

A STUDY OF THE ANTIGENIC RELATIONSHIP OF PARAINFLUENZA VIRUSES
WITH PARTICULAR REFERENCE TO SENDAI AND HA-2

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Abstract

The antigenic relationships among the parainfluenza viruses, Sendai, HA-2, CA, HA-1, mumps and NDV were investigated by various serologic tests. For these studies sera from guinea pigs infected intranasally with these viral agents, and sera from rabbits hyper-immunized by inoculation with these agents were used. Complement fixation (CF) tests and haemagglutination inhibition (HI) tests were performed on the guinea pig convalescent sera. However, on the hyper-immune rabbit sera, only HI and neutralization tests were performed, since preliminary experiments showed that sera from rabbits injected with crude virus fluid, or with zinc acetate precipitated virus, contained CF antibody to host tissue in which the viral agents had been propagated.

For the study of immune response to Sendai virus infection, two groups of guinea pigs were selected. One group was selected on the basis of a negative CF antibody test to the viral agents under study. The other group was similarly selected by means of HI tests. It was found that the HI test was a better criterion than the CF test since HI antibody-free animals were shown always CF antibody-free whereas the reverse was not true. Comparison of the immune response of these two groups of animals indicated that only a small proportion of animals with pre-existing HI antibody showed a homologous or heterologous rise in HI titres though all animals in this group showed rises in CF antibody to Sendai and HA-2, and in a few instances also to HA-1. Animals with no HI or CF antibody, on the other hand, showed rises in both CF and HI titres to Sendai, but only 50% of animals gave heterotypic CF and HI responses to HA-2.

No development of crossing antibody to HA-1 was observed.

A similar study was made of the immune responses to HA-2 infection. Like the response to Sendai, animals with pre-existing HI antibody showed only a homologous CF response with a small number showing a rise in homologous or heterologous HI titres. The incidence of heterotypic responses was less than that observed after Sendai infection; here only 2 in a group of 7 gave crossing CF and HI titres to Sendai. There were 4 showing crossing CF titres to HA-1. All 8 animals, demonstrated antibody-free at the time of HA-2 infection, had responses to HA-2 and Sendai by both CF and HI tests. Two of these animals showed a rise of HI titres to HA-1. It was concluded that HI antibody-free animals were better suited for the present study than CF antibody-free animals as their response was demonstrable by both CF and HI tests.

Four groups of guinea pigs were selected on the basis of negative HI tests. All the animals were subsequently shown to be negative by the CF test with the exception of two. Each group was infected with HA-1, CA, NDV or mumps virus. With the exception of 3 animals, the responses as shown by CF and HI tests were specific. Two animals in a group of 10 infected with HA-1 showed heterotypic rises of HI titre to mumps. These were 2 animals with a low level of CF antibody to HA-1 and HA-2 at the time of infection. One animal infected with CA gave a heterotypic CF response to Sendai and HI responses to Sendai and mumps. The results indicated that these 4 agents were antigenically distinct.

An experiment was undertaken to test whether the presence of the small portion of guinea pigs showing no reciprocal crossing antibody to Sendai and HA-2 could be accounted for by the presence

of a similar antigen in the viral agents and guinea pig tissues. Hyperimmune sera were prepared in guinea pigs and in rabbits against guinea pig lungs, spleen, liver and kidney. No increase of antibody to the viral agents under study was demonstrated in these immune sera.

Studies on the hyperimmune response of rabbits to this group of viruses by HI tests showed reciprocal cross reactions between Sendai and HA-2 on the one hand and between mumps and CA on the other. Rabbits immunized against Sendai gave crossing titres to HA-1. However, by neutralization tests only Sendai sera reacted with HA-2 and mumps sera reacted with CA.

The effect of absorption of animal and human sera with Sendai virus was studied. Sendai virus removed Sendai HI antibody, present as a result of homologous responses, from guinea pig convalescent sera. Similar antibodies to HA-2 were also removed. Absorption with Sendai virus had no effect on the titres to CA and HA-1 produced as a result of a homologous response. However, mumps and CA antibody formed as an heterologous response to Sendai infection were absorbed. From Sendai hyperimmunized rabbit sera, Sendai virus removed only antibody to Sendai and had no effect on the heterotypic antibodies to HA-2 and HA-1. From a group of human sera containing HI antibody to Sendai, HA-2, HA-1 and CA, Sendai absorption removed all these antibodies. These results illustrate the complexity of the parainfluenza virus antigens in different animal species, and serve to emphasize the difficulties in comparing serological data between humans and experimental animals.

Sendai virus was shown to have the ability to sensitize

erythrocytes for subsequent agglutination by homologous guinea pig immune sera. The altered erythrocytes were agglutinated by immune sera to CA, HA-2, HA-1 viruses and guinea pig organs, though in all cases pre-infection sera produced no agglutination. The agglutinins were stable to heating at 56°C for 30 minutes and were not removed by Kaolin. However, the agglutinins were removed by absorption with Sendai virus. As a large number of sera from patients with autoimmune disease showed a similar ability to agglutinate such altered erythrocytes, and furthermore, as there appeared to be no association between the agglutination titres and the anti-viral titres in immune animal sera, the reaction was considered unrelated to the immune response to viral infection.

A new serological test, the cell blocking test (CBT), for this group of viruses is described. Cell cultures were inoculated, and after 30 minutes of viral absorption, the inoculum was removed. The cultures were then overlaid with maintenance medium containing convalescent guinea pig serum. After incubation for four days the cultures were examined by haemadsorption. Infected cultures maintained in the presence of homologous viral antiserum gave negative haemadsorption tests. Haemadsorption could be obtained, however, on further incubation after replacing the antibody-containing medium from the cultures with medium free of antibody. The antibody reacting in the CBT was shown to be different from those in CF, HI, neutralization and haemadsorption inhibition tests. An attempt was made, without success, to locate the site of interaction between the antibody and the infected cells in CBT by means of electron microscopy using ferritinized antibody. Antigenic analysis by means of CBT showed that HA-1, CA, mumps and

NDV were specific whereas a reciprocal crossing was demonstrated between Sendai and HA-2 with guinea pig sera, a portion of which had been shown negative by CF and HI tests.

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INTRODUCTION

The isolation of the myxovirus "Sendai" by means of embryonated eggs inoculated with lung tissue extract from mice which had been infected intranasally with human lung tissue from a fatal case of pneumonitis was first reported in 1953 (Kuroya, Ishida and Shiratori 1953). Since 1955, increasing reliance has been placed on the use of cell cultures for virus isolations, in place of fertile eggs. This has led to the detection of several new myxoviruses. The first of these agents, Croup-Associated (CA) virus, unlike influenza and mumps viruses, proved difficult to adapt to the egg, although it multiplied in human and monkey cells in culture, producing cytopathic effects either initially or upon serial passage (Chanock 1956; Beale, McLeod, Stackiw and Rhodes 1958; Chany, Daniel, Robbe-Fossat and Vialette 1958; McKinnay, England and Froade 1959). The adaptation of viral haemagglutination to cell culture techniques (haemadsorption) at the time of the influenza pandemic in 1957, greatly facilitated isolation of similar agents. This enabled Chanock and his co-workers to recover two new viruses, Haemadsorption Type 1 (HA-1) and Haemadsorption Type 2 (HA-2), from the respiratory tract of children (Chanock, Parrott, Cook, Andrews, Bell, Reichelderfer, Kapikian, Mastrota and Huebner 1958). With the same technique, Johnson and co-workers isolated another haemadsorption virus, M-25, from the respiratory tract of children (Johnson, Chanock, Cook and Huebner 1960). Recently a human haemadsorption virus, DA, was reported to have been recovered from human blood in 1961 (Hsiung 1961).

For these new agents, the designation "Parainfluenza Viruses" has been proposed (Andrews, Bang, Chanock, and Zhdanov 1959; Hsiung

1963), namely, Parainfluenza 1, 2, 3, 4, 5 for Sendai and HA-2, CA, HA-1, M-25 and DA respectively. The parainfluenza viruses share with mumps and Newcastle disease viruses several properties that distinguish them from the influenza and fowl plague viruses. These points of distinction may be summarized as follows: (1) The viruses of the parainfluenza group are larger than those of the influenza group (Horne and Wildy 1961) and of different structure (Waterson 1963). (2) The parainfluenza viruses are unrelated serologically to influenza A, B, C or fowl plague viruses. They are also distinct from one another, as demonstrated by the homologous serological response of infected guinea pigs and of young children after primary infection. However, there appear to be some antigens shared among the Parainfluenza viruses judging from the varied heterotypic antibody response of children and adults to infection with these agents (Cook, Andrews, Fox, Turner, James and Chanock 1959; Health, Tyrrell and Peto 1962). (3) There is some evidence that multiplication of Sendai, mumps and Newcastle disease viruses occurs entirely in the cytoplasm, whereas the soluble antigens of fowl plague and influenza A have been demonstrated in the nucleus (Breitenfeld and Schafer 1957; Traver, Northrop and Walker 1960). (4) Viruses of the parainfluenza group are capable of haemolyzing erythrocytes from a variety of animal species, whereas influenza viruses do not possess a haemolysin (Cook et al. 1959). (5) The parainfluenza viruses often induce the formation of syncytia in primary cell cultures and in continuous cell lines while influenza viruses do not.

The parainfluenza group of viruses presents a number of interesting features. Firstly, except in Japan and Russia, Sendai

virus has never been isolated from humans since its initial recovery in 1953, despite the apparent world-wide distribution of human antibody that reacts with Sendai virus (Kuroya et al. 1953; Gorbunova, Gerngross, Gnorizova and Bukrinskaya, 1957; Zhdanov, Ritova and Golygina 1957; Jensen, Minuse and Ackermann 1955; Brown, Oligschlaeger, Legier, Schmit 1960; Gardner, Stanfield and Wright 1960; DerVeen and Smeur 1961; LA NACA and Moscovici 1961; Wilt, Hildes, Parker and Delaat 1961; Jensen, Peeles and Dulworth 1962; Health, Tyrrell and Peto 1962). In studies of the antigenic relationships among NDV, Sendai and mumps viruses, two contradictory observations have been made. On the one hand, antigenic relationship is demonstrated (among the three viruses) when convalescent sera from patients who have suffered NDV or mumps infections are tested (Kilham, Jungherr and Lunginbuhl 1949; Jungherr, Lunginbuhl and Kilham 1959; Evans 1954; Bang and Foard 1956; Jordan and Feller 1950; De Meio and Walker 1957; Gardner 1957; Gardner et al. 1960). On the other hand, no relationship has been found among these viruses when immune and convalescent sera from laboratory animals are used. The sole exception in this respect is the observation of Bartolomei-Corsi (1959). She immunized guinea pigs by intraperitoneal inoculation with live mumps virus. In these sera she demonstrated the presence of antibodies by complement fixation, haemagglutination inhibition and neutralization tests, with both mumps and Sendai.

Secondly, some parainfluenza viruses cause appreciable respiratory disease, although unlike influenza virus they have not caused pandemics. The clinical condition from which parainfluenza viruses are most frequently isolated, is acute laryngotracheobronchitis,

one of the causes of croup (Laxdal, Robertson, Braaten and Walker 1963). In children these agents, especially HA-1 virus, may cause bronchiolitis and pneumonia (McKinney et al. 1959; Parrott, Vargosko, Luckey, Kim, Gunning and Chanock 1962; Lelong, Vialette, Cotlenko, Chang and Nodot 1959). When serological tests were used for diagnosis the role of these viruses in the aetiology of respiratory diseases in children appeared even more prominent (Chanock, Bell and Parrott 1961).

The role of these viruses in causing respiratory disease in adults is at present less clear. They have been recovered from adults with respiratory disease, and HA-1, HA-2 and CA have induced common cold-like symptoms in adult volunteers (Reichelderfer, Chanock, Craighead, Huebner, Turner, James and Ward 1958; Tyrrell, Bynoe, Birkum, Peterson, Sutton and Pereira 1959; Taylor-Robinson 1963).

Thirdly, the role of humoral antibody in preventing infection and illness presents another interesting feature in this group of viruses. It is apparent from the data on natural infection of children with HA-1 and exposure of adult volunteers to HA-2, HA-1 and CA viruses that infection occurred in some individuals with a high titre of neutralizing antibody. These individuals had respiratory disease, virus was recovered from the pharynx and a subsequent increase in antibody, in some cases, was demonstrated (Chanock et al. 1961; Reichelderfer et al. 1960; Tyrrell et al. 1959; TAYLOR-ROBINSON).

In three different outbreaks of respiratory illness due to HA-1 virus in a welfare nursery this agent was recovered on two separate occasions from 16 to 20% of the affected children, though third infections were not observed (Chanock et al. 1961). It is uncertain whether this was due to reinfection or to persistence of

the virus in the host's tissues. It is of some interest that persistent infection of cells in culture with HA-1 has been reported (Henle, Deinhardt, Bergs and Henle 1958; Chany 1961). Moreover, it has been shown that it has been possible to maintain a line of infected KB cells for over two years, and, despite the presence of hyperimmune antiserum in the medium for six months, HA-1 virus could be repeatedly isolated from chronically infected, but intact cells upon removal of the antiserum (Daniel and Chany 1962). However, it must be noted that only a few viruses have been shown persistent in vivo compared to the number of viruses that have been demonstrated capable of chronically infecting tissue cultures.

Finally, although two detailed serological analyses have been reported (Cook et al. 1959; Bukrinskaya 1960) on this group of viruses, the interpretation of these results was made difficult by the fact that the sera employed in these studies were prepared by injection of "purified" viral material into animals which had been previously infected. It is noted that immunization of guinea pigs with normal chorioallantoic fluid has been shown to give rise to antibody reacting in haemagglutination inhibition tests with mumps virus and in complement fixation tests with Sendai, NDV and mumps viruses (Blaskovic et al. 1961). Nevertheless, data in these two studies substantiated in part the serological observations made incidentally to the reports of initial and subsequent isolations.

Accordingly, an investigation was undertaken firstly, to elucidate the antigenic structure of the parainfluenza viruses, secondly, to demonstrate the sharing of common antigens, if any, among these viruses, and thirdly, to study the interaction between these viruses and their homologous antibody in a tissue culture system.

Review of LiteratureInitial Isolation, Characterization
and Subsequent Isolation of Sendai Virus

Sendai virus was first reported to have been isolated in Japan in 1952 from the lungs of a child who had died of pneumonia (Kuroya, Ishida and Shiratori 1953). Isolation by tissue culture was unsuccessful and the primary isolation of the agent was made by intranasal instillation of the lung tissue into mice. Upon the first passage in mice, influenza-like lesions of the lung developed. When extracts of infected mouse lung were transferred to embryonated eggs, both amniotic and allantoic fluids caused agglutination of chicken erythrocytes in the pattern characteristic of influenza virus. Like influenza virus, Sendai was shown to be adsorbed to red blood cells at 4°C and eluted at 37°C. In the receptor gradient, Sendai fell between Newcastle disease virus (NDV) and the FM₁ strain of influenza A. Using haemagglutination inhibition tests, Sendai was shown to be antigenically distinct from mumps, NDV, influenza A, A-prime, B and C viruses. Electron microscopic study revealed that Sendai virus was spherical and was 150 to 200 m μ in diameter.

"Perbronchial" inoculation of Sendai virus into an eight-year old girl resulted in pneumonia with much less severe symptoms than those of the infant from whom the viral agent was initially recovered. The virus was recovered from the girl's throat washings and specific antibody was demonstrated in her convalescent serum. Kuroya and his co-workers considered the disease a new form of virus pneumonitis and termed it "Newborn Virus Pneumonitis (Type Sendai)".

Later in 1952, Misao (Misao 1953) isolated the Akitsugu strain

in mice from an adult human during an epidemic of influenza A-prime. Again, in 1953, Chashi (Chashi 1953) obtained the Tochigi and three other strains by inoculation of throat washings into embryonated eggs during an epidemic of influenza A-prime. In 1954, Fukumi and co-workers reported the isolation of 12 strains of a virus, known as "pneumotropic virus of mice" (PVM), during mouse passage of influenza viruses. These strains were shown to be antigenically and biologically similar to Sendai virus (Fukumi, Nishikawa and Kitayama 1954). Further work indicated that this virus was endemic in mice in Japan (Nishikawa and Fukumi 1954), and was also the cause of a febrile disease of pigs (Sasahara et al. 1954). Also, natural infection with Sendai virus in mice was observed in Russia (Bukrinskaya, Ho Yun-de and Gorbunova 1962), and in guinea pigs and mice in China (Chin-Hsieu 1960; Sun and Fang 1960; Sun, Fang and Wong 1960).

However, by direct egg inoculation with throat washings, in an outbreak of influenza-like illness in Vladivostok in 1956, Gerngross (Gerngross 1957) isolated five strains of Sendai virus from adult humans. In the following year, Zhdanov recovered four more strains of Sendai virus in embryonated eggs in an outbreak in Moscow Children's Hospital (Zhdanov, Ritova, Golygina 1957). In the same year four other isolations were reported from various localities in Russia, (Gorbunova 1958).

Initial Isolation, Characterization and Subsequent
Isolations of Group Associated (CA) Virus

In 1956 Chanock (Chanock 1956) reported the isolation of a new strain of haemagglutinating virus from two of 12 infants with croup in Cincinnati, using monkey kidney tissue cultures inoculated with throat-swab material. Chanock gave the name Group Associated, or CA, to this new virus. It produced a cytopathic effect, causing the cells to form focal syncytia that progressed to sponge-like masses. A period of 10 to 15 days was required for the appearance of the cytopathic effect when the culture was inoculated with throat-swab material. This period was shortened to 3 to 5 days when inoculated with first passage fluid. Multiplication of CA virus occurred in the amniotic cavity though not in the allantoic cavity of embryonated eggs. By filtration, the size of CA was estimated to be between 90 and 135 m μ . Like influenza, CA was sensitive to ether. It agglutinated human and fowl erythrocytes and the cell receptors for CA agglutination were destroyed by treatment with Vibrio cholerae filtrate containing receptor destroying enzyme (RDE). Using specific serum from guinea pigs that had been infected intranasally with infected tissue culture medium, in haemagglutination inhibition and complement fixation tests, CA virus was shown to be distinct antigenically from influenza A, B and C viruses, Sendai virus, mumps virus, Adenovirus, Herpes, Distemper and Reovirus.

CA virus agglutinated one-day-old chick red cells at 4°C. The agglutinated red cells dissociated readily at 37°C, but re-suspension and sedimentation of the same cells at 4°C restored the positive pattern. In the receptor gradient, CA virus was found to

precede NDV, Sendai and influenza A and B viruses, since erythrocytes whose receptors had been removed by CA virus were fully agglutinable by the latter four viruses.

In 1958 Beale and co-workers (Beale, McLeod, Stackiw and Rhodes 1958) reported the isolation of ten strains of an agent associated with croup in fifteen infants in Toronto in 1955. The ten strains proved to be serologically identical with CA virus. Other strains isolated from infants with respiratory diseases in North America were reported by Shelokov, by Vargosko, by Cramblett and by McLean (Shelokov, Vogel and Chi 1958; Vargosko, Chanock, Huebner, Luckey, Kim, Cuning and Parrott 1959; Cramblett 1958; McLean, Edward, McQueen, Petite 1962; Kapikian, Bell, Mastrota, Huebner, Wong and Chanock 1963). Isolation of CA from adults with respiratory disease was reported by Mogabgab in North America and by Craighead in the Canal Zone (Craighead, Shelokov, Peralta and Vogel 1961; Mogabgab, Dick and Holmes 1961). Elsewhere, CA strains were isolated from children in England by Gardner and by Pereira, in Australia by Forbes and in Russia by Morozenko and by Bukrinskaya (Gardner, Stanfield, Wright, Court and Green 1960; Pereira and Fisher 1960; Forbes 1960; Morozenko 1961; Bukrinskaya and Blyumental 1962).

Initial Isolations, Characterization andSubsequent Isolations of HaemadsorptionVirus Type 2 (HA-2) and Haemadsorption Virus Type 1 (HA-1)

Using the then new haemadsorption technique in tissue culture, Chanock and his co-workers reported the isolation of two new viruses from the throat swabs of infants with croup in 1958 (Chanock, Parrott, Cook, Andrew, Bell, Reichelderfer, Kapikian, Mastrota and Huebner 1958). The viruses were given the names Haemadsorption type 1 (HA-1) and Haemadsorption type 2 (HA-2).

Initial characterization of these viruses showed that they were both sensitive to ether. Two of the seven HA-1 strains multiplied in the amniotic cavity of seven-day-old embryonated eggs but only one of the three HA-2 strains multiplied in the same manner. HA-1 and HA-2 produced different effects in monkey kidney tissue culture; HA-1 infected cells showed detachment from the cell sheet followed by an elongation of the cells while HA-2 infection was characterized by the appearance of small scattered round cells, followed by a focal loss of cells. A further difference was demonstrated between HA-1 and HA-2 in their preferential red cell agglutinations. Though both viruses agglutinated fowl and guinea pig erythrocytes, HA-1 agglutinated guinea pig red cells at higher titres whereas HA-2 gave higher titres with chicken red cells. RDE from Vibrio cholerae filtrate removed receptors on erythrocytes for both viruses. Using hyperimmune rabbit sera in haemagglutination inhibition and neutralization tests, HA-1 and HA-2 were shown to be serologically distinct from each other.

Using CF tests on the human sera of cases from which the viruses were recovered, HA-1 sera showed no cross-reaction with Sendai, mumps

or CA while HA-2 sera gave a reaction with Sendai virus only. Similar serological reactions were demonstrated in CF as well as HI tests with sera from immunized animals. At the same time, both HA-1 and HA-2 were shown to be completely distinct serologically from influenza A, B and C and from NDV.

The initial recovery of HA-2 was from 3 infants with croup. In a subsequent study of 1654 children with respiratory illness, HA-2 was isolated from 3.6% (Chanock et al. 1959). Meanwhile, in Denmark, a virus named Copenhagen 222 was recovered from a child with an influenza-like illness (Petersen Magnus 1958). This virus was later shown to be identical with HA-2 (Sulton, Clarke, Tyrrell 1959). In England, between 1959 and 1960, 3 strains of HA-2 were recovered (Holland, Tamer, Pereira and Taylor 1960) and recovery of one strain from a child with upper respiratory illness was reported in Japan (Fukumi, Nishikawa, Sugiyama, Yamaguchi, Nanba, Masuura and Oikawa 1959). In Russia, isolation from children with croup was reported by Bukrinskaya and by Kallinskova (Kallinskova and Morozenko 1961; Bukrinskaya and Blyumental 1962). In Canada, isolation from children with croup was reported by McLean and by Laxdal (McLean, Edward, McQueen, and Petite 1962; McLean, Roy, O'Brien, Wyllie and McQueen 1961; Laxdal, Robertson, Braaten and Walker 1963).

Subsequent to their initial isolation of HA-1 virus, Chanock and his co-workers, in a study of 1654 cases of respiratory illness in children, reported isolation of HA-1 virus from 2.6% compared with 0.14% from the control group with no apparent respiratory symptoms (Chanock et al. 1959). In California, two strains of HA-1 virus were recovered from adults with influenza-like diseases (McKinney, England

and Froede 1959). Elsewhere, isolations of the virus from patients with croup were reported in Australia (Ferris 1960), in England (Sulton, Clarke, Tyrrell 1959), in France (LeLong, Vialette, Gotlenko, Chany and Nodot 1959; Chany, Daniel, Robbe-Fossat, Vialette, Lepine and LeLong 1958), and in Canada (McLean et al. 1961; McLean et al. 1962; Laxdal et al. 1963).

A virus, named Mill's agent, isolated repeatedly from uninoculated HeLa-cell cultures, was shown to be identical with HA-1 (Chanock et al. 1958). In France, an agent identical with HA-1 was isolated from uninoculated primary monkey kidney tissue culture (Chany et al. 1958). These agents have no known aetiological significance as a cause of respiratory illness.

Another viral agent, SF-4, recovered from cattle with shipping fever, has been shown to possess properties similar to HA-1 virus (Reisinger, Heddleston and Manthei 1959). The original comparison of HA-1 and SF-4 viruses indicated that they were antigenically indistinguishable (Abinanti and Huebner 1959). Further studies, however, showed that the viruses could be differentiated by the use of convalescent guinea pig sera (Abinanti, Chanock, Cook, Wong and Warfield 1961). Over the past few years, bovine isolates obtained from different geographic localities have been shown to be similar to the prototype strain of SF-4 (Abinanti, Byne, Watson, Poelma 1960; Bako and Dinter 1960; Ketler, Hamparian and Hilleman 1961; Gale and King 1961).

Initial Isolation and Characterization of M-25

Isolation of a viral agent from throat swab material from a young adult with respiratory illness was reported by Johnson and co-workers (Johnson, Chanock, Cook and Huebner 1960). This agent was given the name M-25. Initial characterization of M-25 established its sensitivity to ether. As with CA virus, haemagglutination of guinea pig erythrocytes by M-25 at 4°C and at 22°C was reversed at 37°C. M-25 virus grew readily in primary monkey kidney cells, and also in primary kidney cells from bovines and hamsters. Unlike the influenza viruses, M-25 did not grow in embryonated eggs. By filtration, the size of M-25 was estimated to be 200 to 250 m μ . By haemagglutination inhibition and CF tests, with rabbit and guinea pig sera, M-25 virus was shown to be serologically distinct from influenza, Sendai, HA-2, CA, HA-1, Vaccinia and Herpes viruses and also NDV and Reoviruses 1, 2 and 3. However, a titration of 7 pairs of acute and convalescent sera from adults with clinical mumps infection showed a rise in titres for M-25 virus by CF and neutralization tests.

The role of M-25 virus in respiratory disease is not clear at the present time. Johnson, who isolated 30 strains (of M-25) in 1958 reported no further isolations in the 5 subsequent years of a continued survey (Johnson 1963). However, a causal relationship with human disease was suggested by the report of the isolation of M-25 virus from children with respiratory illness but none from the control group (Parrott 1963).

Initial Isolation and Characterization of DA Virus

A viral agent was isolated by Hsiung in 1959 from the post-mortem blood sample of a patient who had died of acute viral hepatitis (Hsiung 1961). The agent, designated DA virus, was found to have biological properties similar to those of the mumps-NDV group, and was classified as a myxovirus (Hsiung, Isacson and McCollum 1962). DA virus grew in the amniotic cavity of fertile eggs, and in a variety of primate renal cell cultures including those from humans, rhesus, patas and grivet monkeys and baboons. DA virus was ether-sensitive and its size was estimated to be between 100 m μ and 300 m μ . It agglutinated chick, goose, guinea pig, sheep and human red cells, and showed a higher haemagglutination titre at 4°C than at 37°C.

