eight RS viruses isolated were found in this age group (Table 11.1).

All the virus isolations except one were made from cases with lower respiratory illness (Table 11.2).

The monthly distribution of virus isolations (Table 11.3) showed that RS virus was not isolated after February, although this could be a result of the small numbers of cases tested.

Comparative study of Children with Respiratory and Diarrhoeal Disease, October, 1963 - April, 1965

Cases were selected each weekday morning from those admitted to Ruchill Hospital within the previous 24 hours. Children with respiratory illness were included in the study only if they were under 10 years of age and could be matched for age (within a month if under 3 months old, within 6 months if aged 3 - 12 months, within 2½ years if over 1 year) and time of admission (within 1 week) with children with mainly diarrhoeal symptoms.

After selection, the cases were immediately visited in the ward and throat and nose swabs collected. Initially (before April, 1964) throat and nose swabs were centrifuged and inoculated into tissue cultures separately; for reasons already described (Chapter 6) these practices were discontinued. All swabs were inoculated into two tubes each of primary or secondary RMK cells, BH cells and HEKF held under conditions already described (Chapter 2). All specimens were inoculated within 2 hours of collection and without prior freezing. Blood sera were also collected from some of the children with respiratory disease.

The 113 children with respiratory illness included in the study were thus a selected group which represented only 13% of the children under 10 years of age entering the hospital during the 19-month period. The group comprised 64 males and 49 females aged between 3 weeks and 6 years, with an average of 17.8 months. This compared well with the diarrhoeal group which consisted of 61 males and 52 females aged between 1 week and 4½ years with an average

of 17.4 months (Table 11.4).

The viruses isolated from the throat and nasal swabs of the two groups are summarized in Table 11.5. Sixty-four viruses were isolated from 59 (52%) of 113 children in the respiratory group and 31 viruses from 29 (26%) of 113 children in the diarrhoeal group. The difference in RS virus isolation rate between the two groups is highly significant ($X^2 = 23.6$, $p < 0.001$; Yates' correction). Parainfluenza viruses were isolated from nine children in the respiratory group (two type 1 and seven type 3) and two (type 3) in the diarrhoeal group which is not a significant difference between the two groups ($X^2 = 3.45$, $p > 0.05$; Yates' correction). Rhinoviruses (one M type and nine H type) were isolated with equal frequency from both groups. Herpes simplex virus was not isolated significantly more often from the respiratory than the diarrhoeal cases ($X^2 = 3.29$, $p > 0.05$; Yates' correction) and serological studies showed that in five of the infected children from whom paired sera were available there was no rise in CF antibody against herpes virus. Adenoviruses (two type 1, two type 2 and one type 3) were isolated from five children with respiratory illness and from eight (two type 1, three type 5 and one each of types 2, 3 and 7) children with diarrhoeal illness, showing no significant difference between the groups ($X^2 = 0.30$, $p > 0.3$; Yates' correction). Similarly there was no significant difference in enterovirus isolations between the two groups ($X^2 = 1.16$, $p > 0.2$; Yates' correction); coxsackievirus B4 and eachovirus 9 were isolated from children in the respiratory group; two

two strains of coxsackievirus B2, one strain each of poliovirus 2, echoviruses 8 and 9 and an untyped enterovirus were isolated from children in the diarrhoeal group.

Most of the specimens from children with respiratory illness were collected within a week of the onset of illness (Table 11.6), but there were 10 cases which had been ill longer and 5 whose duration of illness could not be ascertained because of incomplete records. An unexpected finding was that the total isolation rate was higher from patients who had been ill 4-7 days (71%) than from those who had been ill less than 4 days (50%); this trend was seen with each group of viruses isolated except herpes simplex. The overall difference however, was not statistically significant ($X^2 = 3.42$, $p > 0.05$; Yates' correction).

The age distribution of the virus isolations (Table 11.7) shows that the RS virus isolation rate fell from 23% in children under 6 months to 3% in children over 2 years. Isolations of herpes simplex virus were rare from children under 1 year. The other virus groups did not show marked age-associated trends although adenoviruses and enteroviruses were most often isolated from children between the ages of 6 and 24 months.

Virus isolations according to illness are shown in Table 11.8. The diagnosis used is the final diagnosis of the illness with which the patient was admitted. All but one of the RS virus isolations were made from children with lower respiratory illness. Parainfluenza and rhinoviruses were isolated from a slightly higher proportion of children with upper

than of those with lower respiratory disease. Herpes viruses were found with equal frequency in upper and lower respiratory illnesses. There was no obvious difference between the isolation rates of viruses from the children with bacterial or non-bacterial diarrhoea.

The monthly distribution of the virus isolations(Table 11.9) shows two sharply defined winter outbreaks of RS virus and a more sporadic occurrence of the other virus groups.

Children with Respiratory Disease, December 1964 - April 1966

The children in this study comprised those under 16 years entering Ruchill Hospital with respiratory illness between 1 p.m. on Sunday and 1 p.m. on Friday of each week. Throat and nasal swabs from each child were collected within 24 hours of admission and placed in the same bottle of transport medium. Specimens were transported to the laboratory and inoculated into tissue cultures of RMK, BH and HEKF within 2 hours of collection. Some specimens were also tested in WI-38 or BW HEL-7 cells. Paired blood sera collected in the acute and convalescent phases of illness were obtained from some of the children.

A total of 250 children were included in the study and comprised 162 males and 88 females aged between 12 days and 10 years with an average of 16.6 months. The age and sex composition of the population samples (Table 11.10) is different from that of the respiratory group in the comparative study; in this group 65% were males, 57% of children were

under 1 year and 15% were between 1 and 2 years; in the respiratory group of the comparative study 57% were males, 44% were under 1 year and 23% were between 1 and 2 years.

A total of 113 viruses were isolated from 104 (42%) of 250 children; there were nine cases from which two viruses were isolated (Table 11.11). RS virus accounted for 42% of the isolations and was associated with 22% of the illnesses. Rhinoviruses accounted for 19% of the isolations and were associated with 9% of the illnesses. Influenza and parainfluenza viruses comprised 7% and 10% respectively of the isolations and were associated with 3% and 4% respectively of the illnesses. Thus, 78% of the viruses isolated were either myxoviruses or rhinoviruses and these viruses were found in 38% of the illnesses. Herpes simplex virus, adenoviruses and enteroviruses represented 6%, 7% and 9% respectively of the viruses isolated and were associated with 3%, 3% and 4% of the illnesses.

The day of illness on which specimens were collected (Table 11.12) did not appear to affect the total virus isolation rate appreciably, although the isolation rate of RS virus and rhinoviruses fell from 20% and 12% respectively in the first 3 days of illness to 14% and 4% respectively between the fourth and seventh days of illness.

The percentage isolation rates of various virus groups from different age-groups of children (Table 11.13) showed marked differences. RS virus isolation rates decreased sharply with increasing age. Influenza and

herpes simplex viruses were not isolated from children under one year of age. Parainfluenza viruses were not isolated from children over 2 years old but in younger children there was little variation in isolation rate with age. Rhinoviruses were isolated with similar frequency from each of four age groups but least often from children under 6 months. Adenoviruses and enteroviruses were isolated most often from children between 6 and 24 months of age.

Analysis of the virus isolations by illness (Table II.14) showed that all except four of the RS virus isolations were from lower respiratory illness, although the isolation rate from lower respiratory illness (20%) is not much higher than that from upper respiratory illness (16%). Influenza viruses were associated with less than 2% of lower respiratory illness and like enteroviruses were found more often in upper respiratory tract infections. Parainfluenza, herpes simplex and adenoviruses were found in 3-4% of both upper and lower respiratory illness. Rhinoviruses were found in 9% of lower but only 4% of upper respiratory disease.

The most striking aspect of the monthly distribution of virus isolations was the defined outbreaks of RS infections (Table II.15). Isolations of other viruses occurred throughout the year although the numbers are not adequate to show small differences.

Rhinovirus infections

Rhinoviruses were isolated from 32 (9%) of 363 children in hospital with respiratory illness and ten (9%) of 113 children in hospital with diarrhoeal illness. After RS virus, rhinoviruses were the most common viruses in children with respiratory disease. However, the isolation of these viruses from a similar number of children without respiratory symptoms suggests either that such children may be simply carrying rhinoviruses which play little or no part in the aetiology of their disease, or that rhinoviruses also cause diarrhoeal illness.

Further studies were made on the cases with rhinovirus infection, including attempts to isolate the viruses from faces, serological studies and identification of rhinovirus serotypes, in order to obtain more information about the aetiological role of these viruses.

Faecal extracts from seven of the ten diarrhoeal cases whose throat and nasal swabs yielded rhinoviruses were inoculated into HEKF under conditions for rhinovirus isolation. Two viruses were isolated; a coxsackievirus A 9 and an untyped virus which was acid-stable. The faecal specimens had, however, been stored at -20°C for periods up to 18 months which might have inactivated any rhinoviruses they contained.

Paired blood sera were obtained from 19 of the 32 respiratory cases with rhinovirus infection. These sera were tested for CF antibodies against RS virus, adenovirus (group) and parainfluenza (group), for HI antibodies against parainfluenza virus types 1, 2 and 3 and for neutralizing

antibodies against the rhinovirus isolated (Table 11.16).

Evidence of simultaneous RS virus infection was obtained in four cases (Cases 3 and 8 by isolation, Cases 4 and 15 by fourfold or greater CF titre rises). The two cases (3 and 8) from which RS virus was isolated showed no serological evidence of infection with this virus but did show greater than fourfold rises in neutralizing antibody against their homologous rhinoviruses. Of the two cases with serological evidence of RS virus infection, one (Case 15) showed no serological response to the rhinovirus, possibly because the second serum was collected only 9 days after onset of illness and the other (Case 4) showed only a small increase in rhinovirus antibodies.

There was evidence for simultaneous parainfluenza virus infection in two cases (12 and 18). Case 12 yielded parainfluenza type 1 virus and showed a rise in parainfluenza 1 HI antibodies of more than 16-fold; the neutralizing antibody rise to the rhinovirus in this case was detectable, but less than four-fold. Serological evidence of infection (four-fold or greater titre rise) with parainfluenza virus was obtained by both CF and HI tests in Case 18; there was also a significant rise in neutralizing antibodies to the infecting rhinovirus.

Evidence for simultaneous adenovirus infection was obtained in two cases (4 and 7). Case 4 showed a significant CF titre rise and has already been considered under RS virus infections. Adenovirus type 2 was isolated from Case 7 which showed no serological response to it or to the infecting

rhinovirus.

The only other rhinovirus-infected case with evidence of multiple infection (Case 9) yielded herpes simplex from the throat and echovirus 7 from the faeces. CF antibody titres against herpes fell from 64 to 32 suggesting the isolation was a result of the reactivation of a latent infection rather than a new infection of aetiological significance. The echovirus 7 was also considered to be irrelevant to the bronchitis in this case. Neutralizing antibodies against the infecting rhinovirus rose more than 16-fold.

If Case 9 is discounted there were seven cases (3, 4, 7, 8, 12, 15, 18) out of the 19 rhinovirus-positive cases studied which had evidence of simultaneous infection with another virus or viruses which could have caused the illness. In addition, there was one case from whom both RS and a rhinovirus were isolated, but which is not included in Table 11.16 because paired sera were not collected. In two cases (3 and 8) from which RS virus and a rhinovirus were isolated there was a serological response to the rhinovirus but not to the RS virus.

There were 12 cases with no evidence of infection with RS virus, adenovirus or parainfluenza viruses. Of these, eight (1, 2, 5, 6, 9, 11, 16, 17) showed significant neutralizing antibody titre rises against rhinoviruses, three (13, 14, 19) showed less than fourfold titre rises and one (Case 10) was not tested because there was insufficient serum.

Significant serological responses to rhinovirus infection were found in 11 cases, detectable responses in five cases and no detectable response in two cases; one case was not tested. The three cases with M strain rhinovirus infection had significant serological responses but only eight of 15 H strain infections induced such a response. Responses varied even within a serotype: rhinovirus 4411-65 produced a significant response in one case (18) but not in two others (12 and 13); rhinovirus 2030-65 produced significant responses in two cases (3 and 11) but probably not in two others (4 and 7).

The various rhinovirus serotypes found in the 32 children with respiratory illness and the ten with diarrhoea are shown in Table 11.17. Of the 42 strains 23 were neutralized, seven by six prototype antisera and 16 by six antisera made at Ruchill Hospital. The three serotypes found in the children with diarrhoea were also found in children with respiratory illness.

Serological Studies

Complement Fixation.

Paired blood sera were obtained from 193 of the children in hospital with respiratory illness. All these sera were tested by CF against RS, parainfluenza 1 and adenovirus group antigens by Dr. Ross.

A fourfold or greater rise in titre against RS virus was found in 38 cases, from 16 of which RS virus had been isolated. More of the cases

from which RS virus was isolated were under 1 year (10 of 16) than of those without virus isolation (6 of 22). Paired sera were received from 32 of the 73 cases from which RS virus was isolated; fourfold or greater titre rises to RS virus were found in only 16 of these. Eleven of the 16 children who failed to show a CF antibody response were aged 3 months or less. Of the cases with CF titre rises against RS virus, but no RS virus isolation, rhinoviruses were isolated from two, one of which also showed a CF titre rise against the adenovirus group, herpes simplex from one and CF titre rises were found against parainfluenza group in two cases.

Evidence of adenovirus infection was found by the CF test in eight cases from two of which adenovirus had been isolated. Of the six infections diagnosed only by the CF test, one was associated with rhinovirus isolation and RS virus CF rise, one with parainfluenza 3 isolation and one with a CF titre rise against the parainfluenza group. Paired sera were obtained from seven of the 14 children from whom adenovirus was isolated; in only 2 cases were significant CF titre rises found.

There were seven parainfluenza virus infections diagnosed by the CF test, parainfluenza type 3 virus was also isolated from one of them, RS virus from another and a rhinovirus from a third case. In addition, CF titre rises were found against RS virus in two cases and against adenovirus in one case. Paired sera were received from 12 of the 20 cases from whom parainfluenza viruses were isolated, only one of these showed evidence of

infection by the CF test.

Neutralizing Antibodies.

Neutralizing antibodies against the Randall strain of RS virus diluted in 25% unheated rabbit serum were measured in 70 pairs of sera collected between December 1964 and January 1966.

Neutralizing antibody levels in the initial specimens (taken less than 5 days after the onset of illness) are shown according to age in Fig. 11.1. All but one of the 23 sera from children aged 4 months or less had detectable antibody, presumably maternal. In contrast, only three of the 18 sera from children aged 5 to 11 months contained any RS antibody. Thereafter the proportion of children with antibody increased with age so that only 1 of 12 sera from children aged 3 years and older had no detectable antibody. If neutralizing antibody were protective most RS virus infections might be expected to occur in the second 6 months of life, in fact, the highest RS isolation rate (Tables 11.7 and 11.13) is in children under 6 months. Possible explanations of this anomaly are that maternal antibody does not protect children from infection or that RS virus is more difficult to isolate from older children.

Evidence of RS virus infection (either isolation or fourfold or greater CF or neutralizing antibody titre rises) was correlated with neutralizing antibody levels in the initial specimen (Table 11.18). Of 26 children with no detectable antibody in their initial sera 19 (73%) were infected with RS virus and of 44 children with antibody 19 (43%) were infected. Children

with evidence of RS virus infection possessed antibody in 19 (50%) of 38 cases; those with no evidence of infection had antibody in 25 (78%) of 32 cases. Of the 19 children with RS virus antibody who were infected, 11 (58%) were under 5 months (Table 11.19 and Fig. 11.2).

Although the data on RS neutralizing antibodies were obtained from a selected population of sick children they might reveal if there was any association between RS virus isolation and antibody levels or age of the children (Table 11.19 and Fig. 11.2). The geometric means of the initial antibody titres showed no significant difference between the three groups of children under 6 months. There were too few children in the group which were RS-positive by serology only to allow proper analysis. The highest mean titre was in children who were RS-positive by isolation. In children over 6 months old the mean initial antibody titre of the group who were RS-positive by isolation was significantly lower than the mean of the group who were RS-negative and substantially, though not significantly lower than the mean of the group who were RS-positive by serology only. RS virus neutralizing antibody was found in all ten children under 6 months who were RS-positive by isolation but in only two of 11 similar children over 6 months. Thus, isolation of RS virus was associated with a deficiency of neutralizing antibody in children aged 6 months and older but not in younger children.

Fourfold or greater rises in neutralizing antibody titres against RS virus

were found in 31 (44%) of the 70 cases tested. In 21 of these cases, infection with RS virus was also shown by other methods: by isolation only in four cases, by fourfold or greater CF titre rises only in seven cases and by both isolation and CF methods in ten cases. Paired sera from 21 cases with RS virus isolation showed fourfold or greater rises in neutralizing antibodies in 14 (67%), the seven cases which failed to show this serological response were all aged 3 months or less. All but one of 18 cases which showed fourfold or greater CF titre rises also showed similar neutralizing titre rises.

Multiple Infections

Dual isolations from throat and nose swabs were made from 16 children in hospital. Evidence of infection by more than one agent was obtained in a further nine cases by serological tests (Table 11.20).

Influenza viruses were found in association with other infections twice, RS virus 14 times, parainfluenza viruses 9 times, rhinoviruses 10 times, herpes simplex 5 times, adenoviruses 10 times and enteroviruses once. It is difficult to express these figures in a comparative way since serological tests were not available for all virus groups and paired sera were available from only about half the children studied. However, with these limitations, the multiple infections with a given virus or virus group may be expressed as a percentage of the total number of infections with that agent or group of agents detected by either isolation or CF tests.

On this basis 45% of adenovirus infections and 32% of parainfluenza infections were associated with another virus; between 22% and 25% of herpes, rhinovirus and influenza infections were multiple ones but less than 15% RS virus and 6% enterovirus infections were associated with another virus.

CHAPTER 12

A SUMMARY OF VIRUSES ISOLATED FROM 800 CASES, JANUARY 1962 to APRIL 1966

Techniques for isolating respiratory viruses tend to be cumbersome and the scale of my studies on respiratory disease was therefore limited. As a result the numbers of cases in some of the groups were too small to allow firm conclusions to be drawn. In this Chapter, the results described in Chapters 9, 10 and 11 are summarized together and various observations are made about the relative frequency of various viruses among the population groups, the relationship of viruses isolated to illness among children, the effect of age on the pattern of viruses isolated from respiratory disease, the seasonal distribution of viruses isolated, the association of various rhinovirus serotypes with the various population groups and, finally, the monthly distribution of rhinovirus serotypes. There are problems in combining results obtained from different populations at different times but I shall attempt to make only such observations as are justified within these limitations.

A total of 302 viruses were recovered from the respiratory tract of 800 persons with or without respiratory disease (Table 12.1). The population groups from which these viruses were isolated and the frequency with which they were found are shown in Table 12.1 and Fig. 12.1. Viruses were isolated from 50% of non-bronchitic adults with respiratory disease and from 18% of respiratory episodes in chronic bronchitics, from 47% of

children in hospital and 32% of children at home with respiratory disease. The non-bronchitic adults were younger than those with chronic bronchitis and this could explain the lower isolation rate from the latter group. Similarly the children in hospital were generally younger than those at home. Viruses were recovered from 28% of children in hospital with diarrhoea and 13% of symptomless children at home. Influenza viruses were recovered only from people with respiratory illness and most frequently from children and adults at home although the virus isolation rates of 15% and 8% respectively may be abnormally high because a disproportionate number of specimens were collected from these two groups during the influenza B outbreaks in 1962 and 1966. Parainfluenza viruses were found slightly more often in children than in adults with respiratory disease but never in more than 5% of cases; these viruses were also found in 2% of children in hospital with diarrhoeal illness. RS virus was found only in children and, apart from one child at home with respiratory illness and one child in hospital with diarrhoea, all isolations were made from children in hospital with respiratory illness; RS virus was associated with 20% of such illness. Rhinoviruses appear to be the most ubiquitous of the viruses. They were found in all six population groups: in 8% to 13% of children and 16% to 40% of adults. The rhinovirus isolation rates in children with respiratory disease and in those without are similar. Herpes simplex virus was found most often in children in hospital (5% of

those with respiratory illness and 4% of those without). This virus was also isolated from two children at home with respiratory illness and from one chronic bronchitic. Apart from one adenovirus isolated from a child with respiratory illness at home, all the adenoviruses and enteroviruses were isolated from children in hospital and with similar frequency from those with respiratory and those with diarrhoeal illnesses.

The frequency of viruses isolated from various illnesses of children (Fig. 12.2.) has been calculated by combining results obtained from children in hospital and children at home. Most of the upper respiratory illnesses were in children at home who tended to be older and possibly of higher socioeconomic groups than children in hospital. Such differences must be emphasised when interpreting the following analysis. As there were only nine cases of croup, results in this group are unreliable. Influenza viruses were isolated most frequently from croup (22%) and upper respiratory illness (12%), only rarely from pneumonia and bronchitis and not at all from children without respiratory illness. Parainfluenza viruses were isolated most often from croup (11%), then pneumonia, upper respiratory illness and bronchitis; 1% of children without respiratory illness also yielded parainfluenza viruses. RS virus was isolated from 22% of both bronchitis and pneumonia cases; only 4% of children with upper respiratory illness and 1% of those without respiratory illness yielded RS virus. Rhinoviruses were isolated most often from children with croup, upper respiratory illness or

no respiratory illness (10% to 11%) but only slightly less often from children with bronchitis and pneumonia. Herpes simplex was found in 11% of croup cases, 9% of pneumonia cases, and 2 - 3% of bronchitis cases,upper respiratory illnesses and children without respiratory illness. Adenoviruses were found in 11% of children with croup, 6% of children with pneumonia and children without respiratory disease and 2% of children with bronchitis or upper respiratory illness. Enteroviruses were isolated from 4% of children without respiratory disease and 2% to 3% of children with respiratory illnesses, except croup. Viruses were most frequently isolated from the croup (66%) and pneumonia (53%) cases then from bronchitis (41%) and upper respiratory illness (37%) and, finally, from children without respiratory illness (25%).

The effect of age on the frequency of viruses isolated has been observed by combining the results obtained from 656 persons with respiratory disease (Fig. 12.3). The dangers of drawing false conclusions from such combined results must again be emphasised. For example, most of the young children had more severe illnesses and most of the older adults were chronic bronchitics; in these cases different age groups also correspond to different population groups. Three virus groups appear to be most markedly affected by age: these are influenza viruses, RS virus and rhinoviruses. Influenza viruses were first isolated from children between 1 and 5 years (3%) the isolation rate rise sharply to 37% from children aged 6 - 15 years and then declined to 4% and below in persons over 15 years. The very high frequency

in the 6 -15 years age-group may be partly related to the collection of a disproportionate number of specimens from schoolchildren during the influenza B outbreak of 1966. RS virus was isolated from 30% of children under 1 year and the isolation rate declined rapidly with increasing age to 0% in children over 5 years. Rhinoviruses were isolated from 5% to 10% of children under 16, but the isolation rate rose rapidly with age to 48% in young adults 16 - 30 years old. Thereafter, there was a decline to 14% in adults aged 46 - 60 years and 0% in the few cases over 60 years old. None of the remaining virus groups had isolation rates above 10% in any age group. Parainfluenza viruses were found in 5% to 7% of children under 5 years and less often with increasing age. Herpes simplex virus was not isolated from children under 1 year and was most frequently isolated from 1 - 15 year-old children. Adenovirus isolations rose from 4% in the first year to 7% in the second year and then declined with increasing age and were not found in persons over 15 years old. Enteroviruses were found in 2% of children under 2 years and not at all in people over 5 years old. All of the seven virus groups studied were found in children 1 - 5 years old. Influenza and herpes simplex were not found in children under 1 year. Five of the seven groups were found in children aged 6 - 15 years, three or four in adults between 16 and 45 years and only two (influenza and rhinoviruses) in persons over 45 years.

The seasonal variation in the frequency of viruses isolated from the 800 persons studied between January 1962 and April 1966 has been obtained by combining the results of 4 years and 4 months (Figures 12.4 and 12.5). The most marked seasonal variation is shown by influenza viruses, RS virus, rhinoviruses and enteroviruses (Fig. 12.4). Influenza virus isolations were most frequent in January (16%) and apart from one isolation in June, were only made in the first three months of the year. RS virus was isolated almost entirely during the winter months, September to March, with a peak in November (20%). Rhinoviruses were isolated from 6% or more of the cases tested throughout the year. They were isolated least often in the winter months (November to February) and in August. There appear to be two definite peaks: May (29%) and October (18%). The high isolation rate for July (33%) is unreliable as only 9 cases were examined. The enteroviruses were most frequently isolated in the summer months with a peak in June (17%). Parainfluenza viruses, herpes simplex virus and adenoviruses were not isolated from more than 8% of the cases in any month (Fig. 12.5) and they therefore show only small seasonal variations. Parainfluenza viruses were most commonly isolated in the Spring (April and May) and early winter (November). Herpes simplex and adenoviruses were isolated from 2% to 5% of the cases in most months and most frequently in September (8%).

Thirty-seven of the 95 rhinoviruses isolated were not neutralized by any of the 40 antisera used. Twenty of the remaining 58 were neutralized by 11 prototype antisera and 38 by 13 Ruchill antisera (Table 12.2). Not more than six strains of any one serotype were isolated between January 1962 and April 1966 and it is therefore, not possible to draw any definite conclusions about individual serotypes. However, some general observations are justified on the relationship of various serotypes to particular disease groups and the seasonal occurrence of the serotypes.

Thirteen of the 14 rhinoviruses isolated from patients with chronic bronchitis were identified serologically and belonged to 11 different serotypes (Table 12.2). Only three of these serotypes (1321-62, 130-63 and 1376-64) were not also isolated from mild respiratory disease in adults or children at home. Fourteen of the 32 rhinoviruses isolated from severe respiratory illness of children in hospital were unidentified, the remaining 18 strains belonged to 12 serotypes (Table 12.2). Five of the 12 types were isolated only from children in hospital with respiratory disease.

The monthly incidence of rhinovirus serotypes (Fig. 12.6) indicates that at most times there are several different serotypes circulating in the population simultaneously. Two or more strains of several serotypes were isolated only during one period of 5 - 6 months during the four years and four months of study, for example 179E, 68, 16/60, 11757, 4704-62, 130-63, 2030-65, suggesting minor outbreaks. Single strains of other

serotypes were isolated at intervals of 3 years (FEB, 363, 1192-65), 2 years (4574-62, 4462-63, 4411-65) or 1 year (B632). Serotype 1833-63 was isolated sporadically over a period of 2 years. If 4574-62 and 1767-65 are considered to be the same serotype (see Chapter 4 and Table 4.10) there is evidence that two outbreaks may have occurred separated by two years. Thus, no clear pattern of seasonal occurrence emerges.

CHAPTER 13

DISCUSSION.

The epidemiology of each of the groups of viruses isolated during this study is unique to itself and it therefore seems most convenient to consider separately each of the seven groups. The particular aspects which have been investigated in this work are the relative frequency with which the viruses have been isolated, the age and population groups in which they were found, the diseases with which they were associated and their seasonal prevalence.

Influenza Viruses

During this study there were four outbreaks of influenza in Glasgow (Grist, Ross and Stott, 1967; Taylor, Ross and Stott, 1967): due to type A in 1963 and 1965, type B in 1962 and types A and B in 1966. Viruses were isolated from each of these outbreaks. Influenza A2 was isolated once in 1963, twice in 1965 and once in 1966. Influenza B was isolated six times in 1962, once in the summer of 1965 and 18 times in 1966. Thus, influenza A2 was isolated infrequently, possibly because there were few infections in the age groups which I studied or because the techniques which I used were insensitive. Representative strains isolated during these outbreaks were similar to strains isolated in previous years in Britain (Pereira, 1966).