DA was shown to share two distinctive properties with the mumps-NDV sub-group of myxoviruses. Like mumps and NDV, DA virus gave a higher titre on reshaking and resettling of virus-erythrocyte mixture after their initial settling. When erythrocytes on which DA viral agglutinin had been absorbed and eluted were washed and added to fresh untreated erythrocytes, agglutination was observed. Like mumps and NDV, DA virus was shown to sensitize erythrocytes to agglutination by high dilutions of sera from animals immunized against DA virus.

DA virus was shown to be antigenically identical with SA virus (Schultz and Habel 1959), a myxovirus isolated from human nasal washings in fertile eggs, and with SV5 virus, an agent obtained from normal uninoculated rhesus monkey kidney cultures (Hull, Minner and Smith 1956). Initial characterization showed that specific hyper-immune animal sera prepared against influenza, HA-2, HA-1, CA and M-25

viruses, did not react with DA virus (Hsiung, Isacson, McCollum 1962). Recent evidence indicates a close antigenic relationship between mumps and DA virus, and in addition primates immunized with DA virus have been shown to yield antibody reacting with HA-2, CA and HA-1 virus (Hsiung, Chang, Guadrado and Isacson 1965).

Nomenclature and Classification

When Kuroya and his co-workers (Kuroya et al. 1953) reported the isolation of a new viral agent causing pneumonitis in new-born infants, no name was given to the agent, and it was subsequently referred to by the Japanese investigators as Newborn Pneumonitis Virus. When strains were isolated from mice, they were given the name Haemagglutinating Virus of Mice, (Fukumi, Nishikikawa and Kitayama 1954). In 1956, the Nomenclature Committee of the Society of Japanese Virologists adopted the name Haemagglutinating Virus of Japan, (HVJ) for this agent (Tadoko, Suzuki and Fukazawa 1958). Since that time Japanese investigators have used this nomenclature. Another name, Type D Influenza, was suggested for this agent on the basis of its similarity to mumps, NDV and influenza viruses (Jensen, Minuse and Ackermann 1955).

In 1959, Andrews and co-workers (Andrews, Bang, Chanock and Zhdanov 1959) proposed to include these "newer" viruses in the Myxovirus group and to designate them as parainfluenza viruses. In this proposal, Sendai and HA-2 were assigned to a group designated Myxovirus parainfluenzae 1 (Parainfluenza 1) on the basis of an antigenic crossing shown between these two viruses (Cook, Andrews, Fox, Turner, James and Chanock 1959). CA and HA-1 viruses were designated Myxovirus parainfluenzae 2 (Parainfluenza 2) and parainfluenzae 3 (Parainfluenza 3) respectively. Myxovirus parainfluenzae 4 (Parainfluenza 4) was the name suggested for M-25 virus by the authors who reported its initial isolation (Johnson et al. 1960). Similarly, the authors reporting the initial isolation of DA virus (Hsiung et al.) suggested, at the VIIIth International Congress for Microbiology in 1962, the name Myxovirus parainfluenza 5 for the group

consisting of DA, SA and SV5 viruses.

Though these proposals were approved by the Virus Subcommittee of the International Nomenclature Committee, they have not met with universal acceptance. The main objection appears to be to the grouping of Sendai and HA-2 viruses together under the designation of Myxovirus parainfluenzae 1. Zhdanov, one of the co-authors of the original proposal, pointed out that the biological and ecological differences between the two viruses were great enough to justify separate names, in spite of the antigenic crossing (Zhdanov and Bukrinskaya 1960). In a comparative study of Sendai and HA-2 viruses, the Japanese investigators (Fukumi and Nishikawa 1961) substantiated the differences pointed out by the Russian workers, and contended that the two viruses should be separated from each other. Further work by the Russian investigators revealed the antigenic heterogeneity among strains of Sendai and strains of HA-2. Two variants of Sendai virus, the Japanese variant and the Vladivostok variant, were recognized on the basis of an analysis of 24 strains of Sendai virus isolated in the U.S.S.R., Japan and China in the period from 1950 to 1959 (Ho Yun-de and Gorbunova 1961). On the basis of the CF test, using sera against the Japanese variant of Sendai virus, HA-2 strains were divided into two groups, the North American strains which gave a positive reaction, and the Moscow strains which gave a negative reaction (Bukrinskaya, Ho Yun-de and Gorbunova 1962). Furthermore, neither the American nor the Moscow strains of HA-2 virus reacted with antisera against the Vladivostok variant of Sendai virus. Thus, it appears that the antigenic crossing, which formed the basis for the designation of both Sendai and HA-2 as Myxovirus parainfluenza 1, is not universal.

In this thesis, the viruses, for the sake of clarity, will be referred to by the names given on initial isolation, except in the case of Newborn Pneumonitis Virus which will be referred to as Sendai virus.

Properties of Sendai Virus

Initial studies of the Sendai agent indicated it to be roughly spherical in shape and to measure between 150 and 180 m μ in diameter (Kuroya et al. 1953). Further work showed that its morphology was similar to that of mumps and NDV. It was of irregular shape, sometimes elongated like a finger, or sometimes with a tail-like appendix when suspended in Ringer's or saline solution, while in distilled water or fixed in formalin, it appeared as a flattened cube (Nishikawa and Fukumi 1954). The Svedberg sedimentation constant (S_{20}) of Sendai virus was found to be 1200 (Nishikawa et al. 1954, Fukumi 1956; and Tadokoro 1958).

Sendai virus grows rapidly in the amniotic sac as well as the allantoic cavity of 10 to 11-day-old fertile eggs without killing the embryo. However, injection of the virus into the chorio-allantoic cavity of 5 to 6-day-old eggs kills the embryo in 48 hours (Fukae and Suzuki 1955). Inocula of high multiplicity have been reported to yield virus fluids with a low infectivity to haemagglutination ratio (10^2) compared with the high ratio (10^6) in harvests from small inocula, after 48 hours of incubation, suggesting the production of incomplete virus when inocula of high multiplicity are used (Fukai et al. 1955). This observation has been confirmed in a tissue culture system (Bukrinskaya and Zhdanov 1961; and Zhdanov and Bukrinskaya 1962). Also, incomplete Sendai virus has been produced in tissue cultures treated with proflavine (Zhdanov, Bukrinskaya and Azadova 1961).

A study of the growth of the virus in fertile eggs showed that when undiluted chorioallantoic fluid was used as the inoculum, the maximal infectivity titre was attained in 36 hours, compared with a

maximal titre in 48 hours when eggs were inoculated with chorio-allantoic fluid diluted 10^{-7} (Tadokoro 1958). No decrease of infectivity to haemagglutination ratio in the infected fluid was observed when the incubation time was shortened to 36 hours and passages were made with undiluted virus fluid, although other biological changes in the virus were noted. After 20 passages the virus was more readily inactivated by repeated freezing and thawing than was the original virus, and showed less haemolytic activity. Infected fluid produced lesions on the chorioallantoic membrane. These were small and resembled the type of pocks produced by herpes simplex (Jensen, Minuse and Ackermann 1955). Like infected chorioallantoic fluid, extracts of infected membrane were infective and haemagglutinating. Sendai virus has been found to grow well in a variety of primary kidney cell tissue cultures, namely human, monkey, bovine, swine, lamb, dog, mouse, chicken and guinea pig, also in primary kidney tissue cells from tortoise (Shimizu, Ishizaki, Konno, Kumagai, Arai, Sasahara, Ishii and Matumoto 1955; Heath and Tyrrell 1959; Dossena and Bellelli 1961; Shindarov 1962). It has been shown to multiply in a large variety of continuous line cells (Traver, Northrop and Walker 1960; Ho Yun-de 1962a). All Japanese strains of Sendai virus gave cytopathic effects in tissue culture (Fukumi and Nishikawa 1961). The infected cells become elongated and the nucleus shows irregular staining. Large syncytia were formed in all continuous line cells of human origin (Bukrinskaya and Zhdanov 1961) except in the 720 line of human melanoma cells. However this was the only established line in which the cells formed syncytia after infection with the Far Eastern strains 960 and 390, two of the five strains isolated in Vladivostok (Ho Yun-de 1962a).

The growth characteristics of Sendai virus have been studied in HeLa, FL, L and other continuous line cells, and also in primary kidney cells, with respect to the production of infectious particles, haemagglutinins and antigens (Heath and Tyrrell 1959; Ishida and Homma 1960; Lotte and Kirillova 1960; Osato and Ishida 1961). Tissue culture cells infected with Sendai virus, as with other myxoviruses, yielded intracellularly first, complement fixing antigen, haemagglutinins and finally, infectious particles. In mouse fibroblast L cells, complement fixing antigen appeared intracellularly 6 hours after infection. Measurable intracellular haemagglutinins appeared at the 9th hour, with a logarithmic increase in titre which continued up to the 14th hour, after which the titre remained unchanged until the 24th hour. Infectious particles first appeared at the 10th hour and increased at a logarithmic rate up to the 20th hour (Ishida et al. 1960).

Haemadsorption was positive at the 7th hour, 2 hours before the detection of haemagglutinin inside the cells. No soluble complement fixing antigen was detectable in the fluid until 96 hours. Haemagglutinin release was detectable at 18 hours and continued at a logarithmic rate for a period of 60 hours after infection. Release of infectious particles, however, was not detectable until 24 to 36 hours and the maximum titre was reached at 48 hours when the ratio of infectivity to haemagglutinin was greatest. Changes in the morphology of cells began at the time intracellular infectious particles were first detected. When the intracellular infectious titre reached a maximum all cells revealed basophilic margination of the cytoplasm. Essentially similar growth characteristics were

observed in primary kidney cells of various species, including human, monkey, bovine embryo, guinea pig, except that a longer period was required for the release of virus and haemagglutinin into the medium. A still longer period was observed with lung cell cultures of beef, chicken, mouse and hamster.

By the use of specific Sendai-immune horse serum conjugated with fluorescein it was shown that, in cultures of monkey kidney cells Sendai antigen first appeared at 6 hours in the perinuclear region of the cytoplasm. At 18 hours, a high concentration of viral antigen was found in the cytoplasm, in the cytoplasmic inclusions, in the nuclear membrane and in some nuclei. The maximum accumulation of antigen in the cell was observed at 24 hours, at which time antigen was demonstrated also on the cellular membrane (Lotte et al. 1960). However, no antigen was found in the nucleus when a study was made of chick embryo lung and human conjunctival cells infected with Sendai and stained with convalescent animal sera conjugated with fluorescein (Traver et al. 1960). Indeed, only cytoplasmic staining was observed in cells infected with Sendai, NDV or mumps, a feature distinguishing them from cells infected with influenza viruses, which exhibit nuclear staining. The absence of nuclear staining in cells infected with Sendai was observed also in mouse fibroblast cells stained with hyperimmune rabbit or goat fluorescein-conjugated sera (Osato et al. 1961). Sendai antigen appeared in the cytoplasm at 4 hours, in an area adjacent to the Golgi zone, increased in concentration and then became scattered at the periphery of the cells by 16 to 18 hours. However, other workers using the fluorescent antibody technique in primary monkey kidney cells demonstrated antigen in the cytoplasm only, when the

inoculum was 1 EID₅₀ per cell whereas antigen was observed in both the nucleus and the cytoplasm when a larger inoculum of 100 EID₅₀ per cell was used (Zhdanov et al. 1961).

The dynamics of the displacement of the nucleic acid components of P³² or uracil C¹⁴ labelled Sendai virus in infected tissue culture cells was studied by autoradiography (Bukrinskaya, Zhdanov and Ramenskaya 1961). Radioactive grains appeared early after the infection, mainly in the nucleolus, then in the extra nucleolar part of the nucleus, and it was not until 2 hours after infection that radioactive grains were demonstrable in the cytoplasm. Autoradiography showed that less than 10 per cent of the cells in the culture had incorporated viral components. In autoradiograms of cultures infected with methionine-S³⁵ or cysteine-S³⁵ labelled Sendai virus, the radioactive grains were uniformly distributed on the surface of 90 to 97 per cent of the cells (Zhdanov, Bukrinskaya and Ramenskaya 1963). Infection with methionine-S³⁵ or cysteine-S³⁵ labelled virus at 4°C instead of 37°C did not decrease labelling of the cells. However, the radioactivity of the cells was markedly reduced when inoculated at 4°C with virus labelled with uracil-C¹⁴, a specific precursor of nucleic acids. Since analysis of S³⁵ labelled virus showed 55.5 per cent radioactivity bound to viral protein and only 10.4 per cent in the soluble antigen (Bukrinskaya et al. 1961), these studies suggest that, upon infection, the soluble antigen penetrated the cells while the protein coat of the virus remained on the cell surface.

When a large infectious dose of virus was inoculated on susceptible cells, a large portion of the virus was adsorbed on the cell surface (Zhdanov and Bukrinskaya 1961; Zhdanov and Bukrinskaya 1963). After a short time, most of the virus was eluted. When the virus was

brought into contact with susceptible cells in divided portions, the total amount of virus penetrating the cell was greater than on single contact with the whole dose of virus. The rate of virus absorption was higher at 37°C than at 4°C. At 37°C 60 per cent of the inoculum was absorbed in 15 minutes. Compared with culture cells in suspension, monolayer cells absorbed four times more virus inoculum.

Sendai virus has been shown to agglutinate erythrocytes of the following species: chicken, guinea pig, mouse, rabbit, cow, rat, hamster, horse, monkey, sheep and ferret (Fukumi, Nishikawa and Kitayama 1954; Jensen et al. 1955). The haemagglutination titre varied with erythrocytes from different species of animals; chicken and guinea pig red cells gave the highest titre and hamster red cells the lowest. At temperature of 4°C and 22°C, the haemagglutination titres showed less than two-fold differences when determined with red cells of the chicken, guinea pig and monkey. However, when human or mouse red cells were used, a lower titre was obtained at 4°C than at 22°C. In the case of erythrocytes from the hamster, ferret, sheep or horse, the reverse was noted, higher titres being obtained at the lower temperature.

The agglutination of red cells by Sendai virus has been considered to be associated with an enzymatic reaction, since erythrocytes treated with Vibrio cholerae filtrate containing receptor destroying enzyme were not agglutinable by the virus (Kuroya et al. 1953; Jensen et al. 1955). Virus fluid containing 4000 haemagglutinating units has been shown to liberate 50 ug. of sialic acid from either haemagglutinin inhibitor of chorioallantoic membrane or virus inhibitor prepared from bovine serum (Sokol, Blaskovic and Krizamova 1961). After haemagglutination, Sendai virus was eluted

from the red cells on prolonged incubation at room temperature. The haemagglutinating ability of the virus was closely related to its self-eluting ability and both were destroyed by heating at 50°C for ten minutes, by treating with M/25 potassium periodate for 30 minutes, by ultra violet irradiation, or by trypsin digestion (Fukai and Suzaki 1955). However, periodate at low concentrations destroyed the self-eluting ability at a higher rate than the haemagglutinating ability, and an indicator virus was prepared by treating Sendai virus with potassium periodate below M/50 (Jensen et al. 1955; Fukai et al. 1955). Casamino acid in 2% solution, polypeptone in 1% solution or M/1.5 phosphate buffered saline stabilized the haemagglutinin to heating (Tadokoro, Suzuki and Fukazawa 1958).

On treatment with ether, Sendai virus particles disintegrated. One component had a sedimentation coefficient of 90S. It appeared to be spherical and measured between 40 and 50 m μ in diameter. Another component had a sedimentation coefficient of 37S. The latter was observed in the form of chains and rosaries measuring between 15 and 20 m μ in width (Hosaka, Hosokawa and Fukai 1960; Sokol, Blaskovic and Rosenberg 1961). The 90S Component was shown to be haemagglutinin, since the haemagglutinin fraction, prepared by absorption and elution of ether-disintegrated Sendai virus with fowl red cells, was found to have a similar sedimentation pattern. It was suggested that the 37S component is the soluble antigen, and that it be referred to as the "g-antigen" (Blaskovic, Sokol and Kociskoya 1961). The haemagglutinin subunit, although not infectious, was similar to the whole virus particle with respect to adsorption on and elution from red cells of the virus. It may be noted that infectious RNA has been isolated from tissues infected with Sendai virus (Ugoleva 1965).

This gives support to the presumptive ribonucleoprotein nature of the "g-antigen".

Extensive studies by the Russian workers have shown that the soluble (S) antigen of Sendai is synthesized in the nucleoli of infected cells (Zhdanov, Azadova, Uryvayev 1965) whereas the viral (V) antigen is synthesized in the perinuclear zone of the cytoplasm. By labelling S and V antibodies with p-aminophenylmercuryl-acetate, endoplasmic reticular channels were shown to transport the S antigen to the cytoplasm (Zhdanov, Azadova, Kulberg 1965a). At the cell periphery, the S and V components are assembled and mature virus particles formed. It was found that addition of proflavine did not affect the synthesis of either antigen, but fixed S antigen in the nucleoli and prevented its transportation to the cytoplasm.

All Sendai strains, except Far Eastern strains 960 and 390 (Bukrinskaya et al. 1961), haemolyse red cells. The haemolytic action increased with increase in virus concentration (Fukai et al. 1955). By photometric measurement, a direct proportional relationship between the virus concentration and the haemoglobin release was found, and an equation was derived by which the virus concentration could be readily estimated (Neurath and Sokol 1962). Also, haemolysis by Sendai virus was inhibited either by pretreatment of the red cells with other myxoviruses or by simultaneous treatment of the red cells with Sendai and another myxovirus. A similar inhibition of haemolysis was observed when Sendai haemagglutinin subunits were substituted for the other myxovirus. Using the ability of other myxoviruses, and also of their haemagglutinins, to inhibit haemolysis by Sendai virus, the myxoviruses were arranged in a receptor gradient which was the same as that obtained by the classical adsorption and elution

method.

The general character of haemolysis by Sendai virus is similar to that caused by mumps and NDV (Hosaka 1958a; Hosaka 1958b; Ho Yunde 1961). The haemolytic activity of infected chorioallantoic fluid is not high. However, the activity is markedly increased after dialysis, repeated freezing and thawing, sonic vibration, or change of osmotic pressure. Haemolytic activity is also enhanced by chemical agents such as ethylene diamine tetraacetate or sodium citrate (Fukai et al. 1955).

The exact mechanism of the haemolytic action of Sendai virus has not been determined. Studies on the effect of antiserum and low-temperature on Sendai virus haemolysis suggested a two-stage reaction: an initial reaction which is sensitive to the inhibitory actions of both antiserum and low temperature, and a second reaction which is sensitive to neither (Tadokora et al. 1958). Before haemolysis took place erythrocytes were agglutinated by the virus, indicating a close association between haemolytic and haemagglutinating activities. However, the two phenomena are evidently functions of different viral components, since the haemagglutinating activity (and the eluting activity) was not changed by heating in casamino acid or polypeptone solution whereas the haemolytic activity was destroyed, (Tadokora et al. 1958; Neurath and Sokol 1962). Also, isopropyl alcohol or methanol was found to destroy only the haemolytic activity, leaving the virus with haemagglutinating activity (Fontages, Garrigue, Odobert 1964). Subunits derived by ether-disintegration of Sendai virus retained both haemagglutinating and eluting activity, but lost haemolytic activity when treated by ether or other fat solvents (Sokol, Blaskovic and Krisamova-

Laucikova 1961; Colobert and Fontanges 1959). The optimum pH for haemolysis was between 7.0 and 9.0. Haemolysis was inhibited by calcium ions. Lecithinase has been suggested as the mechanism of haemolysis by Sendai virus (Hosaka 1958a). However, the virus has been shown to haemolyse sheep and goat red cells, neither of which contain lecithin (Neurath *et al.* 1962).

The species of red cells susceptible to agglutination by Sendai virus were found to be susceptible to its haemolytic action (Ho Yun-de 1962b). However, chicken erythrocytes, which are the most susceptible to agglutination were only moderately sensitive to haemolysis. Mouse and guinea pig erythrocytes were the most sensitive to the haemolytic action of Sendai whereas rabbit red cells were least sensitive.

Paper chromatography of the phospholipid extract from fowl red cells haemolyzed by Sendai virus showed a decrease in concentration of sphingomyelin (Hosaka 1958b). This decrease was also shown by quantitative chemical analysis. As with mumps virus, it was observed with fowl cells only (Moberry, Marinetti, Witter, and Morgan 1958; Soule, Marinetti and Morgan 1959). Non-nucleated red cells of human or of guinea pig, showed no decrease in sphingomyelin. No change was noted in the sphingomyelin content of Erhlich ascites tumour cells infected with Sendai and showing fusion and lysis of cells.

As mentioned earlier, not all strains of Sendai virus possess haemolytic activity. Among the haemolytic strains there are quantitative differences in activity (Ho Yun-de 1962b). The haemolytic activity of different strains is proportional to the syncytium-forming activity (Ahdanov and Bukrinskaya 1962). Non-haemolytic

strains such as Far Eastern strains 960 and 390 do not induce syncytium formation; moderately haemolytic strains such as EMV (isolated during mouse passage of influenza in China) and LM-1 (One of the five isolates in the Vladivostok outbreak in 1956) exhibit less syncytium-forming activity than the more haemolytic strains isolated in Japan. A number of other similarities between haemolysis and syncytium formation have been noted. Both processes required whole virus particles; neither could be produced by ether-treated virus. Both processes were inhibited by pretreatment of cells with either RDE or other myxoviruses. The time relationships for both activities appeared to be similar. Haemolysis reached a maximum in 60 to 80 minutes. Cynomolgus "monkey heart" cell membranes were disintegrated after 40 minutes contact with the Japanese strain of Sendai, and after 60 to 90 minutes cells began to fuse and form syncytia.

The initial stage of virus-cell interaction in haemolysis was temperature-sensitive, and the later stage independent of temperature (Hosaka 1958a) while the reverse was observed with syncytium formation. Cell membranes were disintegrated during incubation at 37°C regardless of whether the absorption of virus on the cells was carried out at 4, 22 or 37°C, and syncytia were observed only when the absorption was at 37°C but not at 22 or 4°C. Also, unlike haemolysis, the activity of which was enhanced by dialysis or freezing and thawing of the infected fluid, the syncytium-forming activity was reduced by dialysis and was completely removed by freezing and thawing ten times. Formation of syncytia by Sendai virus was completed within a few hours at the time when no infectious virus was detectable and was preceded by the destruction of cell membranes, in contrast

to syncytia formation by viruses such as measles, CA and HA-1, which take a minimum of 3 days in cell culture, and the development of which coincided with the period of rapid increase in virus. Despite the apparent inadequate evidence, it was suggested that the haemolytic activity and the syncytium-forming activity were associated with an enzymatic reaction. The lytic enzyme responsible for both processes was designated "cell wall destroying enzyme" (Bukrinskaya and Zhdanov 1961; Okada, Suzuki and Hosaka 1957). Recently, absorption of Sendai virus on red blood cells has been shown insufficient to induce haemolysis (Neurath 1965) and the latter is preceded by a rapid total loss of potassium and by swelling (Klempere 1960). By analysis of the release of both potassium and haemoglobin from red cells attacked by Sendai virus, the interaction between virus and cell has been characterized as colloid-osmotic haemolysis (Neurath 1965a).

Croup-Associated (CA) Virus

CA virus grows in primary tissue cultures derived from human and simian kidney, and in human amnion. It also grows in continuous line cells such as HeLa, Hep-2, KB and Salk "monkey heart". It does not multiply in fibroblast cultures of either human or chick origin (Smorodinstev 1962). Generally, CA multiplies poorly in embryonated eggs. However, one strain of CA (recovered from lung materials obtained at autopsy of a newborn) was reported to grow only in the chick embryo amniotic cavity, and unlike the prototype, this strain failed to grow in human or monkey renal cell culture (Von Euler, Kantor, Hsiung 1963). Also, one strain of CA has been adapted successfully to grow in the allantoic cavity (De Meio 1963).

In primary kidney cultures CA virus produced weakly acidophilic (eosinophilic) to moderately basophilic cytoplasmic inclusions (Brandt 1961; Trombetta, Vitali-Mazza 1961). Using immunofluorescence techniques and haematoxylineosin staining in hamster cells infected with CA virus, the eosinophilic cytoplasmic inclusion body has been shown to contain antigen (Chien Liu 1963). Indeed, the cytoplasmic inclusion body was the only cellular component which has been shown to contain antigen, since no antigen has been demonstrated in the nucleus or in the cytoplasm (Chanock 1963; Liu 1963). Freezing and thawing of the infected cells yielded higher infectious titres than tissue culture fluid (Smorodinstev Jr. 1962), presumably due to the release of virus from the inclusion body.

In susceptible cell cultures, CA strains induce formation of syncytia. However, syncytial effects are variable and isolates have been recognized by the haemadsorption technique when syncytium formation was not observed, (Lepine, Chany, Droz and Robbe-Fossat 1959).

While most primary isolation cultures do not become haemadsorption positive until after the 10th day, susceptible tissue cultures inoculated with laboratory strains become haemadsorption positive on the 2nd day, and increasingly so between the 3rd and 5th day when a maximum infectious titre of 10^{-6} to 10^{-7} is obtainable in the infected culture fluid.

CA virus agglutinates chicken, guinea pig and human red cells. The haemagglutination titre was shown to vary with red cells from different chickens (Smorodinstev Jr. 1962). Generally, the haemagglutination titre with chicken erythrocytes is highest at 4°C . Treatment of CA virus with ether increased the agglutination titre for guinea pig red cells. Exposure to ultrasonic vibration also increased the titre for guinea pig red cells but not for chick red cells and did not change the infectious titre of the virus (Hermodsson 1960). Treating CA virus with ether in the presence of Tween-80 has been reported to increase the haemagglutinating titre (John and Fulginiti 1966). Ether treated virus was not infectious. It also gave a higher haemagglutination inhibition titre than whole virus suspension. As with influenza virus and NDV, trypsin exerted no effect on the infective or haemagglutinating titre of CA virus (Gresser, Enders 1961). Like other myxoviruses, erythrocyte receptors for CA are susceptible to the action of RDE (Chanock 1956). Erythrocytes, treated with CA for the removal of receptors, are agglutinable by all other myxoviruses (Darrell, Howe 1964). Unlike the other parainfluenza viruses, CA virus could not be converted to the indicator state by heating at 56°C .

Natural infection of infants with CA virus is often associated with croup (Chanock 1956; Beale et al. 1958; Kim et al. 1961; Parrott

et al. 1962). Infection may, however, be asymptomatic. In one study only one case of croup was observed in 31 children whose throat washings yielded virus (Kapikian et al. 1963). In adult human volunteers only a mild respiratory illness was observed after experimental infection with CA virus (Riechelderfer et al. 1958; Taylor-Robinson 1963). CA virus multiplies readily in the lung tissue of guinea pigs and hamsters after intranasal instillation (Chanock 1963; Chien Liu 1963). Virus was recovered from the lungs 48 hours after infection in quantities of 10^7 TCD₅₀ per gram of tissue although no gross signs of illness were observed.

Haemadsorption Type 1 (HA-1) and
Haemadsorption Type 2 (HA-2) Viruses

Initial characterization showed that both HA-1 and HA-2 viruses agglutinated fowl and guinea pig red cells. The receptors for both viruses on chicken and guinea pig red cells were removed by the action of RDE. Both HA-1 and HA-2 viruses were ether sensitive. However, there are three main points of distinction. First, HA-1 virus agglutinated guinea pig cells to higher titres whereas HA-2 gave higher titres with chicken red cells. Secondly, in monkey kidney cell cultures, HA-1 virus produced cytopathic changes leading to dislodgement of the infected cells, whereas only a slight cytopathic effect was induced by HA-2. Finally, immune rabbit sera to HA-1 and HA-2 did not cross react in either HI or neutralization tests.

A number of strains of both HA-1 and HA-2 have been found to grow in the amniotic cavity of fertile eggs. All HA-1 and HA-2 strains grow in primary renal cells of human and monkey origin. Fibroblast cell cultures from humans and chickens are not susceptible to either virus. However, HA-1 virus was shown to have a wider tissue culture range than HA-2; human amnion cells were susceptible to HA-1, but not to HA-2 (Smorodinstev Jr. 1962). Among established cell lines, only HeLa was susceptible to HA-2 virus, whereas Hep-2, HeLa, FL, LLC-MK2 and KB supported the growth of HA-1 (Fukumi *et al.* 1959; Marston, Vaughan 1960; Diebel, Hotchin, 1961; Massah Loh 1962; Falke, Schmidt 1963).

In primary monkey renal cell culture, CPE induced by HA-2 was minimal and consisted of a rounding and detachment of a few cells from the glass surface. Eosinophilic cytoplasmic inclusions were

common. On inoculation in primary MK cells, HA-2 virus was maximally absorbed at 5 hours; some liberation of infective virus was noted at 11 to 15 hours, with a maximal titre being reached at 60 hours and maintained through 132 hours. Haemagglutinin was first detected at 30 hours and reached a maximal level at 60 hours. The ratio of infectivity to haemagglutinin titre was relatively constant. In HeLa cells a slower rate of growth was observed, infectious virus being detected first at 132 hours and reaching a maximal titre at 240 hours, (Chanock et al. 1958; Petersen 1959; Dick, Mogabgab 1961).

The infectivity of HA-2 virus is completely destroyed at 50°C in 2 hours (Dick et al. 1961). Unlike influenza, NDV and DA viruses, HA-2 loses its infectivity when treated with trypsin. The haemagglutinin, on the other hand, was found to be more thermostable; at 44°C, the haemagglutinin titre was stable for a period of 100 hours. Trypsin exerted no effect on the haemagglutinins (Gresser et al. 1961). The haemagglutinin titre varied with temperature and with different species of red cells. With human or guinea pig erythrocytes, the haemagglutinin titres were 2 to 4 times higher if the cells were allowed to settle at room temperature instead of at 4°C. With rooster, sheep or rabbit red cells, a different temperature relationship was noted, the titre at room temperature usually being lower than that attained at 4°C (Dick et al. 1961a).

HA-2 virus has been shown to infect guinea pigs, hamsters, ferrets and monkeys by the intranasal route (Petersen 1959; Cook et al. 1959; Marine 1964). Although no signs of illness were observed, animals yielded virus at a concentration of 10^6 TCD₅₀ per gram of lung homogenate on the second day of infection. In children substantial evidence that HA-2 virus causes respiratory disease

has been accumulated (Petersen et al. 1958; Holland et al. 1960; Fukumi et al. 1963). Little information is, however, available concerning the capacity of this virus to cause naturally occurring disease in adults. Volunteer studies conducted in the United States and in England have shown that perpharyngeal instillation of HA-2 results in upper respiratory infection in adults (Reichelderfer, Chanock, Craighead, Huebner, Turner, James, Ward 1958) (Tyrrell et al. 1959).

In primary tissue cultures, HA-1 virus exhibits only minimal CPE during initial passage (Chanock et al. 1958). Upon serial passages, the virus induces formation of spindle-shaped cells, followed by complete loss of the cell sheet. In infected renal cell cultures, eosinophilic cytoplasmic inclusions have been seen.

A wide range of established cell lines have been shown to support the growth of HA-1 virus (Marston and Vaughan 1960). However, there is variation in cell response to infection in different cell lines. In HeLa (Lepine, Chany, Droz, Robbe-Fossat 1959) FL, Lohi and KB cell cultures (Diebel and Hotchin 1961; Miller, Brackett 1963; Falke and Schmidt 1963) HA-1 infection leads to the formation of syncytia. In rabbit cell lines, such as CRP, CRE and ERK, no cytological change was observed though growth was indicated by haemadsorption. Growth of HA-1 virus with no cytological change was observed in established human amnion cell lines although in primary human amnion culture, HA-1 infection always induces the formation of syncytia (Warren, Jensen and Mason 1962). In the monkey cell line LLC-MK-2, cytological changes consisted of rounding, increase in granularity and increase in cytoplasmic basophilia.

In Lohi cells, logarithmic increase of released virus was observed at the 8th hour after infection, reaching a maximum at the

11th hour. Intracellular virus multiplication was similar, except that it appeared two hours earlier and consistently yielded more virus. A slightly different growth pattern was demonstrated in non-syncytia-forming LLC-MK-2 cells. Intracellular virus was not greater than released virus (Massab, Loh, Philip 1962). The maximal release was not attained until the 20th hour.

Immuno-fluorescent studies (Massab et al. 1962; Chien Liu 1963; Chanock 1963b) demonstrated the first appearance of viral antigen in the cytoplasm, particularly in the perinuclear region. The area of viral antigen increases in size, and by the middle of the logarithmic phase, specific fluorescence is seen in the cytoplasm towards the periphery of the infected cells. At the time of maximal virus release, aggregates of fluorescent material are seen at the periphery of the cells. As with HA-2, Sendai and mumps viruses and NDV, no antigen has been demonstrated in the nuclei of the cells infected by HA-1 virus (Waterson 1962; Hilleman 1962; Chanock 1963) except in one study (Cohen, Bullivant, Edwards 1961). This single observation, however, requires confirmation.

Limited observations on the multinucleated cells induced by HA-1 infection have shown that syncytia are formed (Chany, Cook 1960; Cohen et al. 1961), preceding the appearance of new virus in the culture fluid.

Among the parainfluenza viruses, the aetiological role of HA-1 in respiratory diseases of children has been best established (Chanock et al. 1958; Chanock et al. 1963; Gernez-Rieuss et al. 1962). Also in adults, HA-1 has been associated with upper respiratory tract infections (Evans 1960; Bloom, Johnson, Jacobsen, Chanock 1961). Volunteer studies (Tyrrell 1960; Kapikian et al. 1961) showed that

HA-1 infection may produce a mild upper respiratory illness in adults.

M-25 Virus

Since the initial isolation of M-25 virus from the Washington area (Johnson et al. 1960) there has been only one further report. This was on the isolation of 25 strains from throat-washings from children in the same locale, though by a different group of workers (Canchola, Vargosko, Kim, Parrott, Christmas, Jeffries, Chanock 1964) and suggested that M-25 viruses may be divided into 2 groups, subtypes A and B, on serological grounds. Antisera to subtype A do not neutralize subtype B while antisera to B do neutralize A. By CF tests the 35 strains recovered between 1959 and 1961 belong also to B. However, only 2 of the 25 strains recovered in 1962 belong to B, the other 23 being subtype A. This single observation suggests that M-25 undergoes antigenic variation, but more data are required to determine if this member of the parainfluenza group would show a continuing variation as do the influenza viruses.

Unfortunately, recovery of M-25 is made difficult by the fact that rhesus monkey kidney cultures in extremely good condition are required. In a three-year survey by the investigators who made the original isolation, no additional recovery of M-25 was made (Johnson 1963).

DA, SA, and WB Viruses and SV5

The DA virus, named according to the initials of the fatal case from whose blood the virus was isolated (in rhesus monkey kidney cultures) (Hsiung 1961) is serologically identical with SA virus (Hsiung, Isacson, McCollum 1962), a myxovirus isolated from human throat washings (Schultz, Habel 1959). Both DA and SA viruses have subsequently been found to be serologically indistinguishable from SV5, a virus latent in normal rhesus monkey kidney cell cultures (Hull, Minner, Smith 1956). The WB virus, which was initially recovered in rhesus MK cultures inoculated with urine and blood of humans during the convalescent phase of experimentally induced, as well as naturally acquired, infectious hepatitis, is serologically related to SV5 (Liebhaber, Krugman, Giles, McGregor 1964). Though sera from animals immunized against WB virus gave cross-reactions with SV5 virus, no rise of WB antibody was observed in infected guinea pigs infected with SV5 (Liebhaber, Krugman, McGregor, Giles 1965).

Whether or not DA, SA and WB viruses are from the ecologic stand-point, primarily human agents is an interesting question, but one which lies beyond the scope of the present work. What concerns us here is their possible occurrence as contaminants in the MK tissue culture used in the study, since crossing antibody between SV5 and CA viruses (Chanock et al. 1961) and between mumps and CA (Hsiung 1963) have been demonstrated.

SV5, SA and DA viruses grow in primary MK tissue cultures without causing CPE. Their presence may be demonstrated by haemadsorption with guinea pig RBC. In latency in MK cultures, SV5 is not revealed by haemadsorption but may be "unmasked" by prolonged incubation

(Chan, Hsiung 1965). These agents multiply in both the amniotic and allantoic cavities of embryonated eggs with the production of haemagglutinins. They grow also in a number of cell lines. In FL cells, SV5 induces the formation of syncytia after 2 passages (Wong 1965). On BHK 21F cells (Wistar's suckling hamster kidney cell line), SV5 also produces large, vacuolated syncytia (Compans, Holmes, Dales, Choppin 1966). In MK cells where no structural alteration is induced, the yield of infectious virus is higher than that from the lines in which SV5 infection results in the formation of syncytia. Like other parainfluenza viruses, these agents are ether-sensitive and the receptor sites on erythrocytes are susceptible to the action of RDE. It was proposed at the VIII International Congress for Microbiology that the DA-SA-SV5 group of viruses be designated as parainfluenza virus type 5 (Chan, Hsiung 1962). This designation has not been generally accepted and SV5 has since been still generally considered a subtype of Parainfluenza 2 (Chanock 1963).

The WB virus shows a number of properties similar to DA, SA and SV5. It grows in primary MK culture without CPE, whereas its multiplication in cell lines such as WGM-1 and HeLa leads to the formation of syncytia indistinguishable from those induced by SA virus. However, WB does not grow in eggs. The authors responsible for its isolation and characterization grouped WB with CA and SV5 viruses (Liebhaber et al. 1964).

By antibody surveys, these agents have been found to be widely disseminated among common laboratory animals with the exception of rabbits. In two extensive surveys, using DA haemagglutinating antigen, the incidence of antibody was more than 50% in hamsters, guinea pigs, cows and monkeys (Hsiung 1963b; Hsiung et al. 1965).

A survey of guinea pig colonies in different places in the United States showed that SV5 antibody is present in this species of animal throughout the continent (Severs, personal communication). High titres of antibody to WB virus have also been found in "normal" guinea pig sera (Liebhaber et al. 1965).

Antigenic Relationships

The antigenic relationships among the parainfluenza viruses are at present not clear. This is due partly to the contradictory findings between human sera and immune animal sera, but mainly to the conflicting reports from individual investigators. Using immune guinea pig sera Cook and associates (Cook et al. 1959) showed reciprocal cross-reactions between Sendai and HA-2 in CF and HI tests. The animals were immunized by intranasal instillation of virus, followed by intraperitoneal injection of infected culture fluid treated with fluorocarbon. In neutralization tests, they showed that anti-Sendai sera had low titres against HA-2. So closely were Sendai and HA-2 shown to be related that Cook suggested that they be considered subtypes of the same virus. However, evidence from the Soviet Union indicated that Sendai virus was not more closely related to HA-2 than to other parainfluenza viruses (Bukrinskaya 1960). Indeed, with the same strains and similar techniques in guinea pig sera production, Bukrinskaya found no cross-reacting CF antibody between Sendai and HA-2. Furthermore, he found only homologous neutralizing antibody in Sendai hyperimmune sera from rabbits and from rats. The Russian findings were substantiated by investigators in New Orleans (Dick, Mogabgab, Hohmes 1961). No cross-reactions between Sendai and HA-2 viruses were demonstrated in HI tests with convalescent ferret sera (Jensin, Peeler, Dulworth 1962). A further report from the Soviet Union (Bukrinskaya, Ho, Gorbunova 1962), however, revealed the heterogeneity of the antigenic structure of both HA-2 and Sendai viruses. By means of CF and HI tests with immune guinea pig sera, antigenic variation of Sendai strains was satisfactorily demonstrated.

Similar variation was noted in HA-2 strains. Whereas the Japanese variant of Sendai virus cross-reacted with the American variant of HA-2, no common antigens were demonstrated in the Vladivostok variant of Sendai virus and the Moscow variant of HA-2 virus. Cross-reactions were not observed between the strains of the Moscow variant of HA-2 virus and antisera against the Japanese variant of Sendai virus. However, antiserum to the Moscow variant reacted only with certain strains of the Japanese variant of Sendai virus.

In confirmation of the close relationship between Sendai and HA-2 viruses, Cook and Chanock (Cook, Chanock 1963) demonstrated that prior Sendai infection in hamsters rendered the animals resistant to challenge with HA-2, as demonstrated by resistance of the lungs to growth of the challenge virus. Prior HA-2 infection was shown to influence only to a slight extent the course of subsequent Sendai infection. It is interesting to note that Sendai virus has been found to stimulate the production of interferon (Gresser 1961) and it calls to mind an early observation by Francis (Francis 1934) who pointed out that immunization with one strain of influenza virus would not protect against infection by a heterologous strain of virus even though both strains might belong to the same type. This observation has been repeatedly substantiated, and more recently confirmed, in a study in which guinea pigs immunized against the influenza A, PR-8 strain, were shown to be susceptible to challenge with influenza A1, FM-1 strains (Tong, Fong 1964).

The antigenic relationship of mumps to other parainfluenza viruses, especially to Sendai and NDV, has been described in a series of conflicting reports. Intranasal infection of guinea pigs with mumps virus was found to give rise only to homologous antibody,

as demonstrated in CF, HI and neutralization tests (Cook et al. 1959). Similar results were shown with NDV in the same study. Also, immunization of guinea pigs with haemagglutinin subunits, released by the treatment of mumps with ether in the presence of Tween 80, elicited only homologous antibody responses (Sokol, Blaskovic, Krizamova-Lancikova 1961), as shown in CF, HI and neutralization tests. Ferrets also gave a specific antibody response upon intranasal mumps infection (Marine 1964). These findings were in agreement with the earlier reports based on analysis of immune sera from laboratory animals immunized with crude infectious egg fluids (Kilham et al. 1949); Wenner, Jensen, Monley 1952; Kuroya et al. 1953; Bang and Foard 1956; Jensen, Minuse, Ackermann 1955; De Meio; Walker 1957).

There have, however, been a few studies reporting heterologous antibody responses in animals immunized with these agents. Guinea pigs given intraperitoneal injections of live mumps virus were shown to yield sera reacting with both mumps and Sendai viruses (Bartolomei-Cors 1959). A study from the Soviet Union (Bukrinskaya 1960) reported that, whereas immunization of rabbits and guinea pigs against mumps gave a homologous response only, immunization of rats elicited HI antibody reacting to the same titre against mumps, Sendai and NDV. Similar heterologous antibody was demonstrated in rabbits and rats immunized against NDV. It is interesting to note that Ishida and co-worker's, working with immune fowl and guinea pig sera, reported that cross reacting antibody, both HI and neutralizing, appeared only late in the course of immunization, and that the apparently cross reacting sera were serologically specific upon analysis by CF tests with S antigens (Ishida, Hiruma, Numazaki, Homma 1959; Numazak 1960).

The human response to complex antigens is often broader than that found after inoculation of animals. As this is quite pronounced with the influenza group (Jensen 1957) it comes as no surprise that antigenic sharing among parainfluenza viruses, especially between Sendai and mumps, is readily demonstrated in man. The reports describing the cross reaction shown in human sera are substantial and have been comprehensively reviewed (Cook et al. 1959; Hilleman 1962; Heath, Tyrrell, Peto 1962; Chanock et al. 1963; Stack, Heath, Peto 1964). Following mumps infection, rise of heterotypic antibody to Sendai has been observed (De Meio et al. 1957; Gardner 1957; Ishida et al. 1959; Lennette, Jensen, Guenther, Magoffin 1963) and to HA-1 and CA (McKinney et al. 1959; Gardner et al. 1960). It is of interest that, despite the apparent antigenic relationship between Sendai and HA-2 viruses, the sera of mumps patients have commonly shown antibody to Sendai but rarely to HA-2 (Lennette et al. 1963). Mumps convalescent sera have also been found to react with M-25 virus (Johnson et al. 1960). Tests of sera obtained after infection of human volunteers, showed that 8 of 9 individuals infected with HA-2 virus had a heterotypic response to Sendai, and 2 of 10 persons infected with HA-1 responded to HA-2 and CA.

The frequency of heterotypic antibody responses following natural infection by viruses of this group is well illustrated in a study which reported the immune responses of 110 children from whom parainfluenza viruses had been recovered (Parrott et al. 1962). By CF tests, 56 showed only a homotypic response, 7 showed only a heterotypic response, 22 yielded cross reacting antibody, and 25 had no response. Prior experience with parainfluenza viruses appears to govern the type of immune response. Parrott and associates showed

that children with pre-existing antibody to HA-1 gave heterotypic rises to HA-1 after HA-2 infection. Heterotypic responses to M-25 virus were observed in adults, but not in infants, after mumps infection (Johnson et al. 1960), a finding which is not surprising in view of the adults' extensive previous experience with the parainfluenza viruses. Indeed, it is unusual to find individuals without antibody to at least one of the parainfluenza viruses after the first year of life, (Heath et al. 1962), and the sera of most children contain antibody to all parainfluenza viruses by the age of 11 years (Stark et al. 1964).

The presence of naturally-occurring antibody to parainfluenza viruses in guinea pigs apparently renders the animals refractory to antigenic stimuli by HA-1 and HA-2 (Chanock et al. 1958). A survey of guinea pigs from several colonies in the eastern region of the United States (Cook et al. 1959) showed a high incidence of CF antibody to all parainfluenza viruses. By means of HI tests, cows, goats, monkeys and guinea pigs were found to have antibody reacting with HA-2, CA, HA-1, DA and mumps viruses while rabbits were shown to have antibody reacting with HA-2, CA and mumps (Hsiung 1963b). At the same time, hamsters were found free of antibody, except to the DA virus. Testing of three commercial sources of pooled guinea pig complement revealed the presence of high titres of HI antibody to all parainfluenza viruses, especially to mumps and DA (Hsiung 1963a).

Selecting animals that showed a negative HI test to HA-2, HA-1, CA, mumps and DA, at 1:10 dilution of serum, Hsiung and associates administered mumps or DA virus to hamsters, rabbits and guinea pigs by intranasal and intraperitoneal routes (Hsiung, Chang, Cuadrado,

Isacson 1965). The hamsters remained refractory to the first dose of DA virus, but gave a homotypic response to the second dose. In contrast, a prompt homotypic response to mumps was observed after the initial dose of live mumps virus. By passively immunizing hamsters against HA-1 with hyperimmune guinea pig plasma and subsequently inoculating them with live HA-1 virus, McHugh and co-workers (McHugh, Andersen, Harellick, Frankel 1963) showed that the immunized animals gave a mean HI titre of 1:6 in contrast to 1:1536 given by the non-immunized hamsters. It appears reasonable to assume that the sensitivity of the test used for animal selection in Hsiung's study was low and the failure of response to the first DA inoculation may well reflect the prior experience of the hamsters with this agent. The view was supported by a 29% incidence of DA antibody in the hamster colony from which the selection was made for this study. Rabbits, which had shown no incidence of DA antibody but 100% incidence of mumps antibody, gave immediate rises of titres to mumps upon initial DA inoculation. The second dose of DA virus produced a rise of titre to DA. On the other hand, mumps elicited a homotypic response in rabbits. Guinea pigs, which had shown a high incidence of antibody to both agents, were shown to give a heterotypic response to DA and to mumps after inoculation of either virus. Thus DA and mumps would be considered closely related by the reciprocal crossing shown in guinea pigs, whereas the same two viruses, when tested only in hamsters, would be regarded as antigenically distinct. Similarly, Sendai and mumps appeared to be related when tested in rats (Bukrinskaya 1960) or in rabbits; but not when tested in humans or guinea pigs. Antigenic crossing between Sendai and HA-2, though readily demonstrated

in guinea pigs, (Cook et al. 1959) was pronounced in rabbits (Bukrinskaya 1960) and not detectable in ferrets (Jensen et al. 1962).

Cook and co-workers showed that guinea pigs gave homologous responses to HA-1, CA, mumps and NDV, but not to Sendai or HA-2 after a single intranasal infection followed in two weeks by an intraperitoneal injection (Cook et al. 1959). Using the same species of animals with the same method of immunization reciprocal crossing between Sendai and SF-4 was shown, in CF tests, but not between Sendai and HA-2 and HA-1 (Spurrier Robinson 1965). Antisera to HA-1 reacted with Sendai and also antisera to SV5 reacted with CA. CF tests using untreated virus infected egg fluid and TC fluid as antigen showed more extensive crossings although reciprocal crossings were still only between Sendai and SF-4. Here, antisera to HA-2 reacted with Sendai, antisera to CA reacted with HA-1 and SF-4, antisera to SV5 reacted with CA and reciprocal intratypic crossings were shown between HA-1 and SF-4.

By HI tests, reciprocal crossings were shown between Sendai and HA-2 and between Sendai and SF-4. Antisera to Sendai reacted with SF-4, antisera to CA reacted with HA-1 and SF-4 and antisera to HA-1 reacted with Sendai (Spurrier, Robinson 1965). In ferrets, after intranasal infection (Marine 1964), Marine found homologous responses in HI tests to mumps, Sendai, HA-2, HA-1, CA and NDV. Upon second intranasal infection, while a booster-effect was observed in ferrets with prior experience of mumps. CA, HA-1 and NDV, sera from ferrets infected with Sendai and those with HA-2 lost their specificity and showed reciprocal HI antibody to each other. A further booster effect was observed in these animals upon injection of the respective viruses suspended in Arlacel A. Sera from these animals

remained specific with the exception of those immunized with Sendai and HA-2. Sera from ferrets which had been twice infected with Sendai, and originally showed heterotypic antibody to HA-2 virus only, became reactive also with HA-1 after the second infection. At the same time, sera from HA-2 infected ferrets showed a broadened heterotypic response by reacting with mumps as well as to Sendai.

It has been suggested that with parainfluenza viruses, as with influenza A, infection with a given strain results in an antibody response directed primarily against the serotype of the initial infection rather than against the serotype causing the immediate infection (Hsiung et al. 1965). As supporting evidence for this hypothesis, Hsiung and her associates showed that chickens previously immunized against NDV, then infected intranasally with DA virus at a time when no detectable HI antibody was present, gave earlier and higher antibody rises to NDV than to DA virus. Indeed, sera taken two weeks after the intranasal instillation of DA virus showed antibody to NDV only, and only in the sera taken four weeks after infection was antibody to the challenge virus demonstrated. Similar results were obtained with mumps intranasal infection following NDV. In this case, one chicken gave a response only to NDV.

Response to the first antigenic stimulus was also observed in 2 of 3 ferrets infected first with mumps and then with Sendai (Marine 1964). However, a similar response to mumps was observed only in 1 of 5 ferrets superinfected with CA and in 1 of 3 ferrets superinfected with HA-1. No recall antibody response to mumps was observed in ferrets superinfected with HA-2. When ferrets were initially infected with Sendai, HA-2, NDV and CA and subsequently infected with mumps, no recall antibody response was demonstrated except in 1 of the 3 infected animals.

These studies of the serial infections of ferrets, although providing little to substantiate the hypothesis of "original antigenic exposure", demonstrated nevertheless the effect of prior antigenic experience on the immune response. Thus, apparently antigenically "virgin" ferrets gave only homotypic responses to mumps, Sendai, HA-2, HA-1, CA and NDV. Reciprocal crossing between Sendai and HA-2 viruses was shown in ferrets after two homologous re-infections, and in ferrets first infected with mumps and subsequently infected twice with Sendai or HA-2. Animals which were infected twice with Sendai virus and then given an injection of the virus in adjuvant showed heterotypic antibody to HA-1. Similar heterotypic responses were observed in ferrets initially infected with mumps then by Sendai virus infections. On the other hand, heterotypic antibody to mumps, observed in the serum of ferrets infected twice with HA-2 followed by an injection of HA-2 in adjuvant was not observed in ferrets infected first with mumps followed by 2 instillations of HA-2 virus. The homotypic response of ferrets to CA, HA-1, mumps and NDV remained unchanged upon serial infections of the respective viruses. Previous infection with mumps did not alter the specificity of the ferrets' response to subsequent infections with CA or HA-1 virus except in one of five CA reinfected and one of three HA-1 reinfected animals which showed an anamnestic response to mumps. Previous parainfluenza infection did not alter the homotypic response of ferrets to mumps infection. An anamnestic response was observed only in one of three animals infected initially with CA.

Intranasal infection of guinea pigs with HA-2, CA, HA-1 and mumps was reported to give only homotypic responses (Veen, Sonderkamp 1965). CF antibody which gave a cross reaction with HA-2 virus,

but not HI antibody, was shown by animals infected with Sendai. One month after the first infection, the animals were infected with the homologous virus. No heterotypic antibody was observed in guinea pigs infected with CA virus. Upon a second mumps infection only 2 of the 5 animals gave homotypic responses by CF tests, and 3 of 5 by HI tests. Two of these animals showed cross-reacting CF antibody to CA. In a group of 6 guinea pigs infected twice with HA-2, although all gave a homotypic response by HI tests, only 4 showed a booster response by CF. However all 6 showed CF but not HI heterotypic antibody to Sendai. In the case of second Sendai infections, homotypic booster responses were observed in all animals, by both CF and HI tests. Crossing antibody to HA-2 was shown by all 5 animals by CF tests though only 3 by HI. Whereas no other cross-reaction was shown by CF tests, by HI test one animal gave a heterotypic response to CA and two to HA-1. Second HA-1 infection gave rise to booster homotypic response to CA and two to HA-1. Second HA-1 infection gave rise to booster homotypic responses in all 7 guinea pigs by CF and HI tests. In addition, heterotypic antibody was shown to HA-2 in 4 by CF and none by HI, to Sendai in 4 by CF and 3 by HI, and to CA in 2 by CF and one by HI.