Influenza viruses were found most frequently in children between 6 and 15 years old but this is because a disproportionately large number of

schoolchildren of this age-group were examined during the type B influenza epidemics. It does, however, appear that these viruses cause only a small proportion of lower respiratory illnesses in younger children.

Influenza viruses were isolated only from patients with respiratory illness; thus, there was no evidence of symptomless infections. These viruses were isolated from 15% of children and 8% of adults at home in this study but these figures are much higher than those of Higgins and colleagues (1966) and are probably due to the higher proportion of cases which I examined during influenza outbreaks. There may be other differences between the mainly rural population studied by Higgins and co-workers and the urban population of Glasgow.

Evidence of influenza infections in chronic bronchitics was sought during the type A outbreak of 1963 and the type B outbreak of 1962. However, no influenza viruses were isolated and only one of 47 exacerbations investigated was associated with a significant CF antibody rise against influenza A or B. This result is surprising since influenza viruses are known to cause severe illnesses in patients with chronic bronchitis (Stuart-Harris and Hanley, 1957) and Stark and co-workers (1965) found 6.1% of exacerbations were associated with influenza B infections. Possible explanations of this are that only a small proportion of chronic bronchitics suffer the severe distress described by Stuart-Harris and Hanley or that the outbreaks of influenza investigated by us were unusually mild.

Influenza viruses were isolated from less than 2% of children in hospital with respiratory disease during the whole study, but, during the outbreak in January and February 1966, influenza viruses were found in 5 (28%) of the 18 children studied. These observations are closely similar to those of Chanock and Parrott (1965a) and Holzel and colleagues (1965a).

Most of the influenza viruses were isolated from people with colds or, more often, mild influenza-like illness. In children in hospital however, two cases of croup and four other lower respiratory tract illnesses were associated with influenza infections; an association between influenza A2 and croup has been previously reported (Forbes, 1958).

All but one of the influenza viruses were isolated in the first three months of the year. This closely defined seasonal distribution appears to be characteristic of influenza virus outbreaks (Stuart-Harris, 1965).

Parainfluenza Viruses

Only 27 parainfluenza viruses were isolated during this study; seven type 1, one type 2 and 19 type 3. No strains of parainfluenza type 4 were isolated; possibly because all haemadsorption tests were done at 4°C which is not the optimal temperature for this type (Canchola et al. 1965). It is also possible that only types 1 - 3 were present in Glasgow during the period of study. There was no evidence of any outbreak of parainfluenza virus infections like those reported in general practice (Banatvala et al. 1964) and

in children in hospital (Lewis et al. 1961a; McLean et al. 1963).

Parainfluenza viruses were found most often in children under 6 years old in hospital but infections, probably reinfections, occurred in adults. This difference may not reflect a true difference in infection rate since it is more difficult to isolate parainfluenza viruses from adults (Bloom et al. 1961).

Parainfluenza viruses were isolated from only two children who did not have respiratory symptoms and one of these developed lower respiratory illness three days later, suggesting that symptomless infections in this age-group are rare. Although in this study an association of parainfluenza viruses with respiratory disease could not be proved statistically, larger studies (Bell et al. 1961; Chanock and Parrott, 1965b) have shown such an association in children.

Parainfluenza viruses were isolated from 2.7% of children and 1.7% of adults at home with respiratory disease; the isolation rate from children is lower than the 5% of Higgins and colleagues (1966) but the rate for adults is almost the same as those in other studies (Hamre et al. 1966; Higgins et al. 1966). Parainfluenza virus infections in chronic bronchitics have been reported (Carilli et al. 1964; Stark et al. 1965) but they were associated with respiratory illnesses without exacerbations as often as with exacerbations. Parainfluenza virus was isolated only once from the bronchitics we studied and was not associated with an exacerbation. The isolation rates of parainfluenza viruses from children in hospital with

respiratory disease were 1.0% for type 1, 0.2% for type 2 and 3.7% for type 3. This is the same order of frequency as that found in larger studies although I found type 1 only half as often as other workers (Chanock and Parrott, 1965a; Holzel et al. 1965a).

One of the two adults from whom parainfluenza viruses were isolated had an influenza-like illness and the other a cold. Six of the 25 children with parainfluenza infections had an upper respiratory illness, one (type 2) had croup, seven had bronchitis, nine had pneumonia and two had no respiratory illness. These represent isolation rates of 4% from upper respiratory illness, 3% from bronchitis and 6% from pneumonia. Thus, our isolation rate from upper respiratory illnesses is lower and from lower respiratory illnesses is higher than those in other reports (Chanock and Parrott, 1965a; Holzel et al. 1965a).

No conclusions about the seasonal distribution of parainfluenza virus infections can be drawn from only 27 virus isolations. Results from larger studies suggest that parinfluenza viruses are endemic and most common in the colder months (Parrott et al. 1963b; Chanock and Parrott, 1965a; Higgins et al. 1966). In our study there was a suggestion that parainfluenza viruses were most common in the Spring and they might therefore be partly responsible for the irregularity in the curve of notifications of pneumonia in children in Glasgow which follows the main RS virus outbreak (Grist et al. 1967).

Respiratory Syncytial Virus

Respiratory syncytial virus was isolated from 83 cases. Infections were detected in sharply defined outbreaks in each of the four winters during which children in hospital were investigated.

The age association of RS virus is unique in that it was most commonly found in children under one year (30%) and not at all in persons over 5 years old. The population group from which this virus was isolated is also sharply defined since only one child from whom the virus was isolated was not in hospital. These observations agree with those of other workers in this country and the U.S.A. (Chanock and Parrott, 1965a; Holzel et al. 1965a; MRC, 1965b; Higgins et al. 1966).

RS virus has been associated with exacerbations of chronic bronchitis (Sommerville, 1963; Carilli et al. 1964). However, we found no evidence of RS virus infection in chronic bronchitics in Glasgow although this virus caused outbreaks of respiratory illness in children in the City during three of the winters when chronic bronchitics were being investigated.

RS virus infection was nearly always associated with respiratory illness. The only case which did not have respiratory symptoms at the time the virus was isolated developed lower respiratory tract signs three days later. Seventy-six (92%) of the 83 RS virus infections were associated with lower respiratory illness; this is a higher proportion than has been found in other studies (Chanock and Parrott, 1965a; Holzel et al. 1965a).

It is difficult to prove that RS virus infections are rare in older children and adults; they may simply be more difficult to detect. However, the predilection of RS virus for children under six months and severity of the illness which it causes, often in the presence of high levels of circulating antibody, are unique characteristics. In this context it is interesting that children injected intramuscularly with a killed RS virus vaccine had more RS virus infections and more severe infections than an unvaccinated control group, although the vaccine induced low level neutralizing antibody responses (Parrott et al. 1967; Mufson, 1967). A possible explanation of this is that the vaccine "sensitized" the children. Another possible explanation is that the vaccine induced the production of antibody which combined with the virus but did not neutralize it; such antibody-coated virus particles might then be less susceptible to neutralization by more "active" antibody. A similar situation has been shown to occur with Neisseria gonorrhoeae where Ig G antibodies, which are only weakly bactericidal, reduce the activity of the bactericidal Ig M antibodies (Cohen, Norins and Julian, 1967). The complement-requiring RS virus neutralizing antibodies which were reported in Part I could be relevant to such a mechanism. On this basis it is also possible to speculate that maternal antibodies may aggravate an RS virus infection and thus explain the high frequency and severity of RS virus infections in children under six months. This latter hypothesis does not explain the repeated, severe

infections which do occur in children, often with strains antigenically identical (Beem, 1965).

The presence of RS neutralizing antibody in the acute sera of children infected with this virus has been observed previously (Beem et al. 1960; Chanock et al. 1961). However, a comparison of the neutralizing antibody levels in acute sera of children with RS infection with those of children without such infection does not appear to have been reported. In children under six months, whose antibody is most probably maternal, there was little difference between the antibody levels of infected and those of uninfected children. In children over six months, who would have little or no maternal antibody (Beem et al. 1964) antibody was present in 67% of children not infected but only 31% of children who were infected. Another difference between the children whose antibody was presumably maternal and those whose antibody was presumably actively acquired as a result of infection, was the method of diagnosis of the RS virus infection. In children under six months with antibody, diagnosis was by virus isolation in ten of 11 infected cases. In children over six months, with antibody, virus was isolated from only two of eight infected cases and these had antibody titres of only 4 and 8 respectively. A possible explanation of these observations is that during an RS virus infection antibody is produced into the upper respiratory secretions and that this is the antibody which is important in protection. This has been shown to be the case in parainfluenza 1 infections

in volunteers (Smith, Purcell, Bellanti and Chanock, 1966). The importance of local antibody secretion could explain why maternal antibody afforded no apparent protection and why virus was isolated less frequently from older children who had evidence of a previous RS virus infection. It is also true that CF antibody levels rise slowly in very young children (Ross, Stott, McMichael and Crowther, 1964) and that some of the infections diagnosed serologically may have been acquired in hospital and not present when the admission swabs were collected (Chanock et al. 1961). These factors might also contribute to differences in methods of diagnosis.

Outbreaks of RS virus occurred every winter they were sought. This observation is similar to those of other workers in large urban communities (Chanock and Parrott, 1965a; Holzel et al. 1965a) and is another of the characteristics unique to RS virus. This may be because it only takes one year to produce a sufficiently large population of susceptible children. The annual outbreaks are certainly not caused by different antigenic strains (Coates et al. 1966).

Rhinoviruses

Rhinoviruses were isolated from 95 cases and were, therefore, the most commonly isolated group of viruses. Since 58 of these viruses belonged to 24 different serotypes and 37 strains were not identified it is difficult to draw any conclusion about the epidemiology of individual serotypes. However, consideration of these viruses as a group reveals

some interesting findings, particularly since few other workers have investigated the distribution of rhinoviruses in such varied population and age groups during the same period in the same area.

Rhinoviruses were isolated from adults with respiratory disease much more frequently than from children. The isolation rate was 5% to 10% in persons under 16 years old, rose to a peak of 48% in adults aged 16 to 30 years and thereafter declined with increasing age. Other workers who have investigated both children and adults also found a higher isolation rate from adults (Reilly et al. 1962; Bloom et al. 1963; Higgins et al. 1966) but did not record a more detailed break-down into age groups. The population group from which rhinoviruses were most frequently isolated were the adults at home. The isolation rates from the four groups of children were similar (between 8% and 13%) whether children had respiratory illness or not and whether they were in hospital or at home. It is difficult to compare these results with those of other workers since children at home have not previously been compared with those in hospital and our isolation rates are consistently higher than those so far reported, probably because of the greater sensitivity of HEKF cells reported in Part I.

Rhinovirus infection of children was associated with a wide range of illnesses or none at all. Rhinoviruses were isolated as often from children at home with respiratory illness as from their symptomless contacts. The rhinovirus isolation rate from children in hospital was the same whether they had respiratory or diarrhoeal illnesses. On the evidence of these

studies it was not possible to establish a causal relationship between rhinovirus infection and respiratory diseases of children. However, a statistically significant difference between the isolation rates from children with respiratory illness and those without has been shown in larger studies (Bloom et al. 1963). The absence of such differences in my studies may be due to insufficient numbers. It is also possible that the choice of contacts as controls is a more severe test and that rhinoviruses might also be associated with diarrhoeal illness. The latter suggestion seems unlikely since rhinoviruses are rarely isolated from faeces and I was unable to isolate them from the faeces of the children with rhinovirus infection and diarrhoeal illness. Although some of the control children developed respiratory illness some days after swabs were collected, there is little doubt that symptomless rhinovirus infections do occur in children. Rhinovirus infections in children were associated with 10% of upper respiratory illness, 8% of bronchitis and bronchiolitis and 7% of pneumonia and bronchopneumonia. The isolation of rhinoviruses from lower respiratory disease in children has been reported previously (Reilly et al. 1962; Bloom et al. 1963; Chanock and Parrott, 1965a) but the difficulty of establishing a causal role has been emphasised by Portnoy and colleagues (1965) who found 5 (38%) of 13 children with lower respiratory disease and rhinovirus infection also had serological evidence of infection with RS or parainfluenze type 3 viruses. In this study there were 17 children with lower respiratory illness from whom rhinoviruses were isolated and paired sera were received; six

(35%) of these 17 also had evidence of simultaneous infection with para-influenza viruses, RS virus or adenoviruses which could also have caused the illness. Thus, the isolation of a rhinovirus from a child with lower respiratory illness is not always evidence of an etiological association. It does, however, seem likely that rhinoviruses can cause lower respiratory illness in children.

The homologous neutralizing antibody response of 18 children in hospital with rhinovirus infection was investigated. Three of the six children aged four months or less had antibody in their acute sera (although two of these acute sera were collected more than 5 days after the onset of illness). Only one of the 12 children over 6 months had such antibody. The presence of antibody, presumably maternal, in very young children has been previously observed (Hamparian et al. 1964). The three children infected with M strains all had strong antibody responses; only eight of the 15 children infected with H strains had fourfold or greater antibody titre rises. The ability of M strains to induce more and greater antibody responses than H strains in children has been previously reported (Taylor-Robinson et al. 1963). As in this study, other workers have found that about half the children infected with H strains show significant serological responses (Taylor-Robinson et al. 1963) but some reports show responses in a higher proportion of children (Hamparian et al. 1964; Portnoy et al. 1965). These differences might be due to time of collection of second sera. In this study only three convalescent sera were collected 3 weeks or more after the onset of illness.

A control group of adults without respiratory illness was not investigated

in this study and therefore, no evidence was obtained about an etiological association of rhinovirus infection with respiratory illness in adults. Other studies have established such an association. The difference between isolation rates from adults with respiratory illness and those without is much greater than the difference in children (Bloom et al. 1963; Hamparian et al. 1964; Hamre et al. 1966). All the rhinovirus infections in normal adults in this study were associated with upper respiratory illness; this is consistent with findings in other studies (Hamparian et al. 1964). However, only one of 14 rhinovirus infections in chronic bronchitics was not associated with an exacerbation; this suggests that rhinoviruses may infect the lower respiratory tract of bronchitics. Further evidence for this is the presence of rhinoviruses in sputa, often in high titre and in the absence of virus in throat swabs collected at the same time. There is also experimental evidence that rhinoviruses in a fine particle aerosol can infect the lower respiratory tract of normal adults (Cate et al. 1965). As rhinoviruses were isolated from 13 (25%) of 53 exacerbations investigated, it is likely that they are a significant cause of respiratory distress in chronic bronchitics. Similar observations have been made by Stenhouse (1967); but rhinoviruses were not isolated by the only other workers who have sought them in chronic bronchitics (Carilli et al. 1964). Rhinoviruses did not appear to persist as latent infections causing successive exacerbations of bronchitis since different serotypes were isolated during separate exacerbations of the same bronchitic.

The homologous neutralizing antibody responses during the 13 rhinovirus infections associated with exacerbations in bronchitics were greater and more frequent than those in normal adults in most other reports (Taylor-Robinson et al. 1963; Gwaltney and Jordan, 1964; Hamre et al. 1964; Phillips et al. 1965b). This could be further evidence that rhinovirus infections in bronchitics were more severe and widespread than in normal adults. Persistance of rhinovirus neutralizing antibody in detectable amounts for over 4 years after an infection has been reported but reinfection could have occurred in the intervening period (Hamparian et al. 1964). The results of my serological studies on bronchitics indicate that rhinovirus antibody does persist in the absence of evidence of reinfection.

The seasonal variation in rhinovirus isolation rates found in this study, namely periods of high isolation rates in spring and autumn alternating with lower rates in summer and winter appears to be characteristic (Bloom et al. 1963; Hamparian et al. 1964; Hamre et al. 1966; Spigland et al. 1966). This suggests that most rhinovirus infections occur in the spring and autumn.

Rhinoviruses isolated in Britain have seldom been identified using prototype antisera (Higgins 1966a). However, identification does allow observations to be made on the spread of rhinoviruses and on the association with illness and seasonal distribution of individual serotypes.

The same serotype was isolated from different members of a family

during a respiratory outbreak on three occasions. This suggests that person to person spread occurs. However, there was one family outbreak and four episodes among laboratory workers when different serotypes were isolated. Thus, when an outbreak of respiratory illness occurs in a family or among a small group of workers it cannot be assumed that all are infected with the same virus. In this context it is interesting that an epidemiological analysis of the spread of colds among office workers and their families showed that only a small proportion of the infections were a consequence of known exposure to infected individuals (Lidwell and Williams, 1961).

Most of the rhinovirus serotypes isolated from exacerbations of bronchitis in adults were also found in adults or children with common colds. Types 1321-62 and 1833-63 were also isolated from children with lower respiratory illness. Most of the rhinoviruses which have been serologically identified by other workers were isolated from adults or children with colds or no respiratory illness. However, B632 has been isolated from a child with bronchiolitis (Portnoy et al. 1965) and 204 from a child with croup (Ketler et al. 1962) and two of three strains of 5986 isolated by Ketler and colleagues (1962) were from children with lower respiratory illness. It is therefore interesting that one of the two B632 strains, the 204 strain and the 5986 strain which I isolated were from children with croup or lower respiratory illness. Thus, it does seem

possible that certain serotypes may cause lower respiratory illness in children more readily than others.

In this study some serotypes occurred only during one season and others were present during several successive seasons. Similar findings have been reported in the U.S.A. (Hamparian et al. 1964; Hamre et al. 1966). In one study, nine strains of type 68 were isolated during a three-month period but none during the succeeding two years (Hamparian et al. 1964). The three strains of type 68 I isolated were found only during a six-month period out of the four years and four months they were sought. The three strains of 179E which I isolated occurred only during a four-month period. Strain 179E has occurred in a sharp outbreak in the U.S.A. (Mufson et al. 1966). However, Hamre and colleagues (1966) reported isolations of 179E throughout an 18-month period and Higgins (1966a) found four of six M strains isolated between October 1962 and December 1963 were type 179E. It is interesting that these isolations were made by Higgins (1966a) during the same period in which this virus was prevalent in Glasgow. Thus, a rhinovirus serotype may occur in widely separated areas at the same time.

It is clear that many more rhinovirus strains must be identified before any conclusions can be reached about their individual epidemiology.

Herpes Simplex Virus

Herpes simplex virus was isolated from 26 individuals. Although most of them had respiratory illness, there was no evidence of an etiological association.

Herpes virus was not isolated from children with respiratory illness under 1 year but was found in 8% of children in the second year of life. Thereafter, the isolation rate declined with increasing age. These results would agree with the observation that herpetic gingivostomatitis is most prevalent between one and three years of age (Scott and Tokumaru, 1965).

Herpes was isolated most commonly from children in hospital and most, though not all of them, had clinical evidence of stomatitis.

The isolation rates from children with respiratory illness and from those without were not significantly different. Serological evidence suggested that the herpes infections were latent ones probably reactivated by the respiratory illness. Thus, there was little evidence that herpes virus infection was a cause of respiratory disease. This conclusion would agree with that of Albanese, Bynoe and Tyrrell (1965) who were unable to induce colds in volunteers by infecting them with herpes simplex virus isolated from a person with respiratory illness.

Adenoviruses

Adenoviruses were isolated from 24 children but no adults. There were six strains of type 1, five of type 2, four of type 3, seven of type 5 and two of type 7.

Adenoviruses were isolated most commonly from children under 2 years

as in other studies (Chanock and Parrott, 1965a).

I isolated adenoviruses from 4% of children in hospital with respiratory disease which is a similar figure to that of Holzel and colleagues (1965a) but rather less than the 8% of Chanock and Parrott (1965a).

Since the same adenovirus serotypes were found more often in children with diarrhoeal illnesses than in those with respiratory illness and approximately 45% of adenovirus infections in children with respiratory disease were associated with another virus infection, adenovirus infection cannot be shown in this study to cause respiratory disease. Adenovirus types 1, 2, 3 and 5 were, however, isolated from children with lower respiratory illness. There was no marked seasonal distribution of adenovirus isolations. Adenoviruses, therefore, contributed little to the etiology of the respiratory disease which I studied.

Enteroviruses

Enterovirus infections were detected in 18 children in hospital. Twelve strains were isolated from children with respiratory illness; four strains of coxsackievirus B5, three strains of coxsackievirus B1, two untyped enteroviruses and one strain each of echoviruses 3 and 9 and coxsackievirus B4. These represent an isolation rate of 3% which is similar to that found in other studies of children in hospital with respiratory illness (Clarke et al. 1964; Holzel et al. 1965a). Six strains were isolated from children with diarrhoeal illness: two strains of coxsackievirus B2 and one

strain each of echoviruses 8 and 9, poliovirus 2 and an untyped enterovirus. Since enteroviruses are known to cause both respiratory and diarrhoeal illness it is not possible to show any etiological relationship in this study. However, it is interesting that eight of the 12 viruses from children with respiratory disease were coxsackieviruses of the B group which have previously been associated with respiratory disease (Cramblett et al. 1964). The enteroviruses were isolated only from children under six years old and were found most often in the summer months. These findings are compatible with the established epidemiology of enteroviruses.

The results which I have reported indicate that the recognized epidemiology of certain respiratory viruses was reproduced in Glasgow between 1962 and 1966. In addition, some new information was obtained about RS virus and the rhinoviruses. However, many unsolved problems remain in the epidemiology of known respiratory viruses, and the role of certain recently discovered viruses has yet to be established. Prolonged epidemiological studies of the kind described here are essential if such questions are to be answered.

REFERENCES

ABRAHAM, A.S. (1962) Proc. Soc. exp. Biol. Med. 109, 855.

ALBANESE, M., BYNOE, M.L., and TYRRELL, D.A.J. (1966) Arch. ges. Virusforsch. 18, 356.

ALMEIDA, J.D., and TYRRELL, D.A.J. (1967) J. gen. Virol. 1, 175.

ANDERSON, J.M., and BEEM, M.O. (1966) Proc. Soc. exp. Biol. Med. 121, 205.

ANDERSON, S.G. (1947) Aust. J. exp. Biol. med. Sci. 25, 243.

ANDERSON, S.G., and BURNET, F.M. (1947) Aust. J. exp. Biol. med. Sci. 25, 235.

ANDREWES, C.H., CHAPRONIERE, D.M., GOMPELS, A.E.H., PEREIRA, H.G., and RODEN, A.T. (1953) Lancet ii, 546.

ANDREWS, B.E., and McDONALD, J.C. (1955) Br. med J. ii, 992.

BANATVALA, J.E., ANDERSON, T.B., and REISS, B.B. (1964) Br. med. J. i, 537.

BEEM, M., WRIGHT, F.H., HAMRE, D., EGERER, R., and OEHME, M. (1960) New Engl. J. Med. 263, 523.

BEEM, M., WRIGHT, F.H., FASAN, D.M., EGERER, R., OEHME, M. (1962) J. Pediat. 61, 864.

BEEM, M., EGERER, R., and ANDERSON, J. (1964) Pediatrics, Springfield 34, 761.

BEEM, M.O. (1965) J. Pediat. 67, 941.

BEEM, M.O., OEHME, M., and CHANEY, S. (1965) Proc. Soc. exp. Biol. Med. 118, 975.

BEHBEHANI, A.M., MELNICK, J.L., and DeBAKEY, M.E. (1965) Proc. Soc. exp. Biol. Med. 118, 759.

BELL, J.A., HUEBNER, R.J., ROSEN, L., ROWE, W.P., COLE, R.M., MASTROTA, F.M., FLOYD, T.M., CHANOCK, R.M., and SHVEDOFF, R.A. (1961) Am. J. Hyg. 74, 267.

BELL, J.A., ROWE, W.P., and ROSEN, L. (1962) Am. J. Publ. Hlth 52, 902.

BELL, T.M., TURNER, G., MacDONALD, A., and HAMILTON, D.A. (1960) Lancet ii, 1327.

BENNETT, C.R., and HAMRE, D. (1962) J. infect. Dis. 110, 8.

BERGLUND, B., VIHMA, L., and WICKSTRÖM, J. (1965) Am. J. Epid. 81, 271.

BERKOVICH, S., and KIBRICK, S. (1964) J. Pediat. 65, 368.

BLOOM, H.H., JOHNSON, K.M., JACOBSEN, R., and CHANOCK, R.M. (1961) Am. J. Hyg. 74, 50.

BLOOM, H.H., JOHNSON, K.M., MUFSON, M.A., and CHANOCK, R.M. (1962) J. Am. med. Ass. 179, 120.

BLOOM, H.H., FORSYTH, B.R., JOHNSON, K.M., and CHANOCK, R.M. (1963) J. Am. med. Ass. 186, 38.

BLOOM, H.H., FORSYTH, B.R., JOHNSON, K.M., MUFSON, M.A., TURNER, H.C., DAVIDSON, M.A., and CHANOCK, R.M. (1964) Am. J. Hyg. 80, 328.

BROWN, P.K., and TYRRELL, D.A.J. (1964) Br. J. exp. Path. 45, 571.

BROWN, P.K., and TAYLOR-ROBINSON, D. (1966) Bull. Wld Hlth Org. 34, 895.

BUCKLAND, F.E., BYNOE, M.L., ROSEN, L., and TYRRELL, D.A.J. (1961) Br. med. J. i, 397.

BUKRINSKAYA, A.G., and PAKTORIS, E.A. (1960) Acta virol., Prague, 4, 184.

BUKRINSKAYA, A.G., YUN-De, H., and GORBUNOVA, A.S. (1962) Acta. virol., Prague, 6, 352.

BURROWS, M.T. (1910) J. Am. med. Ass. 55, 2057.

CANCHOLA, J., VARGOSKO, A.J., KIM, H.W., PARROTT, R.H., CHRISTMAS, E., JEFFRIES, B., and CHANOCK, R.M. (1964) Am. J. Hyg. 79, 357.

CANCHOLA, J.G., CHANOCK, R.M., JEFFRIES, B.C., CHRISTMAS, E.E., KIM, H.W., VARGOSKO, A.J., and PARROTT, R.H. (1965) Bact. Rev. 29, 496.

CARILLI, A.D., GOHD, R.S., and GORDON, W. (1964) New Engl. J. Med. 270, 123.

CARREL, A. (1912) J. exp. Med. 15, 516.

CATE, T.R., COUCH, R.B., and JOHNSON, K.M. (1964) J. clin. Invest. 43, 56.

CATE, T.R., COUCH, R.B., FLEET, W.F., GRIFFITH, W.R., GERONE, P.J., and KNIGHT, V. (1965) Am. J. Epid. 81, 95.

CELL CULTURE COLLECTION COMMITTEE (1965) Registry of animal cell lines. First supplement. U.S. Department of Health, Education and Welfare, Bethesda.

CHANOCK, R.M. (1956) J. exp. Med. 104, 555.

CHANOCK, R., and FINBERG, L. (1957) Am. J. Hyg. 66, 291.

CHANOCK, R., ROIZMAN, B., and MYERS, R. (1957) Am. J. Hyg. 66, 281.

CHANOCK, R.M., PARROTT, R.H., COOK, K., ANDREWS, B.E., BELL, J.A., REICHELDERFER, T., KAPIKIAN, A.Z., MASTROTA, F.M., and HUEBNER, R.J. (1958) New Engl. J. Med. 258, 207.

CHANOCK, R.M., KIM, H.W., VARGOSKO, A.J., DELEVA, A., JOHNSON, K.M., CUMMING, C., and PARROTT, R.H. (1961) J. Am. med. Ass. 176, 647.

CHANOCK, R.M., HAYFLICK, L., and BARILE, M.F. (1962) Proc. natn. Acad. Sci. U.S.A. 48, 41.

CHANOCK, R.M., PARROTT, R.H., JOHNSON, K.M., KAPIKIAN, A.Z., and BELL, J.A. (1963) Am. Rev. resp. Dis. 88, no. 3 pt. 2, 152.

CHANOCK, R.M. and PARROTT, R.H. (1965a) Pediatrics, Springfield 36, 21.

CHANOCK, R.M., and PARROTT, R.H. (1965b). In Viral and rickettsial infections of man (ed. F.L. Horsfall and I. Tamm), chap. 31, pp. 741-754. Lippincott, Philadelphia.

CHANY, C., LÉPINE, P., LELONG, M., LE-TAN-VINH, SATGÉ, P., and VIRAT, J. (1958) Am. J. Hyg. 67, 367.

CHAPPLE, P.J., and HARRIS, W.J. (1966) Nature, Lond. 209, 790.

CLARKE, S.K.R., CORNER, B.D., GAMBIER, D.M., MACRAE, J., and PEACOCK, D.B. (1964) Br. med. J. i, 1536.

COATES, H.V., and CHANOCK, R.M. (1962) Am. J. Hyg. 76, 302.

COATES, H.V., KENDRICK, L., and CHANOCK, R.M. (1963) Proc. Soc. exp. Biol. Med. 112, 958.

COATES, H.V., ALLING, D.W., and CHANOCK, R.M. (1966) Am. J. Epid. 83, 299.

COHEN, I.R., NORINS, L.C., and JULIAN, A.J. (1967) J. Immun. 98, 143.

COMMITTEE ON THE ENTEROVIRUSES (1957) Am. J. publ. Hlth 47, 1556.

COUCH, R.B., CATE, T.R., FLEET, W.F., GERONE, P.J., GRIFFITHS, W.R., and KNIGHT, V. (1964) J. clin. Invest. 43, 1286.

CRAMBLETT, H.G., ROSEN, L., PARROTT, R.H., BELL, J.A., HUEBNER, R.J., and McCULLOUGH, N.B. (1958) Pediatrics, Springfield 21, 168.

CRAMBLETT, H.G., MOFFET, H.L., MIDDLETON, G.K., BLACK, J.P., SCHULENBERGER, H., and YONGUE, A. (1962) Archs intern. Med. 110, 574.

CRAMBLETT, H.G., MOFFET, H.L., BLACK, J.P., SHULENBERGER, H., SMITH, A., and COLONNA, C.T., (1964) J. Pediat. 64, 406.

DALLDORF, G., and SICKLES, G.M. (1948) Science, N.Y. 108, 61.

DALLDORF, G., SICKLES, G.M., PLAGER, H., and GIFFORD, R. (1949) J. exp. Med. 89, 567.

DALLDORF, G., and MELNICK, J.L. (1965). In Viral and rickettsial infections of man (ed. F.L. Horsfall and I. Tamm), chap. 19, pp. 474 - 512. Lippincott, Philadelphia.

DARNELL, J.E., and SAWYER, T.K. (1959) Virology 8, 223.

DICK, E.C., MOGABGAB, W.J., and HOLMES, B. (1961) Am. J. Hyg. 73, 263.

DOGGETT, J.E. (1963) Personal communication.

DOGGETT, J.E. (1965) Bull. Wld Hlth Org. 32, 849.

DOGGETT, J.E., and TAYLOR-ROBINSON, D. (1965) Arch. ges. Virusforsch. 15, 601.

DOUGLAS, R.G., CATE, T.R., and COUCH, R.B. (1966) Proc. Soc. exp. Biol. Med. 123, 238.

DOWDLE, W.R., and ROBINSON, R.Q. (1966) Proc. Soc. exp. Biol. Med. 121, 193.

EADIE, M.B., STOTT, E.J., and GRIST, N.R., (1966) Br. med. J. ii, 671.

EAGLE, H. (1955) Proc. Soc. exp. Biol. Med. 89, 362.

ELVEBACK, L.R., FOX, J.P., KETLER, A., BRANDT, C.D., WASSERMAN, F.E., and HALL, C.E. (1966) Am. J. Epid. 83, 436.

ENDERS, J.F., WELLER, T.H., and ROBBINS, F.C. (1949) Science, N.Y. 109, 85.

EVANS, A.S., DICK, E.C., and NYSTUEN, K. (1963) Archs envir. Hlth 6, 748.

FANTES, K.H. (1962) J. Hyg., Camb. 60, 123.

FELDMAN, H.A., and WANG, S.S. (1961) Proc. Soc. exp. Biol. Med. 106, 736.

FELL, H.B. (1951) Meth. med. Res. 4, 233.

FENTERS, J.D., GILLUM, S.S., HOLPER, J.C., and MARQUIS, G.S. (1966) Am. J. Epid. 84, 10.

FIALA, M., and KENNY, G.E. (1966) J. Bact. 92, 1710.

FLEET, W.F., COUCH, R.B., CATE, T.R., and KNIGHT, V. (1965) Am. J. Epid. 82, 185.

FORBES, J.A. (1958) Med. J. Aust. 2, 75.

FORSYTH, B.R., BLOOM, H.H., JOHNSON, K.M., and CHANOCK, R.M. (1964) Am. J. Hyg. 80, 343.

FOX, J.P., ELVEBACK, L.R., SPIGLAND, I., FROTHINGHAM, T.E., STEVENS, D.A., and HUGER, M. (1966) Am. J. Epid. 83, 389.

FRANCIS, T. (1934) Science, N.Y. 80, 457.

FRANCIS, T. (1940), Science, N.Y. 92, 405.

FRANCIS, T., QUILLIGAN, J.J., and MINUSE, E. (1950). Science, N.Y. 112, 495.

FRANCIS, T., and Maassab, H.F. (1966). In Viral and rickettsial infections of man (ed. F.L. Horsfall and I. Tamm) chap. 30, pp 689-740. Lippincott, Philadelphia.

FRY, J. (1958) Br. med. J. i, 259.

FRY, J. (1959) Br. med. J. ii, 135.

FRY, J. (1961) Br. med. J. i, 745.

FUKUMI, H., NISHIKAWA, F., MIZUTANI, H., YAMAGUCHI, Y., and NANBA, J. (1958) Jap. J. med. Sci. Biol. 11, 129.

FUKUMI, H., NISHIKAWA, F., SUGIYAMA, T., YAMAGUCHI, Y., NANBA, J., MATSUURA, T., and OIKAWA, R. (1959) Jap. J. med. Sci. Biol. 12, 307.

FUKUMI, H., NISHIKAWA, F., SONOGUCHI, T., and SHIMIZU, T. (1961) Jap. J. med. Sci. Biol. 14, 21.

FUKUMI, H., NISHIKAWA, F., SONOGUCHI, T., and SHIMIZU, T. (1962) Jap. J. med. Sci. Biol. 15, 145.

GELFAND, H.M. (1959) Sth. med. J., Nashville 52, 819.

GERBER, P., WOOLRIDGE, R.L., SEAL, J.R., and ZIEGRA, S.R. (1952) Proc. Soc. exp. Biol. Med. 81, 624.

GEY, G.O., COFFMAN, W.D., and KUBICEK, M.T. (1952) Cancer Res. 12, 264.

GOFFE, A., and BELL, E.J. (1963) Proceedings of the IX Symposium of the European Association against Poliomyelitis and Allied Diseases, 311. Brussels.

GOFFE, A. (1965) Arch. ges. Virusforsch. 16, 469.

GRIST, N.R. (1955) Br. med. J. ii, 994.

GRIST, N.R., ROSS, C.A.C. and STOTT, E.J. (1967) Br. med. J. i, 456.

GWALTNEY, J.M., and JORDAN, W.S. (1964) Bact. Rev. 28, 409.

GWALTNEY, J.M. (1966) Proc. Soc. exp. Biol. Med. 122, 1137.

GWALTNEY, J.M., and JORDAN, W.S. (1966) Am. Rev. resp. Dis. 93, 362.

GWALTNEY, J.M., HENDLEY, J.O., SIMON, G., and JORDAN, W.S. (1966) New Eng. J. Med. 275, 1261.

HAFF, R.F., WOHLSEN, B., FORCE, E.E., and STEWART, R.C. (1966) J. Bact. 91, 2339.

HAMBLING, M.H. (1964) Br. med. J. i, 1223.

HAMBLING, M.H. (1966) J. Path. Bact. 91, 625.

HAMPARIAN, V.V., KETLER, A., HILLEMAN, M.R., REILLY, C.M., McCLELLAND, L., CORNFELD, D., and STOKES, J. (1961a) Proc. Soc. exp. Biol. Med. 106, 717.

HAMPARIAN, V.V., KETLER, A., and HILLEMAN, M.R. (1961b) Proc. Soc. exp. Biol. Med. 108, 444.

HAMPARIAN, V.V., LEAGUS, M.B., HILLEMAN, M.R., and STOKES, J. (1964) Proc. Soc. exp. Biol. Med. 117, 469.

HAMPARIAN, V.V. (1966) Personal Communication.

HAMRE, D., LASHOF, J.C., MARSHALL, J.A., CASSIDY, J.E., SMITH, M., and BENNETT, C.R. (1961) Am. Rev. resp. Dis. 83, 38.

HAMRE, D., and PROCKNOW, J.J. (1961) Br. med. J. ii, 1382.

HAMRE, D. (1963) Am. Rev. resp. Dis. 88, No 3 Pt 2, 306.

HAMRE, D. (1964) Bact. Proc. 132.

HAMRE, D., CONNOLLY, A.P., and PROCKNOW, J.J. (1964) J. Lab. clin. Med. 64, 450.

HAMRE, D., CONNELLY, A.P., and PROCKNOW, J.J. (1966) Am. J. Epid. 83, 238.

HAMRE, D., and PROCKNOW, J.J. (1966) Proc. Soc. exp. Biol. Med. 121, 190.

HARRISON, R.G. (1907) Proc. Soc. exp. Biol. Med. 4, 140.

HAUSCHKA, T.S., MITCHELL, J.T., and NIEDERPRUEM, D.J. (1959) Cancer Res. 19, 643.

HAYFLICK, L., and MOORHEAD, P.S. (1961) Expl Cell Res. 25, 585,

HAYFLICK, L., JACOBS, P. and PERKINS, F. (1964) Nature, Lond. 204, 146.

HAYSLETT, J., McCARROLL, J., BRADY, E., DUESCHLE, K., McDERMOTT, W., and KILBOURNE, E.D. (1962). Am. Rev. resp. Dis. 85, 1.

HEGGIE, A., SCHULTZ, I., GUTEKUNST, R.R., ROSENBAUM, M., and MILLER, L.F. (1960) Am. J. publ. Hlth 50, 1342.

HERDERSCHEE, D. (1963) Antonie v. Leeuwenhoek, 29, 154.

HUEBNER, R.J., COLE, R.M., BEEMAN, E.A., BELL, J.A., and PEERS, J.H. (1951) J. Am. med. Ass. 145, 628.

HUEBNER, R.J., ROWE, W.P., and CHANOCK, R.M. (1958) A. Rev. Microbiol, 12, 49.

HIGGINS, P.G. (1966a) Proc. R. Soc. Med. 59, 48.

HIGGINS, P.G. (1966b) Mon. Bull. Minist. Hlth 25, 223.

HIGGINS, P.G. (1966c) Mon. Bull. Minist. Hlth 25, 283.

HIGGINS, P.G., ELLIS, E.M., and BOSTON, D.G. (1966). Mon. Bull. Minist. Hlth 25, 5.

HILLEMAN, M.R., and WERNER, J.H. (1954). Proc. Soc. exp. Biol. Med. 85, 183.

HILLEMAN, M.R., HAMPARIAN, V.V., KETLER, A., REILLY, C.M., McCLELLAND, L., CORNFELD, D., and STOKES, J. (1962) J. Am. med. Ass. 180, 445.

HOBSON, D., and SCHILD, G.C. (1960) Br. med. J. ii, 1414.

HOLZEL, A., PARKER, L., PATTERSON, W.H., CARTMEL, D., WHITE, L.L.R., PURDY, R., THOMSON, K.M., and TOBIN, J. O'H. (1965a) Br. med. J. i, 614.

HOLZEL, A., SMITH, P.A., and TOBIN, J. O'H. (1965b) Acta paediat. Scand. 54, 168.

HOOFT, C., NIHOUL, E., LAMBERT, Y., and VALCKE, R. (1963) Helv. pediat. Acta 18, 230.

HOORN, B., and TYRRELL, D.A.J. (1965) Br. J. exp. Path. 46, 109.

HOORN, B., and TYRRELL, D.A.J. (1966) Arch. ges. Virusforsch. 18, 210.

HORSTMANN, D.M., and HSIUNG, G.D. (1963) Clin. Pediat. 2, 378.

HOUSE, W., and WADDELL, A. (1967) J. Path. Bact. 93, 125.

HSIUNG, G.D., ISACSON, P., and McCOLLUM, R.W. (1962) J. Immun. 88, 284.

JACKSON, G.G., MULDOON, R.L., and COOPER, G.S. (1961) J. clin. Invest. 40, 1051.

JACKSON, G.G., MULDOON, R.L., JOHNSON, G.C., and DOWLING, H.F. (1963) Am. Rev. resp. Dis. 88, no. 3 pt 2, 120.

JAHN, C.L., FELTON, O.L., and CHERRY, J.D. (1964) J. Am. med. Ass. 189, 236.

JOHNSON, K.M., CHANOCK, R.M., COOK, M.K., and HUEBNER, R.J. (1960) Am. J. Hyg. 71, 81.

JOHNSON, K.M., CHANOCK, R.M., RIFKIND, D., KRAVETZ, H.M., and KNIGHT, V. (1961) J. Am. med. Ass. 176, 663.

JOHNSON, K.M., BLOOM, H.H., CHANOCK, R.M., MUFSON, M.A., and KNIGHT, V. (1962a) Am. J. publ. Hlth 52, 933.

JOHNSON, K.M., BLOOM, H.H., MUFSON, M.A., and CHANOCK, R.M. (1962b) J. Am. med. Ass. 179, 112.

JOHNSON, K.M., BLOOM, H.H., MUFSON, M.A., and CHANOCK, R.M. (1962c) New Engl. J. Med. 267, 68.

JOHNSON, K.M., and ROSEN, L. (1963) Am. J. Hyg. 77, 15.

JOHNSON, K.M., BLOOM, H.H., FORSYTH, B.R., and CHANOCK, R.M. (1965) Am. J. Epid. 81, 131.

JORDAN, W.S., BADGER, G.F., CURTISS, C., DINGLE, J.H., GINSBERG, H.S., and GOLD, E. (1956a) Am. J. Hyg. 64, 336.

JORDAN, W.S., STEVENS, D., KATZ, S., and DINGLE, J.H. (1956b) New Engl. J. Med. 254, 687.

JORDAN, W.S., (1958) Archs intern. Med. 101, 54.

JORDAN, W.S., BADGER, G.F., and DINGLE, J.H. (1958) New Engl. J. Med. 258, 1041.

KAPIKIAN, A.Z., BELL, J.A., MASTROTA, F.M., JOHNSON, K.M., HUEBNER, R.J., and CHANOCK, R.M. (1961a) Am. J. Hyg. 74, 234.

KAPIKIAN, A.Z., CHANOCK, R.M., REICHELDERFER, T.E., WARD, T.G., HUEBNER, R.J., and BELL, J.A. (1961b) J. Am. med. Ass. 178, 537.

KASEL, J.A., CRAMBLETT, H.G., and Utz, J.P. (1958) Proc. Soc. exp. Biol. Med. 99, 703.

KASEL, J.A., and KNIGHT, V. (1963) Proc. Soc. exp. Biol. Med. 113, 602.

KASEL, J.A., ROSEN, L., LODA, F., and FLEET, W. (1965) Proc. Soc. exp. Biol. Med. 118, 381.

KAY, H.E.M., PEPPERCORN, M.E., PORTERFIELD, J.S., McCARTHY, K., and TAYLOR-ROBINSON, C.H. (1964) Br. med. J. ii, 166.

KELLY, S. and SANDERSON, W.W., (1962) Am. J. publ. Hlth 52, 455.

KENDALL, E.J.C., RIDDLE, R.W., TUCK, H.A., RODAN, K.S., ANDREWS, B.E., and MacDONALD, J.C. (1957) Br. med. J. ii, 131.

KENDALL, E.J.C., COOK, G.T., and STONE, D.M. (1960) Br. med. J. ii, 1180.

KENDALL, E.J.C., BYNOE, M.L., and TYRRELL, D.A.J. (1962) Br. med. J. ii, 82.

KETLER, A., HAMPARIAN, V.V., and HILLEMAN, M.R., (1962) Proc. Soc. exp. Biol. Med. 110, 821.

KILBOURNE, E.D., SUGIURA, A., and WONG, S.C. (1964) Proc. Soc. exp. Biol. Med. 116, 225.

KISCH, A.L., WEBB, P.A., and JOHNSON, K.M. (1964) Am. J. Hyg. 79, 125.

KRAVETZ, H.M., KNIGHT, V., CHANOCK, R.M., MORRIS, J.A., JOHNSON, K.M., RIFKIND, D., and Utz, J.P. (1961) J. Am. med. Ass. 176, 657.

KUROYA, M., ISHIDA, N., and SHIRATORI, T. (1953) YOKOHAMA med. Bull. 4, 217.

LA PLACA, M., and MOSCOVICI, C. (1962) J. Immun. 88, 72.

LENNETTE, E.H., FOX, V.L., SCHMIDT, N.J., and CULVER, J.O. (1958) Am. J. Hyg. 68, 272.

LERNER, A.M., KLEIN, J.O., LEVIN, H.S., and FINLAND, M. (1960) New Engl. J. Med. 263, 1265.

LEWIS, F.A., LEHMANN, N.I., and FERRIS, A.A. (1961a) Med. J. Aust. ii, 929.

LEWIS, F.A., RAE, M.L., LEHMANN, N.I., and FERRIS, A.A. (1961b) Med. J. Aust. ii, 932.

LIDWELL, O.M., and WILLIAMS, R.E.O. (1961) J. Hyg., Camb. 59, 321.

LIKAR, M. (1961) Pathologia Microbiol. 24, 415.

LOVELOCK, J.E., and BISHOP, M.W.H. (1959) Nature, Lond. 183, 1394.

McDONALD, J.C., MILLER, D.L., ZUCKERMAN, A.J., and PEREIRA, M.S. (1962) J. Hyg., Camb. 60, 235.

McLEAN, D.M., ROY, T.E., O'BRIEN, M.J., WYLLIE, J.C., and McQUEEN, E.J. (1961) Can. med. Ass. J. 85, 290.

McLEAN, D.M., BACH, R.D., LARKE, R.P.B., and McNAUGHTON, G.A. (1963) Can. med. Ass. J. 89, 1257.

MAGILL, T.P. (1940) Proc. Soc. exp. Biol. Med. 45, 162.

MATUMOTO, M. (1963) Am. Rev. resp. Dis. 88, No 3 pt. 2, 46.

MEDICAL RESEARCH COUNCIL (1960) Br. med. J. ii, 1665.

MEDICAL RESEARCH COUNCIL (1965a) Lancet i, 775.

MEDICAL RESEARCH COUNCIL (1965b) Br. med. J. ii, 319.

MEENAN, P.N., CLARKE, M., and TYRRELL, D.A.J. (1959) Lancet ii, 98.

MELNICK, J.L., SHAW, E.W., and CURNEN, E.C. (1949) Proc. Soc. exp. Biol. Med. 71, 344.

MELNICK, J.L., and ÅGREN, K. (1952) Proc. Soc. exp. Biol. Med. 81, 621.

MELNICK, J.L. (1965). In Viral and rickettsial infections of man (ed. F.L. Horsfall and I. Tamm), chap. 20, pp. 512-545. Lippincott, Philadelphia.

MERRYMAN, H.T. (1962) Natn. Cancer Inst. Monogr. No. 7.

MIDULLA, M., WALLIS, C., and MELNICK, J.L. (1965) J. Immun. 95, 9.

MOFFET, H.L., and CRAMBLETT, H.G. (1962) New Engl. J. Med. 267, 1213.

MOGABGAB, W.J., and HOLMES, B. (1961) J. infect. Dis. 108, 59.

MOGABGAB, W.J. (1962) J. Bact. 83, 209.

MONTO, A.S., and JOHNSON, K.M. (1966) Proc. Soc. exp. Biol. Med. 121, 615.

MOORE, A.E., SABACHEWSKY, L., and TOOLAN, H.W. (1955) Cancer Res. 15, 598.

MORRIS, J.A., BLOUNT, R.E., and SAVAGE, R.E. (1956) Proc. Soc. exp. Biol. Med. 92, 544.

MOSS, P.D., ADAMS, M.O., and TOBIN, J. O'H. (1963) Lancet i, 298.

MUFSON, M.A., KAWANA, R., JAMES, H.D., GAULD, L.W., BLOOM, H.H., and CHANOCK, R.M. (1965) Am. J. Epid. 81, 32.

MUFSON, M.A., BLOOM, H.H., FORSYTH, B.R., and CHANOCK, R.M. (1966) Am. J. Epid. 83, 379.

MUFSON, M.A. (1967) Personal Communication.

MULDER, J., BRAUS, L.M., and MASUREL, N. (1956). Studies on the Antigenic composition of the Influenza A strains. Monograph of the Institute of Preventive Medicine No. 15. Leyden, H.E., Stenfert Kroese, Leyden. Cited by Stuart-Harris, C.H. (1965) in Influenza and other infections of the respiratory tract, 2nd ed. Edward Arnold, London.

NAKANO, M. (1959) Jap. J. med. Sci. Biol. 12, 79.

NEVA, F.A., and WELLER, T.H. (1964) J. Immun. 93, 466.

ORMSBY, H.L., FOWLE, A.M.C., and DOANE, F. (1957) Am. J. Ophthal. 43, 17.

PAFFENBERGER, R.S., BERG, G., CLARKE, N.A., STEVENSON, R.E., POOLER, B.G., and HYDE, R.T. (1959) Am. J. Hyg. 70, 254.

PAL, S.R., McQUILLIN, J., and GARDNER, P.S. (1963) J. Hyg., Camb. 61, 493.

PARKER, W.L., WILT, J.C., and STAKIW, W. (1961) Can. J. publ. Hlth 52, 246.

PARKMAN, P.D., MUNDON, F.K., McCOWN, J.M., and BUESCHER, E.L. (1964) J. Immun. 93, 608.

PARROTT, R.H., KIM, H.W., VARGOSKO, A.J., and CHANOCK, R.M. (1962a) J. Pediat. 61, 205.

PARROTT, R.H., VARGOSKO, A.J., KIM, H.W., BELL, J.A., and CHANOCK, R.M. (1962b) Am. J. publ. Hlth 52, 907.

PARROTT, R.H., KIM, H.W., ARROBIO, J.O., CANCHOLA, J.G., BRANDT, C.D., DeMEIO, J.L., JENSEN, K.E., and CHANOCK, R.M. (1967) First International Conference on Vaccines against Viral and Rickettsial Diseases of Man.

PARSONS, R., BYNOE, M.L., PEREIRA, M.S., and TYRRELL, D.A.J. (1960) Br. med. J. i, 1776.

PAYNE, A.M. - M. (1958) Proc. R. Soc. Med. 51, 1009.

PEACOCK, D.B., and CLARKE, S.K.R. (1961) Lancet ii, 466.

PELON, W., MOGABGAB, W.J., PHILLIPS, I.A., and PIERCE, W.E. (1957) Proc. Soc. exp. Biol. Med. 94, 262.

PEREIRA, H.G. (1966) Personal communication.

PEREIRA, M.S., and PEREIRA, H.G. (1959) Lancet ii, 539.

PEREIRA, M.S., HAMBLING, M.H., McDONALD, J.C., and ZUCKERMAN, A.J. (1963) J. Hyg., Camb. 61, 471.

PEREIRA, M.S., PEREIRA, H.G., and CLARKE, S.K.R. (1965) Lancet i, 21.

PHILLIPS, C.A., MELNICK, J.L., and GRIM, C.A. (1965a) Proc. Soc. exp. Biol. Med. 119, 843.

PHILLIPS, C.A., RIGGS, S., MELNICK, J.L., and GRIM, C.A. (1965b) J. Am. med. Ass. 192, 277.

PHILIPSON, L. (1958) Arch. ges. Virusforsch. 8, 318.

PHILIPSON, L., and WESSLÉN, T. (1958) Arch. ges. Virusforsch. 8, 77.

PLOTKIN, S.A. (1964) J. Am. med. Ass. 190, 265.

PORTERFIELD, J.S. (1962) Nature, Lond. 194, 1044.

PORTERFIELD, J.S., and ASHWOOD-SMITH, M.J. (1962) Nature, Lond. 193, 548.

PORTNOY, B., ECKERT, H.L., and SALVATORE, M.A. (1965) Pediatrics, Springfield 35, 899.

PRICE, W.H. (1956) Proc. natn. Acad. Sci. U.S.A. 42, 892.

REICHELDERFER, T.E., CHANOCK, R.M., CRAIGHEAD, J.E., HUEBNER, R.J., TURNER, H.C., JAMES, W., and WARD, T.G. (1958) Science, N.Y. 128, 779.

REILLY, C.M., HOCH, S.M., STOKES, J., McCLELLAND, L., HAMPARIAN, V.V., KETLER, A., and HILLEMAN, M.R. (1962) Ann. intern. Med. 57, 515.

REILLY, C.M., STOKES, J., HAMPARIAN, V.V., and HILLEMAN, M.R. (1963) J. Pediat. 62, 538.

REPORT of W.H.O. EXPERT COMMITTEE on RESPIRATORY VIRUS DISEASES (1959) Tech. Rep. Ser. Wld Hlth Org. 170.

ROBBINS, F.C., and ENDERS, J.F. (1950) Am. J. med. Sci. 220, 316.

ROBBINS, F.C., ENDERS, J.F., and WELLER, T.H. (1950) Proc. Soc. exp. Biol. Med. 75, 370.

ROBBINS, F.C., ENDERS, J.F., WELLER, T.H., and FLORENTINO, G.L. (1951) Am. J. Hyg. 54, 286.

ROBERTS, A.N., RENDTORFF, R.C., and HALE, B.D. (1965) Am. J. Epid. 82, 123.

ROBINSON, R.Q., HOSHIWARA, I., SCHAEFFER, M., GORRIE, R.H., and KAYE, H.S. (1962) Am. J. Hyg. 75, 18.

ROSE, H.M., MOLLOY, E., and O'NEILL, E. (1945) Proc. Soc. exp. Biol. Med. 60, 23.

ROSEN, L., JOHNSON, J.H., HUEBNER, R.J., and BELL, J.A. (1958) Am. J. Hyg. 67, 300.

ROSEN, L. (1960) Am. J. Hyg. 71, 120.

ROSEN, L., HOVIS, J.F., MASTROTA, F.M., BELL, J.A., and HUEBNER, R.J. (1960a) Am. J. Hyg. 71, 258.

ROSEN, L., HOVIS, J.F., MASTROTA, F.M., BELL, J.A., and HUEBNER, R.J. (1960b) Am. J. Hyg. 71, 266.

ROSEN, L., EVANS, H.E., and SPICKARD, A. (1963) Am. J. Hyg. 77, 29.

ROSEN, L., KERN, J., and BELL, J.A. (1964) Am. J. Hyg. 79, 163.

ROSS, C.A.C., STOTT, E.J., McMICHAEL, S., and CROWTHER, I.A. (1964) Arch. ges. Virusforsch. 14, 553.