Veen and Sonderkamp reported also on the response of guinea pigs infected first with Sendai followed in a month's time with HA-1, and vice versa. In a group of 15 animals, 13 gave rises of titre to the second infecting HA-1 virus by both CF and HI and the same number of animals showed an anamnestic response to the first infecting Sendai virus by CF and HI tests. In a group of 13 animals, only 8 showed a response to the second virus by CF and 7 by HI while 7 showed an anamnestic response to HA-1 by CF and 9 by HI tests.

However, in the case of serial infections first with mumps followed by HA-1, and vice versa, no recall of antibody was shown to the first infecting virus. The response to the second infecting virus in both cases was 100% both by CF and HI test. In addition, 3 gave responses to CA by CF and HI in a group of 11 mumps-HA-1 infected animals; 4 by CF and 7 by HI to CA in a group of 15 HA-1-mumps infected animals.

Prior antigenic experience in chickens appears to effect a loss of specific immune response. Chickens which were immunized with live NDV and inoculated a year later with mumps gave heterotypic responses to HA-2, HA-1 and CA in addition to an anamnestic response to mumps (Hsiung 1965). In man, the frequency of heterotypic responses to Sendai, HA-2, CA and HA-1 after mumps infection appeared to increase markedly with age (Leunette et al. 1963). Thus it is difficult, if not impossible, to evaluate the reports on the antigenic crossing among this group of viruses on the basis of human convalescent serum studies (Chanock et al. 1958; Cook et al. 1959; McKinney et al. 1959; Gardner et al. 1960; Hsiung 1963). Johnson and co-workers found that cross reactions with M-25 virus occurred only with sera from adults convalescent from mumps infection (Johnson et al. 1960). Also heterotypic responses to HA-1 in persons infected with HA-2 virus were demonstrated to be related to prior experience with HA-1 virus (Chanock et al. 1960).

Hsiung in her two reports (Hsiung 1963b; Hsiung et al. 1965) of studies on serum absorption indicated that heterotypic HI antibody was not removed by absorbing with the immunizing viruses. Thus, in a monkey serum which showed HI antibody to HA-2, CA, HA-1, DA and mumps upon immunization with DA virus, absorption with DA removed only the DA antibody, and absorption with HA-1 removed only

the HA-1 antibody. Another serum absorption study was reported by Marine (Marine 1964) who showed that Sendai did not remove HI antibody from convalescent ferret sera showing homologous responses to HA-1 infection. Similarly, HA-2 did not remove Sendai or mumps HI antibody as a result of homologous response.

However, antibody titres to Sendai as a result of a heterologous response after repeated HA-2 absorption dropped from 1:128 to less than 1:8 in one case and from 1:16 to 1:8 in another, while their respective HA-2 titres were reduced from 1:512 to 1:8 and from 1:2048 to 1:512. On the other hand, in convalescent sera showing heterologous HI antibody to HA-2 and HA-1 as a result of repeated Sendai infections, absorption with Sendai virus removed all of both homologous and heterologous antibody. The results suggest some basic difference between antibody resulting from a homologous response and antibody resulting from a heterologous response, reflected in their differential susceptibility to removal by absorption with viral agents other than the one used in immunization.

Summary of Antigenic Relationships

In summary, serial infections in animals showed that prior antigenic experience with parainfluenza viruses exerted an effect on the immune response which varied in different species of animals, and which varied also in individual animals within the same species. In ferrets, with no detectable antibody to this group of viruses upon infection, only homologous or homotypic responses were demonstrated. However, in rabbits and guinea pigs, evidence has been presented showing antigenic relationships between Sendai and mumps, and between mumps and CA. The evidence has been by no means unequivocal. Indeed, the published data on the antigenic crossings are often conflicting. Thus, the antigenic relationship among Sendai, HA-2, CA, HA-1 and mumps viruses is still unsettled.

Outline of Experimental ApproachIntroduction

Published reports of antigenic studies indicate that crossing reactions among the parainfluenza viruses are more readily demonstrable by CF tests than by HI tests (reviewed earlier under the heading "Antigenic Relationships"). Although intimate association of normal host material with purified myxoviruses has been well documented with influenza virus (Knight 1946; Hoyle 1952; Smith, Belyavin, Sheffield 1955; Kates Allison, Tyrrell, James 1961), no direct evidence for such an association has been presented for the parainfluenza group of myxoviruses with the exception of DA virus which, when grown in rhesus monkey cells has been shown to contain a B antigen similar to that found in uninfected rhesus monkey kidney cells (Isacson, Koch 1965). However, this blood group B antigen was found to be incorporated on the non-haemagglutinating site of the viral envelope and had no influence on the antigenicity of the viral haemagglutinin. Presumably, this particular host antigen would exert no effect on the HI test. Nevertheless, the demonstration of this single host antigenic component on the parainfluenza DA virus raised the question of the possible presence of other as yet undetected host antigenic material on these viruses. The possibility was given credence by the example of host-controlled variation (Drake, Lay 1962; Matsumoto, Maeno 1962; Stenback, Durand 1963), and the suggestion has been made that the variation may be a reflection of the incorporation of host material into the virus particle. Therefore, the early part of the present study was devoted to trying to demonstrate host antigen in an egg grown virus.

Preliminary experiments

Three groups of rabbits were immunized, one group with crude allantoic fluid from embryonated eggs infected with mumps virus (CAF mumps), one group with mumps virus suspended in phosphate buffered saline after two cycles of high speed and one of low speed centrifugation (PBS mumps), and one group with normal allantoic fluid (NAF). Pre-immunization and post-immunization sera were titrated in CF tests against CAF mumps, CAF Sendai, CAF influenza A-Jap and NAF. A rise in CF titres to all four antigens was shown by sera of rabbits immunized against CAF mumps and by sera of rabbits immunized against PBS mumps. A rise in CF titres to influenza A-Jap and NAF was shown also by sera of animals immunized against NAF. One rabbit in this group showed an increase in titre to mumps and Sendai.

The possible presence of a host antigenic component in myxovirus PR-8 purified by zinc acetate precipitation (Hansler, Dick 1960) was next investigated. Cross titrations in CF tests with purified PR-8, CAF PR-8 and NAF against rabbit anti-NAF serum, showed that the purified virus reacted to the same titre as the crude virus and both gave the same end-point as NAF.

The preliminary investigation thus indicated that the host antigenic component, presented either as a part of viral materials or as a contaminant in the antigen, may render the results of the CF test on sera of immunized animals difficult to interpret. Accordingly, attention was given to the preparation of sera devoid of antibodies to the host tissue materials. To this end, animals were infected with live virus given intranasally rather than by injecting material by other routes.

Experiments on antigenic analysis

The first experiment was a study of the antigenic response following intranasal infection, with Sendai virus, of two groups of guinea pigs. The animals were selected on the basis of showing no antibody to the viruses under study, one group by means of CF tests at 1:4 serum dilution and another group by means of HI tests at 1:10 serum dilution. The 14-day convalescent sera were titrated in CF and HI tests against the homologous antigen and four heterologous antigens. In the second experiment, involving six groups of guinea pigs, one group, selected by CF tests, was intranasally infected with HA-2, and the other groups, chosen for showing negative HI titres, were each infected with one of HA-2, HA-1, CA, mumps or NDV. The convalescent sera were titrated for CF and HI antibodies.

The third experiment investigated possible antigenic crossing between the guinea pig organs and the viruses under study. Sera from guinea pigs and rabbits hyperimmunized with guinea pig organs were examined for viral antibody. The hyperimmune response of rabbits to this group of viruses is reported in Experiment 4. Absorption of convalescent and hyperimmune animal sera, and of convalescent human sera with Sendai virus, is reported in Experiment 5. The ability of Sendai virus to alter the erythrocytes for subsequent agglutination by convalescent animal and human sera was investigated in Experiment 6. Finally, in Experiment 7, a new serological test for parainfluenza viruses is described. An antigenic analysis of the viruses under study using this test is reported.

General Materials and Methods

Viruses

The Japanese strain of Sendai virus, isolated originally by Kuroya and co-workers, was obtained from Dr. K. E. Jensen and was maintained in our laboratory by serial allantoic passages in 10 to 11-day-old eggs. The Enders strain of mumps was obtained from the Laboratory of Hygiene, Ottawa. It was maintained by serial allantoic passages in 7 to 8-day old eggs. Newcastle Disease Virus was obtained from the American Type Culture Collection (ATCC). It was maintained by serial allantoic passages in 9 to 10-day old eggs. The Greer strain of CA virus, the C234 strain of HA-1 and the C35 strain of HA-2 were obtained from the ATCC. These three strains were originally isolated by Chanock and co-workers. They were maintained in our laboratory by serial passages in primary monkey kidney cell culture.

Primary monkey kidney

Rhesus monkey kidney was received as a cell suspension (approximately 300,000 cells per ml.) from the Connaught Medical Research Laboratories, Toronto. The cells were suspended in medium consisting of Hanks Balanced Salt Solution (HBSS)*, 0.5% lactalbumin hydrolysate and 2% calf serum. Cells were dispensed in 1.8 ml. volumes in 16 x 150 mm. pyrex test tubes. After six days incubation at 37°C in a stationary position, a confluent cell sheet was obtained. The growth medium was replaced with 1.8 ml. of maintenance medium HB597** with 100 units penicillin and 75 mg. streptomycin per ml. of medium.

*Appendix 1

**Appendix 2

Complement

Fresh serum from individual guinea pigs was used as the source of complement. Different lots of lyophilized, pooled guinea pig serum from a commercial source, and from the Laboratory of Hygiene, Ottawa, were found unsuitable as they exhibited anti-complementary effects with a number of immune rabbit sera as well as a number of convalescent guinea pig sera. In addition, they all fixed complement with parainfluenza antigens, especially with CA virus. Accordingly, a group of guinea pigs whose sera had no anticomplementary effects with the test sera and antigens was selected for preparation of complement. Preceding each batch of CF tests, anticomplementary controls on the test reagents were performed.

Sheep red blood cells

Sheep red blood cells were collected in Alsever solution from the jugular vein of the animal. Sheep cells obtained in this way and stored at 4°C remained suitable for approximately one month before visible haemolysis occurred. Cells for CF tests were stored for at least 2 days before use.

Guinea pig red blood cells

Guinea pig red blood cells obtained by cardiac puncture were collected in Alsever solution. Blood stored at 4°C remained usable until the occurrence of visible haemolysis.

Amboceptor

Amboceptor was purchased from Markham Laboratories, Chicago, U.S.A.

Complement fixation test

The technique of the CF test was adopted from that described by Delaat (Delaat 1964). Briefly, an antigen pool was prepared for each of the five viruses under study. For mumps and Sendai, the source of antigen was infected allantoic fluid. HA-2, HA-1 and CA antigens were prepared by pooling 5 day infected tissue culture fluid. Initially, the block titration to determine the CF antigen unit was done with hyperimmune guinea pig sera purchased from Microbiological Associates, Baltimore. Subsequent titrations were done with convalescent guinea pig sera after intranasal infection. Antigen medium controls consisted of normal allantoic fluid and uninoculated tissue culture fluid harvested in the same way and used in the same concentration as the viral antigen.

The test was performed in perspex plates. Veronal buffered Saline (VBS)* was used as diluent throughout. The total volume of the test reagents was 0.4 ml. consisting of four equal parts: 0.1 ml. containing 2 CF units of viral antigen, 0.1 ml. of diluted serum, 0.1 ml. of guinea pig serum containing $2 \frac{1}{2}$ HD₅₀ of complement and 0.1 ml. of 2% sensitized sheep cells. Fixation of complement was allowed to proceed overnight at refrigeration temperature. Before adding sensitized sheep cells, the plates were warmed to 37°C for 15 minutes. A period of 30 minutes at 37°C was allowed for the sensitized cells to react with any unbound complement. The serum dilution at which 50 per cent of the cells were lysed was considered the end point. The CF titre was expressed as the reciprocal of the end point serum dilution.

* Appendix 3

Haemagglutination Inhibition test (HI test)

Infected monkey kidney tissue culture fluids were used as antigens for HA-2, HA-1 and CA viruses and infected allantoic fluids were employed for mumps, Sendai and NDV. The cultures were incubated at 37°C for five days after inoculation with 500 to 1000 TCD₅₀ of the respective viruses. The fluids were then harvested and stored at -20°C. Antigens for Sendai and NDV were prepared by harvesting 2 days after allantoic inoculation of 9 to 10 day developing chick embryos with approximately 100 EID₅₀ of virus. Mumps allantoic fluid antigens were obtained by allantoic inoculation of 7 day developing chick embryos with 100 to 500 EID₅₀ of virus followed by incubation for 7 days at 37°C.

Titration of antigen

Serial 2-fold dilutions were made in phosphate buffered saline (PBS*) in 0.5 ml. amounts, 8 to 10 tubes. Kahn tubes 13 x 75 mm. were used. Then 0.5 ml. of 0.25% guinea pig red cell suspension was added, and the tubes were shaken. Racks were allowed to stand at room temperature until the cells in the saline control tubes had settled. The results were read by examining the bottoms of the tubes. Individual tubes were usually graded as positive or negative. As a rule, the end-points were sharp, changing from diffuse film in one tube to a completely negative sharply outlined button in the next. The concentration of virus suspension (before addition of red cells) in the last positive tube was taken as the titre, and this is defined as containing one haemagglutinating unit. Usually, Sendai allantoic fluid had a titre of 1:128 to 1:256, mumps and NDV titres of 1:64 to 1:128, while HA-2 tissue culture fluid had a titre of 1:16 to 1:32, CA a titre of 1:32 and HA-1 a titre of 1:128.

* Appendix 4

Preparation of standardized antigen

The viruses were diluted in PBS so that 0.25 ml. contained 4 haemagglutinating units. Since the preliminary titrations were carried out with 0.5 ml. volumes, it was necessary to adjust the concentration of virus to permit the use of 4 units in 0.25 ml. volume. The standardized antigen dilution was obtained by dividing the reciprocal of the titre of virus suspension by eight.

The standard virus suspension was retitrated routinely on the day of each test. This was performed by making serial 2-fold dilutions, mixing 0.5 ml. of the prepared dilution with 0.5 ml. PBS in each of 5 tubes and adding 0.5 ml. of 0.25% red cell suspension of the day. Four units per 0.25 ml. gave a positive pattern in the first three tubes.

Treatment of sera to destroy non-specific inhibitor

An equal volume of 25% suspension of acid-washed kaolin in saline was added to a 1:5 dilution of serum. The mixture was allowed to stand at room temperature for 20 minutes. The kaolin was sedimented by centrifugation at 2,000 rpm for 10 minutes, and the recovered serum was considered to be diluted ten times.

Sera were further treated to remove red cell agglutinins. To 1.0 ml. of 1:10 serum was added 0.1 ml. of a 50% suspension of washed guinea pig red cells. Absorption was allowed to proceed at 4°C for one hour. Red cells were removed by centrifugation at 1,200 rpm for 10 minutes. Before absorption the sera were inactivated at 56° for 30 minutes.

The test

Two-fold dilutions of sera, 0.25 ml. per dilution, were made. When all sera had been diluted and distributed, 0.25 ml. of the standard virus suspension was added to each series, and the racks were shaken vigorously and allowed to react at room temperature for 30 minutes. For each serum, a control for non-specific haemagglutination was included containing 0.25 ml. of the highest serum concentration and saline was substituted for virus. The 0.25% suspension of guinea pig erythrocytes was then added in 0.5 ml. volumes to all tubes and the racks shaken to ensure even dispersal of red cells. The reading of the test was done when the concurrent titration of the adjusted standard virus tubes had settled to a distinct button, usually in an hour. The titre was taken as the concentration of serum in the last negative tube.

Virus Assays

All virus infectivity titrations were performed by inoculating serial 10-fold dilutions of the virus into monkey kidney culture tubes. The inoculum volume was 0.2 ml. per tube culture containing 1.8 ml. maintenance medium HB 597. The titration end-point was determined by the haemadsorption technique on the fifth day of incubation (Vogel, Shelokov 1957). For this purpose, the cultures were decanted, washed once with PBS and then tested with 1.0 ml. of 0.1% guinea pig erythrocytes in PBS. Examination for haemadsorption was made following incubation at 4°C for 30 minutes. The TCD_{50} (50% tissue culture infectious dose) was calculated by the method of Reed and Muench.

Serum Neutralization Test

With the exception of sera prepared against CA virus, all sera were routinely inactivated at 56°C for 30 minutes*. In the test, serial 2-fold dilutions of serum were incubated for one hour at room temperature with an equal volume of virus diluted to contain approximately 100 TCD₅₀. The inoculum was 0.2 ml. of the virus-serum mixture. The end-point was the highest initial concentration of serum which effected complete inhibition of viral proliferation as measured by the haemadsorption technique on the fifth day of incubation in monkey kidney cell cultures at 37°C.

Immunization of rabbits

Rabbits were immunized by a weekly intravenous injection of 2 ml. of virus, from the respective virus antigen pools, for 4 weeks, and the animals were bled 7 days after the last injection. For hyperimmunization, rabbits were given two 2 ml. intramuscular injections per week for 4 weeks. A sample bleeding was done 1 week after the last injection. After a 3-week rest period, the same immunizing schedule was repeated. Animals were bled 7 days after the last injection.

Intranasal instillation

Groups of guinea pigs were inoculated intranasally, under light ether anaesthesia, with 0.2 ml. of undiluted tissue culture fluid containing a minimum of 10⁵ TCD₅₀ or allantoic fluid containing a

*Sera against CA were not heat inactivated because heating at 56°C for 30 has been reported to cause a 4-fold or greater reduction in titre (Chanock 1956).

minimum of $10^{4.5}$ EID₅₀. Prior to instillation of virus, a blood sample was taken by cardiac puncture. In most cases, animals were bled out 14 days after infection.

Guinea pigs infected intranasally were kept in an isolated room that was washed and disinfected with "Wescodyne" between experiments with different viruses. Between experiments, the isolation room was left empty for at least three days.

Concentration of virus by centrifugation

The crude virus fluid was centrifuged at 22,000 rpm for 30 minutes, and the pellets resuspended in PBS to 1/10 of the original volume. After sedimentation of the extraneous materials by centrifugation at 3,000 rpm for 15 minutes, the supernatant was again centrifuged at 22,000 rpm for 30 minutes. The pellet containing the virus was resuspended to 1/10 of the supernatant volume.

Zinc acetate precipitation of virus

Virus in crude allantoic fluid was purified by zinc acetate precipitation according to the method described by Hausler and Dick (Hausler, Dick 1960). The crude virus fluid was chilled to 2°C and 0.1 M zinc acetate solution added drop by drop to give a final concentration of 5 ml. per litre of fluid. The material was kept at 2°C in an ice-bath for 10 minutes, and then the zinc-protein complex was sedimented at 6000 rpm for 12 minutes at 4°C. The remaining procedure was performed throughout at 4°C. The zinc-protein complex was resuspended in the centrifuge cup with a minimum amount of 1.0 M glycine, and the contents transferred to a separatory funnel. The centrifuge cup was again washed with glycine to remove as much of the sediment as possible.

To the partially dissolved zinc-protein complex in the funnel was added 100 ml. of 1.0 M glycine. After vigorous shaking, the contents were transferred to an exchange resin column (Amberlite 1R-120 standard grade supplied in sodium form from Fisher Chemical Co.) for removal of zinc.

The resin effluent, which contained protein dissociated from the complex, was concentrated by ultracentrifugation at 22,000 rpm for 30 minutes. The pellets were resuspended in 1.0 M glycine to one half of the original volume. Removal of gross particles was accomplished by centrifugation at 3,000 rpm for 10 minutes. The supernatant was again centrifuged at 22,000 rpm for 30 minutes. The resulting pellet was resuspended in 0.1 M phosphate buffer at pH 7.0 and diluted to a volume approximately one-tenth of the original crude fluid.

Experiment 1: Immune Response of Guinea Pigs to Sendai Virus InfectionIntroduction

It has been shown that antibody-free guinea pigs, upon intranasal instillation of parainfluenza viruses followed by an intraperitoneal injection, gave rise to homotypic antibody, and in some cases, to heterotypic antibody (Cook et al. 1959). In guinea pigs not selected on the basis of serology intranasal infection alone has been found to produce homotypic and heterotypic antibody (Veen et al. 1965). The following experiment shows the immune response of two groups of guinea pigs to Sendai infection; one group selected on the basis of showing a CF antibody titre of <4 and the other group on the basis of showing an HI antibody titre of <10 .

Experimental

From a colony of 56 guinea pigs (supplied by the Deer Lodge Hospital) 15 animals were chosen which were negative by CF at 1:4 dilution to Sendai, HA-1, HA-2, mumps and CA (Group I). The CF and the HI antibody distribution of this whole colony is shown in Table 1. In another colony of 48 guinea pigs (supplied by this laboratory) using HI as the criterion 5 guinea pigs provided negative sera at 1:10 dilution to the 5 viruses (Group II). The distribution of HI antibody of this colony is shown in Table 1.

Both groups were inoculated intranasally under light ether anaesthesia with 0.2 ml. of undiluted Sendai allantoic fluid with TCD_{50} of $10^{5.6}$ and an EID_{50} of $10^{6.3}$ per 0.2 ml. Animals from Group I were bled after 2 weeks and exsanguinated after 4 months. Animals from Group II were bled after 2 weeks and exsanguinated after 29 days.

Five convalescent sera randomly selected from Group I were cross

titrated in doubling dilutions ranging from 1:4 to 1:64 with NAF by the CF test. The same sera, in the same doubling dilutions were tested for complement fixation with normal monkey kidney fluid.

All 14-day convalescent sera from both groups were titrated against the antigen using both CF and HI tests. Four-month convalescent sera from Group I were tested by both CF and HI reactions against Sendai and HA-2 antigens.

Results

The five convalescent sera selected for cross titration against NAF were #1, #3, #4, #9 and #14. No CF reaction was demonstrated in serial dilutions of these sera ranging from 1:4 to 1:64 against serial dilutions of NAF ranging from undiluted to 1:32. Similarly, no CF reaction was shown with the same serial dilutions of these sera against diluted monkey kidney culture fluid.

The 14 day CF response of Group I guinea pigs to Sendai infection is shown in the upper part of Table 2. Two animals #13 and #14 showed no response. The remaining thirteen animals gave titres between 64 and 1024 against Sendai. Of these 13 animals giving a homologous response to Sendai, 11 showed a heterologous response to HA-2 and 3 to HA-1. No heterologous responses to mumps and CA were observed. In Group II animals in the lower part of Table 2, the homologous CF titres varied between 256 and 512. Two animals in this group gave heterologous titres of 32 to HA-2. With the exception of guinea pig #16 (which was the only animal in this group subsequently shown to contain CF antibody prior to infection, in this case a titre of 4 to mumps and titrated at 8 after infection) no animals gave any crossing response to mumps, HA-1 or CA by CF

test.

The 14-day homologous CF titres of Group II guinea pigs were maintained at the same level 29 days after infection (Table 3) whereas the low heterologous titres to HA-2 showed a decrease from 32 to <4 with serum #16 and from 32 to <4 with serum #18. A sharp decrease of homologous CF titres of group I guinea pigs was observed with 5 of 8 sera four months after infection (compared with the titre 14 days after infection as shown in Table 4). However, the decrease was not consistent, since serum #2 showed an unchanged titre of 128 and serum #11 which was 256 at 14 days became 64 after 4 months.

The pre-infection and post-infection HI titres against the five viral antigens of both Group I and Group II guinea pigs are shown in Table 5. In Group I consisting of 15 animals, selected on the basis of showing <4 CF titre to the five viral antigens, there were only 3 guinea pigs #3, #5, and #15 shown to be free of HI antibodies to these antigens at 1:10 serum dilution. These three animals showed a high homologous rise of HI titre from <10 to 320 or 640, contrasting with the remaining 12 animals, only two of which gave 4-fold rises of homologous HI titre, #8 and #13. The homologous HI titres in all 5 animals in Group II were >320.

For the purpose of comparing HI heterotypic responses, #3, #5 and #15 animals were added to the 5 animals in Group II which had been selected in the first place on the basis of negative HI antibody. Thus, we have now two groups of guinea pigs; one group of eight without HI antibodies to the five viral antigens prior to Sendai infection, and one group of twelve with pre-existing HI antibodies. The number of animals in these 2 groups giving heterotypic rises to each of the viral antigens under study is tabulated

in Table 6. Of the 12 guinea pigs with pre-infection HI antibodies against one or more parainfluenza viruses, only two showed a homologous rise of HI titre upon infection, whereas all eight animals without pre-infection HI antibody showed a homologous response. On the other hand, heterologous HI responses in the two groups occurred with about the same frequency. The titres against Sendai ranged from 320 to 2560 and the heterologous titres from 40 to 160.

A slight decrease of HI titres was observed from the 14th day to the 29th day after infection (Table 3). Four months after infection, 6 of 8 animals in Group I (Table 4) still showed low HI titres to Sendai.

Discussion

The high incidence of CF and HI antibody to HA-1 and CA shown in the screening of guinea pigs suggested possible epizootic infection with these agents in the animal colonies. For the screening of guinea pigs, the HI test appears to be better, since CF negative pre-infection sera of Group I yielded positive HI results, whereas HI negative pre-infection sera of Group II were CF negative with the exception of one serum (#16) which had a low CF titre of 4 to mumps. In Table 1, in the case of pigs with antibody against HA-1 and CA the CF test appeared to be more sensitive; with Sendai and HA-2, the HI test appeared to be more sensitive.

By the CF test, no crossing antibody to CA and to mumps was demonstrated in these animals with the exception of #16 guinea pig. This is in agreement with the observations of Cook (Cook et al. 1959) and with those of Bukrinskaya (Bukrinskaya 1960). With the exception of three sera #1, #2, and #8, there was no crossing CF antibody to

HA-1. Low level CF antibody to HA-1 was also demonstrated by Cook et al. in their pooled guinea pig sera against Sendai. However, the present heterologous CF titre to HA-1 varied only between 16 to 32, and in view of the fact that 50% of animals from this colony had CF antibody to HA-1, the inclination is to reject these low heterologous titres as evidence of crossing antigen shared by Sendai and HA-1.

On the other hand, of the 18 animals giving homologous titres to Sendai 15 gave heterologous titres to HA-2. No apparent correlation was noted between the titre to Sendai and the appearance of crossing CF antibody to HA-2. Furthermore, there was no correlation between the level of homologous antibody and the level of crossing antibody. Homologous CF antibody remained approximately of the same titre in 29 days and was detectable after 4 months. On the other hand, heterologous CF antibody to HA-2 became <4 in 29 days. The proportion of animals yielding a heterologous response was large enough to suggest an antigenic crossing between Sendai and HA-2. In addition, a similar pattern was observed using the HI test: here the number of animals showing a 4-fold or greater rise to Sendai was 10 and to HA-2 was 8 (Table 6). These data correspond with those reported by Cook et al.