ROSS, C.A.C., McMICHAEL, S., EADIE, M.B., LEES, A.W., MURRAY, E.A., and PINKERTON, I. (1966) Thorax 21, 461.

ROWE, W.P., HUEBNER, R.J., GILMORE, L.K., PARROTT, R.H., and WARD, T.G. (1953) Proc. Soc. exp. Biol. Med. 84, 570.

SCADDING, J.G. (1959) Lancet i, 323.

SCHERER, W.F., and HOOGASIAN, A.C. (1954) Proc. Soc. exp. Biol. Med. 87, 480.

SCHIEBLE, J.H., LENNETTE, E.H., and KASE, A. (1965) Proc. Soc. exp. Biol. Med. 120, 203.

SCHILD, G.C., and HOBSON, D. (1962) Br. J. exp. Path. 43, 288.

SCHMIDT, N.J., FOX, V.L., and LENNETTE, E.H. (1961) Proc. Soc. exp. Biol. Med. 107, 63.

SCHMIDT, N.J., HO, H.H., and LENNETTE, E.H. (1965) Am. J. clin. Path. 43, 297.

SCOTT, T.F. McN., and TOKUMARU, T. (1965) in Viral and rickettsial infections of man (ed. F.L. Horsfall and I. Tamm), chap. 41, pp. 892 - 914. Lippincott, Philadelphia.

SELZER, G. (1963) Lancet ii, 336.

SICKLES, G.M., MUTTERER, M., and PLAGER, H. (1959) Proc. Soc. exp. Biol. Med. 102, 742.

SMITH, C.B., PURCELL, R.H., BELLANTI, J.A., and CHANOCK, R.M. (1966) New Engl. J. Med. 275, 1145.

SMITH, W., ANDREWES, C.H., and LAIDLAW, P.P. (1933) Lancet ii, 66.

SOMMERVILLE, R.G. (1963) Lancet ii, 1247.

SPICKARD, A., EVANS, H., KNIGHT, V., and JOHNSON, K.M. (1963) J. clin. Invest. 42, 840.

SPIGLAND, I., FOX, J.P., ELVEBACK, L.R., WASSERMANN, F.E., KETLER, A., BRANDT, C.D., KOGON, A. (1966) Am. J. Epid. 83, 413.

STARK, J.E., HEATH, R.B., and PETO, S. (1964) Arch. ges. Virusforsch. 14, 160.

STARK, J.E., HEATH, R.B., and CURWEN, M.P. (1965) Thorax 20, 124.

STEINHARDT, E., ISRAELI, C., and LAMBERT, R.A. (1913) J. infect. Dis. 13, 294.

STENHOUSE, A. (1967) Br. med. J. 3, 461.

STERN, H., and TIPPETT, K.C. (1963) Lancet i, 1301.

STRANGEWAYS, T.S.P. (1924) Technique of tissue culture "in vitro". Heffer, Cambridge.

STUART-HARRIS, C.H. (1953) Influenza and other infections of the respiratory tract, 1st ed. Edward Arnold. London.

STUART-HARRIS, C.H. and HANLEY, T. (1957) Chronic bronchitis, emphysema and cor pulmonale. Wright, Bristol.

STUART-HARRIS, C.H. (1965) "Influenza and other virus infections of the respiratory tract", 2nd ed. Edward Arnold, London.

STULBERG, C.S., RIGHTSEL, W.A., PAGE, R.H., and BERMAN, L. (1959) Proc. Soc. exp. Biol. Med. 101, 415.

SUTO, T., YANO, N., IKEDA, M., MIYAMOTO, M., TAKAI, S., SHIGETA, S., HINUMA, Y., and ISHIDA, N. (1965) Am. J. Epid. 82, 211.

SUTTON, R.N.P., CLARKE, S.K.R., and TYRRELL, D.A.J. (1959) Lancet i, 395.

SUTTON, R.N.P. (1962) J. Hyg., Camb. 60, 51.

SVEHAG, S-E., and MANDEL, B. (1962) Virology 18, 508.

SWIM, H.E., HAFF, R.F., and PARKER, R.F. (1958) Cancer Res. 18, 711.

TAKANO, K., YAMADA, M., and HIROKAWA, Y. (1961) Jap. J. Med. Sci. Biol. 14, 27.

TANG, F-F., and LAING, Y-K. (1957) Antigenic studies of influenza virus isolated from the 1957 Epidemic in China. Proc. 3rd Intern. Meeting, Biol Stand. Opatija. p. 89. Cited by Stuart-Harris, C.H. (1965) in Influenza and other infections of the respiratory tract, 2nd ed. Edward Arnold, London.

TAYLOR, J.C., ROSS, C.A.C., and STOTT, E.J. (1967) Br. med. J. 3, 406.

TAYLOR, R.M. (1949). Am. J. publ. Hlth 39, 171.

TAYLOR, R.M. (1951) Arch. ges. Virusforsch. 4, 485.

TAYLOR-ROBINSON, D., and TYRRELL, D.A.J. (1962) Br. J. exp. Path. 43, 264.

TAYLOR-ROBINSON, D., and BYNOE, M.L. (1963) J. Hyg., Camb. 61, 407.

TAYLOR-ROBINSON, D., and DOGGETT, J.E. (1963) Br. J. exp. Path. 44, 473.

TAYLOR-ROBINSON, D., JOHNSON, K.M., BLOOM, H.H., PARROTT, R.H., MUFSON, M.A., and CHANOCK, R.M. (1963) Am. J. Hyg. 78, 285.

TAYLOR-ROBINSON, D., and BYNOE, M.L. (1964) Br. med. J. i, 540.

TAYLOR-ROBINSON, D. (1965) Bull. Wld Hlth Org. 32, 833.

THOMPSON, D., EBIN, C.E., and CANTAB, D.P. (1914) Proc. R. Soc. Med. 7, 21.

TOMLINSON, A.J.H., DAVIES, J.R., and MADIGAN, E.M. (1964) Mon. Bull. Minist. Hlth 23, 116.

TYRRELL, D.A.J., BALDUCCI, D., and ZAIMAN, T.E. (1956) Lancet ii, 1326.

TYRRELL, D.A.J., BYNOE, M.L., PETERSEN, K.B., SUTTON, R.N.P., and PEREIRA, M.S. (1959) Br. med. J. ii, 909.

TYRRELL, D.A.J., BYNOE, M.L., HITCHCOCK, G., PEREIRA, H.G., and ANDREWES, C.H. (1960) Lancet i, 235.

TYRRELL, D.A.J., and PARSONS, R. (1960) Lancet i, 239.

TYRRELL, D.A.J. (1961) Personal communication.

TYRRELL, D.A.J., and BYNOE, M.L. (1961) Br. med. J. i, 393.

TYRRELL, D.A.J., BYNOE, M.L., BUCKLAND, F.E., and HAYFLICK, L. (1962) Lancet ii, 320.

TYRRELL, D.A.J. (1963) Am. Rev. resp. Dis. 88, No 3 Pt 2, 77.

TYRRELL, D.A.J., and CHANOCK, R.M. (1963) Science, N.Y. 141, 152.

TYRRELL, D.A.J. (1964) In Cellular biology of myxovirus infections A Ciba Foundation Symposium. pp 182-184. Churchill, London.

TYRRELL, D.A.J., and BYNOE, M.L. (1965) Br. med. J. i, 1467.

TYRRELL, D.A.J., and BYNOE, M.L. (1966) Lancet i, 76.

ULSTRUP, J.C. (1964) Nord. Med. 72, 1324.

VAN der VEEN, J., and van der PLOEG, G. (1958) Am. J. Hyg. 68, 95.

VAN der VEEN, J., DEI, K.G., and PRINS, A. (1960) Ned. Tijdschr. Geneesk. 104, 617.

VAN der VEEN, J., and SMEUR, F.A.A.M. (1961) Am. J. Hyg. 74, 326.

VAN der VEEN, J., and DIJKMAN, J.H. (1962) Am. J. Hyg. 76, 149.

VAN der VEEN, J. (1963) Am. Rev. resp. Dis. 88, No 3 Pt 2, 167.

VARGOSKO, A.J., KIM, H.W., PARROTT, R.H., and CHANOCK, R.M. (1962) Am. J. Dis. Child. 104, 539.

VARGOSKO, A.J., JEFFRIES, B.C., CHANOCK, R.M., KIM, H.W. and PARROTT, R.H. (1964) Bact. Proc. 131.

VOGEL, J., and SHELOKOV, A. (1957) Science, N.Y. 126, 358.

VON EULER, L., KANTOR, F.S., and HSIUNG, G.D. (1963) Yale J. Biol. Med. 35, 523.

WALLACE, R.E. (1964) Proc. Soc. exp. Biol. Med. 116, 990.

WIGAND, R., and SABIN, A.B. (1961) Arch. ges. Virusforsch. 11, 224.

WULFF, H., KIDD, P., and WENNER, H.A. (1964a) Proc. Soc. exp. Biol. Med. 115, 240.

WULFF, H., KIDD, P., and WENNER, H.A. (1964b) Proc. Soc. exp. Biol. Med. 115, 458.

WULFF, H., KIDD, P., and WENNER, H.A. (1964c) Pediatrics, Springfield 33, 30.

YABROV, A.A., GOLUBEV, D.B., and SMORODINTSEV, A.A. (1964) Acta virol., Prague 8, 562.

YOSHINO, K., and TANIGUCHI, S. (1964) Virology 22, 193.

YOSHINO, K., and TANIGUCHI, S. (1966) J. Immun. 96, 196.

APPLICATIONS of TISSUE CULTURE to the STUDY of the EPIDEMIOLOGY of RESPIRATORY VIRUSES

A Thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy

by

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VOLUME II
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CHAPTER 2

MATERIALS AND METHODS

Tables

TABLE 2.1

COMPONENTS OF SIMPLE SALT SOLUTIONS AND MEDIA
(mg/litre)

COMPONENTS	PBS	BSS	LH	TPB
Salts				
$CaCl_2$	-	140	140	-
$MgCl_2 \cdot 6H_2O$	-	100	100	-
$MgSO_4 \cdot 7H_2O$	-	100	100	-
KCl	200	400	400	-
KH_2PO_4	200	60	60	-
NaCl	8,000	8,000	8,000	5,000
Na_2HPO_4	1,150	-	-	2,500
$Na_2HPO_4 \cdot 12H_2O$	-	154	154	-
$NaHCO_3$	-	V	V	-
Miscellaneous				
Tryptose	-	-	-	20,000
Phenol red	20	20	20	-
Dextrose	-	1,120	1,120	5,000
Lactalbumen hydrolysate	-	-	5,000	-

V = Variable

TABLE 2.2

COMPONENTS OF EAGLE'S MEDIA AND OF PARKER'S MEDIUM 199 (mg/litre)

(Continued in Table 2.3)

COMPONENTS	MEM	BM	199
Salts			
$CaCl_2$	200	-	140
$CaCl_2 \cdot 2H_2O$	-	264	-
$MgSO_4 \cdot 7H_2O$	200	200	200
KCl	400	400	400
NaCl	6,800	6,800	6,800
$NaH_2PO_4 \cdot H_2O$	-	140	-
$NaH_2PO_4 \cdot 2H_2O$	150	-	158
$NaHCO_3$	V	V	V
Miscellaneous			
Phenol red	10	17	10
Dextrose	1,000	1,000	1,000
Amino Acids			
L-arginine.HCl	125	21.1	70
L-cystine	24.0	12.0	20
L-histidine.HCl	40	9.6	20
L-isoleucine	-	26.2	-
DL- "	104	-	40
L-leucine	-	26.2	-
DL- "	104	-	120
L-lysine HCl	70	36.5	70
L-methionine	-	7.5	-
DL- "	30	-	30
L-phenylalanine	-	16.5	-
DL- "	64	-	50

TABLE 2.2 (Continued)

COMPONENTS	MEM	BM	199
L-threonine	-	23.8	-
DL- "	96	-	60
L-tryptophane	-	4.1	-
DL- "	20	-	20
L-tyrosine	36	18.1	40
L-valine	-	23.4	-
DL-valine	92	-	50
L-glutamine	292	292	50
Vitamins			
Thiamine HCl	-	-	0.01
Aneurine HCl	1	1	-
Biotin	-	1	0.01
Choline Chloride	1	1	0.50
Folic Acid	1	1	0.01
Inositol	2	2	0.05
Nicotinamide	1	1	0.025
D-calcium pantothanate	1	1	0.01
Pyridoxal H C l	1	1	0.025
Riboflavine	0.1	0.1	0.01

V = Variable

TABLE 2.3

ADDITIONAL COMPONENTS OF PARKER'S MEDIUM 199
(mg/litre) (NOT LISTED IN TABLE 2.2)

COMPONENTS	199
Amino Acids	
DL-alanine	50
DL-aspartic acid	60
L-cysteine HCl	0.1
DL-glutamic acid	150
Glutathione	0.05
Glycine	50
L-hydroxyproline	10
L-proline	40
DL-serine	50
Vitamins	
Ascorbic acid	0.05
Calciferol	0.10
Menaphthene	0.01
Nicotinic acid	0.025
p-aminobenzoic acid	0.05
pyridoxine HCl	0.025
Disodium-α- tocopherol phosphate	0.01
Vitamin A acetate	0.10
Miscellaneous	
Sodium acetate	81.5
D-ribose	0.50
Adenylic acid	0.20
Adenine sulphate	10.0
Guanine HCl	0.30
hypoxanthine	0.30
thymine	0.30
uracil	0.30
xanthine	0.30
sodium adenosine triphosphate	1.04
cholesterol	0.20
tween 80	5.0
$Fe(NO_3)_3 \cdot 9H_2O$	0.72

TABLE 2.4

MAINTENANCE OF TISSUE CULTURES FOR RESPIRATORY VIRUS ISOLATION JANUARY - AUGUST, 1962

Specimen	Tissue	Medium		Conditions
Nasal and Sputum	Primary or secondary RMK	LH	96 ml	Rolled, 33°C
		Calf serum	2 ml	
		$NaHCO_3$ solution	1 ml	
		PSS	1 ml	
	Primary HEK	as above		as above
Throat and Sputum	Primary or secondary RMK	Parker's Medium 199	98 ml	Rolled, 33°C
		$NaHCO_3$ solution	2 ml	
	BH	Eagle's MEM	93 ml	Stationary, 36°C
		Fowl serum	2 ml	
		$NaHCO_3$ solution	4 ml	
		PSS	1 ml	

TABLE 2.5

MAINTENANCE OF TISSUE CULTURES FOR RESPIRATORY VIRUS ISOLATION AND GROWTH

SEPTEMBER 1962 - APRIL 1966*

Tissue	Medium		Conditions
Primary or Secondary RMK	Parkers Medium 199	98 ml	Rolled, 33°C
	$NaHCO_3$ solution	2 ml	
BH	Eagle's MEM	93 ml	Stationary, 36°C
	Fowl or Rabbit Serum	2 ml	
	$NaHCO_3$ solution	4 ml	
	PSS	1 ml	
HEL or HEKF	Eagle's MEM	91 ml	Rolled, 33°C
	Calf serum	1 ml	
	$NaHCO_3$ solution	2 ml	
	TPB	5 ml	
	PSS	1 ml	

* Between September, 1962 and September 1963 throat swabs were inoculated into RMK and BH cells, nasal washings into HEL or HEKF cells and sputa into all three cell types. From September 1963 all specimens were inoculated into each of the three cell types.

TABLE 2.6

HAEMAGGLUTININ TITRES OF PARAINFLUENZA TYPES 1, 2 and 3

WITH VARIOUS ERYTHROCYTE SPECIES AT DIFFERENT TEMPERATURES

Virus Type	Erythrocyte Species	Temperatures (°C)		
		4	18	36
1	Fowl	2	0	0
	Guineapig	8	32	32
	Human	2	16	16
2	Fowl	N	0	0
	Guineapig	2	8	8
	Human	N	N	N
3	Fowl	0	0	0
	Guineapig	4	8	16
	Human	N	2	8

N = Haemagglutinated only when undiluted

CHAPTER 3

PREPARATION AND PRESERVATION OF HUMAN EMBRYONIC TISSUE CULTURES

Tables

TABLE 3.1

RESULTS OF CULTIVATION OF TISSUES FROM 36 HUMAN EMBRYOS

Embryo	Age (weeks) and sex	Source	Trypsinisation time (hours)*	Organ	Termination of Serial Cultivation	
					Passage	Cause
1	14 F	G	2	K	8	D
				L	10	D
2	14 M	G	3	K	0	NG
				L	0	NG
3	22 M	I	16	K	0	NG
4	10 M	I	16	K	6	D
				L	10	D
5	20 M	I	16	K	4	D
				L	8	D
6	12 F	I	16	K	26	D
				L	30	D
7	16 M	I	16	K	22	F
				L	30	D
8	16 M	G[I]	16	K	0	C
				L	0	C
9	16 M	G[I]	16	K	0	C
				L	0	C
10	12 M	I	3	L	9	D
11	16 M	I	4	K	4	D
				L	10	D
12	22 F	G	16	K	8	D
				L	13	D
13	10 M	I	16	K	40	D
14	12 M	G	16	K	24	F
				L	56	D
15	20 M	I	16	K	13	F
				L	12	F

TABLE 3.1 (Continued)

RESULTS OF CULTIVATION OF TISSUES FROM 36 HUMAN EMBRYOS

Embryo	Age (weeks) and sex	Source	Trypsinisation time (hours)*	Organ	Termination of Serial Cultivation	
					Passage	Cause
16	20 M	G	16	K	11	F
				L	12	F
17	8 M	I	16	K	14	F
18	24 F	G†	16	K	0	NG
				L	0	NG
19	14 M	G	16	K	0	NG
				L	0	NG
20	10 M	I	16	L	3	F
21	12 M	I	16	L	4	F
22	20 M	G	16	K	7	D
				L	4	F
23	14 M	I	16	K	9	F
				L	4	F
24	10 M	I	3	K	8	F
				L	4	F
25	16 F	G	16	K	9	F
				L	4	F
26	16 M	I	3	K	9	F
				L	4	F
27	14 M	I	16	K	16	F
				L	4	F
28	15 M	I	3	K	8	D
				L	3	F
29	10 M	I	2.5	K	36	D
				L	3	F
30	16 M	I	2	K	36	D
				L	3	F
31	12 M	I	2.5	K	20	D
				L	4	F
32	10 M	I	2	K	16	D
				L	4	F

TABLE 3.1 (Continued)

RESULTS OF CULTIVATION OF TISSUES FROM 36 HUMAN EMBRYOS

Embryo	Age (weeks) and sex	Source	Trypsinisation time (hours)*	Organ	Termination of Serial Cultivation	
					Passage	Cause
33	16 M	I	2	K	18	D
				L	4	F
34	20 F	I	2	K	15	F
35	20 M	I	2	K	40	D
				L	46	D
36	14 M	I	16	K	30	D
				L	20	D

‡ Fetus held at 4°C overnight before arrival at the laboratory

* 16 hours' trypsinisation was done at 4°C; other times at 37°C

I Inverness, G = Glasgow, K = Kidney, L = Lung

NG = no growth in primary cultures, C = primary cultures contaminated

D = degeneration of cells, F = cells frozen at -70°C.

TABLE 3.2

COMPARISON OF THREE METHODS OF FREEZING HUMAN EMBRYONIC LUNG CELLS

		Freezing Method					
		1		2		3	
		V	M	V	M	V	M
Experiment 1*							
Ampoule	1	23	<10	34	10	33	20
	2	19	<10	17	20	22	20
	3	19	<10	13	<10	18	<10
Experiment 2+							
Ampoule	1	45	<10	50	20	62	20
	2	23	<10	36	20	46	20
	3	30	10	48	20	70	40

* Ampoules thawed 1, 2 and 16 months after freezing.

\+ Ampoules thawed 5 months after freezing.

<10 = Cells failed to form monlayer at 1/10 dilution.

V = Cells not stained by trypan blue as percentage of total cells counted.

M = Reciprocal of highest dilution of cell suspension which formed a monolayer in 7 days.

TABLE 3.3

COMPARISON OF GLYCEROL AND DMS IN THE PREVENTION OF FREEZING DAMAGE TO HUMAN EMBRYONIC LUNG CELLS

		GLYCEROL		DMS	
		V	M	V	M
Experiment 1*					
Ampoule	1	49	40	57	80
	2	56	40	55	40
	3	56	40	69	40
	4	63	80	76	40
Experiment 2+					
Ampoule	1	62	40	72	20
	2	63	20	60	20

* Ampoules thawed 5 months after freezing.

\+ Ampoules thawed 15 months after freezing.

Other abbreviations as in Table 3.2.

CHAPTER 4

STUDIES WITH RHINOVIRUSES. 1

Tables

TABLE 4.1

ISOLATION OF RHINOVIRUSES FROM PATIENTS STUDIED DURING THREE PERIODS.

Period	Cells used	Patients studied	Rhinoviruses isolated	Rhinoviruses isolated per cent
Jan 1962 - Mar 1963	Primary HEK RHEL-1 RHEL-7	171 *	4	3.4 I
Apr 1963 - Sep 1964	RHEKF-13 WI-38	206	14	6.8
Oct 1964 - Apr 1966	RHEKF-30 RHEKF-35 BWHEL-7 WI-38	423	55	13.0
Jan. 1962 - Apr 1966		800	73	9.1 I

* Specimens from 54 of these patients were not inoculated into any human embryonic cells either because no tissue was available (15 patients) or because swabs were inoculated directly into BH cultures only (39 patients).

I Exclusing 54 patients whose specimens were not examined in human embryonic cells.

TABLE 4.2

TITES OF RHINOVIRUS FEB IN SIX CELL STRAINS

Cell Strain	Passage	Titre*
RHEL-7	14	3.2
RHEKF-13	26	4.8
RHEKF-14	14	3.5
RHEL-14	10	3.2
RHEL-15	4	2.5
WI-38	27	4.2

* Log TCD_{50}/0.1 ml

TABLE 4.3

RESULTS OF RE-TESTING SPECIMENS FROM 131 PATIENTS

Cells used in original test	Re-test in RHEKF-30 Patients	RH	Re-test in WI-38 Patients	RH	Total Re-tested Patients	RH
None*	15	4	9	3	15	5
Primary HEK	5	0	5	0	5	0
RHEL-1	8	1	8	1	8	1
RHEL-7	83	11	26	6	83	14
WI-38	20	2	20	2	20	2
TOTAL	131	18	68	12	131	22

RH = rhinoviruses isolated

* Specimens from these 15 patients had not previously been tested in human embryonic cells because no tissue was available.

TABLE 4.4

RHINOVIRUS ISOLATIONS FROM 298 SPECIMENS EXAMINED IN HEKF AND HEL CELL STRAINS

Cell Strains	Specimens Tested	Specimens Yielding Rhinovirus			
		WI-38	RHEKF-30	RHEKF-35	Total
WI-38 + RHEKF-30	167	19	28	....	30
WI-38 + RHEKF-35	131	10	....	12	13
Total	298	29	40		43

TABLE 4.5

EFFECT OF TRYPTOSE PHOSPHATE BROTH
ON APPEARANCE OF RHINOVIRUS FEB MICROPLAQUES

Time (hours)	Average microplaque count per tube	
	with 5% TPB	without TPB
24	0	0
48	3.5×10^2	2×10^1
72	1.3×10^3	9.3×10^1
96	5.7×10^3	3×10^2
$Log_{10}TCD_{50}$ at 7 days	3.8	3.8

TABLE 4.6

EFFECT OF RHINOVIRUS CONCENTRATION ON NEUTRALIZING ANTIBODY TITRES OF PAIRED SERA

Patient's Age	Serum	Virus dose*	Antibody titre	Virus dose*	Antibody titre
10 M	Acute	10	<8	68	<4
	Conv.		32		8
16 M	Acute	32	<4	180	<4
	Conv.		8		<4
3 Y	Acute	10	<8	180	<8
	Conv.		45		11
3.5 Y	Acute	18	<4	100	<4
	Conv.		16		<4
29 Y	Acute	10	<4	180	<4
	Conv.		128		32

* TCD_{50}/0.1 ml

M = months

Y = years

TABLE 4.7

EFFECT OF UNHEATED RABBIT SERUM ON NEUTRALIZING ANTIBODY TITRES

Patient's Age	Serum	PBS Virus dose *	PBS Antibody Titre	URS Virus dose *	URS Antibody Titre
3 M	Acute	68	< 4	32	< 8
	Conv.		23		45
14 M	Acute	18	< 8	18	< 8
	Conv.		23		32
16 M	Acute	32	< 8	10	< 8
	Conv.		8		16
5 Y	Acute	56	< 4	56	< 8
	Conv.		< 4		< 8
29 Y	Acute	145	< 8	32	< 8
	Conv.		32		45

* TCD_{50}/0.1 ml

M = months

Y = years

TABLE 4.8

ANTIBODY TITRES IN GUINEA PIGS AFTER IMMUNIZATION WITH THREE RHINOVIRUSES BY THREE DIFFERENT METHODS.

Virus Inoculated		Method	Antibody Titres		Virus dose I	Typing Dilution**
Strain	Titre*		PR	PO		
4574-62 C	4.8	1	<8	32	68	-
T	5.5	2	<8	256		8
T	5.5	3+	<8	32		-
1647-63 C	2.8	1	<8	32	100	-
T	2.5	2	<8	23		-
T	2.5	3	<8	360		16
4462-63 C	3.5	1	<8	<16	68	-
T	4.5	2	<8	64		-
T	4.5	3	<8	720		16

*Log TCD_{50}/0.1 ml

I TCD_{50}/0.1 ml

** Dilution at which antiserum was used for the identification of unknown rhinovirus serotypes. - = not used for rhinovirus identification.

+ Immunization schedule incomplete. Second injection omitted because of injury to animal, third injection was 3 ml virus intraperitoneally.

C = virus concentrated by $AlPO_4$

PR = pre-immunization serum

PO = post-immunization serum

T = tissue culture fluid which had not been concentrated

TABLE 4.9

ANTIBODY RESPONSES OF GUINEA PIGS INOCULATED WITH 12 RHINOVIRUSES

Virus Inoculated			Antibody Titres			
Strain	Titre*	GP	PR	PO	Virus dose I	Typing Dilution**
409-62	4.5	1	<8	256	32	-
		2	<8	720		16
1321-62	4.0	1	<8	512	68	-
		2	<8	512		16
1746-62	3.2	1	<8	720	32	16
		2	<8	512		-
4704-62	4.0	1	<8	360	32	8
		2	<8	360		-
130-63	4.2	1	<8	720	32	-
		2	<8	1024		32
992-63	4.2	1	<8	720	32	16
		2	<8	256		-
1833-63	5.5	1	<8	256	100	-
		2	<8	512		16
1376-64	4.2	1	<8	360	68	16
		2	<8	360		-
1192-65	4.5	1	<8	1024	32	32
		2	<8	512		-
1767-65	5.2	1	<8	512	100	16
		2	<8	256		-
2030-65	4.5	1	<8	360	320	16
		2	<8	180		-
4411-65	4.5	1	<8	256	180	8
		2	<8	<32		-

* Log TCD_{50}/0.1 ml

I TCD_{50}/0.1 ml

** Dilution at which antiserum was used for the idenficiation of unknown rhinovirus serotypes. - = not used for rhinovirus identification.

GP = number of guinea pig used.

PR = pre-immunization serum

PO = post-immunization serum.

TABLE 4.10

CROSS-NEUTRALIZATION TESTS WITH 15 RHINOVIRUS STRAINS *

(Antiserum titres expressed as reciprocal of dilution causing 50% neutralization)

Virus	Antisera														
	409	1321	1746	4574	4704	130	992	1647	1833	4462	1376	1192	1767	2030	4411
409-62	720	-	256	-	-	-	-	-	-	-	-	-	-	-	-
1321-62	-	512	-	-	-	-	-	-	-	-	-	-	-	-	1024
1746-62	1024	-	720	-	-	-	-	-	-	-	-	-	-	-	-
4574-62	-	-	-	256	-	-	-	-	-	-	-	-	128	-	-
4704-62	-	-	-	-	360	-	-	-	-	-	-	-	-	-	-
130-63	-	-	-	-	-	1024	512	-	-	-	-	-	-	-	-
992-63	-	-	-	-	-	512	720	-	-	-	-	-	-	-	-
1647-63	-	-	-	-	-	-	-	360	-	-	-	-	-	-	-
1833-63	-	-	-	-	-	-	-	-	512	-	-	-	-	-	-
4462-63	-	-	-	-	-	-	-	-	-	720	-	-	-	-	-
1376-64	-	-	-	-	-	-	-	-	-	-	360	-	-	-	-
1192-65	-	-	-	-	-	-	-	-	-	-	-	1024	-	-	-
1767-65	-	-	-	8	-	-	-	-	-	-	-	-	512	-	-
2030-65	-	-	-	-	-	-	-	-	-	-	-	-	-	360	-
4411-65	-	-	-	-	-	-	-	-	-	-	-	-	-	-	256

* Virus dose was 32-320 TCD_{50}/0.1 ml

- = virus not neutralized by antiserum at 'typing dilution' (see Tables 4.8 and 4.9)

CHAPTER 5

STUDIES WITH RHINOVIRUSES. 2.

Tables and Figures.

TABLE 5.1

CHARACTERISTICS OF EIGHT HUMAN EMBRYO CELL STRAINS

Strain	Embryo Age (Wks)	Embryo Sex	Showing Fibroblasts	Frozen Stock	Passage Level Sensitivity Tests	Mycoplasma Tests	Showing "Senescene"
RHEKF-30	16	M	7	9	23 - 32	29, 30	36
RHEL-30	16	M	1	3	8 - 22	16, 17	25+*
RHEKF-33	16	M	9	10	14 - 29	21, 23	31
RHEL-33	16	M	1	3	6 - 21	13, 16	23+*
RHEKF-35	20	M	6	8	19 - 31	25, 29	40
RHEL-35	20	M	1	8	16 - 25	18, 26	46
RHEL-36	14	M	1	6	10 - 24	18, 22	28
WI-38	NK	F	1	NFS	19 - 37	20, 34	40

* Senescence not showing at this passage. Cell strain was not cultured further

NK = Not known

NFS = No frozen stock.

TABLE 5.2

RESULTS OF RETESTING IN EIGHT CELL STRAINS
60 SPECIMENS PREVIOUSLY TESTED FOR RHINOVIRUSES.

Strain	No. Specimens Positive		Total	Per Cent*
	Previously Positive	Previously Negative		
RHEKF-30	26	4	30	79
RHEL-30	13	2	15	39
RHEKF-33	20	4	24	63
RHEL-33	12	3	15	39
RHEKF-35	22	4	26	68
RHEL-35	7	0	7	18
RHEL-36	11	2	13	34
WI-38	19	7	26	68
Total positive in at least one cell strain	31	7	38	100
Total Tested	36	24	60	-

* Total specimens positive in the cell strain expressed as percentage of 38, which is the total specimens positive in at least one of the eight cell strains.

TABLE 5.3

TITRES OF RHINOVIRUSES IN THREE CLINICAL SPECIMENS ASSAYED IN EIGHT CELL STRAINS.

Strain	Specimen number and type		
	1833-63 Sputum	3131-64 T+N/W *	1254-65 Sputum
RHEKF-30	3.5	2.8	2.8
RHEKF-33	2.5	1.2	2.8
RHEKF-35	2.5	1.8	2.8
RHEL-30	2.5	1.2	2.2
RHEL-33	2.2	1.2	1.5
RHEL-35	0.8	< 0.5 I	< 0.5 I
RHEL-36	1.8	1.2	1.8
WI-38	2.8	0.5	< 0.5 I
Virus Serotype	1833-63	1833-63	68

* Throat swab and nasal washing

I No virus detected when specimen inoculated undiluted.

TABLE 5.4

TITRES OF VIRUS POOLS OF SIX PROTOTYPE RHINOVIRUS SEROTYPES IN EIGHT CELL STRAINS.

Virus Pool Serotype	A FEB	A FEB	B 181	C 5986	D 1059	E 11757	F 11757	G 363
RHEKF-30	4.8	4.8	4.8	4.8	4.2	4.8	4.8	3.5
RHEKF-33	4.8	4.8	3.5	4.5	3.5	3.8	4.2	3.8
RHEKF-35	4.8	4.8	5.2	4.8	3.5	4.5	4.5	4.5
RHEL-30	4.5	4.8	3.5	4.8	3.8	4.8	4.5	4.2
RHEL-33	4.5	4.8	3.8	3.8	3.8	4.5	4.2	3.5
RHEL-35	4.5	4.2	4.5	3.5	3.8	4.2	4.8	2.5
RHEL-36	4.5	4.8	4.5	4.8	3.5	5.2	4.2	3.2
WI-38	4.5	4.8	4.2	4.5	4.5	4.8	4.2	4.5
HEKF Mean Titre	4.8	4.8	4.5	4.7	3.7	4.4	4.5	3.9
HEL Mean Titre *	4.5	4.7	4.2	4.4	3.9	4.7	4.4	3.6
Difference between means	0.3	0.1	0.3	0.3	-0.2	-0.3	0.1	0.3

* Excluding RHEL-33. See text for explanation.

TABLE 5.5

TITRES OF VIRUS POOLS OF EIGHT RUCHILL RHINOVIRUS SEROTYPES IN EIGHT CELL STRAINS.

Virus Pool Serotype	H 409-62	I 1321-62	J 130-63	K 4462-63	L 1192-65	M 1767-65	N 4411-65	0 UT
RHEKF-30	4.8	4.5	4.2	4.2	3.8	4.8	4.8	2.8
RHEKF-33	3.8	5.5	3.2	3.2	3.8	5.2	4.5	3.8
RHEKF-35	4.2	5.5	3.8	4.2	4.2	5.5	4.5	4.5
RHEL-30	4.2	5.5	3.2	3.5	4.2	5.2	4.5	3.2
RHEL-33	C	5.2	2.8	C	4.2	5.2	4.5	2.5
RHEL-35	3.5	5.2	3.5	2.5	4.5	4.8	4.5	3.8
RHEL-36	3.5	5.2	1.8	3.5	3.2	5.2	3.5	3.5
WI-38	4.8	5.5	4.5	3.5	4.5	5.2	4.5	3.5
HEKF Mean Titre	4.3	5.2	3.7	3.9	3.9	5.2	4.6	3.7
HEL Mean Titre*	4.0	5.3	3.2	3.3	4.1	5.1	4.3	3.5
Difference between means	0.3	-0.1	0.5	0.6	-0.2	0.1	0.3	0.2

C = No titration because cell strain contaminated with bacteria

* Excluding RHEL-33. See text for explanation.

TABLE 5.6

TITRES OF VIRUS POOLS OF THREE RUCHILL RHINOVIRUS SEROTYPES IN EIGHT CELL STRAINS

Virus Pool Serotypes	P 1647-63	Q 1376-64	R 2030-65	S 2030-65	T 2030-65
RHEKF-30	4.8	4.5	5.2	4.8	4.5
RHEKF-33	3.8	3.8	3.8	4.5	4.5
RHEKF-35	4.2	3.5	4.2	4.8	3.8
RHEL-30	3.5	2.2	2.5	3.2	2.5
RHEL-33	C	C	2.2	2.8	1.5
RHEL-35	1.5	0.8	3.2	2.8	3.5
RHEL-36	2.5	1.8	1.5	2.5	2.2
WI-38	4.2	3.5	3.2	3.5	2.8
HEKF Mean Titre	4.3	3.9	4.4	4.7	4.3
HEL Mean Titre *	2.9	2.1	2.6	3.0	2.7
Difference between Means	1.4	1.8	1.8	1.7	1.6

C = No titrations because cell strain contamined with bacteria.

* Excluding RHEL-33. See text for explanation.

TABLE 5.7

MEAN TITRES OF TITRATIONS OF N AND K VIRUSES IN EIGHT CELL STRAINS.

Cell Strain	Titrations of N virus pools	Titrations of K virus pools
RHEKF-30	4.39 ± 0.17	4.76 ± 0.13
RHEL-30	4.34 ± 0.19	2.78 ± 0.24
RHEKF-33	4.21 ± 0.19	4.08 ± 0.11
RHEL-33	4.09 ± 0.21	ND
RHEKF-35	4.61 ± 0.15	4.10 ± 0.22
RHEL-35	4.16 ± 0.19	2.36 ± 0.52
RHEL-36	4.08 ± 0.28	2.10 ± 0.17
WI-38	4.55 ± 0.12	3.44 ± 0.23

ND = Not calculated because two titrations were bacterially contaminated.

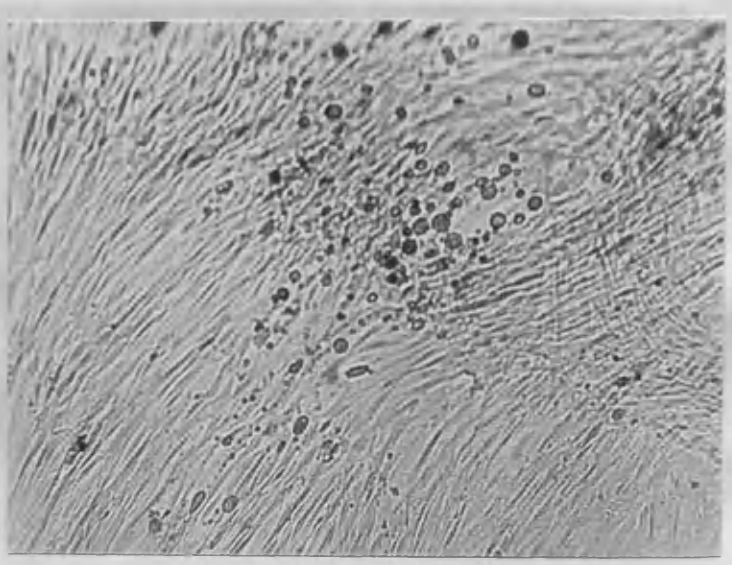

Fig 5.1 Microplaque in RHEKF-30 cells 72 hours after inoculation with rhinovirus FEB. x 100

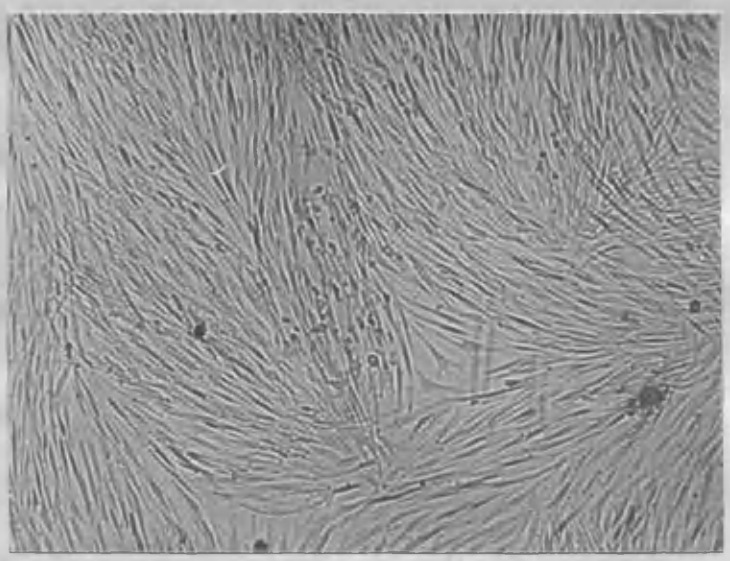

Fig. 5.2 Microplaque in RHEL-30 cells 72 hours after inoculation with rhinovirus FEB. x 100

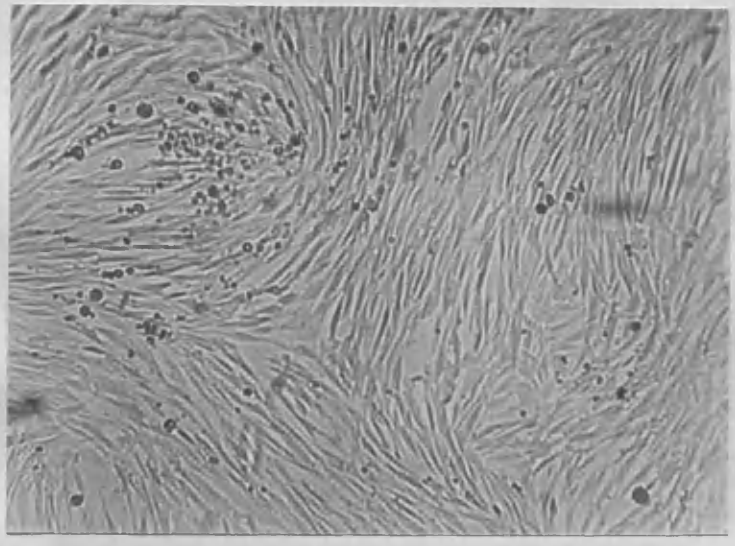

Fig 5.3 Microplaque in RHEKF-33 cells 72 hours after inoculation with rhinovirus FEB. x 100

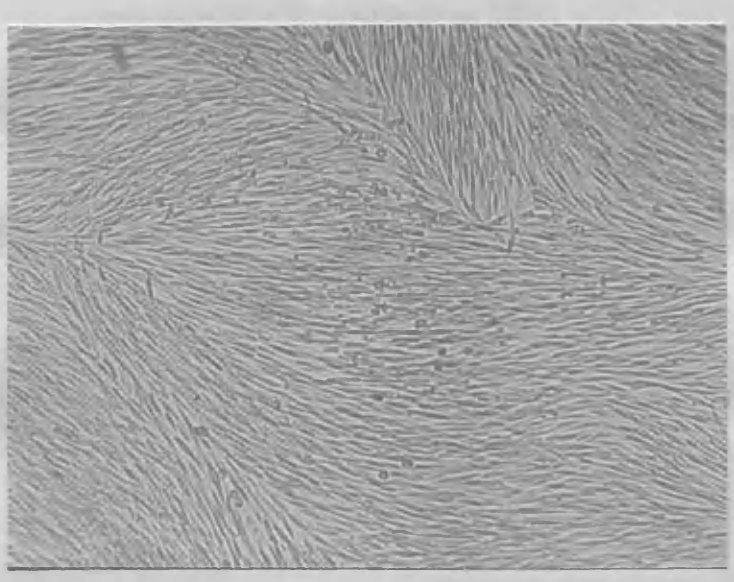

Fig 5.4 Microplaque in RHEL-33 cells 72 hours after inoculation with rhinovirus FEB. x 100

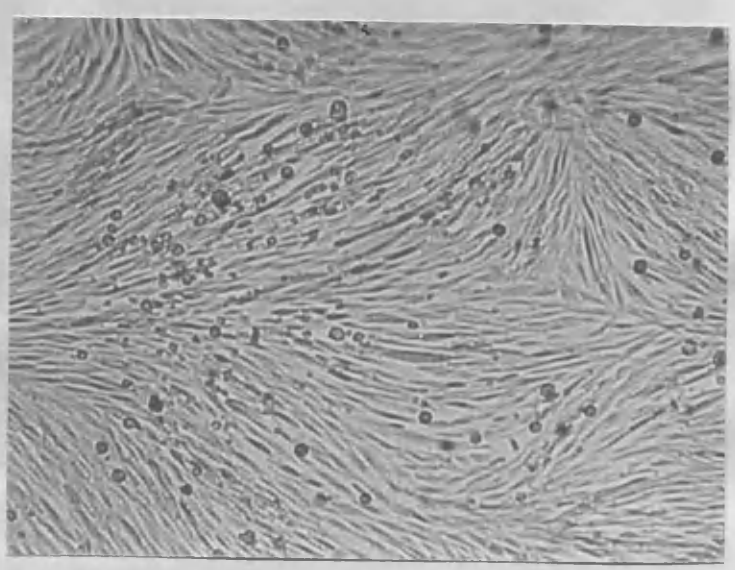

Fig 5.5 Microplaque in RHEKF-35 cells 72 hours after inoculation with rhinovirus FEB. x 100

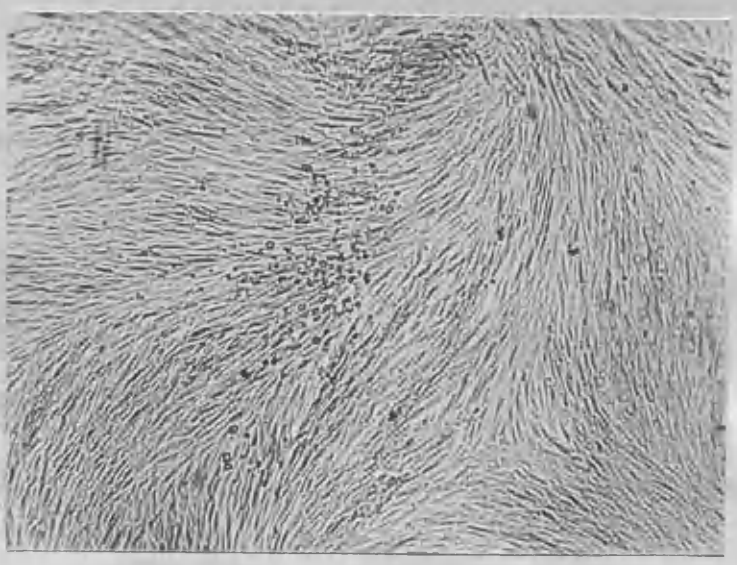

Fig 5.6 Microplaque in RHEL-35 cells 72 hours after inoculation with rhinovirus FEB. x 100

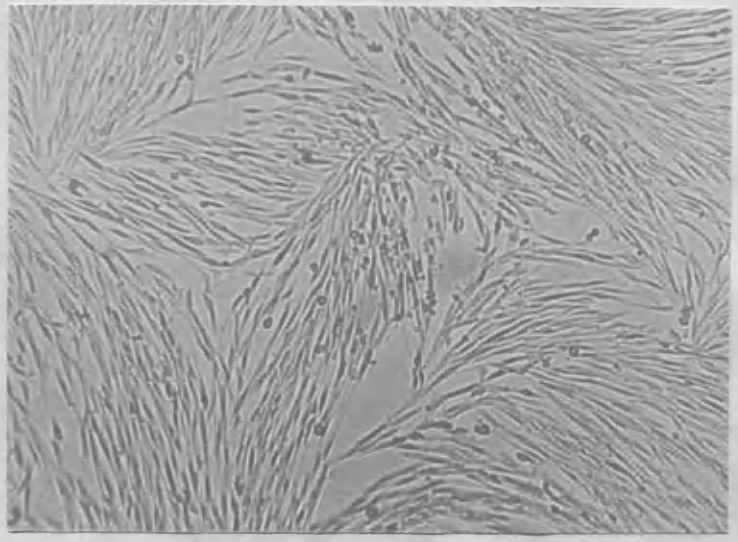

Fig 5.7 Microplaque in RHEL-36 cells 72 hours after inoculation with rhinovirus FEB. x 100

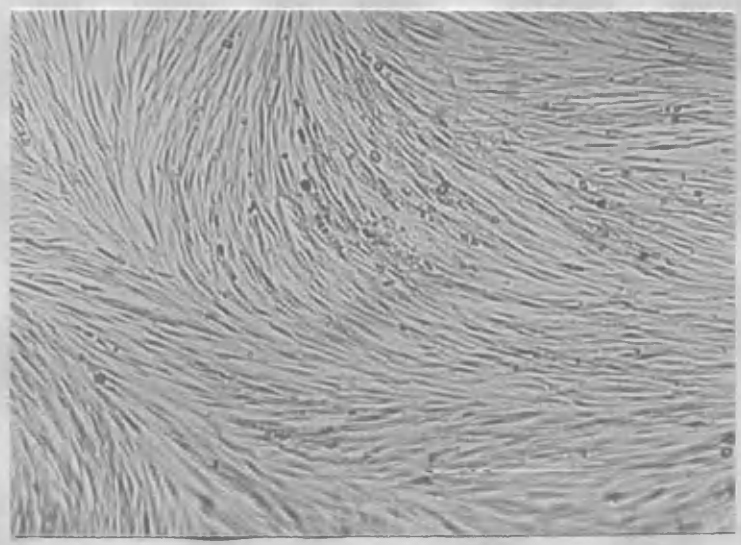

Fig 5.8 Microplaque in WI-38 cells 72 hours after inoculation with rhinovirus FEB. x 100

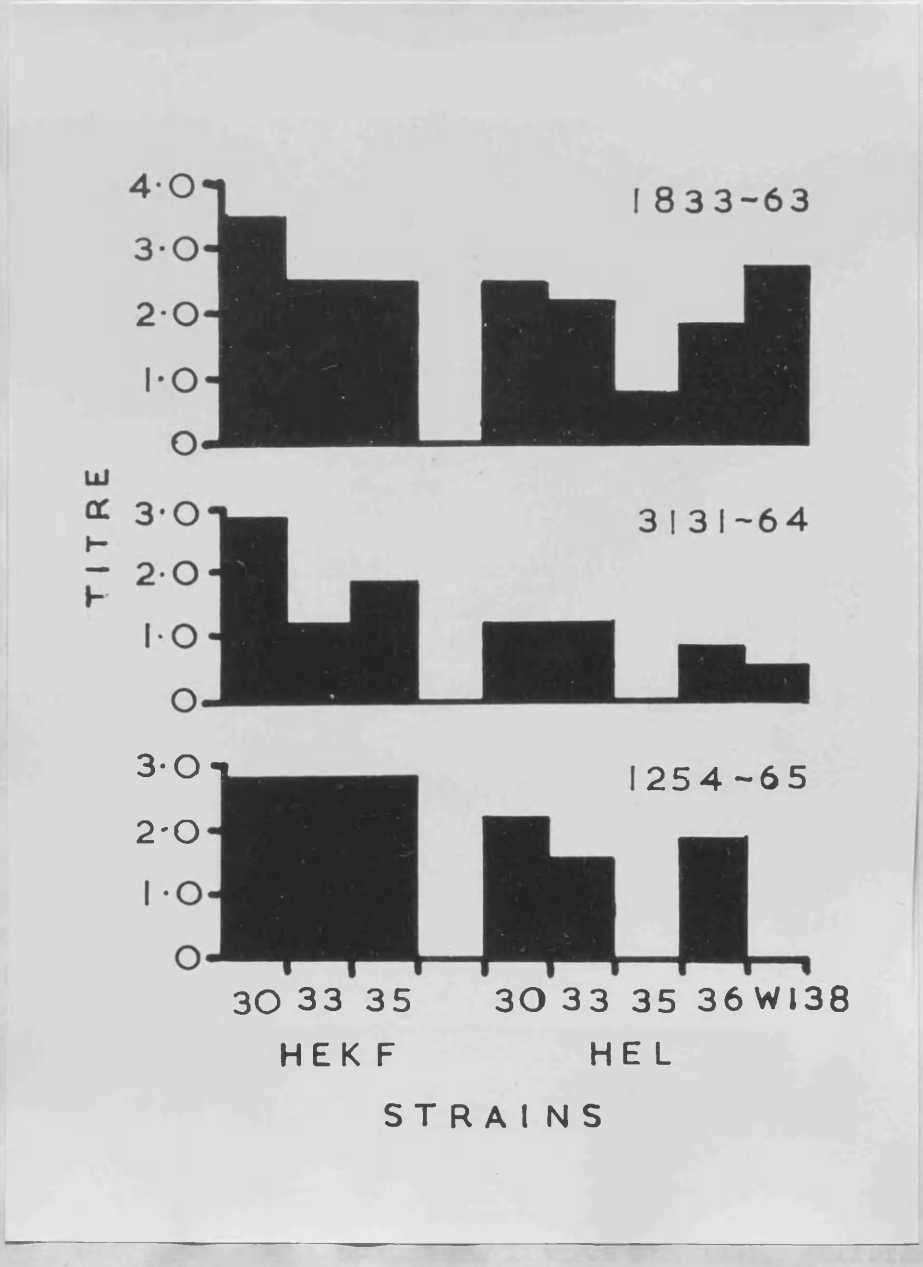

Fig 5.9 Titres of rhinoviruses in three clinical specimens assayed in eight cell strains. Titres expressed as Log TCD_{50}/0.1 ml

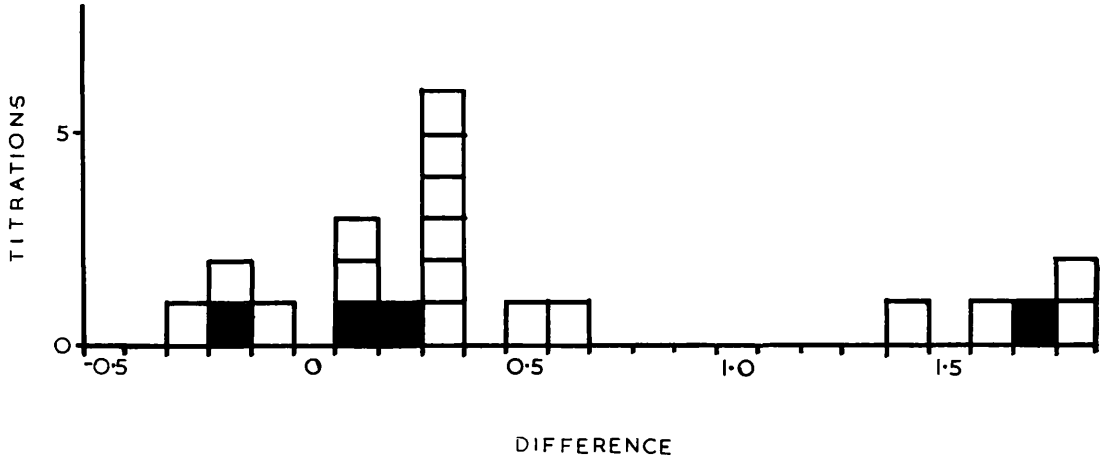

Fig 5.10 Differences between mean titre in three HEKF strains and mean titre in four HEL strains for 21 virus titrations. Differences calculated by subtracting mean titre in HEL strains from mean titre in HEKF strains. ■ Virus passed at least once in HEL cells immediately before titration. □ Virus passed in HEKF before titration.