The results of Sendai infection in guinea pigs indicate that the presence of antibody at the time of infection prevents the animals producing a high level of HI antibody and a predominantly specific response. It is suggested that the suppression of antibody production by pre-existing antibody may be explained on the basis of limited replication in vivo.

Conclusion

1. By CF tests, guinea pigs infected with Sendai virus showed crossing antibody to HA-2 and minimal crossing to HA-1. By HI tests, animals showed only crossing antibody to HA-2. In both tests, no crossing antibody to CA and mumps was shown.
2. The presence of pre-infection antibody reduces the homologous and heterologous response and decreases the specificity.

Table 1

Distribution of CF and HI antibodies against some parainfluenza
viruses in guinea pigs

No. of animals showing antibody to:

Source of animals	Total no. of animals		Sendai	HA-1	HA-2	mumps	CA
		CF*	0	28	0	0	13
Deer Lodge Hospital	56	HI**	11	0	5	0	7
Virus Lab. University of Manitoba	48	HI	15	39	15	0	15

* Sera tested at 1:4 dilution.

**Sera tested at 1:10 dilution.

Table 2

CF titres 14 days after Sendai infection of guinea pigs whose sera prior to infection showed no CF antibody at 1:4 dilution to designated

antigen

	<u>Sendai</u>	<u>mumps</u>	<u>HA-2</u>	<u>HA-1</u>	<u>CA</u>
<u>Group I</u>					
Serum #1	512*	0**	128	16	0
2	128	0	32	32	0
3	128	0	0	0	0
4	256	0	64	0	0
5	256	0	0	0	0
6	1024	0	256	0	0
7	256	0	64	0	0
8	256	0	16	16	0
9	64	0	8	0	0
10	512	0	32	0	0
11	256	0	32	0	0
12	512	0	64	0	0
13	0	0	0	0	0
14	0	0	0	0	0
15	64	0	16	0	0
<u>Group II</u> ^o					
Serum #16***	512	8	32	0	0
17	256	0	0	0	0
18	512	0	32	0	0
19	512	0	0	0	0
20	256	0	0	0	0

*Reciprocal of serum dilution showing complement fixation.

**Not detectable at 1:4 serum dilution.

***Pre-infection serum showed 1:4 CF titre to mumps.

^oAnimal sera prior to infection showed no HI antibody at 1:10 dilution also.

Table 3CF and HI titres of guinea pigs with pre-infection titres (HI) <10.14 days and 29 days after Sendai infection

<u>Sera No.</u>	<u>Post 14 days</u>				<u>Post 29 days</u>			
	<u>CF</u>		<u>HI</u>		<u>CF</u>		<u>HI</u>	
	<u>Sendai</u>	<u>HA-2</u>	<u>Sendai</u>	<u>HA-2</u>	<u>Sendai</u>	<u>HA-2</u>	<u>Sendai</u>	<u>HA-2</u>
16	512	32	2560	160	512	0	1280	160
17	256	0	5120	0	512	0	1280	0
18	512	32	1280	80	256	0	80	160
19	512	0	320	0	128	0	40	0
20	256	0	640	0	256	0	80	0

0 = <4 for CF and <10 for HI

Table 4

CF and HI titres of guinea pigs with pre-infection CF titre <4
14 days and 4 months after Sendai infection

<u>Sera No.</u>	<u>CF</u>		<u>HI</u>		<u>CF</u>		<u>HI</u>	
	<u>Sendai</u>	<u>HA-2</u>	<u>Sendai</u>	<u>HA-2</u>	<u>Sendai</u>	<u>HA-2</u>	<u>Sendai</u>	<u>HA-2</u>
1	512*	128	80	40	-**	-	-	-
2	128	132	80	40	128	0	40	0
3	128	0	640	0	0	0	20	0
4	256	64	40	40	0	0	10	0
5	256	0	320	80	8	0	40	0
6	1024	256	20	40	-**	-	-	-
7	256	64	40	0	-	-	-	-
8	256	16	80	0	8	0	10	0
9	64	8	0	0	4	0	0	0
10	512	32	40	20	-	-	-	-
11	256	32	80	80	64	0	40	20
12	512	64	40	40	-	-	-	-
13	0	0	40	40	-	-	-	-
14	0	0	20	0	16	0	0	0
15	64	16	640	40	-	-	-	-

*Reciprocal of serum dilution

**animal missing

Table 5

HI titres after Sendai infection of guinea pigs with pre-infection

CF titre < 4

<u>Group I</u>	Sendai		Mumps		HA-2		HA-1		CA		
	<u>Sera No.</u>	<u>Pre</u>	<u>Post</u>	<u>Pre</u>	<u>Post</u>	<u>Pre</u>	<u>Post</u>	<u>Pre</u>	<u>Post</u>	<u>Pre</u>	<u>Post</u>
1	80*	80	0**	0	10	40	0	0	40	10	
2	80	80	0	0	20	40	0	0	40	40	
3	0	640	0	0	0	0	0	0	0	0	
4	40	40	0	0	40	40	10	0	10	0	
5	0	320	0	0	0	80	0	20	0	0	
6	20	20	0	0	0	40	0	0	0	0	
7	20	40	0	0	0	0	0	0	40	20	
8	20	80	0	0	0	0	0	0	20	0	
9	40	0	0	0	0	0	0	0	0	0	
10	80	40	0	0	20	20	0	0	40	40	
11	40	80	0	0	20	80	0	0	40	20	
12	40	40	0	0	0	40	0	20	0	0	
13	10	40	0	0	40	40	0	0	0	20	
14	80	20	0	0	0	0	0	0	0	0	
15	0	640	0	0	0	40	0	0	0	0	
<u>Group II</u>											
16***	0	2560	0	40	0	160	0	0	0	160	
17	0	5120	0	0	0	0	0	0	0	0	
18	0	1280	0	0	80	80	0	0	0	0	
19	0	320	0	0	0	0	0	0	0	0	
20	0	640	0	0	0	0	0	0	0	0	

*Reciprocal of serum dilution.

**Not detectable at 1:10 serum dilution.

***Pre-infection serum showed 1:4 CF titre to mumps.

Table 6

Number of guinea pigs showing a 4-fold or greater rise of HI antibody to designated antigen after Sendai infection

Pre-infection HI antibody to:	number of animals	number of animals showing rise of titre to 4 HA units of:			
		Sendai	HA-2	HA-1	CA
Sendai, HA-2, HA-1 and CA	1	0	0	0	0
HA-2, HA-1 and CA	5	1	3	0	1
Sendai, HA-2 and CA	1	0	0	0	0
Sendai and CA	2	1	0	0	0
Sendai	3	0	1	1	0
Total	12	2	4	1	1
No pre-infection HI antibody to Sendai, HA-2, HA-1 or CA	8	8	4	1	1

Experiment 2: Immune Response of Guinea Pigs to HA-2, HA-1, CA, mumps and NDV Virus Infection.

Introduction

Having shown in the previous experiment that sera from guinea pigs 14 days after Sendai intranasal infection contained an adequate level of HI and CF antibodies for antigenic study, and that HI antibody-free animals gave a better homologous response than CF antibody-free animals, 5 groups of guinea pigs were selected on the basis of HI tests. Each group was infected with one of HA-2, HA-1, CA, mumps or NDV. In addition, one group of guinea pigs was selected on the basis of negative CF tests and was infected with HA-2. These sera were examined by means of HI and CF tests against the five viral agents under study.

Experimental

The techniques of infection and bleeding were similar to those described in Experiment 1. Animals were bred and raised in an isolated area, and were cared for by 2 individuals. The proportion of guinea pigs with HI antibody to this group of viruses at the age of approximately 10 weeks was lower than among the animals from Deer Lodge Hospital. For example, 8 guinea pigs in a group of 18 gave negative HI tests to the five viral antigens.

The selected animals, cared for by one individual, were housed in individual cages in an isolated room. After intranasal infection with one member of this group of viruses, the animals were kept for 14 days and were bled out. The room having been washed with Wescodyne, a period of three days was allowed for daily disinfection before another group of animals was housed for infection with another virus.

Results

The results of HI tests on convalescent sera from two groups of guinea pigs intranasally infected with HA-2 are summarized in Table 7. Neither group showed detectable CF antibody prior to infection. One group of 7 animals had pre-infection HI antibody to two or more viral antigens under study (upper five lines of table). Only 2 animals here gave a homologous rise in titre to HA-2; one of these showed also a heterologous rise of HI antibody to Sendai, the other a rise of HI titre to CA. However, by CF tests, all 7 animals showed homologous titre rises upon HA-2 infection (Table 8). In addition, heterologous CF titres were shown to Sendai by two animals, to HA-1 by four and to CA by one animal. The other group of guinea pigs which had no pre-infection antibody showed a 100% homologous seroconversion by HI and CF (Table 7). All 8 animals gave heterologous HI and CF rises to Sendai (Tables 9 and 10). Two of these animals gave titres by HI tests to HA-1 and mumps but not by CF tests. Thus, the result of Experiment 1 on Sendai infection is confirmed in those guinea pigs; higher titres and more specific antibody are produced by pigs lacking pre-immunization antibody.

With the exception of two animals, all guinea pigs for the HA-1 infection experiment (Table 11) were free of HI and CF antibody prior to infection. Guinea pig number 49 showed a pre-immunization CF titre to HA-1 and to HA-2 while number 53 gave a CF titre to HA-1 (Table 11). All ten animals except these two gave only a homologous response both by CF and HI tests, (Tables 11 and 12). The two animals with pre-infection CF antibody gave heterologous rises of HI antibody to mumps to the same titre as the homologous rise to HA-1. There was, however, no heterologous rise in CF titres.

The results of CF and HI tests on sera from animals infected

with CA, mumps and NDV are also shown in Table 11 and 12. With one exception, all animals gave only a homologous response. Guinea pig number C-4 infected with CA gave heterologous titres by CF and HI to Sendai and by HI to mumps.

Discussion

Cook and associates (Cook et al. 1959), using sera from guinea pigs intranasally infected and then injected with Sendai, HA-2, CA, HA-1 and mumps, showed that each serum pool was specific for its homotypic antigen, but that heterotypic reactions were demonstrated on testing individual sera. These antigenic crossings were mainly between Sendai and HA-2 where reciprocal relations were shown by CF tests in every guinea pig serum immunized against Sendai or HA-2. However, by HI tests, reciprocal crossing was shown only in 6 of 14 Sendai sera and 12 of 18 HA-2 sera. A small number of Sendai and HA-2 sera reacted also with CA and HA-1 by both CF and HI tests. Only one of ten animals immunized against CA reacted also with Sendai by HI tests; two of the HA-1 immune sera reacted with Sendai. On the basis of these results and of in vivo challenge experiments in hamsters (Cook, Chanock 1963) where protection to HA-2 infection was shown in Sendai convalescent animals, though no protection to Sendai was apparently conferred on HA-2 convalescent hamsters, Cook and associates proposed that HA-2 and Sendai viruses be classified as subtypes of Myxovirus parainfluenzae type-1. The studies described in the present and in the preceding experiment are in general agreement with their results.

However, a recent report by Dutch investigators (Veen, Sonderkamp 1965) indicates that, while a first Sendai infection in guinea pigs provokes a heterotypic response to HA-2, a first HA-2 infection in

guinea pigs fails to elicit crossing antibody to Sendai. It was only by a second HA-2 infection that CF antibody (but not HI antibody) to Sendai was provoked in all six animals. It is important to note that the animals in their experiment were not selected on the basis of any serological criterion. At this point a recapitulation of the observations on animals showing pre-infection antibody, as against those on animals showing no pre-existing antibody, may help to explain the apparent disagreement between the observations of the Dutch group and those reported by Cook et al. and the present author. All eight animals in Table 7 with no antibody at the time of infection with HA-2 showed crossing CF and HI antibody to Sendai. In comparison, in a group of seven animals in the same table showing HI antibody at the time of infection, one developed Sendai HI antibody and two developed CF antibody; at the same time, a homologous rise of titre to HA-2 was shown in only two animals by HI tests, though in all seven by CF tests. The Dutch workers on the other hand reported a homologous rise of titre to HA-2 in only one animal by HI and five by CF in a group of six guinea pigs 14 days after infection. Their observations are therefore similar to ours on animals with pre-infection antibody. Whereas one instillation of virus was adequate to provoke a heterologous response in our antibody-free guinea pigs, a similar response in the animals studied by the Dutch group was provoked only after two serial instillations.

Thus, the CF responses, and a portion of the HI responses, observed in the present study suggest that HA-2 and Sendai are closely related. The study reported from Russia (Zhdanov, Bukrinskaya 1960) showed that an antigenic relationship between the two viruses could only be demonstrated in the CF tests using guinea pigs, but

not by using rabbit and rat immune sera. Further studies of the Soviet workers (Bukrinskaya, Ho Yun-de, Gorbunova 1962) showed that strain variants exist in both Sendai and HA-2; Vladivostok strains of Sendai gave only homologous reactions, Biryukov and Galkin strains of HA-2 gave heterotypic reactions with the MN Japanese strain of Sendai but not with Vladivostok strains. Similarly, strain variants were reported by American workers (Dick, Mogabgab 1962) who by means of immune rabbit and rooster sera, showed that HA-2 strain E94 did not elicit crossing HI antibody to Sendai, whereas crossing titres were provoked by an HA-2 strain, Copenhagen 222 (Petersen 1958) (which, incidentally, provoked lower heterotypic titres than the N.I.H. strain). It is interesting to note that HA-2 strains #94 and Copenhagen 222 showed reciprocal crossing by HI test with HA-1, and gave no crossing reaction with the N.I.H. strain of HA-2. Possibly, the strain difference was responsible for the recent observation reported by Spurrier and Robinson (Spurrier, Robinson 1965) who showed a one way crossing between HA-2 and Sendai with HA-2 immunized guinea pig sera, using the HA-2 strain C-38 from the World Health Organization Regional Reference Laboratory instead of strain C-35 from ATCC used in the present study.

With the exception of three animals, the response to intranasal infection of guinea pigs with HA-1, CA, mumps and NDV was specific. This is in agreement with the results obtained on guinea pigs, ferrets and hamsters (Cook et al. 1959; Dick et al. 1962; Jensen et al. 1962; Marine 1964; Veen et al. 1965). The two animals that gave crossing reactions to mumps after HA-1 infections, and the single animal which showed heterologous titres to Sendai and mumps after CA infection, may possibly be explained on the basis

of prior exposure to homologous, or antigenically related viruses, rather than to antigen-sharing between HA-1, mumps, Sendai and CA. Hsiung and associates (Hsiung et al. 1965) showed that antibody to NDV was undetectable in chickens four months after three vaccinations with live NDV. Data from our preceding experiment demonstrated that seven guinea pigs in a group of 15 gave a negative homologous HI test four months after Sendai infection. Thus, prior antigenic experience may escape detection by serologic testing. It is known that the incidence and magnitude of serologic cross-reactions may increase if animals are exposed repeatedly to the same antigen or related antigens in influenza infection (Henle, Lief 1963). Such broadening of antibody spectra to parainfluenza virus infections has been well documented in human sera (De Meio et al. 1957; Cook et al. 1959; Jensen et al. 1962; Lennette et al. 1963; Hsiung et al. 1963; Stark et al. 1964). These observations have been recently confirmed in ferrets (Marine 1964) which gave crossing titres to mumps, HA-2 and HA-1 upon two serial Sendai infections. By repeated homologous HA-1 and CA infections, apparent crossing antibodies were demonstrated both between mumps and HA-1, and between mumps and CA. Similar observations on guinea pigs have been recently reported (Veen et al. 1965). Thus, guinea pig number 49 and guinea pig number 53, both showing pre-existing antibody by CF tests, may be considered secondarily infected upon instillation of HA-1. Guinea pig C-4 may have had some antibody that was not measurable at the time of infection.

Conclusions

1. Sendai and HA-2 viruses have a demonstrable common antigen or antigens.
2. HA-1, CA and mumps viruses and NDV appear to be serologically distinct from one another.

Table 7

Number of guinea pigs showing a 4-fold or greater rise of
HI antibody to designated antigen after HA-2 infection

Pre-infection HI antibody to:	number of animals	number of animals showing rise of titre to 4HA units of:				
		Sendai	HA-2	HA-1	CA	mumps
5 agents	3	0	0	0	0	0
Sendai and CA	2	0	1	0	1	0
HA-2 and CA	1	0	0	0	0	0
HA-1 and CA	1	1	1	0	0	0
Total	7	1	2	0	1	0
No pre-infection HI antibody to Sendai, HA-2, HA-1, CA or mumps	8	8	8	2	0	0
Total no. of animals with and without pre- infection HI antibody	15	9	10	2	1	0

Table 8

CF titres of guinea pigs with pre-infection HI antibody to designated antigens but <4 CF antibody to all 5 viral antigens after HA-2 infection

<u>Guinea pig number</u>	<u>HI pre-infect. antibody to</u>	<u>Sendai</u>	<u>HA-2</u>	<u>HA-1</u>	<u>CA</u>	<u>Mumps</u>
18	5 agents	4	32	4	0*	4
23	5 agents	0	16	4	0	0
31	5 agents	0	16	0	0	0
20	Sendai and CA	8	64	8	0	0
27	Sendai and CA	0	8	0	0	0
22	HA-2 and CA	0	16	0	4	0
19	HA-1 and CA	0	16	4	0	0

*0 = <4

Table 9

CF titres of guinea pigs without pre-existing antibody* against
designated antigens after HA-2 infection

<u>Guinea pig serum number</u>	<u>Sendai</u>	<u>HA-2</u>	<u>HA-1</u>	<u>CA</u>	<u>Mumps</u>
16	8	128	0	0	0
17	16	64	0	0	0
21	16	256	0	0	0
24	8	128	0	0	0
25	8	64	0	0	0
28	16	64	0	0	0
29	8	64	0	0	0
30	4	128	0	0	0

* < 4 CF Antibody and < 10 HI antibody to 5 viral antigens.

Table 10

HI titres of guinea pigs without pre-existing antibody* against
designated antigens after HA-2 infection

4 HA units

<u>Guinea pig serum number</u>	<u>Sendai</u>	<u>HA-2</u>	<u>HA-1</u>	<u>CA</u>	<u>mumps</u>
16	40	320	0	0	0
17	20	160	0	0	0
21	40	320	40	0	40
24	20	160	0	0	0
25	40	160	80	0	40
28	80	320	0	0	0
29	40	320	0	0	0
30	20	160	0	0	0

*<4 CF antibody and <10 HI antibody to 5 viral antigens.

Table 11

CF titres after specified infection of guinea pigs whose sera prior to infection showed no CF and HI antibody to designated antigens

<u>Infected with</u>	<u>Guinea pig no.</u>	<u>Sendai</u>	<u>HA-2</u>	<u>HA-1</u>	<u>CA</u>	<u>Mumps</u>	<u>NDV</u>
HA-1	37	0	0	128	0	0	0
	38	0	0	128	0	0	0
	39	0	0	64	0	0	0
	40	0	0	128	0	0	0
	42	0	0	64	0	0	0
	44	0	0	64	0	0	0
	45	0	0	128	0	0	0
	47	0	0	64	0	0	0
	*49	0	8	32	0	0	0
**53	0	0	64	0	0	0	
CA	D-1	0	0	0	64	0	0
	D-2	0	0	0	64	0	0
	D-3	0	0	0	128	0	0
	D-4	0	0	0	64	0	0
	C-1	0	0	0	64	0	0
	C-2	0	0	0	128	0	0
	C-4	16	0	0	64	0	0
	C-6	0	0	0	128	0	0
	C-7	0	0	0	256	0	0
	C-10	0	0	0	64	0	0

Table 11 (continued)

<u>Infected with</u>	<u>Guinea pig no.</u>	<u>Sendai</u>	<u>HA-2</u>	<u>HA-1</u>	<u>CA</u>	<u>Mumps</u>	<u>NDV</u>
NDV	102	0	0	0	0	0	16
	104	0	0	0	0	0	8
	105	0	0	0	0	0	16
	106	0	0	0	0	0	32
	107	0	0	0	0	0	16
<hr/>							
Mumps	71	0	0	0	0	128	0
	72	0	0	0	0	128	0
	74	0	0	0	0	32	0
	76	0	0	0	0	64	0
	77	0	0	0	0	128	0
	78	0	0	0	0	128	0
	79	0	0	0	0	64	0
<hr/>							

*Pre-immunization CF titre = 4 to HA-1; 8 to HA-2

**Pre-immunization CF titre = 4 to HA-1

Table 12

HI titres after specified infection of guinea pigs whose sera prior to infection showed no CF and HI antibody to designated antigens

<u>Infected with</u>	<u>Guinea pig no.</u>	<u>Sendai</u>	<u>HA-2</u>	<u>HA-1</u>	<u>CA</u>	<u>Mumps</u>	<u>NDV</u>
HA-1	37	0	0	160	0	0	0
	38	0	0	80	0	0	0
	39	0	0	80	0	0	0
	40	0	0	80	0	0	0
	42	0	0	160	0	0	0
	44	0	0	80	0	0	0
	45	0	0	160	0	0	0
	47	0	0	320	0	0	0
	*49	0	0	80	0	80	0
	**53	0	0	80	0	40	0

CA	D-1	0	0	0	80	0	0
	D-2	0	0	0	160	0	0
	D-3	0	0	0	160	0	0
	D-4	0	0	0	80	0	0
	C-1	0	0	0	160	0	0
	C-2	0	0	0	80	0	0
	C-4	160	0	0	80	80	0
	C-6	0	0	0	160	0	0
	C-7	0	0	0	320	0	0
	C-10	0	0	0	80	0	0

Table 12 (Continued)

<u>Infected with</u>	<u>Guinea pig no.</u>	<u>Sendai</u>	<u>HA-2</u>	<u>HA-1</u>	<u>CA</u>	<u>Mumps</u>	<u>NDV</u>
NDV	102	0	0	0	0	0	320
	104	0	0	0	0	0	640
	105	0	0	0	0	0	640
	106	0	0	0	0	0	320
	107	0	0	0	0	0	1280
<hr/>							
Mumps	71	0	0	0	0	80	0
	72	0	0	0	0	80	0
	74	0	0	0	0	40	0
	76	0	0	0	0	80	0
	77	0	0	0	0	40	0
	78	0	0	0	0	40	0
	79	0	0	0	0	80	0

*Pre-immunization CF titre = 4 to HA-1; 8 to HA-2

**Pre-immunization CF titre = 4 to HA-1

Experiment 3: Study on a Possible Antigenic Constituent Common to Guinea Pig Tissues and Parainfluenza Viruses.

Introduction

In all the studies reviewed previously on the antigenic relationships among the parainfluenza viruses using animal sera, and in the experiments described above, one consistent feature is noted, namely, the variation of heterologous response in individual animals. These heterologous crossings bear no consistent relationship to the level of the homologous response and are generally not predictable except in the case of Sendai and HA-2. If these are considered to reflect the existence of a common group antigen, then a state of immunological tolerance might be postulated in animals which failed to show an heterologous response. Thus, DA, a member of parainfluenza 5, provoked anti-B isoagglutinins in Patas and African Green monkeys but not in Rhesus monkeys whose organs were shown to contain a B-like substance (Isacson, Holden 1962). Therefore, the following experiment was undertaken to investigate whether antigenic similarity existed between guinea pig tissues and the viral agents under study by examining, for specific viral antibody, sera from animals immunized against an antigen prepared with pooled guinea pig organs.

Experimental

Animals

Guinea pigs and rabbits used in this experiment were bred and raised in the animal quarters and cared for by two individuals.

Preparation of guinea pig organ antigen

Lungs, spleen, liver and kidneys were removed from eight guinea

pigs. A 40% organ suspension was made in HBSS consisting of equal parts by weight of liver, spleen, lung and kidney which had been first homogenized in a "Waring" blender. The suspension was used for hyperimmunization after three cycles of freezing and thawing.

Immunization

Four rabbits approximately four months old were selected on the basis of showing no pre-existing HI antibody to the viral agents under study. The rabbits were given two intraperitoneal injections of 2.0 ml. twice weekly for four weeks followed by weekly 2.0 ml. intramuscular injections for another four weeks. Fourteen days after the last injection, the rabbits were exsanguinated. Sera were inactivated at 56°C for 30 minutes and were tested for HI and neutralizing antibody against the viral agents.

Ten guinea pigs were selected on the basis of showing negative CF tests at 1:4 serum concentration to the viral agents. Two were HI positive to both Sendai and CA. The guinea pigs were immunized with the organ antigen, using the same schedule as that of the rabbits but only a fourth of the dosage. Testing of the guinea pig sera included the CF test in addition to the HI and neutralization tests.

Results

By HI and neutralization tests on rabbit and guinea pig sera, no rise of anti-viral antibody was observed after immunization with guinea pig organs. Guinea pig post-immunization sera showed no rise of CF titres. Four of these sera, were anticomplementary after immunization, up to 1:8 serum dilution. However, they gave negative CF tests at 1:16 serum dilution.

Comment

The results provide no evidence that antigen is shared by guinea pig tissues and the parainfluenza viruses. Thus, if a group antigen exists for these viral agents, the lack of response to this antigen in some guinea pigs is probably not attributable to a state of immunological tolerance.

Experiment 4: Hyperimmune Response of Rabbits to Injections with Live Parainfluenza Viruses.

Introduction

Since conflicting results in different species of animals have been observed when antibody responses were examined after inoculation of parainfluenza viruses (as reviewed earlier) and since these results were obtained by different groups of workers in different geographic areas, as for example, the N.I.H. workers (Cook et al. 1959) in Washington using guinea pigs and the Moscow group (Zhdanov et al. 1960) using rabbits, a study of the immune responses of rabbits to this group of viruses was also made in this laboratory. HI and NT response were used because antibodies against host tissue do not interfere as they do in CF tests.

Experimental

A group of thirteen rabbits was selected on the basis of showing negative HI tests to the immunizing viral agents. The animals were hyperimmunized using the schedule outlined in the section "General Methods and Materials". With the exception of rabbits immunized against CA, all animals were immunized by giving two weekly intramuscular injections of 2.0 ml. for four weeks. In the case of CA, two courses of injections were given. The sera were examined for HI and neutralizing antibodies.

In the process of animal selection, a survey was made of naturally acquired parainfluenza HI antibody in rabbits raised in this laboratory.

Results

Naturally acquired parainfluenza HI antibody in rabbits

Although the rabbits were bred and raised in the animal quarters of this laboratory and handled by only two individuals, they displayed a broad spectrum of previously acquired antibody to parainfluenza viruses. As seen in Table 13, the highest incidence of antigenic experience was shown to be HA-1.