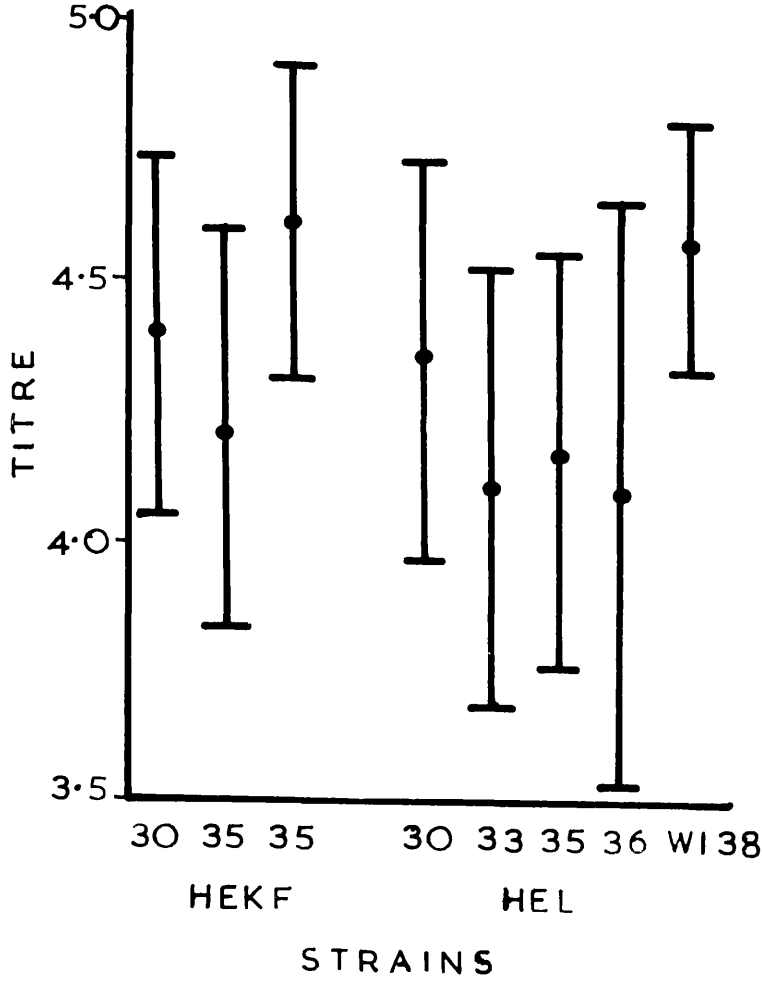

Fig 5.11 Mean titres of 14 titrations of N (normal) rhinoviruses in eight cell strains, showing two standard deviations of the means.

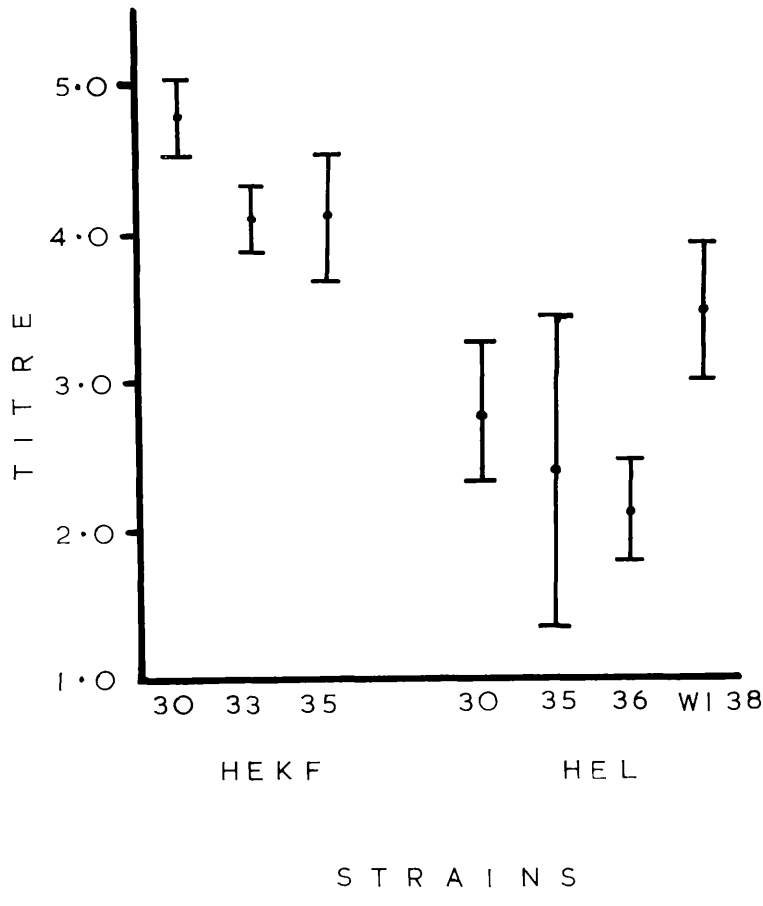

Fig 5.12 Mean titres of five titrations of K (kidney) rhinoviruses in seven cell strains, showing two standard deviations of the means.

CHAPTER 6

STUDIES WITH RESPIRATORY SYNCYTIAL VIRUS

Tables and Figure

TABLE 6.1

EFFECTS OF CENTRIFUGATION AND ANTIBIOTICS ON MICROBIAL CONTAMINANTS

Inoculum	Specimens inoculated	Tubes inoculated	Tubes contaminated *	% contaminated *
Supernate)	40	240	1	0.4
Deposit)		120	24	20.0
Supernate)	40	240	13	5.4
Deposit + PSN)		120	7	5.8

* Within 3 days of inoculation (i.e. before change of medium)

PSN = 100 units penicillin, 100 µg streptomycin and 50 units nystatin

TABLE 6.2

TITRES OF RS VIRUS IN BH CELLS AFTER VARIOUS PERIODS IN CONTINUOUS CULTURE

Thawing	Weeks in culture after thawing	Titre*
Experiment 1	53	2.1
	4	3.5
Experiment 2	55	3.1
	6	4.7
	55 + A	3.3
	6 + A	4.5
Experiment 3	16 + A	2.9
	2 + A	3.9

* Expressed at Log TCD_{50}/0.1 ml. Five tubes were inoculated with each dilution of virus.

A = grown in presence of 50 µg/ml of aureomycin

TABLE 6.3

ISOLATION OF RS VIRUS IN FOUR TYPES OF TISSUE CULTURES

Winter	Cases Tested	Cases yielding RS Virus BH	RMK	HEKF	WI-38	Total
1963-64	110	11	9	4	...	14
1964-65	232	33	22	9	...	38
1965-66	134	21	7	0	4	22
Total	476	65	38	13	(4)	74

TABLE 6.4

EFFECTS OF VIRUS DOSE AND DURATION OF INCUBATION ON RS VIRUS NEUTRALIZING ANTIBODY TITRES

	4 Days *			7 Days *				
	Virus dose ‡			Virus dose ‡				
Serum	320	32	3.2	32,000	3,200	320	32	3.2
1 Acute	<16	<16	<16	<16	<16	<16	<16	<16
Conv	16	64	256	<16	<16	<16	16	64
2 Acute	<8	<8	<8	<8	<8	<8	<8	<8
Conv	32	128	1024	<8	8	8	32	128
3 Acute	...	<16	<16	...	<16	<16	<16	<16
Conv	...	16	128	...	<16	<16	32	256
4 Acute	...	<16	32	...	<16	<16	<16	<16
Conv	...	32	256	...	<16	<16	16	16

* Duration of incubation of test before reading

‡ TCD_{50}/0.1 ml

TABLE 6.5

ANTIBODY TITRES OF PATIENTS' SERA AGAINST TWO STRAINS OF RS VIRUS

Patient's Age (Months)	Complement Fixation Randall		Neutralization Randall (100 TCD_{50})*		Neutralization 329-63 (68 TCD_{50})*	
	Acute	Conv	Acute	Conv	Acute	Conv
10	<4	32	<8	<16	<8	64
5	<8	32	<8	16	<8	45
13	<4	64	<8	23	<8	90
16	<4	128	<8	23	<8	45
12	<4	128	<8	45	<8	180
15	<4	256	<8	32	<8	32
5	<4	256	<8	16	<8	45

* Virus dose

TABLE 6.6

EFFECT OF UNHEATED SERA ON RS VIRUS NEUTRALIZING ANTIBODY TITRES

Sera	Virus diluent		
	PBS	PBS + 1% UGPS	PBS + 25% URS
Acute	<8	<8	<8
Convalescent	23	90	>256
Virus dose *	10	10	10

UGPS = unheated guinea pig serum

URS = unheated rabbit serum

* TCD_{50}/0.1 ml.

TABLE 6.7

ANTIBODY TITRES IN PATIENTS' SERA AGAINST RS VIRUS DILUTED IN PBS AND IN PBS WITH HEATED AND UNHEATED RABBIT SERUM

Patients' Sera	Additional Rabbit serum		
	Unheated	Heated*	None
1 Acute	23	11	11
Conv	64	23	16
2 Acute	<8	<8	<8
Conv	64	16	16
Virus dose +	32	32	32

* $56^{\circ}C$ for 30 min

\+ TCD_{50}/0.1 ml.

TABLE 6.8

EFFECT OF UNHEATED RABBIT SERUM ON NEUTRALIZING ANTIBODY TITRES AGAINST DIFFERENT STRAINS OF RS VIRUS.

1963-64 Winter

Age *	DI	121-64 Strain -US	121-64 Strain +US	Randall Strain -US	Randall Strain +US
2	6	< 8	45	11	32
	16	< 8	64	11	32
5	10	< 8	< 8	<16	<16
	41	< 16	128	16	90
11	4	< 8	< 8	< 8	< 8
	16	< 8	128	16	64
Virus dose ‡		32	10 - 100	32 - 68	68 - 100

1964-65 Winter

Age *	DI	143-65 Strain -US	143-65 Strain +US	Randall Strain -US	Randall Strain +US
4	3	16	23	11	23
	11	23	64	16	64
7	5	< 8	< 8	< 8	11
	12	128	>512	180	>512
7	1	< 8	< 8	< 8	< 8
	12	< 16	23	< 16	45
13	6	< 8	< 8	< 8	< 8
	13	23	90	32	64
Virus dose ‡		32	68 - 100	32 - 68	68 - 100

* Months

DI = Day of illness on which sera were collected

‡ TCD_{50}/0.1 ml

US = Unheated rabbit serum

TABLE 6.9

NEUTRALIZING ANTIBODY TITRES AGAINST

RANDALL AND 329-63 STRAINS

Age*	DI	Randall		329-63	
		-US	+US	-US	+US
2	5	ND	64	ND	45
	38	ND	45	ND	64
2	6	ND	23	ND	32
	16	ND	32	ND	32
	84	ND	16	ND	16
4	3	11	23	11	23
	11	16	45	16	32
34	3	16	32	16	45
	10	>1024	>1024	>1024	>1024
Virus dose ‡		32 - 68	68 - 100	32 - 68	32 - 100

* Months

‡ TCD_{50}/0.1 ml

DI = Day of illness on which sera were collected

US = Unheated rabbit serum

TABLE 6.10

ANTIBODY RESPONSES OF GUINEA PIGS

INOCULATED WITH TWO STRAINS OF RS VIRUS

Serum Number			Antibody titre against virus strain					
			Randall		329-63		Fold Difference *	
			+US	-US	+US	-US	+US	-US
Randall	1	PR	< 4	< 4	< 4	< 4		
		PO	512	512	16	11	32	45
	2	PR	< 4	< 4	< 4	< 4		
		PO	512	256	128	32	4	8
329-63	1	PR	< 4	< 4	< 4	< 4	2	4 ‡
		PO	1024	360	2048	90		
	2	PR	< 4	< 4	< 4	< 4		
		PO	1024	256	2048	256	2	0
Virus dose **			180	68	180	68	...	...

* Between homologous and heterologous titres

‡ Heterologous titre higher than homologous

** TCD_{50}/0.1 ml

US = Unheated rabbit serum

PR = Pre-immunization serum

PO = Post-immunization serum

TABLE 6.11

TITRES AND NEUTRALIZATION INDICES OF TWO RS VIRUS STRAINS IN THE PRESENCE OF VARIOUS SERA

Serum	Virus strain			
	Randall +US	329-63 +US	Randall -US	329-63 -US
Normal	4.7	4.1	4.3	3.9
Randall/1	$\leq$ 0.5	1.7	$<$0.5	3.3
329-63/2	0.9	$<$0.5	1.5	0.9
NI with Randall/1	$>$4.2	2.4	$>$3.8	0.6
NI with 329-63/2	3.8	$>$3.6	2.8	3.0

Titres expressed as Log TCD_{50}/0.1 ml

NI = Neutralization index

TABLE 6.12

ATTEMPTS TO CONCENTRATE RS VIRUS WITH $AlCl_3$ AND Na_2CO_3

	Expt. 1	Expt. 2	Expt. 3
Original virus	4.7	4.9	5.2
Supernate	< 1.5	1.7	2.1
Precipitate	6.3	6.3	5.9

Titres expressed as Log TCD_{50}/0.1 ml

TABLE 6.13

ANTIBODY RESPONSES OF GUINEA PIGS TO VARIOUS RS ANTIGENS

Antigen Type	Titre *	Serum No.	Virus Strain Randall +US	Randall -US	329-63 +US	329-63 -US
Randall						
Normal	5.2	4 PR	<4	<4	<4	<4
		PO	32	11	16	<4
		5 PR	<4	<4	<4	<4
		PO	512	16	<16	4
Concentrated	5.9	6 PR	<4	<4	<4	<4
		PO	<16	<4	<16	4
		7 PR	<4	<4	<4	<4
		PO	45	4	<16	11
329-63						
Normal	4.7	4 PR	<4	<4	<4	<4
		PO	<16	<4	16	<4
		5 PR	<4	<4	<4	<4
		PO	<16	<4	512	<4
Concentrated	6.3	6 PR	<4	<4	<4	<4
		PO	<16	<4	128	6
		7 PR	<4	<4	<4	<4
		PO	128	32	>1024	32
Virus dose ‡			180	100	180	100

* Log TCD_{50}/0.1 ml

‡ TCD_{50}/0.1 ml

US = Unheated rabbit serum

PR = Pre-immunization serum

PO = Post-immunization serum

TABLE 6.14

TITRES OF FOUR RS ANTISERA AGAINST NINE VIRUS STRAINS ‡

Virus		Antisera			
		Randall		329-63	
Strain	Dose *	1	5	2	5
CH 18357	32	256	1024	2048	2048
3892-65	180	256	1024	2048	2048
4410-65	32	512	1024	4096	2048
143-65	180	64	16	1024	256
4414-65	32	64	32	512	< 8
4178-65	32	16	8	256	< 8
121-64	180	16	8	128	<8
Randall	180	512	512	1024	<16
329-63	180	16	<16	2048	512

‡ Virus diluted in 25% unheated rabbit serum in all titrations

* TCD_{50}/0.1 ml

TABLE 6.15

GEOMETRIC MEAN TITRES OF RANDALL AND 329-63 ANTISERA AGAINST 9 VIRUS STRAINS

Virus Strain	Randall Sera 1 + 5	329-63 Sera 2 + 5	Fold Difference *
Randall	512	90	0.2
4178-65	11	11	1
4414-65	45	45	1
121-64	11	23	2
3892-65	512	2048	4
4410-65	720	2880	4
CH 18357	512	2048	4
143-65	32	512	16
329-63	11	1024	90

* Fold difference = $\frac{\text{geometric mean titre of 329-63 sera}}{\text{geometric mean titre of Randall sera}}$

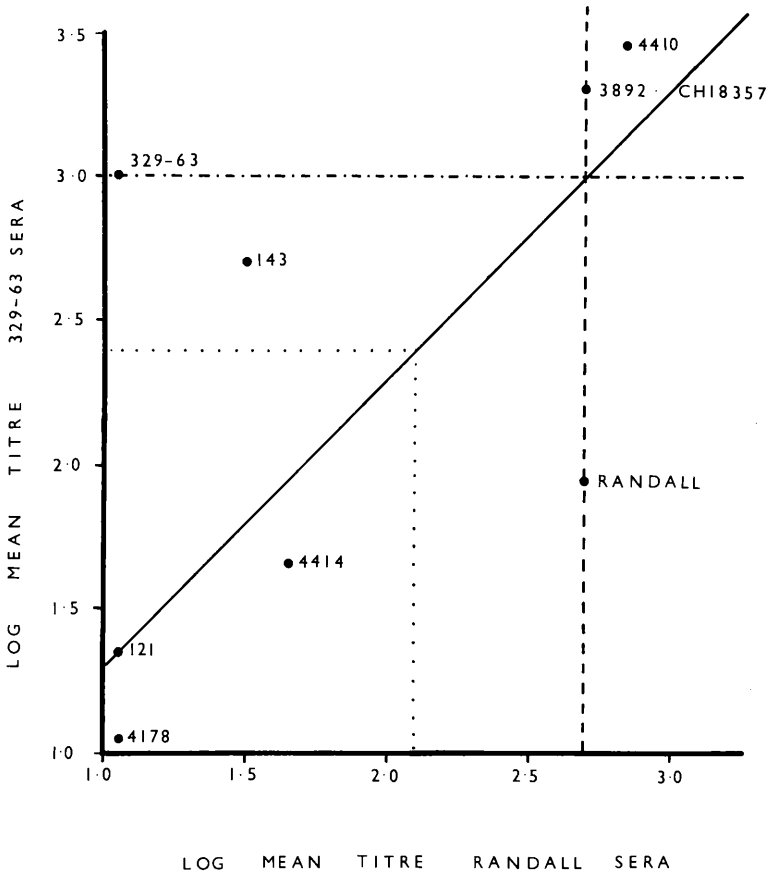

Fig 6.1 Log. mean titre of 329-63 sera plotted against log. mean titre of Randall sera for 9 RS virus strains.
- - - - homologous titre of Randall sera
-. -. - homologous titre of 329-63 sera
. . . . . fourfold less than homologous titres
Diagonal line indicates strains equally related to Randall and 329-63 strains.

PART II

THE EPIDEMIOLOGY OF HUMAN RESPIRATORY VIRUSES ISOLATED IN GLASGOW, 1962 - 1966

CHAPTER 9

ADULTS AND CHILDREN AT HOME AND AT WORK

Tables

TABLE 9.1

AGE AND SEX CHARACTERISTICS AND VIRUS ISOLATIONS OF CASES AND THEIR CONTACTS

	Age (Years)		Sex		Viruses Isolated		
	Range	Mean	M	F	RS	RHM	RHH
31 cases	2-13	5.1	15	16	1	1	3
31 contacts	2-11	5.5	14	17	0	1	3

TABLE 9.2

MONTHLY DISTRIBUTION OF CASES, CONTACTS AND VIRUSES ISOLATED

MONTH	CASES	VIRUSES	CONTACTS	VIRUSES
1962 November	4	-	4	-
December	12	1 RS, 3 RHH	12	1 RHH
1963 January	5	1 RHM	5	1 RHH, 1 RHM
February	6	-	6	-
March	2	-	2	-
April	2	-	2	1 RHH

TABLE 9.3

VIRUSES ISOLATED ACCORDING TO AGE

AGE (Years)	EPISODES	VIRUSES ISOLATED						
		FL		PF		RH		HS
		A2	B	1	3	M	H	
0 - 15	61	0	16	1	0	0	6	1 [x]
16 - 30	34	1	1	0	0	2	16	0
31 - 45	18	0	2	0	1	1	5	0
46 - 60	4	0	1	0	0	0	0	0
Total	117	1	20	1	1	3	27	1 [x]

[x] isolated from a child who also yielded influenza B.

TABLE 9.4

VIRUSES ISOLATED ACCORDING TO SEX

SEX	EPISODES	VIRUSES ISOLATED						
		FL		PF		RH		HS
		A2	B	1	3	M	H	
Male (Adult)	29	0	3	0	0	2	10	0
(Child)	38	0	10	1	0	0	5	1 ×
Female (Adult)	27	1	1	0	1	1	11	0
(Child)	23	0	6	0	0	0	1	0
Total	117	1	20	1	1	3	27	1 ×

× isolated from a child who also yielded influenza B.

TABLE 9.5

VIRUS ISOLATIONS ACCORDING TO ILLNESS

ILLNESS	GROUP	EPISODES	VIRUSES ISOLATED						
			FL		PF		RH		HS
			A2	B	1	3	M	H	
Cold	Adult	39	0	0	0	1	3	20	0
	Child	46	0	11	1	0	0	6	0
Sore Throat	Adult	9	0	0	0	0	0	1	0
Flu-like	Adult	8	1	4	0	0	0	0	0
	Child	15	0	5	0	0	0	0	1[x]
Total		117	1	20	1	1	3	27	1[x]

[x] isolated from a child who also yielded influenza B virus.

TABLE 9.6

MONTHLY DISTRIBUTION OF EPISODES AND VIRUS ISOLATIONS 1962 - 66

MONTH	EPISODES	VIRUSES ISOLATED						HS
		FL		PF		RH		
		A2	B	1	3	M	H	
January	32	0	14	0	0	0	0	1[x]
February	15	1	6	0	0	0	1	0
March	13	0	0	0	0	1	4	0
April	8	0	0	0	1	0	3	0
May	3	0	0	0	0	1	1	0
June	2	0	0	0	0	0	2	0
July	2	0	0	0	0	0	0	0
August	4	0	0	0	0	0	0	0
September	8	0	0	0	0	0	4	0
October	11	0	0	0	0	0	5	0
November	10	0	0	1	0	0	5	0
December	9	0	0	0	0	1	2	0
Total	117	1	20	1	1	3	27	1[x]

Figures for February, March, April include 5 years' observations, figures for other months include 4 years' observations.

[x] child also yielded influenza B virus.

TABLE 9.7

VIRUSES ISOLATED ACCORDING TO DURATION OF ILLNESS

DURATION (Days)	EPISODES	VIRUSES ISOLATED							PER CENT
		FL		PF		RH		HS	
		A2	B	1	3	M	H[x]		
1 - 3	92	1	17	1	1	2	18	1	45
4 - 7	23	0	3	0	0	1	6	0	43
8 - 14	2	0	0	0	0	0	2	0	(100)
Total	117	1	20	1	1	3	26	1	45

[x] One rhinovirus H infection was detected only in a second specimen and is therefore considered negative in this table.

TABLE 9.8

EXAMINATION OF 35 SPECIMENS FROM 13 EPISODES

SPECIMENS	DURATION OF ILLNESS (DAYS)			
	1 - 3	4 - 7	8 - 14	≥15
Examined	11	11	10	3
Rhinovirus positive	10	9	8	0
First appearance of CPE (Mean number of days)	5.8	5.9	6.9	-

TABLE 9.9

RHINOVIRUS SEROTYPES ISOLATED FROM SEPARATE EPISODES OF SIX ADULTS.

ADULT	MONTHS STUDIED	SEROTYPES ISOLATED
1	10	UT , B632, 68, UT
2	28	409-62, UT, 1059
3	15	1833-63, 4574-62, UT
4	6	FEB, UT
5	35	1192-65, UT
6	5	UT, UT

UT = untyped

CHAPTER 10

ADULTS WITH CHRONIC BRONCHITIS

Tables and Figure

TABLE 10.1

CLASSIFICATION OF 15 BRONCHITICS
(MRC, 1965a).

TYPE OF BRONCHITIS	MALE	FEMALE
Simple Chronic Bronchitis	2	5
Chronic or Recurrent Mucopurulent Bronchitis	2	2
Chronic Obstructive Bronchitis	4	0
Total	8	7

TABLE 10.2

CHARACTERISTICS OF 15 BRONCHITICS (MRC, 1960).

CHARACTERISTIC	MALE	FEMALE
Age		
25 - 44 years	3	2
45 - 64 years	5	4
65 - 84 years	0	1
Phlegm Production		
Grade 1	3	3
Grade 2	5	4
Sputum Volume		
Group 0	1	2
1	1	0
2	1	3
3	4	1
4	1	1
Smoking Habits		
Group 0	1	1
1	1	2
2 L	3	3
2 M	2	1
2 H	1	0

TABLE 10.3

CLINICAL CLASSIFICATION AND VIROLOGICAL RESULTS OF 94 RESPIRATORY EPISODES

PATIENTS	CLINICAL GROUPS		VIRUS ISOLATIONS	SEROLOGICAL DIAGNOSIS
Chronic Bronchitics (75)	Colds (42) a) Without exacerbations	21	Rhinovirus 1	Influenza C 1(a) + 1
	b) Preceded or followed by exacerbation	21	Rhinovirus 10 Herpes simplex 1(b)	Influenza C 1(a)
	Influenza-like illness	7	Parainfluenza 1 1	None
	Exacerbations without colds	26	Rhinovirus 1	Influenza A 1 Influenza C 1
Family Contacts (19)	Children Colds	14	Rhinovirus 1 Parainfluenza 1 1 Parainfluenza 3 1 Adenovirus 7 1 Herpes simplex 1(b)	
	Influenza-like illness	1	None	Not tested
	Adults Colds	3	None	
	Influenza-like illness	1	None	

(a) falling titres: evidences suggestive only
(b) probably incidental infection

TABLE 10.4

EXAMINATION OF 187 SPECIMENS FROM 75 EPISODES IN CHRONIC BRONCHITICS

TYPE OF SPECIMEN	NO OF SPECIMENS	VIRUSES ISOLATED			
		PF	HS	RH	TOTAL
Nose	67	1	1	11	13
Throat	69	1	0	3	4
Sputum	51	1	0	5	6

TABLE 10.5

EXAMINATION OF 36 SPECIMENS FROM 19 EPISODES

IN FAMILY CONTACTS

TYPE of SPECIMEN	NO. of SPECIMENS	VIRUSES ISOLATED				
		PF	HS	RH	AD	TOTAL
Nose	17	2	1	1	1	5
Throat	18	1	0	0	1	2
Sputum	1	0	0	0	0	0

TABLE 10.6

DETAILS OF THREE RHINOVIRUS INFECTIONS IN PATIENT 1.

FEMALE AGED 53 YEARS *

DATE	WEEK OF STUDY	INFECTION 1		INFECTION 2		INFECTION 3	
		I	S	I	S	I	S
27.2.62	9		<4		<4		ND
12.3.62	11		<4		ND		8
14.5.62	18		<4		<4		8
3.9.62	36	N	<4		ND		8
10.9.62	37	S	...		...		...
17.9.62	38		<8		<8		ND
29.10.62	44		45	NS	<8		ND
12.11.62	46		64		720		8
10.6.63	76		11		720		8
21.10.63	95		11		360		8
28.2.64	113		ND		720		8
17.3.64	116		4		ND		ND
18.4.64	120		ND		512		8
4.5.64	123		...		...	N	...
4.6.64	127		8		720		128
Type of episode		C → E		C → E		C → E	
Date of onset		2.9 → 10.9		28.10 → 5.11		3.5 → 4.5	
Rhinovirus serotype		4704-62		4574-62		1376-64	

I = specimens from which rhinoviruses were isolated

S = neutralizing antibody titres against homologous rhinovirus

N = nasal washings

S = sputum

C = cold

E = exacerbation

ND = not done

* Age at beginning of investigation

... = no serum collected.

TABLE 10.7

DETAILS OF TWO RHINOVIRUS INFECTIONS IN PATIENT 2.
FEMALE OF 32 YEARS

DATE	WEEK OF STUDY	INFECTION 1		INFECTION 2	
		I	S	I	S
16.1.63	55		<4		ND
22.5.63	73	N	...		...
23.5.63	73	S	...		...
5.6.63	75		8		<4
7.8.63	84		128		ND
21.8.63	86		180		<4
30.10.63	96		32		<4
27.11.63	100		...	N	...
11.12.63	102		32		23
1.4.64	118		32		23
Type of episode		C ——→ E		C ——→ E	
Date of Onset		13.5 →3.6		18.11 → 5.12	
Rhinovirus serotype		1647-63		4462-63	

Abbreviations as in Table 10.6.

TABLE 10.8

DETAILS OF TWO RHINOVIRUS INFECTIONS IN PATIENT 3.

FEMALE OF 29 YEARS

DATE	WEEK OF STUDY	INFECTION 1		INFECTION 2	
		I	S	I	S
13.2.62	7	N	<4		<4
22.2.62	8		4		ND
25.10.62	43		<4		<4
8.11.62	45		<4		ND
12.2.63	59		<4		ND
28.2.63	61		ND		<4
6.6.63	75		ND	NTS	<4
26.6.63	78		<4		256
20.10.63	96		<4		23
15.4.64	120		<4		45
Type of episode		C		C ⟶	E
Date of onset		10.2		31.5 ⟶	6.6
Rhinovirus serotype		409-62		1833-63	

T = throat swab

Other abbreviations as in Table 10.6.