Response to Parainfluenza Immunization

By HI tests, rabbits immunized with Sendai gave crossing titres to HA-2 and HA-1 (Table 14). However, by neutralization tests, only crossing titres to HA-2 were shown (Table 15). Rabbits immunized with HA-2 showed crossing antibody to Sendai by HI tests but not by neutralization tests. Similarly, animals injected with CA gave heterologous titres to mumps by HI tests but not by neutralization tests. On the other hand, animals immunized with mumps gave crossing antibody to CA by both tests. Animals immunized with HA-1 or with NDV showed only homologous responses.

Discussion

The results indicate that Sendai virus possesses antigenic components which provoke in rabbits HI antibodies reacting heterologously with HA-2 and HA-1 viruses. This observation is in agreement with the earlier studies on rabbits by both the Russian and the American groups; (Zhdanov et al. 1960; Dick et al. 1962). Also, ferrets, which yielded only homologous antibody upon Sendai infection, gave crossing HI titres to HA-2 and HA-1 on hyperimmunization (Marine 1964). The close relationship between Sendai and HA-2 is further

evidenced by the crossing HI titres to Sendai in rabbits immunized with HA-2 (Table 14).

The relationship between Sendai and HA-1 is less clearly defined. To begin with, only a small portion of guinea pigs immunized against Sendai virus guinea pigs gave heterologous CF titres to HA-1 (Table 3) and only two of 19 showed HI antibody (Table 6). Similar results were reported in an earlier study (Cook et al. 1959). Although antigenic relationships between these two viruses were given credence by the reciprocal anamnestic response observed in guinea pigs infected first with Sendai followed by HA-1, and vice versa, (Veen et al. 1965), the results from studies on rabbits have been inconsistent. Thus, the Russian workers found heterologous HI titres to HA-1 in one Sendai immune rabbit but not in the other (Zhdanov et al. 1960), whereas Dick and associates showed high titres to HA-1 in all animals immunized with Sendai. In addition, it has been shown (Veen et al. 1965) that in serial infections in guinea pigs with mumps followed by HA-1 or vice versa, instead of showing a recall to the first infecting virus, the animals gave heterologous titres by both CF and HI test to CA. Since an epizootic HA-1 infection (50%) was indicated by the results of the rabbits used for this experiment (Table 13), and in addition, the rabbits immunized with mumps showed crossing antibody to CA, it is probable that the two rabbits showing titres to HA-1 after Sendai immunization reflect an anamnestic response.

In earlier studies (Chanock 1956; Lennette et al. 1963) antibody rises to CA virus were found to occur in patients with mumps virus infection. Serial infections in ferrets (Marine 1964) showed

that, while recall of antibody to mumps was possible as a result of a second infection by Sendai, by CA or by HA-1 in animals initially infected with mumps, a specific anamnestic response was demonstrated only to CA among viruses of this group when mumps was used as the second infecting agent. Thus, the reciprocal HI crossing antibody shown by rabbits immunized with CA, and rabbits immunized with mumps (Table 14), and the one direction heterologous neutralizing antibody to CA (Table 15) serve to substantiate the antigenic relationship between CA and mumps.

Conclusion

The immune response of rabbits to injection with these viruses, using HI and neutralization tests, suggest the following:

1. Sendai and HA-2 share a common antigen.
2. Mumps and CA share a common antigen.
3. HA-1 and NDV are immunogenically distinct from each other and the other parainfluenza viruses.

Table 13

Naturally occurring HI antibodies to parainfluenza viruses
in laboratory rabbits

Positive at 1:10 serum dilution to
 4 HA units of designated antigen

<u>Total number of Rabbits Tested</u>		<u>Sendai</u>	<u>HA-2</u>	<u>CA</u>	<u>HA-1</u>	<u>Mumps</u>	<u>NDV</u>
42	No. of rabbits Positive	6	4	2	21	3	0
	Percent Positive	14	9	5	50	7	0

Table 14

HI titres of sera from rabbits hyperimmunized against
parainfluenza viruses

Rabbits Immune to	Reciprocal serum dilution inhibiting haemagglutination by 4 HA units of designated viral antigen					
	<u>Sendai</u>	<u>HA-2</u>	<u>CA</u>	<u>HA-1</u>	<u>Mumps</u>	<u>NDV</u>
Sendai	1280	40	0	40	0	0
	640	80	0	20	0	0
	640	40	0	0	0	0
HA-2	20	160	0	0	0	0
	20	160	0	0	0	0
CA	0	0	80	0	20	0
	0	0	80	0	20	0
HA-1	0	0	0	2560	0	0
	0	0	0	2560	0	0
Mumps	0	0	40	0	320	0
	0	0	40	0	320	0
NDV	0	0	0	0	0	640
	0	0	0	0	0	640

Experiment 5: Studies of Absorption of Guinea Pig and Human Convalescent Sera with Sendai Virus.

Introduction

From previous experiments, it became apparent that compared with other members of the group, Sendai virus has the broadest spectrum of activity both immunologically and serologically. It is not clear if some of the results might be interpreted as a non-specific rise in antibody against other viruses previously infecting the animal or as the result of antigens shared by Sendai and other viruses. In order to help in resolving this question, absorption studies were carried out with Sendai on convalescent sera from guinea pigs and from humans. Also included were two sera from rabbits immunized with Sendai showing heterologous antibodies to HA-2 and HA-1.

Experimental

Animal sera

Two convalescent sera were selected from each group of guinea pigs infected with Sendai, HA-2, HA-1 and CA respectively in Experiments 1 and 2. They were selected on the basis of showing high HI antibodies. Two rabbit sera, from Experiment 4, showing heterologous titres to HA-2 and HA-1 after Sendai immunization were also selected.

Human sera

Ten human sera collected in February, 1963, in Eskimo Point, North West Territories, during an outbreak of respiratory disease (Hildes, Wilt, Parker, Stackiw 1965) were made available for the

study by Dr. W. L. Parker. This outbreak was attributed to HA-1 infection.

Sendai virus absorption

Sendai infected allantoic fluid was concentrated and resuspended in PBS to contain 15,000 HA units per 0.5 ml. by the method used in mumps concentration described under General Methods and Materials. A similar procedure was repeated on uninfected allantoic fluid to serve as a control for the serum absorption. To 5.0 ml. of serum diluted 1:5 which had been treated with receptor destroying enzyme (Microbiological Associates) to remove non-specific inhibitors, was added 2.0 ml. Sendai virus suspension. Absorption was allowed to proceed at 37°C for 30 minutes and at 4°C overnight. This was repeated with an additional 2.0 ml. virus suspension. Residual virus was removed by repeated absorption with packed washed guinea pig erythrocytes until serum-virus mixtures became haemagglutination negative.

Guinea pig sera Nos. 16 and C-4, and all human sera, were absorbed with control uninfected allantoic fluid preparations.

Results

The effect of Sendai absorption on guinea pig convalescent sera is shown in Table 16. Sendai virus removed Sendai antibodies formed as a result of a homologous or heterologous response. Both types of antibody to HA-2 were also removed. Sendai exerted no effect on the titres against CA and HA-1 produced as a result of homologous response. However, from serum #16, Sendai removed all antibodies including the heterologous antibodies against mumps and CA. Uninfected allantoic fluid concentrate did not remove antibody from sera 16 and C-4.

On Sendai hyperimmunized rabbit sera, Sendai absorption removed only antibody to Sendai and exerted no effect upon the heterologous antibodies to HA-2 and HA-1.

Table 17 shows the distribution of HI antibodies to Sendai, HA-2, HA-1 and CA in the ten human sera after absorption with uninfected allantoic fluid concentrate. These sera showed high titres to HA-1; six showed a moderate level of HI antibody to Sendai, four to HA-2 and two to CA. Six sera (starred in Table 17) were selected for Sendai absorption; four contained antibody to Sendai and two were negative. Unexpectedly, all six became negative to all the four viral antigens used to test them (Sendai, CA, HA-1 and HA-2). Similar absorption was repeated on another set of samples from these 6 sera and negative reactions were again observed on the post-absorption sera.

Discussion

There have been only two studies reported on antibody absorption with parainfluenza viruses. Using monkey sera immunized against DA virus Hsiung and associates (Hsiung 1963b; Hsiung et al. 1965) showed that heterotypic antibody to mumps and HA-1 could be removed by absorption with the specific viruses, but not with the immunizing live virus. This is in agreement with the present result obtained on one CA convalescent serum C-4, from which only heterotypic antibody to Sendai was removed by Sendai absorption. However, the results on serum #16 and serum #18 are not in agreement with Hsiung's observations. Another study was made on sera from ferrets repeatedly infected and vaccinated with one virus to provoke heterotypic antibodies (Marine 1964). For purposes of

comparison, the results of the ferret serum absorption experiment are recapitulated here. Sendai virus was shown to remove homotypic antibody to Sendai as well as heterotypic antibodies to HA-2 and HA-1 which had been produced upon repeated Sendai instillations. In a similar manner, HA-2 was shown to remove homotypic antibody to HA-2 and heterotypic antibody to Sendai elicited as a result of repeated HA-2 infections. However, heterotypic antibody to mumps, provoked by HA-2 vaccination on HA-2 convalescent ferrets, was not removed by HA-2 absorption. In contrast, HA-2 or HA-1 antibody formed as a result of homologous response was not absorbed by Sendai, nor was Sendai homologous antibody susceptible to HA-2 absorption.

It appears that, in ferret sera, there are demonstrable differences between viral antibodies formed as a result of a homologous response and those resulting from a heterologous response, and between the heterologous antibodies provoked by serial infections and those formed as a result of vaccination. These differences were not so clear-cut in the guinea pig sera, where heterotypic antibodies to mumps, HA-2, and CA in the Sendai convalescent serum #16 were removed by Sendai absorption. Unlike ferret antibody, guinea pig homologous HA-2 antibody was absorbed by Sendai virus, but as with ferret antibody homologous guinea pig antibody to HA-1 and CA was not removed by Sendai.

Indeed, the absorption results indicate that there is no difference between the homotypic and the heterotypic antibody active against Sendai and HA-2 produced by guinea pigs after infection. This may serve to substantiate the relationship between

these two viral agents shown by serology in previous experiments. The insusceptibility of HA-2 antibody to Sendai absorption in sera from rabbits immunized with Sendai shows the variation of immune response in different animal species. This is further illustrated by the results of absorption studies on human sera (Table 17) where Sendai removed antibodies to CA, HA-2, and HA-1 produced as a result of HA-1 infection.

Conclusion

- (1) Common antigens in Sendai and HA-2 virus were demonstrated by antibody absorption from guinea pig convalescent sera. Sendai was shown to share no common antigen with CA and HA-1.
- (2) HI antibodies to Sendai, CA, HA-1 and HA-2 in human sera were removed by Sendai absorption indicating basic differences between human and guinea pig HI antibodies.
- (3) There is a difference between the heterotypic antibody to HA-2 from convalescent sera from guinea pigs, and the heterotypic antibody to HA-2 from a vaccinated rabbit; namely, the former was absorbed by Sendai virus and the latter was not.

Table 16

Effect of absorption with Sendai virus on HI antibody titres in
convalescent guinea pig sera

Convalescent from infection with	Titre with 4 HA units of designated antigen						
	Sera No.	Sendai	Mumps	HA-2	HA-1	CA	
Sendai	16	pre*	2560	40	160	0	160
		post**	0	0	0	0	0
	18	pre	1280	0	80	0	0
		post	0	0	0	0	0
HA-2	21	pre	40	40	320	40	0
		post	0	40	0	40	0
	25	pre	40	40	160	80	0
		post	0	40	0	40	0
HA-1	37	pre	0	0	0	160	0
		post	0	0	0	160	0
	47	pre	0	0	0	320	0
		post	0	0	0	320	0
CA	D-1	pre	0	0	0	0	80
		post	0	0	0	0	80
	C-4	pre	160	80	0	0	80
		post	0	80	0	0	80

*Pre-absorption titre

**Post-absorption titre

Table 17

HI titres of human sera collected from Eskimo Point against some
parainfluenza viruses after absorption with uninfected
allantoic fluid

Titre with 4 HA units of designated antigen

<u>Sera Number</u>	<u>Sendai</u>	<u>CA</u>	<u>HA-1</u>	<u>HA-2</u>
1	0	0	160	0
2*	40	40	160	0
3*	40	0	320	0
4*	0	0	320	40
5	10	0	160	10
6*	0	0	160	0
7*	80	20	640	10
8	20	0	160	0
9	0	0	160	0
10*	20	0	320	40

*These sera were absorbed with Sendai, and no detectable HI antibody at 1:10 demonstrable to all 4 antigens after absorption

Experiment 6: Serum Agglutination of Erythrocytes treated
With Sendai Virus.

Introduction

Certain viruses have been shown to possess the ability to sensitize erythrocytes for subsequent agglutination by homologous and heterologous virus immune sera. This was first reported by Burnet and Anderson (1946) who showed that either human or fowl erythrocytes, upon preliminary contact with NDV at 37°C followed by spontaneous elution of virus at the same temperature, were agglutinated by the addition of a high proportion of sera from humans with infectious mononucleosis. The observation has since been confirmed (Evans, Curnen 1948; Florman 1949). Other viruses possessing the same ability are mumps (Burnet 1946) and DA (Isacson, McCollum, Hsiung 1962). Since Sendai has been shown to exhibit many biological properties similar to mumps and NDV, the following experiment was undertaken to determine whether Sendai could similarly sensitize erythrocytes. The experiment also attempted to show the presence of a group antigen by agglutination of Sendai virus altered erythrocytes using immune sera from guinea pigs.

Experimental

Red Cells

Human red cells, type O, were obtained from a donor. The cells were stored at 4°C in Alsever solution and were not used when more than two weeks old.

Sera

Convalescent guinea pig sera from Experiments 1 and 2 were used

in this study. Sendai convalescent guinea pig serum #18 and HA-1 convalescent serum #47 after Sendai absorption (Experiment 5) were tested at 1:20 serum dilution. Also included were human sera showing high Paul-Bunnell titres and some showing high latex fixation titres. These were kindly supplied by Dr. R. Martin of the Manitoba Provincial Health Laboratory. Two sera from guinea pigs, immunized with guinea pig organ tissues in Experiment 5, were also included.

Sensitization of Erythrocytes

The Sendai virus infected egg allantoic fluid used for sensitization was titrated with human O cells in 0.25% suspension at 4°C by the pattern method. To a 1% suspension of red cells, an appropriate amount of virus was added to make a final concentration of 10 HA units per 0.5 ml. of 0.25% erythrocytes suspension. Sensitization was allowed to proceed overnight at 4°C. Cells were washed with normal saline, resuspended to the original volume and incubated at 37°C in a waterbath. The suspensions were agitated, washed, resuspended and allowed to resettle until a negative pattern was attained. The procedure was duplicated with uninfected allantoic fluid substituted for the Sendai virus. For the haemagglutination test, cells were resuspended to 0.5% and were added in equal volume to serial dilutions of pre-infection and convalescent guinea pig sera. Control cell suspensions treated with an amount of virus equivalent to 0.25 HA units (a sub-haemagglutinating dose) and those treated with normal allantoic fluid were tested at a starting serum dilution of 1:10.

Results

While all pre-infection sera, which had been selected on the

basis of giving a negative HI test at 1:10 serum dilution to four HA units of the parainfluenza viruses under study, failed to agglutinate the sensitized cells, their convalescent sera agglutinated the cells at 37°C to titres as shown in Table 18. Sera from guinea pigs immunized with organ tissues were also positive. However, convalescent serum #18 and #47 after Sendai absorption showed no agglutination at 1:20 dilution. No agglutination was observed when these sera were tested on cells treated with uninfected allantoic fluid, nor with cells treated with a sub-haemagglutinating virus dose.

Table 19 shows the haemagglutination titres of the human sera. These sera were tested and shown HI negative to Sendai except serum #1 which titred at 1:20.

Discussion

It is difficult to judge from the results whether agglutination of Sendai treated red cells has any connection with the immune response to parainfluenza viruses. The apparent absence of relationship between the agglutination titres and the level of HI antibody in convalescent sera suggests separate functional components in either the immune sera or the virus. On the other hand, the agglutinin was removed along with the Sendai HI antibody absorption and this supports the concept that the factor in the immune serum giving rise to this reaction was an antibody arising after stimulation by Sendai or related viruses. Should this be the case, then the antibodies for this agglutination and for the HI test would be reacting against distinct components, since Sendai absorption rendered the HA-1 serum negative for agglutination while exerting

no effect on the homologous HI titre (Table 16). Additional evidence of the factor being an antibody are the stability to heating and the insusceptibility to kaolin absorption. An analogy with this type of response is seen in typhus rickettsiae which gives rise to an antibody against an erythrocyte sensitizing antigen distinct from CF antibodies and antibodies agglutinating Proteus OX-19 (Chang 1953).

Although the Sendai treated cells were not agglutinated by the preinfection guinea pig sera, they were agglutinated by all other convalescent animal and human sera, with the exception of two. In addition, sera from guinea pigs immunized with organ tissues showed high agglutination titres. These observations suggest that the antibody for the agglutination is not associated with viral antigenicity.

Since, in vitro, parainfluenza viruses can be absorbed on and eluted from erythrocytes, it may be postulated that similar occurrence takes place in vivo during viremia. The virus is apparently able to produce an antigenic alteration of the host's erythrocytes or tissue cells by virtue of its enzymatic action. It may be that these altered cells become antigenic for the host, and antibodies are produced against them, the phenomenon then being an "autoimmune" one.

Conclusion

Erythrocytes sensitized by Sendai virus react with an array of sera giving no evidence of specificity. Results obtained using this type of agglutination with immune parainfluenza virus sera cannot be used to indicate the existence of a group antigen.

Table 18

Agglutination titre to Sendai sensitized human O cells of guinea
pig convalescent sera from parainfluenza virus infection

<u>Convalescent</u> <u>from</u>	<u>serum number</u>	<u>agglutination titre</u> <u>to sensitized RBC</u>	<u>HI titre to 4 HA units</u> <u>of the infecting virus</u>
HA-2	28	40	320
	30	40	160
Sendai	16	320	2560
	17	160	5120
	18	80	1280
	19	80	320
	20	40	640
HA-1	47	40	320
	49	20	80
CA	C-7	40	320
	C-10	80	80
Immune to guinea pig organ tissues	0-4	320	
	0-5	320	

Titres were expressed as reciprocals of serum-dilutions showing agglutination in the first column and inhibition of viral haemagglutination in the second.

Table 19Agglutination titre to Sendai sensitized RBC in human sera

<u>Serum Number</u>	<u>Paul-Bunnell</u>	<u>Latex Fixation</u>	<u>Agglutination to Sendai sensitized RBC titre</u>
868	5120*		0
11141	2560		80
10416	2560		80
484	320		80
18703	640		40
1		640**	160
2		1280	160
3		80	80
4		640	160
5		80	160
6		80	160
7		80	160
8		320	640
9		40	320
10		320	640
11		80	0
12		640	1280

*reciprocal of dilution serum.

**80 and over usually considered strongly positive.

Experiment 7: The Cell Blocking Test (CBT).Introduction

The use of an antiserum in the culture medium for the purpose of suppressing the growth of an endogenous virus is not a new technique. Indeed, a majority of the biological supply houses in the USA supply cultures of monkey kidney grown and maintained in the presence of specific antiserum against SV5. This method of growth and maintenance of cultures, while usually adequate for the inhibition of haemadsorption, has been found to be inadequate for the suppression of intracellular growth of the latent virus (Wong 1965). In chronically infected cell lines overlaid with a specific immune antiserum, similar inhibition of haemadsorption without inhibition of intracellular viral multiplication was reported in MCN cells infected with mumps or NDV (Cantell 1961), in KB cells infected with HA-1 (Daniel, Chany 1962) and in HeLa cells infected with HA-2 (Yorio 1964).

Exploratory investigations on the parainfluenza viruses under study produced similar results; tissue cultures after viral infection overlaid with homologous viral antiserum, while showing evidence of intracellular virus multiplication, did not adsorb guinea pig red cells. The term cell-blocking test (CBT) was coined to describe a test utilizing this reaction. Preliminary comparison of CBT results with the titres of HI, CF and neutralization antibodies in the sera suggested that the component responsible for a positive CBT was different from these antibodies. The following experiment was undertaken to study the nature of the CBT by more comparisons with HI, neutralization and haemadsorption inhibition

tests, and by attempts to visualize by means of electron-microscopy the site of interaction between the infected tissue culture and ferritinized viral antibody. The CBT also provided another method of investigating antigenic relationships among the parainfluenza viruses.

Experimental

The viruses used in these tests and the technique for neutralization have been described under "General Methods and Materials".

Haemadsorption Inhibition Test

MK culture tubes containing 1.8 ml. maintenance medium were inoculated with 0.2 ml. virus diluted to contain approximately 100 TCID₅₀. The cultures were incubated at 37°C for four days. The fluid was then removed, the culture washed once with PBS, and 0.2 ml. inactivated serum diluted in PBS was added. Three cultures were allowed for each serum dilution ranging from 1:10 to 1:640. The cultures were rotated on a drum at room temperature for one hour. The serum was then removed. To each tube was added 0.5 ml. of 0.5% guinea pig RBC. After a minimal period of 15 minutes at room temperature in a stationary rack, the cultures were examined for haemadsorption. The titre of the serum was the reciprocal of the serum-dilution at which one or more cultures showed no haemadsorption.

Cell Blocking Test (CBT)

Drained cultures were inoculated with 0.2 ml. of virus containing approximately 100 TCID₅₀. The time allowed for virus adsorption at 37°C on a rotating drum was 30 minutes. The cultures were drained and washed once with about 10 ml. HBSS. Serial serum dilutions ranging from 1:10 to 1:320 were made in HB 597. To each infected culture was added 2.0 ml. of HB 597 containing immune serum.

Three cultures were used for each serum dilution. Cultures were incubated for four days before examining for haemadsorption. The titre of CBT was the highest dilution of serum at which no haemadsorption was observed in one or more cultures.

Demonstrations of Antigen-Antibody Complexes.

The culture fluids containing antiserum were examined for complement fixing complexes after 4 days at 37°C. To test for the stability of these complexes, control cultures with the same dilutions of serum were incubated with the addition of (1) an equal volume of undiluted tissue culture viral antigen or (2) an equal volume of undiluted normal TC fluid. To 0.2 ml. of these serum mixtures, in a doubling dilution series in VBS ranging from undiluted to 1:32, was added 2 1/2 HD₅₀ of complement. Fixation of complement was determined by the standard CF test.

Electron Microscopy

The CA system was chosen to demonstrate the site of reaction between the antibody and the infected cells. Globulins from pooled CA convalescent guinea pig sera and globulins from pre-infection sera were conjugated with ferritin by the method originally described by Singer (Singer 1959). Both the conjugated globulins were used in the CBT. The conjugated immune globulins were also used in an haemadsorption inhibition test. These preparations were examined in thin sections using a Philip 200 electronmicroscope together with a normal MK cell control and a CA infected culture.

Preparation of gamma globulin

All pre-infection sera from the CA group in Experiment 4 were pooled, with the exception of serum #C-4. The convalescent sera were

similarly pooled. Gamma globulin was prepared by Kekwick's sodium sulphate precipitation technique with slight modifications. Sodium sulphate was added to the serum to give a final concentration of 18% by weight. The precipitate was recovered by centrifugation and dissolved in sufficient 0.017 M phosphate buffer pH 8.0 to give a volume of 40% of the initial serum. Further precipitation was effected by adding Na_2SO_4 to give a final concentration of 12%. The precipitates recovered by centrifugation were dissolved again in a volume of phosphate buffer equal to 20% of the initial serum volume. Final precipitation was effected with Na_2SO_4 at a concentration of 12%. The gamma globulin recovered by centrifugation was dissolved in a minimal volume of PBS. This was dialyzed against ten volumes of distilled water with four changes. The gamma globulin was then lyophilized.

Conjugation of Globulin with ferritin

To 3 ml. of ferritin, 4 ml. of borate buffer (0.3 M at pH 9.5) was added. Then 5 ml. of distilled water was added to give a final concentration of 0.1 M buffer. To the buffered ferritin, 0.24 ml. of m-xylene di-isocyanate was added. The mixture was stirred at 4°C for 45 minutes then left to stand for 60 minutes. The free di-isocyanate was removed by centrifugation at 10,000 rpm for 30 minutes. The supernatant which contained ferritin-xylene isocyanate complex was added to 180 mg. of lyophilized globulin. Borate buffer 0.1 M at pH 9.5 was added to make a volume of 20 ml. The buffer was added with 0.1168 gm. of NaCl, an amount to give a 0.1 M NaCl concentration. The conjugation was allowed to proceed at 4°C for 48 hours with constant stirring. The conjugated globulin was then dialyzed against

a 0.1 M solution of ammonium carbonate overnight, and was finally dialyzed against 0.1 M phosphate buffer solution at pH 7.5 for 48 hours. After centrifugation at 12,000 rpm for 20 minutes, the conjugated globulin in the supernatant was sterilized by sintered glass filtration. The recovery of the conjugated globulin was roughly estimated by HI testing.

Specimens for Electron Microscopy

Three MK cultures in milk dilution bottles infected with CA were maintained in HB 597 with 1:10 immune globulin solution for four days (CBT). After washing with PBS the cells were removed from the bottles by trypsinization with 0.25% trypsin at 37°C for 15 minutes. The cells were sedimented by centrifugation at 700 rpm for 10 minutes. The cell pellet was then processed for electron microscopy by a technique modified from that originally described by Kellenberger (Thomas 1962). As a control the entire procedure was duplicated with conjugated globulin from the pre-infection serum pool.

Three bottle cultures were infected similarly with CA and incubated at 37°C for four days, and were then overlaid with 1:10 dilution of the solution of conjugated immune globulin (haemadsorption inhibition test). The cells, after washing with PBS, were processed for electron microscopy.

The technique for the preparation of cell sections for electron microscopy may be briefly described as follows (Thomas 1962). The cell pellets were fixed for 1 hour with buffered osmium tetroxide and washed in de-ionized water. To the fixed cells were added a few drops of 2% liquified agar containing 5% formalin. These agar-embedded cells were solidified at refrigeration temperature and cut

into 1 mm³ blocks. The blocks were dehydrated through a graded series of acetone and Vestopal W. The cell blocks were then embedded in gelatin capsules filled with Vestopal containing 1% benzol peroxide and 1% Cobalt naphthenate. Polymerization of the Vestopal was allowed to proceed in ultraviolet light for 12 hours and was completed by incubating at 50^oC for 12 hours. The cell blocks were sectioned for EM examination.

Results

Comparison of CBT with other serologic tests

Pre-infection and Sendai convalescent sera of six guinea pigs from Experiment 1 were titrated against Sendai virus in CBT, neutralization and haemadsorption inhibition tests. These results together with those of CF and HI tests, determined previously, are shown in Table 20. No apparent correlation with any other serologic test was demonstrated by the CBT.

Convalescent guinea pig serum #19 was titrated using CBT and neutralization tests using different infective doses of Sendai. The titres of CBT were found to remain unchanged (Table 21).

Titration of serum-containing medium during CBT

Inactivated convalescent serum #19 diluted 1:10 in HB 597 was used to maintain MK cultures after Sendai infection, and similarly used with uninfected MK cultures. Two cultures were removed from incubation after one day, two days and four days and tested for adsorption of guinea pig RBC. Haemadsorption on all 6 cultures was negative. The cultures were then washed, replenished with serum-free HB 597 and returned for further incubation at 37^oC. Cultures from one day CBT became haemadsorption positive only after 48 hours further

incubation, 24 hours later than the controls; cultures from two days CBT required the same length of time. However, cultures from four days CBT became positive upon only 24 hours additional incubation.

The overlay sera from the cultures removed after each interval were pooled and, after being treated for the removal of non-specific inhibitors, were titrated using the homologous HI tests. The HI titre of the serum incubated for four days over a normal MK culture was unchanged. Surprisingly, this was also the case with the infected cultures. This test was duplicated on a human mumps convalescent serum, using mumps virus. Similarly, no change of HI titres was observed. There was also no change of specificity in the HI antibody observed in convalescent serum #19 overlying infected cultures in the CBT. Also, the pre-infection serum #19 remained negative in HI tests, after overlying infected cultures.

Demonstration of Antigen-Antibody Complexes

Sendai convalescent serum #19 and CA convalescent serum #C-1 were examined for complement fixing complexes after the CBT. Complement fixing complexes were not demonstrated. The controls showed that the complexes, after incubation at 37°C for four days, still fixed complement. In the CA control, the complement fixing endpoint was shown to be at 1:16 dilution and the Sendai control fixed complement to 1:4 dilution.

Electron microscopy (EM)

Examination of the serial sections of the four EM specimens showed no mature virus or nucleocapsids. However, the electron dense ferritinized globulins were plainly visible. In cell sections from the haemadsorption inhibition test, the globulins were seen lining the outer cellular membrane. No globulins were found in the

cytoplasm. A typical picture of the globulin distribution is shown in Figure 1. In contrast to the haemadsorption inhibition test, sections from the CBT performed with ferritinized CA antibody showed no globulins on the cellular membrane. A section of this is shown in Figure 2. Globulins were, however, seen in the cytoplasm, mainly observed in aggregates bound by structures with dense osmiophilic membrane. Some of these are shown in Figure 3. Cell sections from CBT performed with ferritinized globulins from pre-infection sera showed no electron dense deposits either on the cell surface or in the cytoplasm.

Antigenicity of Parainfluenza as shown by CBT

Group I convalescent sera from Experiment 1 and all convalescent sera from Experiment 2 were screened at 1:10 dilution by CBT homologously and heterologously using only one culture tube per serum per virus. With the exception of 15 sera from animals infected with Sendai virus and 15 sera from HA-2 infection, only homotypic reactions were demonstrated with convalescent sera after HA-1, CA, mumps and NDV infection. A 100% reciprocal reaction was shown between the Sendai and the HA-2 sera.

Because heterotypic HI antibody to Sendai in anti-CA guinea pig convalescent serum C-4 was removed by Sendai virus absorption (Experiment 5), and because this heterotypic reaction was not observed in the initial CBT, a duplicate CBT was performed on this serum using a 1:10 dilution for overlying cultures infected with 50 TCID₅₀ or with 100 TCID₅₀ of Sendai. Homologous CBT using CA virus and similar doses was included as a control. For each virus and for each infecting dose, five tissue culture tubes were used. While C-4 serum gave a positive CBT to CA with each of the two

infecting doses, it showed again a negative CBT to Sendai virus. The apparent contradicting results of this serum in virus absorption and in the CBT suggests that two different reactions may be involved: interaction between the viral envelope and the antibody in the case of viral absorption, and between the infected cellular component and the antibody in the case of the CBT.

The sensitivity of the CBT in showing homotypic and heterotypic antibody in the 15 Sendai convalescent sera and the 15 HA-2 convalescent sera was compared with the CF and HI tests in Table 22. In showing homotypic response the CBT was the most sensitive test with both Sendai and HA-2 sera and the HI test the least sensitive. A similar order of sensitivity for the three tests (CBT, CF and HI) was shown for the heterotypic reaction to HA-2 by Sendai sera, and to Sendai by the HA-2 sera. However, to demonstrate heterotypic antibody to HA-1 or CA, the CBT was the least sensitive whereas the CF test was the most sensitive.

Discussion

The data of the foregoing experiment suggest that the antibody responsible for the CBT may not be the HI antibody. This was supported by the observation of the stable HI titres of sera which had been incubated over infected cultures for four days.

Recently, it has been reported from Russia (Zhdanov, Smirnova 1965) that HA activity of Sendai and other myxoviruses is destroyed by extracts of MK cultures. Such activity of the supernatants of the cultures might have made it appear that the HI titre did not

decrease, but if this was the case, non-specific HI titres should have risen in the overlying fluid. The latter was not so. It is therefore thought that the phenomenon reported by Zhdanov and Smirnova did not affect these results.

The antibody responsible for the CBT may be different from that operating in haemadsorption inhibition, since sera negative in the latter test were positive in the former (Table 20). Like influenza infected cell surfaces, which react with ferritinized specific antibody (Duc-Nguyen 1966), CA infected cell surfaces were shown to react with ferritinized antibody when applied as in the haemadsorption inhibition test (Figure 1). However, conjugated antibody was not found to react with the infected cell surface when given in the manner of a CBT (Figure 2). Although caution must be exercised in attempting to draw conclusions regarding dynamic activities from static photographs, the evidence presented in this study suggests the entrance of antibodies into cells when an infected culture is treated with sera, as in the CBT possibly as a result of pinocytosis.

A recent report on the structure of WB virus, a strain of para-influenza 5, showed excellent micrographs of intracytoplasmic internal components and viral filament formation (Prose, Balk, Liebhaber, Krugman 1965). Viral assembly was demonstrated taking place just beneath the cell surface where the internal viral component oriented itself in the form of loose spirals with the long axes of the spirals parallel to the cell surface. The outer cytoplasmic membrane adjacent to the spirals became more electron dense suggesting transformation into viral envelope. This may well be the mechanism responsible for the acquisition of viral antigenic properties by the cell surface

infected with parainfluenza viruses.

Since in the CBT, cell surfaces showed neither haemadsorption nor deposits of ferritinized antibodies, it may be inferred that overlay immune globulin suppresses the antigenic acquisition. Observations on NDV infection of single cells have shown that the antigenic acquisition of sites on the cell surface is not spontaneous, but is progressively accomplished in a period of time (Marcus 1962). Therefore, it is reasonable to envisage a process of stages by which a cellular surface site is converted to a full complement of viral antigen. Since in the CBT, antibody is present from the beginning of cell infection, antigen-antibody reaction would take place at the moment the cell site turns adequately antigenic, probably prior to the final stage of antigenic acquisition. This view is supported by the finding that antibody active in the CBT is different from that functioning in the HI or that in the haemadsorption inhibition tests.

The original aim of the EM experiment was to locate the site of interaction between the antibody in the CBT and the infected cells. In this respect, the experiment failed. The fact that globulins were observed in the cytoplasm (Figure 3) suggests the engulfment by the cell of the overlay antibody together with the antibody-antigen complexes. The absence of complement-fixing antibody-antigen complexes in the overlay medium lends support to this assumption.

Conclusion

Antibody measured in the CBT is of a different specificity to CF and HI antibody against parainfluenza viruses. It is probably an antibody against a component of the maturing viral envelope. This component seems to have the same antigenic structure in Sendai and

HA-2 viruses.

Acknowledgement

The author is grateful to Dr. C. Thomas of Department of Pathology, University of Manitoba, for processing and examining the EM specimens.

Table 20

Homologous titre of pre-infection and convalescent guinea pig sera (Sendai infection) in different serologic tests

<u>Sera No.</u>	<u>CF</u>	<u>HI</u>	<u>Neut</u>	<u>CBT</u>	<u>Haemad. Inhib.</u>	<u>CF</u>	<u>HI</u>	<u>Neut</u>	<u>CBT</u>	<u>Haemad. Inhib.</u>
6	0*	20	0**	0**	0**	1024	20	160	80	20
12	0	40	0	0	0	512	40	80	40	10
13	0	10	0	0	0	0	40	10	40	0
14	0	80	10	0	0	0	40	0	40	0
18	0	0**	0	0	0	512	1280	160	40	80
19	0	0	0	0	0	512	320	160	40	80

*less than 1:4 dilution

**less than 1:10

Table 21

Titre of Sendai convalescent serum #19 by neutralization tests and by CBT against different virus doses

<u>TDC₅₀ Sendai</u>	<u>Neutralization Test</u>	<u>Cell Blocking Test</u>
10	640	80
100	80	80
1000	20	40
10000	0*	80

*less than 1:10 dilution.

Table 22

Comparison of the sensitivity of CBT, CF and HI tests in showing

homotypic and heterotypic response

<u>Conval. sera</u> <u>to infect. by</u>	<u>CFT</u>		<u>HI Test</u>			<u>CBT</u>		
	<u>Homo</u>	<u>Hetero</u> <u>to</u> <u>HA-2</u>	<u>Hetero</u> <u>to</u> <u>HA-2</u>	<u>Hetero</u> <u>to</u> <u>Others</u>	<u>Hetero</u> <u>to</u> <u>Others</u>	<u>Hetero</u> <u>to</u> <u>HA-2</u>	<u>Hetero</u> <u>to</u> <u>HA-2</u>	<u>Hetero</u> <u>to</u> <u>Others</u>
Sendai	13	11	3	5	6	2	15	0
HA-2	15	10	5	10	9	3	15	0
<u>Total</u>	<u>28</u>	<u>21</u>	<u>8</u>	<u>15</u>	<u>15</u>	<u>5</u>	<u>30</u>	<u>0</u>

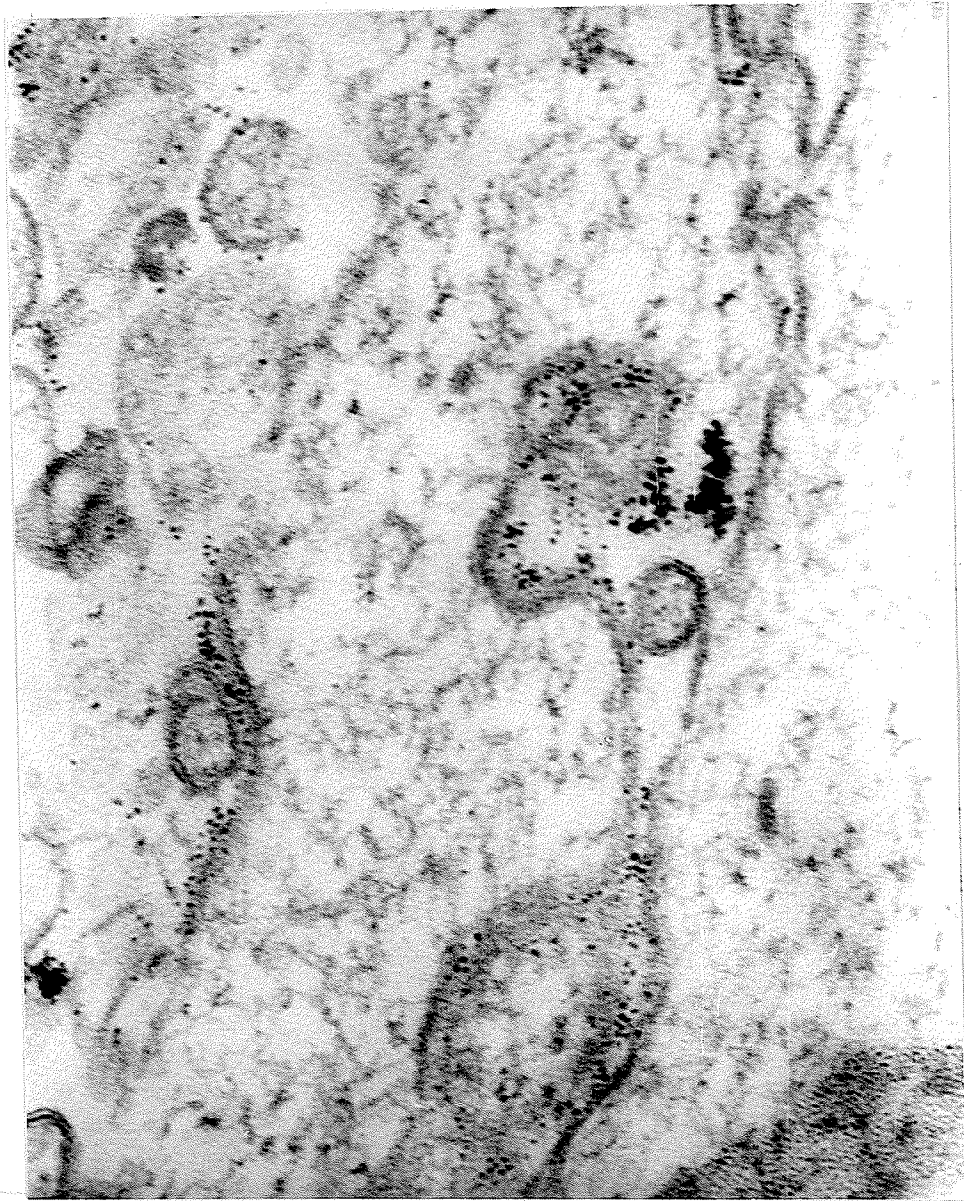


Figure 1

CA virus infected cell haemadsorption inhibition test with
ferritinized globulin from convalescent guinea pig sera.

Note cell membrane lined with globulins.

Magnification: 102,000

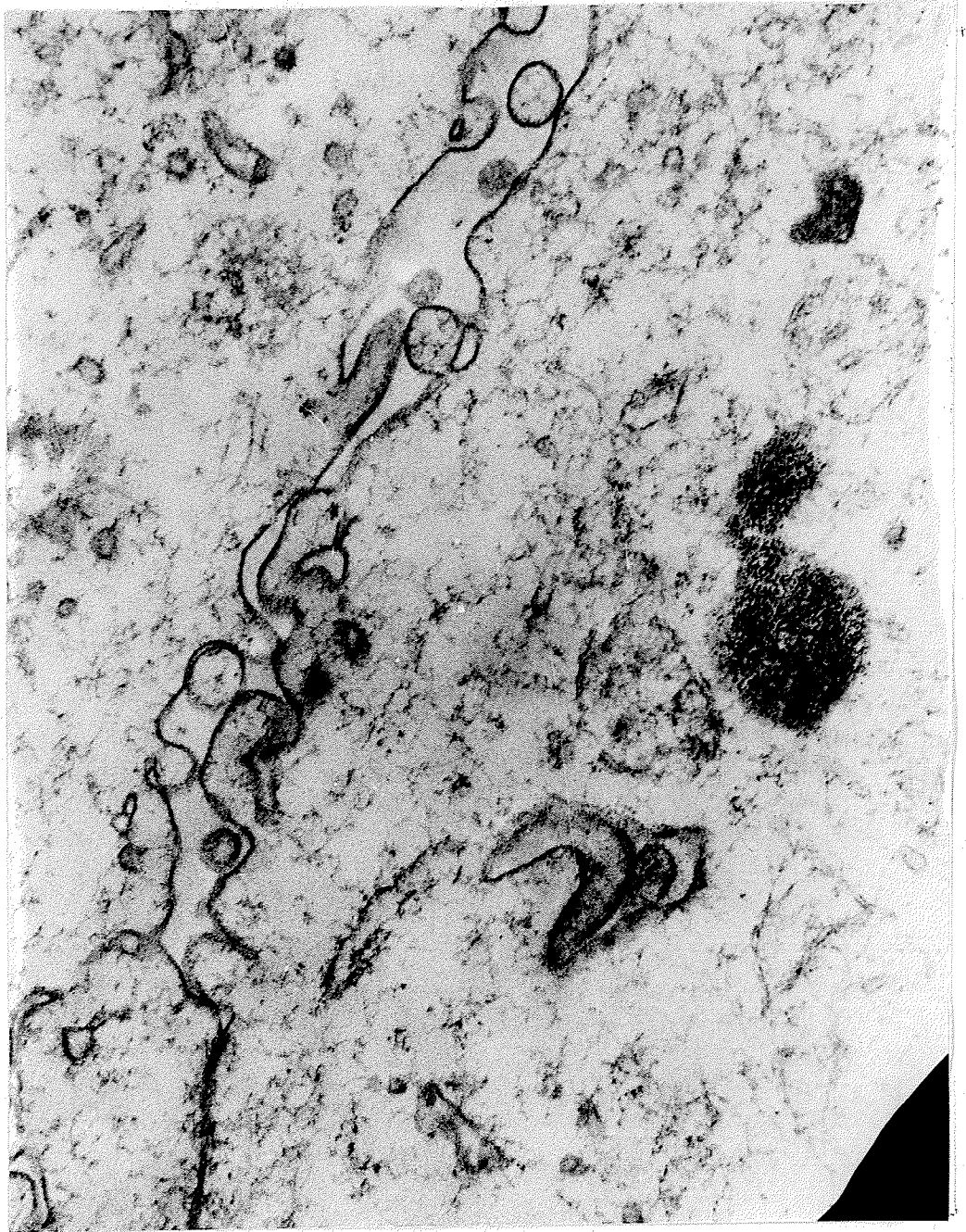


Figure 2

CBT cell section. Note cell membrane surface being free of ferritinized antibody.

Magnification: 91,000

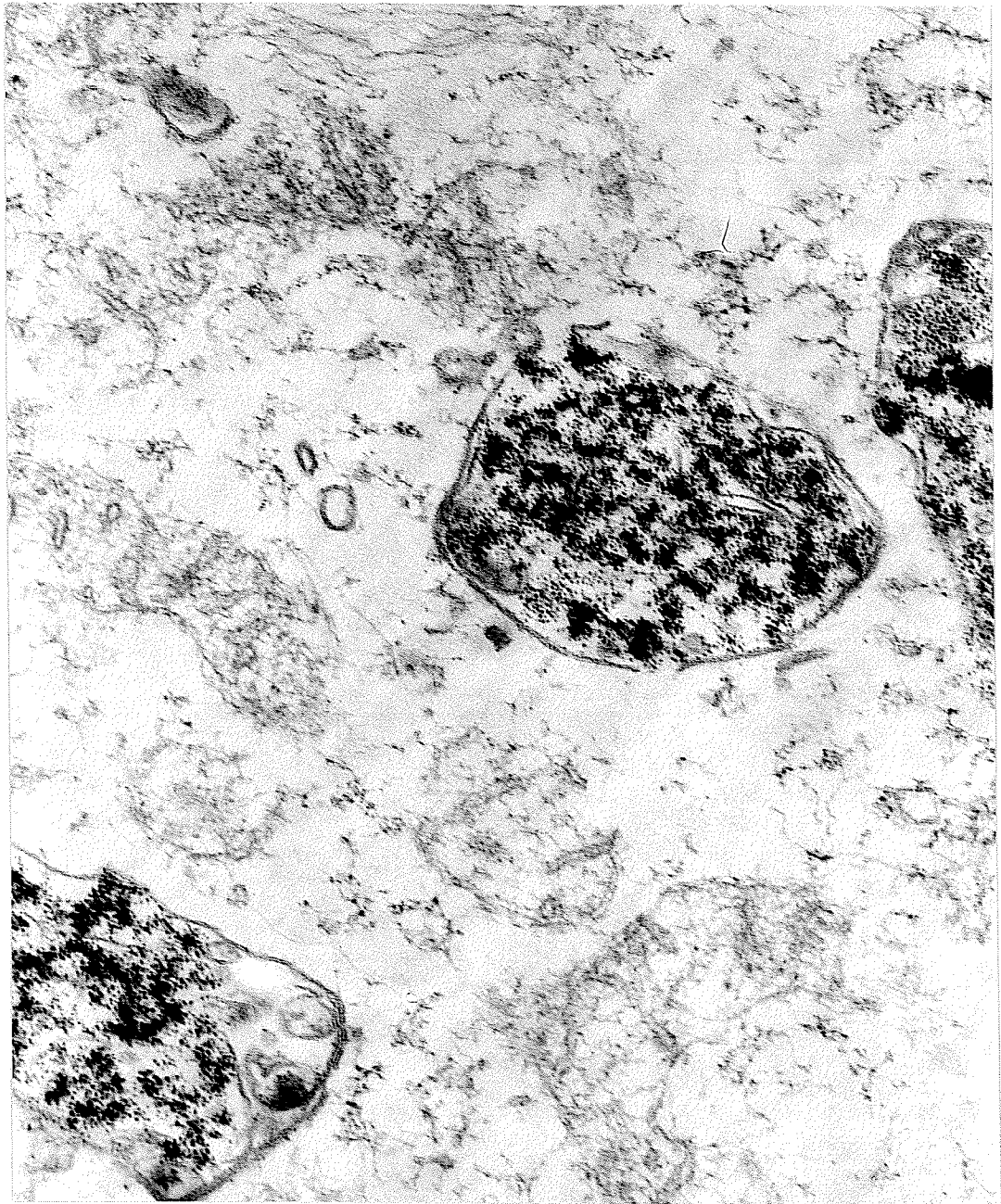


Figure 3

CBT cell section. Note the structures in the cytoplasm containing ferritinized globulins.

Magnification: 91,000

Discussion

The antigenic crossing between Sendai and HA-2 viruses initially shown by Cook and associates with hyperimmune guinea pig sera, was confirmed in the present study with convalescent guinea pig sera. This relationship was further substantiated firstly by the crossings demonstrated with rabbit hyperimmune sera, secondly, by the serum absorption study, and finally by the CBT.

A recent study on Sendai, HA-2, CA, SV5, HA-1 and SF-4 viruses in guinea pigs infected intranasally, followed in 2 weeks by an intraperitoneal injection, gave indications that Sendai was more closely related to HA-1 and SF-4 than to HA-2 (Spurrier, Robinson, 1965). The results of HI tests in that study showed that upon Sendai infection the only heterotypic antibody rise was to SF-4, and that only upon subsequent injection did the animals give rise to Sendai antibody at the same levels as antibody against SF-4. After HA-1 infection, the heterologous response to Sendai antigen was four-fold higher than the homologous response to HA-1. No cross-reacting antibody to SF-4 was found. This was at variance with previous observations (Abinanti et al. 1959, Abinanti et al. 1961; Chanock et al. 1963). Only upon subsequent injection, did the HI titres to HA-1 attain approximately the same level as the titres to Sendai, and at the same time, antibody to SF-4 appeared. A similar pattern of immune response was observed in SF-4 infection; here the heterotypic rise of HI antibody was to Sendai and crossing antibody to HA-1 was found only upon subsequent injection. The general pattern of the response exhibited by these guinea pigs was not unlike the pattern shown by the chickens reported by Hsiung and associates (Hsiung et al. 1965) in their study of the immune response to mumps

and DA viruses in fowls previously given live NDV virus. In this case, the chickens showed rises in HI antibody to NDV and to HA-1, earlier and to higher levels than to the challenge virus, either mumps or DA. The comparison is further justified by the report of Spurrier and associates who found that, after CA infection, guinea pigs produced only heterotypic antibody to HA-1. However, it is important to note that in selecting guinea pigs for their study, animals were chosen on the basis of showing negative HI antibody to the 6 viral agents under their study, with no attention given to mumps or NDV. Serial infection experiments (Veen et al. 1965) have demonstrated that prior parainfluenza virus infection in guinea pigs exerted an effect on the immune response; in the case of Sendai-HA-1 or HA-1 - Sendai infection, a large portion of animals gave a recall antibody rise in addition to the rise of titre against the second virus, but in the case of mumps-HA-1 or HA-1 - mumps infection, instead of a specific anamnestic response, the heterotypic antibody was against CA and appeared earlier than antibody against the virus used in the second infection. In addition, guinea pigs from different geographic areas in the United States have been shown to contain mumps antibody (Cook et al. 1959), and in some colonies, the incidence of mumps antibody was as high as 71% (Hsiung et al. 1965).

The same criticism regarding selection of test animals applies also to the present investigations. The selection of animals was made on the basis of negative antibody tests to the viruses under study, with no attention being given to antibodies to SV5 or WB viruses, prior experience with which may broaden the immune response. Unfortunately, the incidence of SV5 antibody in guinea pigs is high,

varying between 84% and 100% (Hsiung et al. 1965; Liebhaber et al. 1965; Sever 1965, personal communication; Rozee 1965, personal communication). The present author in another study in search of SV5 antibody-free complement found only 3 in a group of 56 guinea pig sera, and these 3 were shown to contain HI antibody against mumps.

On the basis of the results on guinea pig convalescent sera, no antigenic relationship between Sendai and HA-1 can be considered established. In the first place, the 3 guinea pigs showing antibody to HA-1 after Sendai infection had pre-existing HI antibody to Sendai, HA-2 and CA. Also, the crossing titres to HA-1 showed no relationship to the level of homologous antibody nor to the level of heterologous antibody to HA-2. The absence of relationship was further demonstrated by the CBT. Furthermore, when Sendai infection took place in antibody-free guinea pigs, no crossing antibody to HA-1 was noted. Although two rabbits hyperimmunized against Sendai developed low levels of HI antibody against HA-1, interpretation of this as evidence of one-way antigenic crossing must necessarily be cautious, since Sendai absorption which removed homologous as well as heterologous HA-2 antibody from guinea pig sera did not reduce the titres of HA-1 antibody from these rabbit sera. The insusceptibility of heterotypic antibody to absorption by the immunizing virus has been regarded as evidence of the animal's prior antigenic experience with the heterotypic reacting viral agent (Hsiung et al. 1965). Whether or not this was the case with the HA-1 antibody in these two rabbits is purely a matter of conjecture, but supporting evidence for it may be found in the high incidence of naturally occurring HA-1 antibody in this colony of rabbits.

It is apparent from these results that the species of animal used for testing, and the presence or absence of pre-existing antibodies play a major role in determining the degree of antigenic crossings. Thus, CA and mumps gave reciprocal crossing HI antibodies in rabbits, but not in guinea pigs. These crossing antibodies cannot be attributed to hyperimmunization which did not provoke HI titres in rabbits higher than those observed in the CA convalescent guinea pigs, though in the case of mumps the injected rabbits gave an HI titre 4 to 8-fold higher than those from convalescent guinea pigs. Also, it appears from the results that the animal species is an important factor, primarily because of the different pre-existing antibody patterns.