TABLE 10.9

DETAILS OF TWO RHINOVIRUS INFECTIONS IN PATIENT 4,

FEMALE OF 49 YEARS

DATE	WEEK OF STUDY	INFECTION 1		INFECTION 2	
		I	S	I	S
3.3.62	9		32		<4
23.5.62	21	NT	32		ND
30.5.62	22		128		<4
13.10.62	41		1440	N	<4
23.3.63	64		ND		45
9.4.63	67		1024		45
7.9.63	88		512		45
21.9.63	90		512		23
2.11.63	96		512		11
18.4.64	120		512		11
Type of episode		E ⟶ C		E ⟶ C	
Date of onset		13.5 ⟶ 22.5		6.10 ⟶ 11.10	
Rhinovirus serotype		409-62		179 E	

Abbreviations as in Tables 10.6 and 10.8

TABLE 10.10

DETAILS OF RHINOVIRUS INFECTION IN PATIENT 5.

MALE OF 43 YEARS

DATE	WEEK OF STUDY	I	S
25.1.62	4		<4
3.5.62	18	N	<4
17.5.62	20		4
20.12.62	51		<4
28.3.63	65		<4
31.10.63	96		<4
9.1.64	106		<4
Type of episode		E	
Date of onset		1.5	
Rhinovirus serotype		1321-62	

Abbreviations as in Table 10.6.

TABLE 10.11

DETAILS OF RHINOVIRUS INFECTION IN PATIENT 6.

MALE OF 39 YEARS

DATE	WEEK OF STUDY	I	S
3.1.63	53		<4
9.2.63	58		<4
21.3.63	64	N	<4
6.4.63	66		8
11.5.63	71		23
7.9.63	88		4
21.9.63	90		4
19.10.63	94		4
4.4.64	118		4
Type of episode		C ⟶	E
Date of onset		19.3 ⟶ 27.3	
Rhinovirus serotype		130-63	

Abbreviations as in Table 10.6.

TABLE 10.12

DETAILS OF RHINOVIRUS INFECTION IN PATIENT 7.

MALE OF 59 YEARS

DATE	WEEK OF STUDY	I	S
5.12.62	49		<4
14.1.63	55	NTS	...
28.1.63	57		<4
22.4.63	69		32
7.5.63	71		32
9.9.63	89		22
23.9.63	91		45
21.10.63	95		32
6.4.64	119		32
Type of episode		C ⟶	E
Date of onset		11.1 ⟶ 28.1	
Rhinovirus serotype		130-63	

Abbreviations as in Tables 10.6 and 10.8.

TABLE 10.13

CLINICAL CLASSIFICATION AND VIROLOGICAL RESULTS

OF 12 RESPIRATORY EPISODES

CLINICAL GROUPS		RHINOVIRUSES ISOLATED
Colds (a) without exacerbation	2	0
(b) followed by exacerbation	6	2
Exacerbations without colds	4	0

TABLE 10.14

DETAILS OF RHINOVIRUS INFECTION IN PATIENT 8.

MALE OF 25 YEARS

DATE	SPECIMENS FOR ISOLATION	S
24.2.65	T/N +	11
13.3.65	T/N -	1024
8.4.65	T/N -	1024
28.4.65	None	256
Type of episode		C ⟶ E
Date of onset		17.2→24.2
Rhinovirus serotype		UT

+ = rhinovirus isolated
- = no rhinovirus isolated
UT = not neutralized by 40 available antisera
T/N = throat swab and nasal washing combined

Other abbreviations as in Table 10.6.

TABLE 10.15

DETAILS OF RHINOVIRUS INFECTION IN PATIENT 9.

MALE OF 41 YEARS.

DATE	SPECIMENS FOR ISOLATION	S
18.3.65	T/N + S +	<4
25.3.65	T - S +	...
5.4.65	T - S -	...
15.4.65	None	16
Type of episode		C ⟶ E
Date of onset		16.3 → 23.3
Rhinovirus serotype		68

Abbreviations as in Table 10.14.

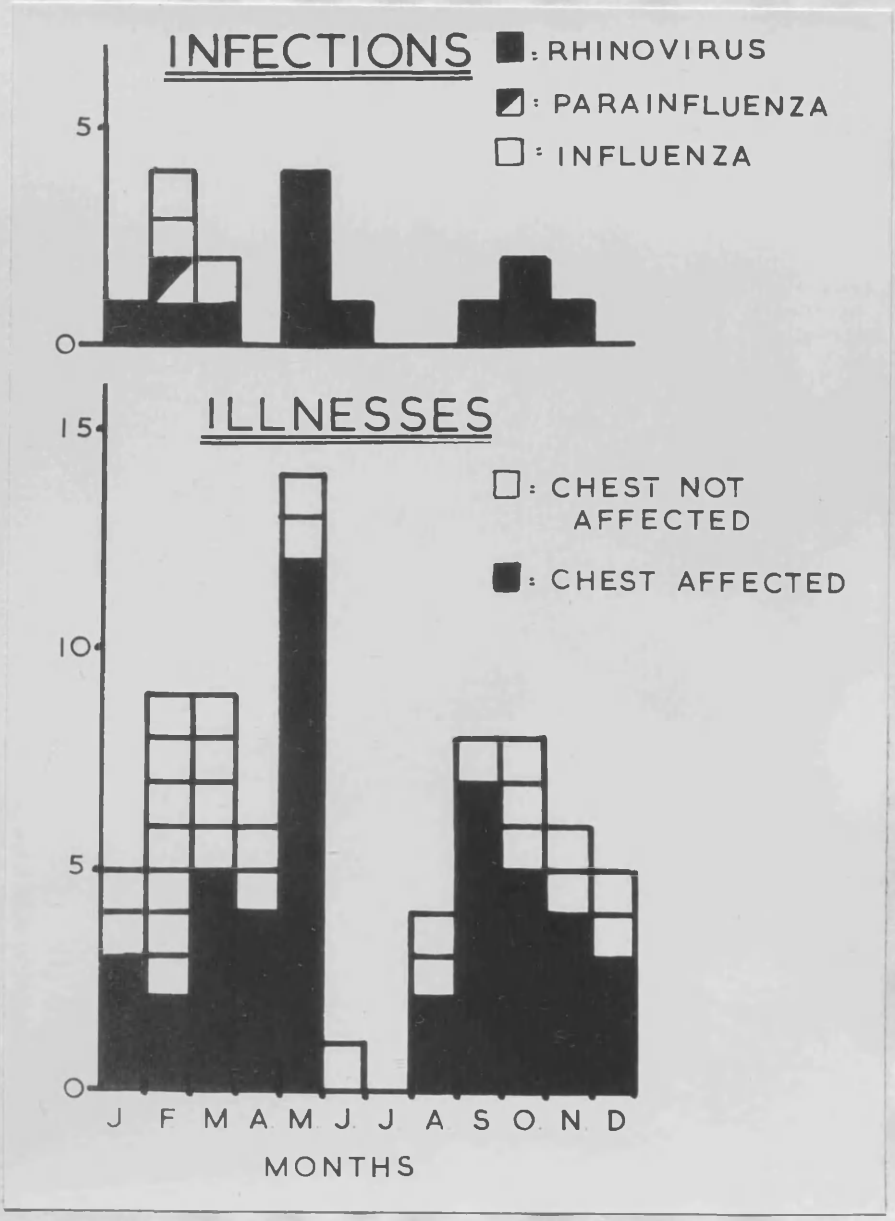

Fig. 10.1 Monthly Distribution of 16 Virus Infections and 75 Respiratory Illnesses found in 15 Chronic Bronchitics. Figures for January - June based on three years', July - December on two years' observations; figures for July unrepresentative because of local holidays.

CHAPTER 11

CHILDREN IN HOSPITAL

Tables and Figures

TABLE 11.1.

VIRUS ISOLATION ACCORDING TO AGE

AGE	CASES	VIRUS ISOLATED		
		RS	AD1	AD5
1 - 5 M	15	4	0	0
6 - 11 M	8	2	1	0
12 - 23 M	14	2	0	1
2 - 5 Y	2	0	0	0
Total	39	8	1	1

M = months

Y = years

TABLE 11.2

VIRUS ISOLATIONS ACCORDING TO ILLNESS

ILLNESS	CASES	VIRUS ISOLATED		
		RS	AD1	AD5
Pneumonia or Bronchopneumonia	18	3	1	0
Bronchitis or Bronchiolitis	13	4	0	1
URT I	8	1	0	0
Total	39	8	1	1

URTI = upper respiratory tract illness

TABLE 11.3

MONTHLY DISTRIBUTION OF VIRUS ISOLATIONS

MONTH	CASES	VIRUS ISOLATED		
		RS	AD1	AD5
December	13	4	0	0
January	8	2	0	0
February	10	2	0	0
March	3	0	1	0
April	3	0	0	1
May	2	0	0	0
Total	39	8	1	1

TABLE 11.4

POPULATION STUDIED

GROUP	SEX	AGE IN MONTHS			TOTAL
		0-11	12-23	24-72	
Respiratory	Male	27	15	22	64
	Female	23	11	15	49
Diarrhoeal	Male	24	16	21	61
	Female	22	14	16	52
Total		96	56	74	226

TABLE 11.5

VIRUSES ISOLATED FROM THROAT AND NOSE SWABS

GROUP	CASES	VIRUS ISOLATED						
		RS	PF	RH	HS	AD	ENT	TOTAL
Respiratory	113	26	9	10	12	5	2	64[x]
Diarrhoeal	113	1	2	10	4	8	6	31[+]
Total	226	27	11	20	16	13	8	95[xx]

[x] Dual isolation in five cases: RS + HS
RS + AD1
PF3+ AD2
RHM+ HS
AD1 + HS

[+] Dual isolation in two cases: RS + PF3
RHH+ AD5

[xx] Dual isolation in seven cases (see above)

TABLE 11.6

VIRUS ISOLATIONS FROM RESPIRATORY ILLNESSES

ACCORDING TO DAY OF ILLNESS

DAY OF ILLNESS	CASES	VIRUS ISOLATED						
		RS	PF	RH	HS	AD	ENT	TOTAL
1 - 3	60	12	4	4	7	2	1	30
4 - 7	38	11	3	5	4	3	1	27
> 7	10	2	1	1	0	0	0	4
Unknown	5	1	1	0	1	0	0	3
Total	113	26	9	10	12	5	2	64

TABLE 11.7

VIRUS ISOLATIONS ACCORDING TO AGE

AGE	CASES	VIRUS ISOLATED						
		RS	PF	RH	HS	AD	ENT	TOTAL
0 - 5 M	74	17	4	5	0	3	1	30
6 - 11 M	23	4	2	3	1	2	2	14
12 - 23 M	56	4	2	5	7	8	3	29
2 - 6 Y	73	2	3	7	8	0	2	22
Total	226	27	11	20	16	13	8	95

M = months

Y = years

TABLE 11.8

VIRUS ISOLATIONS ACCORDING TO ILLNESS

ILLNESS*	CASES	VIRUS ISOLATED						
		RS	PF	RH	HS	AD	ENT	TOTAL
Respiratory								
Pneumonia or Bronchopneumonia	47	13	5	4	6	4	1	33(3)
Bronchitis or Bronchiolitis	51	13	2	3	4	0	0	22(2)
Croup	3	0	0	0	1	0	0	1
URTI	12	0	2	3	1	1	1	8
Diarrhoeal								
Bacterial diarrhoea	55	1	1	6	2	3	2	15(2)
Non-specific diarrhoea	58	0	1	4	2	5	4	16
Total	226	27	11	20	16	13	8	95(7)

* Final diagnosis of illness with which patient was admitted

Figures in brackets refer to dual isolations

URTI = upper respiratory tract illness

TABLE 11.9

MONTHLY DISTRIBUTION OF VIRUS ISOLATIONS

MONTH	CASES	VIRUS ISOLATED						
		RS	PF	RH	HS	AD	ENT	TOTAL
1963 Oct	13(1)[x]	5	0	1	2	0	1	9
Nov	15	2	1	0	0	1	0	4
Dec	12	2	0	0	1	0	1	4
1964 Jan	14(2)	3	0	2	2	3	0	10
Feb	12	1	0	2	0	0	1	4
Mar	14	0	1	1	3	0	0	5
Apr	14(1)	0	2	0	1	1	1	5
May	8	0	0	1	0	1	1	3
Jun	8	1	1	0	1	0	1	4
Jul	0	0	0	0	0	0	0	0
Aug	10	0	0	2	0	0	1	3
Sep	14	0	1	0	2	3	0	6
Oct	18	0	2	2	1	0	0	5
Nov	26(1)	4	2	1	2	1	1	11
Dec	10(1)	2	1	0	0	0	0	3
1965 Jan	4	2	0	0	0	1	0	3
Feb	16	3	0	2	0	0	0	5
Mar	13	2	0	2	1	1	0	6
Apr	5(1)	0	0	4	0	1	0	5
Total	226(7)	27	11	20	16	13	8	95

[x] Figures in brackets indicate dual isolations

TABLE 11.10

AGE AND SEX COMPOSITION OF POPULATION STUDIED

SEX	AGE IN MONTHS			
	0 - 11	12 - 23	24 - 120	TOTAL
Male	96	21	45	162
Female	47	17	24	88
Total	143	38	69	250

TABLE 11.11

VIRUS ISOLATIONS FROM 250 CHILDREN ACCORDING TO SEX

SEX	CASES	VIRUS ISOLATED FL A	FL B	PF 1	PF 2	PF 3	RS	RH M	RH H	HS	AD 1	AD 2	AD 3	AD 5	₵B 1	₵B 5	ECHO 3	UT	TOTAL
Male	162	2	2	2	1	3	35	2	10	6	1	1	2	1	3	2	1	2	76^{x}
Female	88	1	3	0	0	5	12	0	10	1	0	1	0	2	0	2	0	0	37^{+}
Total	250	3	5	2	1	8	47	2	20	7	1	2	2	3	3	4	1	2	113^{xx}

x Dual isolation in eight cases:

FLA + AD1	RS + RHH
FLB + HS	RS + AD3
PF1 + RHH	RS + UT
RS + RHM	RHH + AD2

+ Dual isolation in one case: RS + RHH

xx Dual isolation in nine cases (see above)

₵ = coxsackievirus

UT = untyped

TABLE 11.12

VIRUS ISOLATIONS ACCORDING TO DAY OF ILLNESS

DAY OF ILLNESS	CASES	VIRUS ISOLATED							
		FL	PF	RS	RH	HS	AD	ENT	TOTAL
1 - 3	144(5)	3	5	29	17	1	4	5	64(44%)
4 - 7	81(3)	4	4	11	3	6	3	3	34(42%)
>7	22(1)	1	2	6	2	0	0	1	12(55%)
Unknown	3	0	0	1	0	0	1	1	3
Total	250(9)	8	11	47	22	7	8	10	113

Figures in brackets indicate dual isolations.

TABLE 11.13

VIRUS ISOLATION RATES ACCORDING TO AGE

AGE	TOTAL CASES	TOTAL VIRUSES	PERCENTAGE ISOLATION RATES						
			FL	PF	RS	RH	HS	AD	ENT
0 - 5 M	86(2)	44	0	6	33	7	0	2	3
6 - 11 M	57(2)	25	0	5	19	9	0	4	7
12 - 23 M	38(3)	20	8	8	11	11	3	8	5
2 - 10 Y	69(2)	24	7	0	6	10	9	1	1
Total	250(9)	113	3	4	22	9	3	3	4

M = months

Y = years

Figures in brackets indicate dual isolations

TABLE 11.14

VIRUS ISOLATIONS ACCORDING TO ILLNESS

ILLNESS*	CASES	VIRUS ISOLATED							
		FL	PF	RS	RH	HS	AD	ENT	TOTAL
Pneumonia or Bronchopneumonia	75(2)X	2	4	15	6	6	3	2	38
Bronchiolitis or Bronchitis	144(6)	2	5	28	14	0	3	6	58
Croup	6(1)	2	1	0	1	0	1	0	5
URTI	25	2	1	4	1	1	1	2	12
Total	250	8	11	47	22	7	8	10	113

* Final diagnosis of illness with which patient was admitted

X Figures in brackets indicate dual isolations.

TABLE 11.15

MONTHLY DISTRIBUTION OF VIRUS ISOLATIONS

MONTH	CASES	VIRUS ISOLATED							
		FL*	PF	RS	RH	HS	AD	ENT	TOTAL
1964 Dec	25(3)	0	2	6	2	1	2	2	15
1965 Jan	28(1)	0	1	12	1	1	0	0	15
Feb	14(1)	0	0	4	2	0	2	1	9
Mar	22	2	2	1	3	1	1	0	10
Apr	7	0	0	2	0	0	0	0	2
May	8(1)	0	2	0	3	0	0	1	6
Jun	12	1	0	0	1	0	0	3	5
Jul	7	0	0	0	3	0	0	1	4
Aug	5	0	1	0	0	0	1	0	2
Sep	9	0	0	1	1	1	0	0	3
Oct	11	0	0	2	1	0	0	1	4
Nov	48(1)	0	2	18	3	0	0	0	23
Dec	19	0	1	0	0	0	1	0	2
1966 Jan	12(2)	4	0	0	0	2	1	0	7
Feb	6	1	0	0	0	1	0	0	2
Mar	9	0	0	1	2	0	0	0	3
Apr	8	0	0	0	0	0	0	1	1
Total	250	8	11	47	22	7	8	10	113

Figures in brackets indicate dual isolations

* Two viruses isolated in Mar 1965 and one in Jan 1966 were type A2, the remainder were type B.

TABLE 11.16

DETAILS OF 19 RHINOVIRUS-INFECTED CASES WITH PAIRED SERA

Case	Age in months and sex		Diagnosis	VIRUS ISOLATED			SEROLOGICAL TESTS							
				Rhinovirus Type	Serotype	Other	DI	CF AD	CF RS	CF PF	HI PF1	HI PF2	HI PF3	Neut RH
1	1	M	Bronchopneumonia	M	UT	-	2	8	8	-	10	10	80	3
							11	8	<8	<8	10	10	80	256
2	1	M	Bronchopneumonia	H	UT	-	1	-	-	-	20	-	160	8
							15	<8	<8	<8	20	<10	80	≥256
3	3	M	Bronchitis	H	2030-65	RS	2	-	-	-	<10	20	<10	<4
							13	<8	<8	<8	<10	10	<10	23
4	4	F	Pneumonia	H	2030-65	-	1	<8	<8	-	-	-	-	<8
							12	16	16	<8	<10	<10	<10	8
5	4	F	Bronchitis	H	1767-65	-	10	-	-	-	<10	10	40	16
							24	<8	<8	<8	<10	<10	20	128
6	4	M	Bronchiolitis	H	11757	-	7	-	-	-	<10	<40	160	4
							19	<8	<8	<8	<10	<40	40	90
7	10	M	Bronchitis	H	2030-65	AD2	2	16	-	-	<10	<10	<10	<4
							11	16	<8	<8	<10	<10	<10	<4
8	10	M	Bronchitis	M	UT	RS*	3	-	16	-	<10	<10	<10	8
							13	<8	16	<8	<10	<10	<10	32
9	11	F	Bronchitis	M	B632	HS	9	-	-	-	-	-	40	<8
							18	<8	<8	<8	<10	<10	40	128

TABLE 11.16 (Continued)

Case	Age in months and sex	Diagnosis	VIRUS ISOLATED			SEROLOGICAL TESTS						
			Rhinovirus		Other	DI	CF		HI			Neut
			Type	Serotype			AD	RS	PF	PF1	PF2	PF3	RH
10	12 M	Bronchitis	H	UT	-	8	-	16	-	...	...	...	...
						17	<8	16	<8	...	...	...	...
11	14 F	Bronchitis	H	2030-65	-	3	-	-	-	<10	<10	320	<4
						13	<8	<8	<8	<10	<10	320	23
12	16 M	Bronchiolitis	H	4411-65	PF1	1	-	-	-	<10	<10	<10	<4
						21	<16	<16	<16	160	<10	<10	8
13	21 M	Bronchpneumonia	H	4411-65	-	2	64	16	-	-	-	160	<2
						10	32	16	<16	<10	<10	160	4
14	24 M	Croup	H	11757	-	1	16	-	-	-	-	160	<2
						8	16	<8	<8	<10	<10	160	4
15	24 F	URTI	H	1192-65	-	2	8	<16	<16	<10	<10	40	<8
						9	8	64	16	<10	<10	40	<8
16	36 M	Pneumonia	H	5986	-	1	64	64	-	<10	<10	160	<8
						23	64	64	<8	<10	<10	160	32
17	36 F	Bronchitis	H	UT	-	2	16	128	-	<10	<10	160	<4
						12	16	64	<8	<10	<10	160	256
18	36 F	Bronchitis	H	4411-65	-	3	-	≥512	8	10	40	160	<2
						12	<8	≥512	64	20	40	640	16

TABLE 11.16 (Continued)

Case	Age in months and sex		Diagnosis	VIRUS ISOLATED			SEROLOGICAL TESTS						
				Rhinovirus		Other	DI	CF		HI			Neut
				Type	Serotype			AD	RS	PF	PF1	PF2	PF3	RH
19	60	F	Bronchitis	H	UT	-	1	-	-	32	40	80	640	<4
							10	<8	<8	32	40	80	640	4

DI = day of illness on which sera were collected
Neut = neutralizing antibody titres against infecting rhinovirus
UT = untyped i.e. not neutralized by 40 available antisera
* Echovirus 7 isolated from faeces of this case
+ Only partial neutralization
- = not done
... = not done because sera insuffcient.

TABLE 11.17

RHINOVIRUS SEROTYPES FOUND IN CHILDREN IN HOSPITAL

SEROTYPE	ILLNESS		TOTAL
	Respiratory	Diarrhoeal	
Prototype			
B632	1	0	1
181	1	0	1
204	1	0	1
5986	1	0	1
363	1	0	1
11757	2	0	2
Ruchill			
1321-62	2	1	3
1833-63	1	2	3
1192-65	1	0	1
1767-65	1	0	1
2030-65	4	2	6
4411-65	2	0	2
Untyped	14	5	19
Total	32	10	42

TABLE 11.18

INITIAL RS VIRUS NEUTRALIZING ANTIBODY TITRES AND RS VIRUS INFECTIONS OF CHILDREN IN HOSPITAL

RS VIRUS DIAGNOSIS	INITIAL ANTIBODY TITRE							TOTAL
	<4 or<8	4	8	16	32	64	>64	
Infected	19	4	3	2	7	2	1	38
Not infected	7	3	7	3	8	4	0	32
Total	26	7	10	5	15	6	1	70

TABLE 11.19

INITIAL RS VIRUS NEUTRALIZING ANTIBODY TITRES, AGE AND RS VIRUS INFECTIONS OF CHILDREN IN HOSPITAL.

AGE	VIROLOGICAL DIAGNOSIS	NO.	INITIAL ANTIBODY TITRE RANGE	GM
1 - 5 M	RS negative	11	4 - 32	3.62 ± 0.31
	RS positive-Isol	10	4 - 64	4.0 ± 0.47
	RS positive-Serol	2	<4 - 32	3.0 ± 1.6
6 - 60 M	RS negative	21	<4 - 64	3.33 ± 0.45
	RS positive-Isol	11	<4 - 8	1.82 ± 0.18
	RS positive-Serol	15	<4 - >64	2.73 ± 0.53

M = months

Isol = virus isolation with or without serological confirmation

Serol = fourfold or greater titre rise by CF or neutralization tests, but no virus isolation

GM = geometric mean titre and standard deviation of the mean, expressed as $\log_2$. Titres of <4 were taken as 2, <8 as 4 and > 64 as 128, for these calculations.

TABLE 11.20

MULTIPLE INFECTIONS

ILLNESS	METHOD OF DIAGNOSIS	
	ISOLATION	CF TEST
URTI	RHH	RS
Croup	FLA + AD1	...
BRONCHITIS	RS	PF
"	-	RS+PF
"	-	RS+PF
"	RS+RHM	-
"	RS+RHH	-
"	RS+RHH	...
"	RS+HS	...
"	RS+AD3	RS
"	RHH	PF
"	PF3	AD
"	-	PF+AD
"	RHM+HS	-
"	RHH+AD2	-
BRONCHIOLITIS	PF1+RHH	-
BRONCHOPNEUMONIA	RS+Ad1	RS
"	PF3+AD2	AD
"	HS+AD1	AD
PNEUMONIA	FLB+HS	FLB+HS
"	RS+UT	...
"	RHH	RS+AD

TABLE 11.20 (Continued)

ILLNESS	METHOD OF DIAGNOSIS	
	ISOLATION	CF TEST
Pneumonia	HS	RS
Dysentery	RHH+AD5	...
Gastro-enteritis	RS+PF3	...

- = negative

... = not done

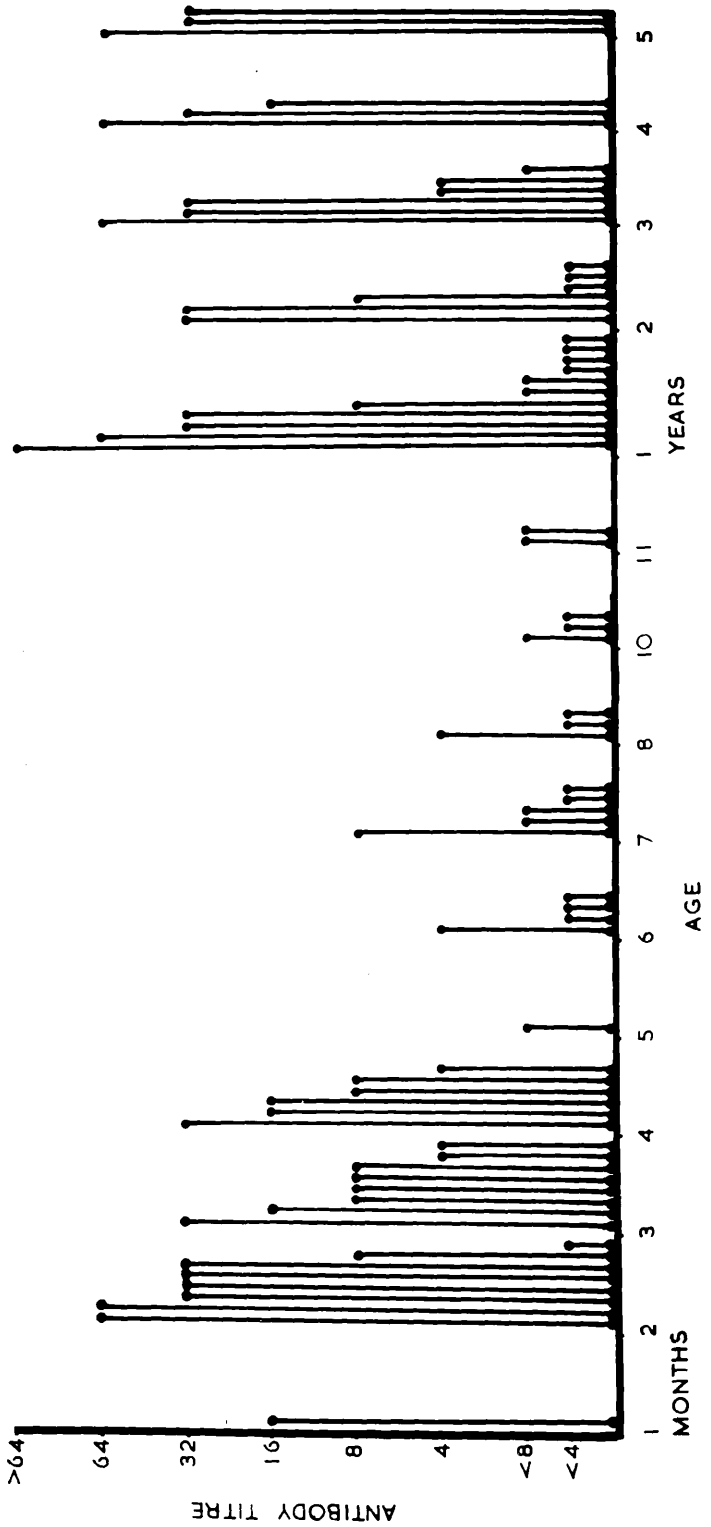

Fig. 11.1 RS Virus Neutralizing Antibody Titres in Children Entering Hospital.

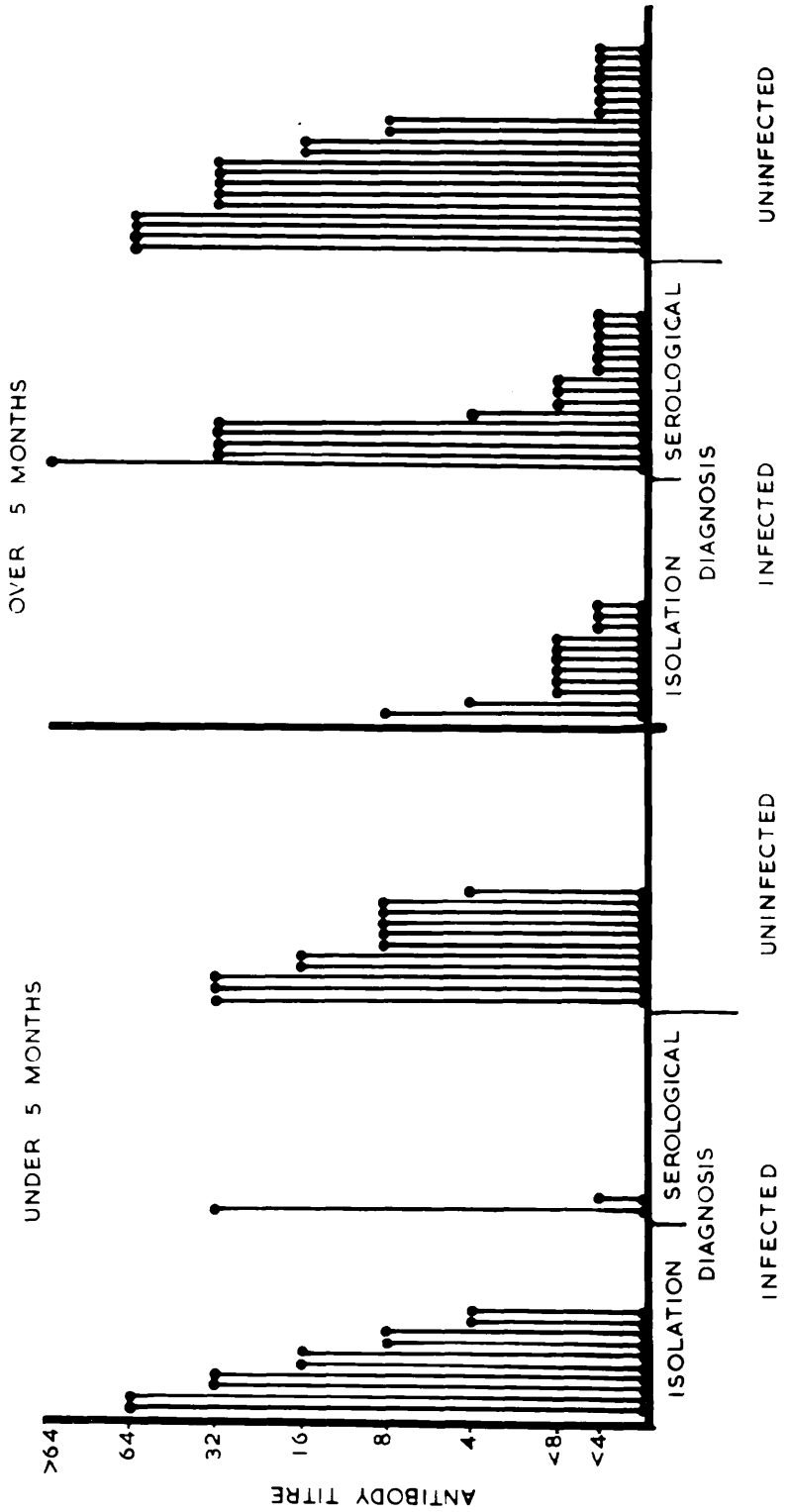

Fig. 11.2 Initial RS Virus Neutralizing Antibody Titres and RS Virus Infections of Children under 5 months and Children over 5 months Entering Hospital. Infected children had an RS virus infection, while in hospital, diagnosed either by virus isolation or by fourfold or greater rise in CF or neutralizing antibody titres. Uninfected children had no such infection.

CHAPTER 12

A SUMMARY OF VIRUSES ISOLATED FROM 800 CASES

Tables and Figures

TABLE 12.1

VIRUSES ISOLATED FROM 800 PERSONS WITH OR WITHOUT RESPIRATORY DISEASE

GROUP AND ILLNESS	CASES STUDIED	VIRUSES ISOLATED FL	PF	RS	RH	HS	AD	ENT	TOTAL
Adults at home									
Acute respiratory	60	5	1	0	24	0	0	0	30
Chronic bronchitis	87	0	1	0	14	1	0	0	16
Children at home									
Acute respiratory	107	16	3	1	11	2	1	0	34
Symptomless	31	0	0	0	4	0	0	0	4
Children in hospital									
Acute respiratory	402	8	20	81	32	19	15	12	187*
Diarrhoeal	113	0	2	1	10	4	8	6	31+
Total	800	29	27	83	95	26	24	18	302**

* Two viruses isolated from each of 14 persons
\+ Two viruses isolated from each of 2 persons
** Two viruses isolated from each of 16 persons

TABLE 12.2

SEROTYPES OF 95 RHINOVIRUSES ISOLATED FROM 800 PERSONS WITH AND WITHOUT RESPIRATORY DISEASE

Serotype	Adults at home		Children at home		Children in hospital		Total
	ARD	CB	ARD	SC	ARD	D	
11 Prototype							
B 632	1	0	0	0	1	0	2
FEB	2	0	0	0	0	0	2
16/60	2	0	0	0	0	0	2
68	2	1	0	0	0	0	3
181	0	0	0	0	1	0	1
204	0	0	0	0	1	0	1
5986	0	0	0	0	1	0	1
363	0	0	1	0	1	0	2
1059	1	0	0	0	0	0	1
11757	0	0	0	0	2	0	2
179E	0	1	1	1	0	0	3
13 Ruchill							
409-62	1	2	0	0	0	0	3
1321-62	0	1	0	0	2	1	4
4574-62	1	1	0	0	0	0	2
4704-62	1	1	0	0	0	0	2
130-63	0	2	0	0	0	0	2
1647-63	1	1	1	0	0	0	3
1833-63	1	1	0	0	1	2	5
4462-63	0	1	1	0	0	0	2
1376-64	0	1	0	0	0	0	1
1192-65	1	0	0	0	1	0	2
1767-65	0	0	2	1	1	0	4
2030-65	0	0	0	0	4	2	6
4411-65	0	0	0	0	2	0	2
Untyped	10	1	5	2	14	5	37
Total rhinoviruses	24	14	11	4	32	10	95
Cases examined	60	87	107	31	402	113	800

ARD = acute respiratory diseases
CB = episodes in chronic bronchitics
SC = symptomless contacts
D = diarrhoeal illnesses

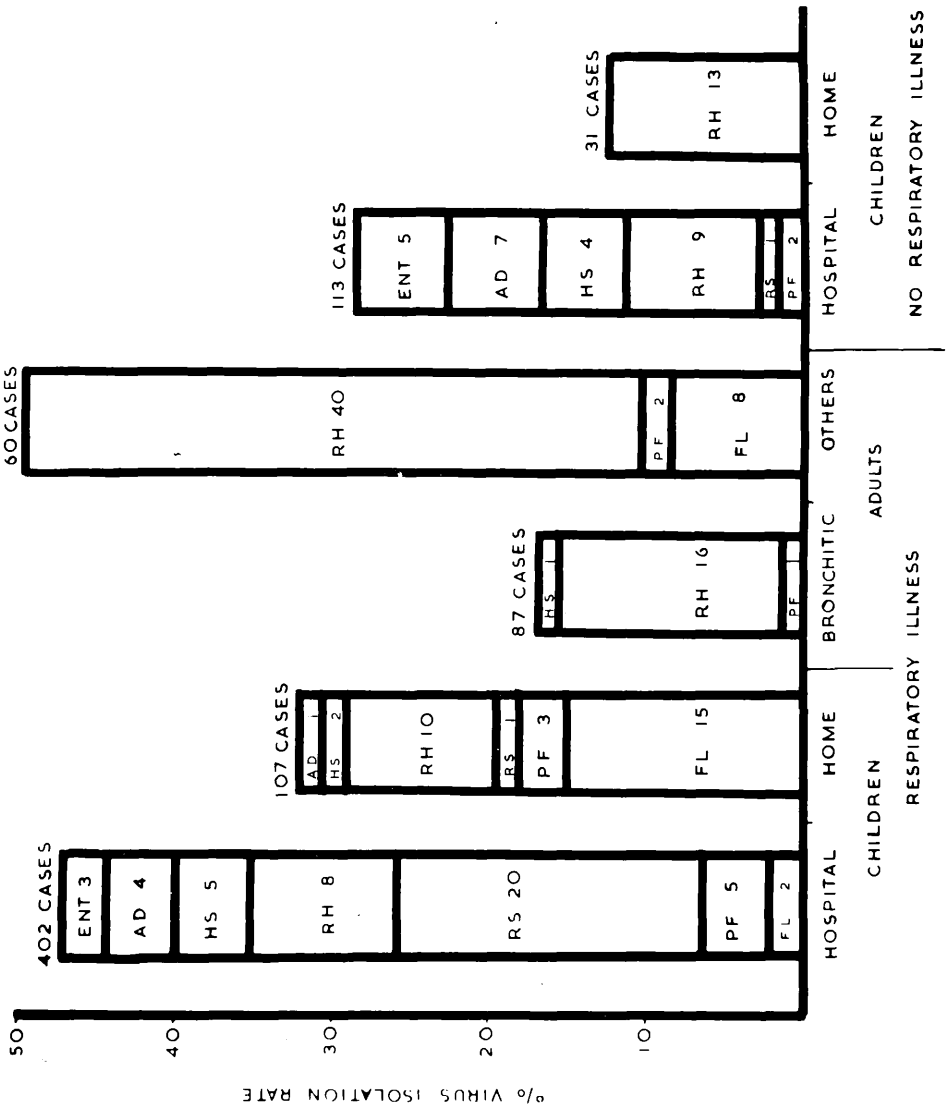

Fig. 12.1. Frequency of viruses isolated from various populations with or without respiratory illness. Based on 302 viruses isolated from 800 persons.

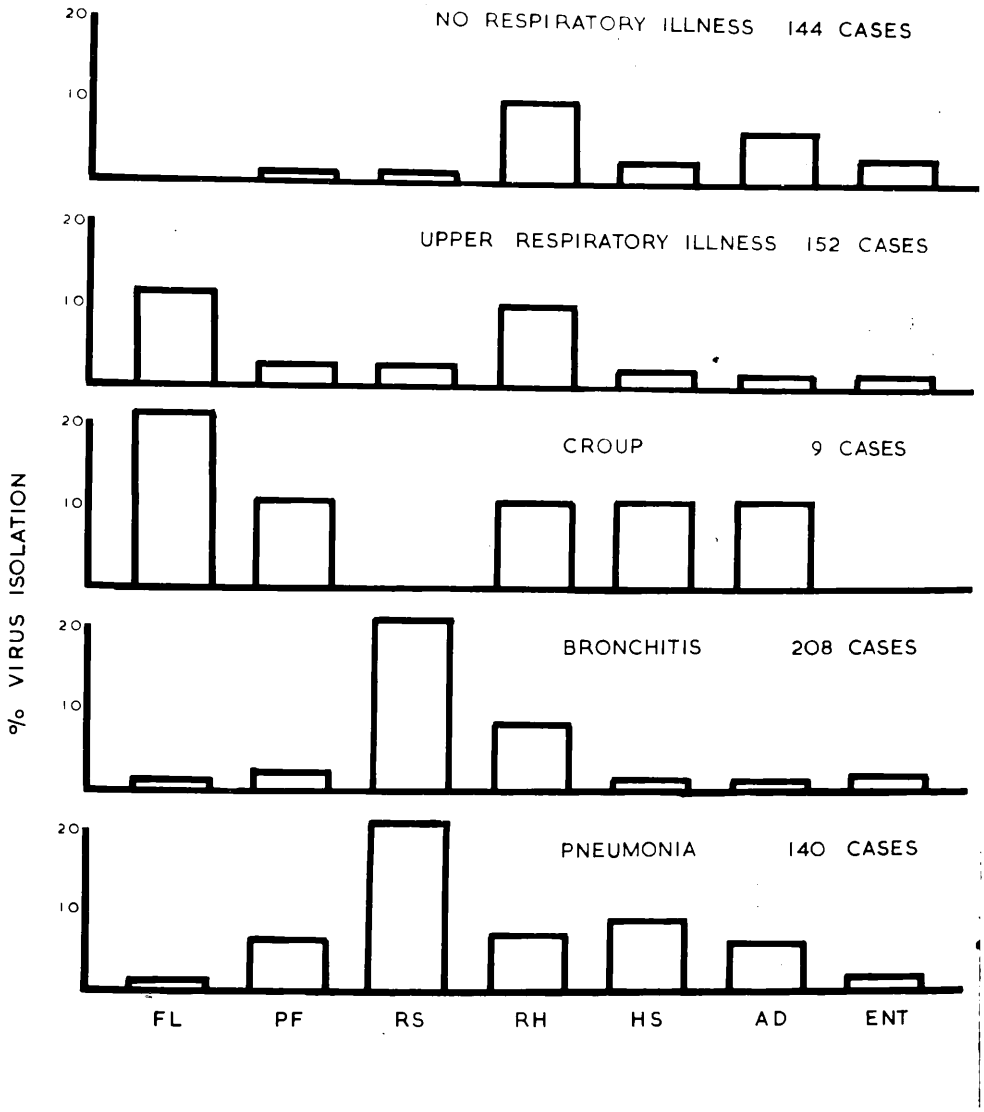

Fig. 12.2. Frequency of viruses isolated from various illnesses of children. Based on 256 viruses isolated from 515 children in hospital and 138 children at home. Bronchitis includes cases of bronchiolitis; pneumonia includes cases of bronchopneumonia.

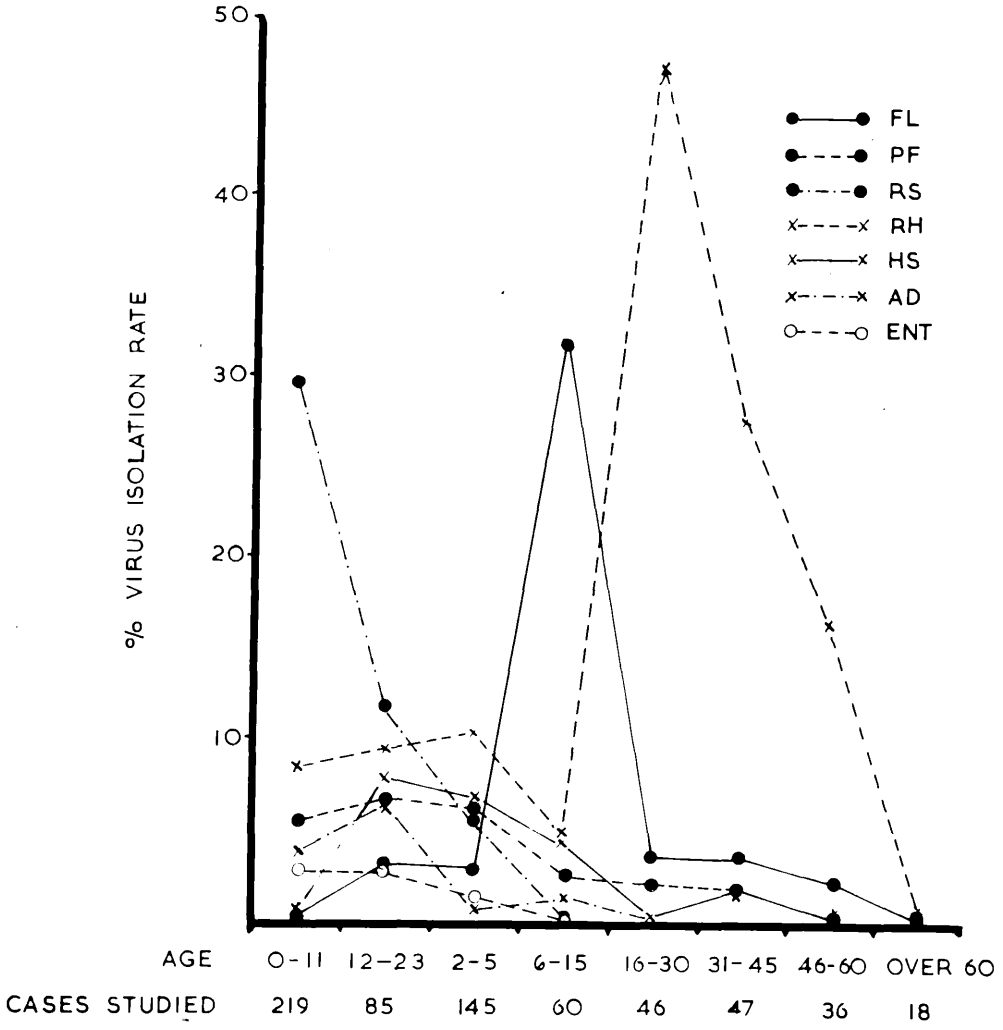

Fig. 12.3. Frequency of viruses isolated from various age groups of 656 persons with respiratory illness. Age groups 0-11 and 12-23 refer to months, others refer to years.

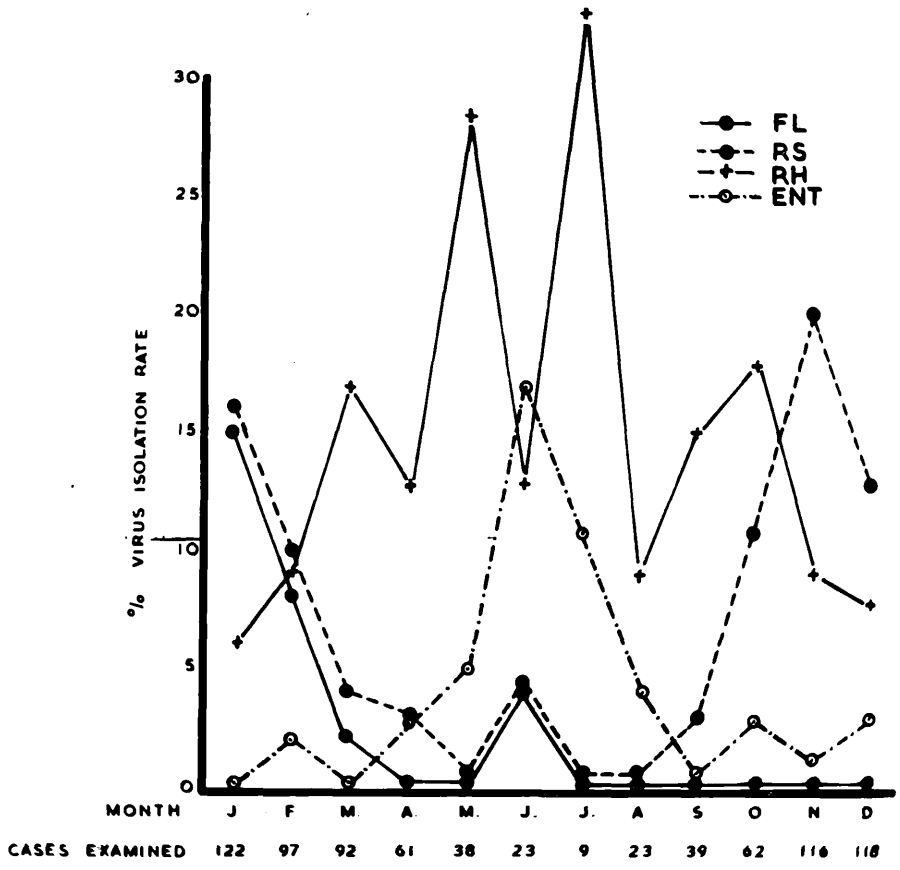

Fig. 12.4. Seasonal distribution of influenza viruses, RS virus, rhinoviruses and enteroviruses isolated from 800 patients, January 1962 to April 1966.

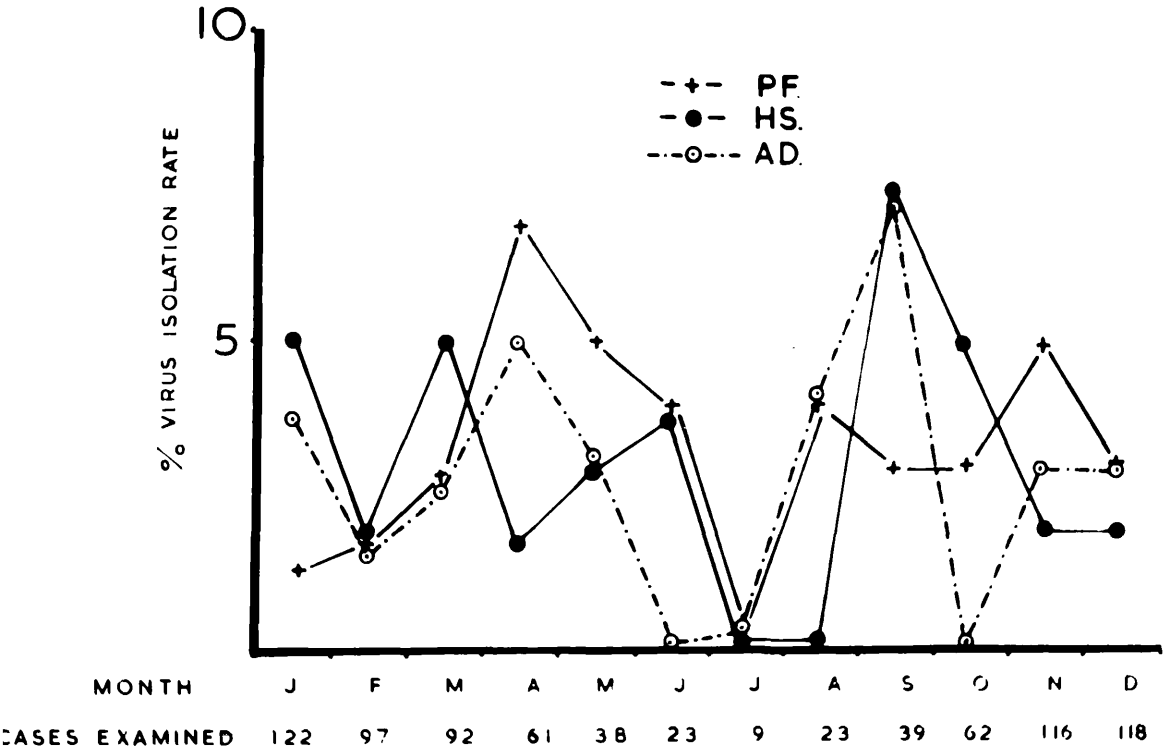

Fig. 12.5 Seasonal distribution of parainfluenza viruses, herpes simplex virus and adenoviruses isolated from 800 patients, January 1962 to April 1966.

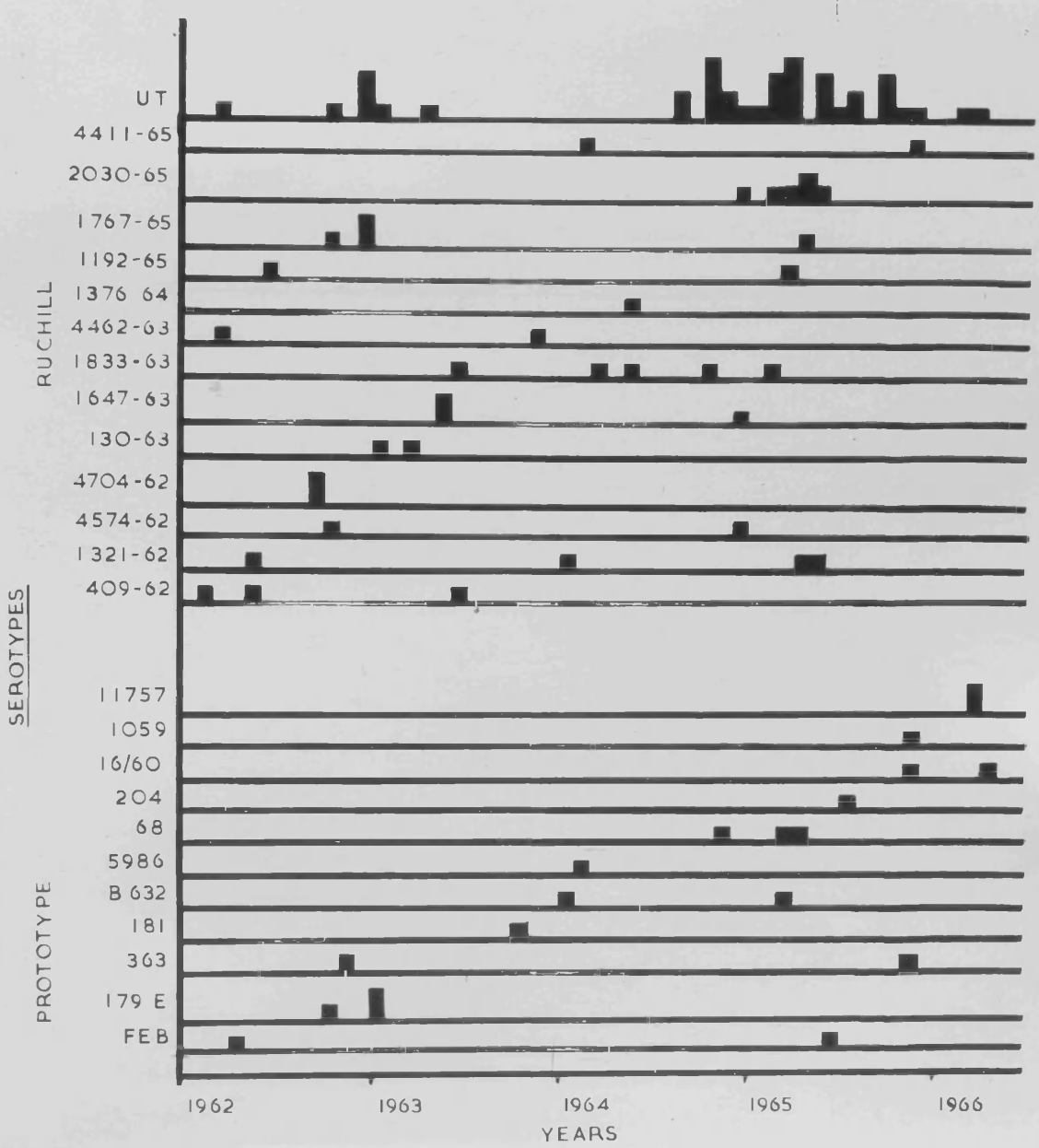

Fig. 12.6 Monthly incidence of 24 rhinovirus serotypes, January 1962 to April 1966. UT = untyped strains.