It is important to remember that instillation of live parainfluenzal virus into a susceptible animal leads to in vivo viral multiplication. In a guinea pig with pre-existing antibody the multiplication in vivo may well proceed in the presence of antibody. Virus propagated in this immune environment apparently induces a less specific immune response in the host suggesting an immunogenic change taking place on in vivo cell passage. Support for this hypothesis may be found in two separate types of recorded observation. Firstly, antigenic variants of Sendai and HA-2 have been reported (Ho Yun-de 1962a). For HA-2 they have been confirmed by American workers (Dick et al. 1962). Antigenic variants of M-25 have also been reported (Canchola et al. 1964). Secondly, host controlled variation has been reported for HA-2, HA-1 and CA. The variants acquired the ability to destroy ciliated epithelia after several passages in organ culture of human tracheal epithelium (Tyrell, Hoorn 1965). Host controlled variation, though not well understood, has been extensively

used for viral attenuation. Thus, mumps after serial chick embryo passages was shown avirulent for humans (Smorodinstev 1961) and after 30 passages induced only antibody to S antigen (Henle, Stokes, Burgoon, Bashe, Burgoon, 1961). What remains is to establish an immunogenic change induced by the passage through an immune host. This would offer an explanation for the increasing incidence of heterotypic responses to a parainfluenza virus infection in humans from infancy to adulthood.

Thus, it is suggested that further work on the stability of these viruses in serial immune and non-immune host passages would be useful. In this respect, the CBT offers a working model in vitro.

Conclusion

The data presented in this thesis confirms the sharing of common antigens by HA-2 and Sendai, and further justifies grouping them as a single type of parainfluenza virus. The results also suggest that mumps and CA share antigens. The remainder of the viruses tested -- HA-1, CA, and NDV -- do not appear to share antigens with one another or with those previously mentioned.

This work illustrates the various factors which make the antigenic analysis of the parainfluenza viruses difficult. There is a high incidence of naturally occurring antibodies against parainfluenza viruses in experimental animals. The presence of such antibodies tends to reduce the titre of specific antibody produced on further antigenic stimulation and to broaden the spectrum of the response -- not a new phenomenon in virology, but a very troublesome one. The interpretation of heterologous responses thus becomes very difficult. Finally, the worker seems to be denied the useful tool of antibody absorption to a very considerable extent, because of differences in the character of homologous and heterologous antibody with respect to absorption, though not with respect to reaction in HI tests.

The results with the cell blocking test suggest that it may be a useful addition to the methods of antigenic analysis. Within the parainfluenza group of viruses at least, it has one characteristic which is probably a great advantage -- it offers a higher degree of specificity in comparison to the CF and the HI test.

Summary

The antigenic relationships among the parainfluenza viruses; Sendai, HA-2, CA, HA-1, mumps and NDV were investigated. Preliminary experiments dealt with the methods used to prepare serum devoid of antibody to host tissue antigen. It was concluded that guinea pig convalescent sera were best suited for the study.

Comparison between the CF and the HI tests as criteria for the selection of guinea pigs free of prior antigenic experience showed that the HI test was more reliable. Six groups of animals were accordingly selected and were each infected with a virus under study. The results of CF and HI tests on individual convalescent guinea pig sera suggested that with the exception of antigenic crossing between Sendai and HA-2, these six viral agents were serologically distinct. A large proportion of sera gave reciprocal crossings between Sendai and HA-2.

To account for the small proportion of guinea pigs showing no crossing antibody between Sendai and HA-2, an experiment was done to show whether the viral agents and guinea pig tissues shared antigens. The results did not indicate such a relationship.

A study on the immune response of rabbits by means of HI and neutralization tests confirmed the reciprocal crossings between Sendai and HA-2. In addition, reciprocal HI crossing was demonstrated between mumps and CA.

Sendai virus absorption of guinea pig, rabbit and human sera did not yield unequivocal results. Sendai removed homologous antibody as well as heterologous antibody to HA-2 and CA from the serum

of guinea pigs convalescent from Sendai infection. From HA-2 convalescent sera, Sendai removed homologous HA-2 antibody and heterotypic antibody to Sendai and HA-1. Sendai exerted no effect on other guinea pig convalescent sera. From the rabbit immune sera Sendai removed only homologous HI antibody, exerting no effect upon the heterotypic antibodies to HA-2 and HA-1. On the other hand, Sendai absorbed HI antibody to Sendai, HA-2, HA-1 and CA from human sera.

Red blood cells, having been agglutinated by Sendai which was subsequently eluted, were agglutinated by homologous guinea pig immune sera. A study of this phenomenon with immune sera against other parainfluenza viruses and with selected human sera indicated no relationship between this reaction and the antigenic response to viral infection.

A new serological test, the cell blocking test, CBT, was described, and the mechanism of its reaction was investigated by electron microscopy using ferritin conjugated antibody. Antigenic analysis by CBT substantiated the reciprocal crossing between Sendai and HA-2 and gave further evidence of the absence of antigenic relationship between HA-1 and NDV, and with Sendai and HA-2. Antigenic crossing between mumps and CA which was demonstrated with immune rabbit sera was, however, not substantiated by the CBT.

Appendix 1Hanks' Balanced Salt Solution (HBBS) 10% (Hanks and Wallace, 1949)

This solution is stored as a 10% stock, which is diluted ten-fold for use. The 10% stock solution is prepared as follows:

Solution I

CaCl ₂	1.4 gm.
Ion-exchange water	200.0 ml.

Solution II

Glucose	15.0 gm.
NaCl	80.0 gm.
KCl	4.0 gm.
MgSO ₄ .7H ₂ O	2.0 gm.
KH ₂ PO ₄	0.6 gm.
Na ₂ PHO ₄ .2H ₂ O	0.6 gm.
Phenol red (water soluble)	0.2 gm.
Ion-exchange water	800.0 ml.

Mix the solution separately. When the salts have dissolved completely, combine the two solutions. Add 2.0 ml. of chloroform. Store in tightly stoppered bottle at 4°C.

For use add 1 part of 10% stock to 9 parts of water. Sterilize by autoclaving. When cool store at 4°C. Immediately before use, add 0.25 ml. of antibiotics mixture to each 100 ml. of salt solution. To adjust pH add 7.5 percent NaHCO₃, using phenol red colour standards as a guide.

Supplemented with 0.5 percent lactalbumin hydrolysate (Melnick and Riorden, 1952) this may be used as a growth medium, Hanks' lactalbumin hydrolysate (HLa).

Appendix 2

HB 597 (C.M.R.L.) consists of 1 part HBSS and 3 parts of medium 597 with .084% sodium bicarbonate. Two antibodies are added to the medium in the following concentration per ml. 200 units of streptomycin sulfate and 40 units of neomycin sulfate.

The 597 medium contains the following ingredients in weight ratio:

dl-glutamic acid monohydrate	150 grams
dl-leucine	120
l-arginine monohydrochloride	70
l-lysine	70
dl-threonine	60
dl-aspartic acid	60
dl-phenylalanine	50
dl-serine	50
dl-valine	50
dl-alpha alanine	50
Glycine	50
dl-isoleucine	40
l-proline	40
dl-methionine	30
l-histidine monohydrochloride	20
dl-tryptophane	20
l-hydroxyproline	10
l-glutamine	100
Sodium acetate	81.5
Phenol red (water soluble)	20

Appendix 3Veronal Buffered Saline (VBS)

Dissolve in 2,000 ml. distilled water, 85.0 grams sodium chloride
 5.75 grams diethyl barbituric acid
 3.75 grams sodium barbitone.

This is used as a stock solution and diluted 1:5 for use. To each 1,000 ml. of final solution is added 1.0 ml. of 10% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1.0 ml. of 1.5% CaCl_2 . The stock solutions are stored at refrigeration temperature after they have been autoclaved at 15 lbs. pressure for 20 minutes.

- Abinanti, F. R., Huebner, R. J., (1959). The Serological Relationship of Strains of Parainfluenza 3 Virus Isolated from humans and cattle with respiratory disease. *Virology* 8: 391 - 394.
- Abinanti, F. R., Bynne, R. J., Watson, R. L., Pollma, L. J., Lucas F. R. and Huebner, (1960). Observation on Infections of cattle with myxovirus parainfluenza. *Am. J. Hyg.* 71: 52 - 58.
- Abinanti, F. R., Chanock, R. M., Cook, M. K., Wong, D. and Warfield, M., (1961). Relationship of human and bovine strain of myxovirus parainfluenza 3. *Proc. Soc. Exptl. Biol. Med.* 106: 466 - 469.
- Andrews, C. H., Bang, F. B., Chanock, R. M. and Zhdanov, V. M., (1959). Parainfluenza viruses 1, 2 and 3. Suggested name for recently described myxoviruses. *Virology* 8: 129 - 130.
- Bakos, K., and Z. Dinter, (1960). Identification of a bovine mucosal-disease virus isolated in Sweden as myxovirus parainfluenza 3. *Nature*, 185: 549.
- Bang, F. B., and M. Foard, (1956). The serology of Newcastle virus infection II. The antigenic relationships of Newcastle virus. *J. Immunol.* 76: 348.
- Bartolomei-Corai, O., (1959). Ricerche su parentele antigeni fra virus parotitico e virus dell influenza D - Sendai. *Lo sperimentale* 109: 76.
- Beale, A. J., McLeod, D. L., Stackiw, W., and Rhodes, A. J., (1958). Isolation of cytopathogenic agents from the respiratory tract in acute laryngotracheobronchitis. *Brit. Med. J.* 1: 302 - 303.
- Blaskovic, D., Sokol, F., and D. Kociskova, (1961). Subunit of Myxoviruses III Antigenic Properties of Newcastle Disease Para-Influenza 1 (Sendai) and mumps viruses, of their haemagglutinin and "g-antigens" *Acta virol.* 5: 294 - 304.
- Bloom, H. H., Johnson, K. M., Jacobsen, R. and Chanock, R. M., (1961). Recovery of parainfluenza viruses from adults with upper respiratory illness. *Am. J. of Hygiene* 74: 50 - 59.
U.S. Eav. Med. Field Res. Lab. Res. Proj. MR005.09 - 1204.4.4.50-9.

- Blumental, K. V. and Bukrinskaya, A. G., (1962). Krup paragripozoni etiologii. Group of parainfluenza etiology. *Petiatriya* 40: 43 - 48
- Brandt, Carl, D., (1961). Cytopathic action of myxoviruses on cultivated mammalian cells. *Virology* 14: 1 - 10
- Breitenfeld, P. M. and W. Schafer (1957). The formation of Fowl Plague Virus Antigen in Infected Cells as Studied with Fluorescent Antibodies. *Virology* 4: 328
- Brown, G. O., Oligschlaeger, D., Legier, M., and R. R. Schmidt (1960). Studies of the Epidemiology of Influenza as Demonstrated by Serum Pools. *A.M.A. Arch. Int. Med.* 106: 496 - 512
- Bukrinskaya, A. G., (1960). Sensitivity of Various tissues to parainfluenza viruses and some special diagnostic features of parainfluenzal diseases. *Vop. virusol.* 5: 744 - 747.
- Bukrinskaya, A. G., Zhdanov, V. M., and Ramenskaya, G. P., (1961). Autoradiographic study of penetration of Sendai virus into tissue culture cells II. Use of virus preparations labelled with ³²P. *Vop. virusol.* 6: 593 - 599
- Bukrinskaya, A. G. and Zhdanov, V. M., (1961). Formation of giant cells in continuous cell cultures infected with Sendai virus. *Vop. virusol.* 6: 352 - 356.
- Bukrinskaya, A. G., and Blyumental, K. V., (1962). The role of parainfluenza viruses in the etiology of pseudo croup. *Vop. Virusol.* 7: 567 - 572.
- Burnet, F. M., and S. G. Anderson (1946). Modification of human red cells by virus action; agglutination of modified human red cells by sera from cases of infectious mononucleosis. *Brit. g. Exper. Path.* 27: 236 - 244.
- Canchola, J., Vargosko, A. J., Kim, H. W., Parrott, R. H., Christmas, E., Jeffries, B., Chanock, R. M., (1964). Antigenic variation among newly isolated strains of parainfluenza type 4 virus. *Am. J. of Hyg.* 79: 357 - 364.

- Cantell, Karl, (1961). Mumps virus. Advances in virus research. 8: 123 - 164.
- Chang, P. W., and G. D. Hsiung (1962). International Congress for Microbiology Abstracts VIII - 95, 1962.
- Chang, P. W., and Hsiung, G. D., (1965). Experimental infection of parainfluenza virus type 5 in mice, hamsters and monkeys. J. of Immunol. 95: (4) 591 - 601.
- Chang, S. M. (1953). Serologically active erythrocyte sensitizing substance from typhus rickettsiae: isolation and titration. J. Immunol 70: 212 - 214.
- Chanock, R. M., (1956). Association of a new type of cytopathogenic myxovirus with infantile croup. J. Exp. Med., 104: 555 - 576.
- Chanock, R. M., Parrott, R. H., Cook, M. K., Andrews, B. E., Bell, J. A., Reichelderfer, T., Kapikian, A. Z., Mastrota, F. M., and Huebner, R. J., (1958). Newly recognized myxovirus from children with respiratory disease. New England J. Med., 258: 207 - 213.
- Chanock, R. M., Vargosko, A. J., Luckey, A. H., Cook, M. K., Kapikian, A. Z., Reichelderfer, T. and Parrott, R. H., (1959). Association of haemadsorption viruses with respiratory illness in childhood. J.A.M.A. 169: 548 - 553.
- Chanock, R. M., Bell, J. A. and R. H. Parrott (1961). Natural History of Parainfluenza Infection, in Perspective in Virology 2, 126.
- Chanock, R. M., (1963b). Am. Rev. of Resp. Disease (3) 88: 195.
- Chany, C., Daniel, P., Robbe-Fossat, Vialatte, J., Lepine, P., et M. LeLong (1958). Isolement et etude d'un virus syncytial non identifie associe a des affections respiratoires aiguës du nourrisson. Annales de l'Institut Pasteur 95, 721.
- Chany, C. and M. K. Cook (1960). A virus-induced factor producing the formation of syncytium in cell culture. Am. Inst. Pasteur (Par) 98: 920 - 924.

- Chany, C. (1961). An Interferon-like Inhibition of Viral Multiplication from Malignant cells. *Virology* 13: 485.
- Chien Lui (1963). Conference on newer Respiratory Disease Viruses. *Thw Am. Rev. Resp. Dis.* 88 (3) 197.
- Chin-Hsien F. (1950) Adenovirus epidemic among Peking children in 1958. *Chin. Med. J.* 80: 331-339.
- Christmas, E., Jeffries, B., Chanock, R. M., (1964). Antigenic variation among newly isolated strains of parainfluenza type 4 virus *Am. J. of Hygiene.* 79 (3) 357 - 364.
- Cohen, S. M., Barllivant, S., Edwards, G. A., (1961). A morphologic study of FL cells infected with parainfluenza 3 virus. *Arch ges Virusforsch. Band XI. Heft 4.* 493 - 515.
- Colobert, L., and Gontanges, R. (1959). Titrage de l'activite haemagglinante et ce l'activite enzymatique de myxovirus parainfluenzae 1 (virus Sendai). *C. R. Soc. Biol. (Paris)* 153: 1382.
- Committee on Standard Aeteogical Procedures in Influenza studies (1950). *J. Immunol.* 65: 347.
- Cook, M. K. Andrews, B. E., Fox, H. H., and Turner, H. C. (1959). Antigenic relationship among the "newer" myxoviruses (parainfluenza). *Am. J. Hygiene* 69: 250.
- Cook, M. K., Chanock, R. M. (1963). In vivo antigenic studies of parainfluenza viruses. *Amer. J. Gygiene* 77: 150 - 159.
- Craighead, J. R., Shelokov, A/, Oeralta, P. H. and Vogel, J. E., (1961). Croup Associated virus infection in adults: Report of two cases. *New England J. Med.* 264: 136 ---137.
- Cramblett, H. G., (1958). Isolation of a cytopathogenic agent resembling the CA virus from an infant with croup. *Pediatrics.* 22: 56 - 59.
- Compans, R. W., Holmes, K. V., Bales, S. and P. W. Choppin (1966) Simian virus 5--an elctron microscopic study of moderate and virulent virus--cell interactions. *Fed. Proc.* 25: (2) 311.
- Daniel, P. et C. Chany (1962). Etude d'une lignes de cellules KB infect es chromiquement par le virus para--influenzae type 3. 1. Aspect de las production virale. *Can. J. Microbiol* 8: 709 - 718.
- Darrell, Richard W., and Calderon, Howe (1964). The neuraminidase of para-influenza virus (type 2). *Pro. Soc. Exptl. Biol. Med.* 116: (4) 1091 - 1094.

- Deibel, R., (1959). Plaque formation in monolayers of FL cells infected with the haemadsorption type I virus. *Virology* 8: 262 - 263.
- Deibel, R., and J. Hotchin (1961). Growth characteristics of haemadsorption virus type I (myxovirus parainfluenza 3) in tissue culture. *Virology* 14 (1) 66 - 73.
- Delaat, Adrian, N. C., (1964). The complement fixation test in virus antibody studies. *Can. J. of Med. Technology*, 26: 35 - 41.
- DeMeio, J. L. and D. L. Walker (1957). Demonstration of antigenic relationship between mumps virus and haemagglutinating virus of Japan. *J. Immunol* 78. 465 - 471.
- Dinter, Z., Hermodsson and Bakos, K., (1960). Studies on variants of a bovine strain of parainfluenza 3 virus.
1. Isolation and growth characteristics. *Acta. Path. et microbiol. Scandinavica* 49: 485 - 471.
- DeMeio, J. L., (1963), Adaptation of parainfluenza 2 (Croup-associated) virus to the embryonated hen's egg. *J. Bact.* 85: 943 - 944.
- Dick, E. C. and Mogabgab, W. J., (1961). Characteristics of parainfluenza 1 (HA-2) virus III Antigenic relationships, growth, interaction with erythrocytes and physical properties. *J. Bact.* 83 (3). 561 - 571.
- Dick, E. C. and Mogabgab, W. J., (1961a). Characteristics of parainfluenza 1 (HA-2) virus. II Haemagglutination, haemagglutination-inhibition and neutralization. *Am. J. Hyg.* 73: 273 - 281.
- Doorschodt, H., Kepsenberg, J. G., and Gispen, R., (1962). Parainfluenza virus infection. *Ned. Tijdschr. Geneesk.* 106: 2479 - 2485.
- Dossena, G. and Bellelli, E., (1961). Coltura dei virus parainfluenzai en cellule umane ed animall. *Igiene Moderna* 54 (7/8) 494 - 502.
- Drake, J. W. and Lay, P. A., (1962). Host controlled variation in NDV. *Virology*. 17: 56 - 64.
- Duc-Nguyen, H., (1966). An electron microscopic study of changes at the surface of influenza infected cell. *Virology* 28: 404 - 412.

- Evans, A. S., and E. C. Curnen. (1948). Serological studies on infection mononucleosis. *J. Clin Investigation* 27: 106 - 110.
- Evans, A. S., (1954). Newcastle disease neutralizing antibody in human sera and its relationship to mumps virus. *Am. J. Hyg.* 60: 204 - 213.
- Evans, A. S., (1960). Infections with haemadsorption virus in University of Wisconsin students. *New England Journ. Med.*, 263: 233 - 237.
- Falke, D., and Schmidt, G., (1963). Riesenzlltypen durch parainfluenza virus 3 in kulturzellen. *Zentr. Bakteriolog. Parasituek. Abt. Orig.* 188 (2): 149 - 159.
- Ferris, A. A. (1960). The Croup Viruses - Bacteriological Aspects. *M. J. Australia (Nov. 12)*, 1960.
- Florman, A. L., (1949). Agglutination of human erythrocytes modified by treatment with Newcastle disease and influenza virus. *J. Bact.* 57: 31 - 38.
- Fontanges, R. J., Garrigue and Colobert, L., (1964). Effet de la trypsine sure les properieties hemolytique et neuraminietasique de myxovirus parainfluenza I. *An. Inst, Pasteur*, 106 (6): 894 - 901.
- Forbes, J. A., (1960). Croup: Clinical aspects. *Med. J. Aust.* 47, 2: 769
- Francis, T., Jr., (1934). Transmission of influenza by a filterable virus. *Sci.* 80: 457 - 459.
- Francis, T., (1955). The current status of the control of influenza. *Ann. Intern. Med.* 43: 534 - 538.
- Fukai, K. and Suzuki, T., (1955). On the characteristics of a newly-isolated haemagglutinating virus from mice. *Med. J. of Osaka University*, 6: 1 - 15.
- Fukumi, H., Nishikawa, F. and Kitayama, T., (1954). A pneumotropic virus from mice causing haemagglutination. *Jap. J. of Med. Sci. and Biol.*, 7: 345 - 363.
- Fukumi, H., (1956). Characteristics and class of HVJ (in Japanese) 2nd congress of Virologists in Eastern Japan. Cited by Hosaka, Y., Horsokawa, Y. and Fukai, K., (1960). Structure of HVJ. 1. Two kinds of sub-units of HVJ, *Biken's Journal* 3: (1) 27 - 40.

- Fukumi, H., Nishikawa, F., Sugiyama, T., Yamaguchi, Y., Nauba, J., Matsuura, T., and Oikawa, R., (1959). An epidemic due to HA-2 virus in an elementary school in Tokyo. *Jap. J. M. Sc. and Biol.*, 12: 307 - 317.
- Fukumi, H. and Fumio, Nishikawa, (1961). Comparative studies of Sendai and HA-2 viruses. *Jap. J. Med. Sci. and Biol.* 14: 109 - 120.
- Gale, C. and King, N. B., (1961). Isolation of a virus from clinical shipping fever in cattle. *J. Am. Vet. Med. Assoc.* 138: 235 - 238.
- Gardner, P. S., (1957). Serologic Evidence of Infection With Sendai Virus in England, *Brit. Med. J.* 1: 1143
- Gardner P. S., Stanfield, J. P., Wright, A. E., Court, S.D.M., and C. A. Green (1960). Viruses, Bacteria and Respiratory Disease in Children. *Brit. Med. J.* 1: 1 - 11.
- Gardner, P. S., Stanfield, J. P., Wright, A. E., Court, S.D.M., and Green, C. A., (1961). Viruses, Bacteria and respiratory disease in children. *Brit. Med. J.* 1: 1 - 11.
- Gernez-Rieux, C. H., Breton, A., Kamaille, J. and LeLong, M., (1962). Enquete serologique sur la frequences des myxovirus parainfluenzae au cours des infections respiratoires de l'enfant. *Helv. Paediat. Acta.* 17 (3): 197 - 206.
- Gerngross, O. G., (1957). Peculiarities of the 1958 influenza outbreak in Vladivostok due to D virus. *Problem of Virology.* 2: 71 - 75.
- Gorbunova, A. S., Gergross, O. G., Gnorizova, V. M., and A. G. Bukrinskaya, (1957). Strains of influenza virus of type D isolated in Vladivostok, and their role in the aetiology of the 1956 outbreak. *Probl. Virol.* (N.Y.) 2 (2): 76 - 84.
- Gorbunova, A. S., (1958). The variation of influenza virus in relation to the development of the 1957 pandemic. *Vop. virisol.* 3: 193 - 201.
- Gresser, J., (1961). Production of interferon by suspensions of human leucocytes. *Proc. Soc. Exptl. Biol. Med.* 108 (3): 799 - 803.
- Hamar, M., and Rosenberg, M., (1958). Morphology of type D influenza virus (Sendai strain). *Acta Virologica Prah* 2: 65 - 68.

- Harboe, A., (1962). An anamnestic antibody increase in ferrets cross-infected with influenza A2 virus after myxoviruses not belonging to the A-group. *Acta Path. et microbiol. Scand.* 55: 425 - 436.
- Harboe, H., (1963). The influenza virus haemagglutination inhibition by antibody to host material. *Acta Path. et microbiol Scand.* 57: 317 - 330.
- Hausler, W. J. Fr., and E. C. Dick (1960). The Purification and concentration of influenza A virus (PR8) by a method of continuous extraction with glycine of a zine-virus precipitate. *J. Infect. Dis.* 107: 189 --194.
- Heath, R. B., Tyrell, D. A. J., and Pato, S., (1962). Serological studies with Sendai virus. *Brit. J. Expt. Path.* 43: 444 - 450.
- Heath, R. B., Tyrell, D. A. J., (1959). The behaviour of some influenza viruses in tissue cultures of kudney cells of various species. *Arch. ges. Virus forch.* 8: 577 - 591.
- Henle, G., Stokes, J, Jr., Burgoon, J. S., Bashe, W. J. Jr., Burgoon, C. F., and Henle, W., (1961). Studies on the prevention of mumps IV. The effect of oral spraying of attenuated active virus. *J. Immunol.* 66: 579 - 594.
- Henle, G., Deinhardt, F., Bergs, V. V., and W. Henle, (1958). Studies on Persistent Infections of Tissue Cultures. *J. Exp. Med.* 108: 537.
- Henle, W., Henle, Gertrudes, Deinhardt, F., and Bergs, V. V., (1959). Studies in persistent infection of tissue culture. *J. Expt. Med.* 110: (4) 525 - 554.
- Henle, W. and Lief, F. S., (1963). *Am. Rev. Resp. Dis.* 88: Part 2, 379.
- Hermodsson, S. (1960). Effect of Ultrasonic Vibration on the Haemagglutinating Activity of Some Para-influenza Viruses. *Nature* 188: (4757): 1214.
- Hildes, J. A., Wilt, J. C., Parker, W. L., Stackiw, W. and Delaat. A/, (1965) Surveys of respiratory virus antibodies in an arctic Indian population. *Can. Med. Assoc. J.* 93: 1015 - 1018.
- Holland, W. W., Tanner, E. I., Pereira, M. S. and Taylor, C. E. (1960). A study of the aetiology of respiratory disease in a general hospital. *Brit. Med. J.* 5190: 1917 - 1922.

- Horne, R. W., and P. Wildy (1961). Symmetry in Virus Architecture. *Virology* 15: 348.
- Hosaka, Yasuhiro (1958a). On the Hemolytic Activity of HWJ
I. An analysis of the Hemolytic Reaction. *Biken's J.* 1: 70 - 89.
- Hosaka, Yasuhiro (1958b). On the Hemolytic Activity of HWJ II. Decrease of Sphingomyelin in Red Cells Hemolysed by HWJ *Biken's J.* 1: 90 - 99.
- Hoyle, L., (1952). Structure of influenza virus. The relation between biological activity and chemical structure of virus fractions. *J. Hyg.* 50: 229 - 245.
- Ho Yun-de, and Gorbunova, A.S., (1961). Antigenic structure of 24 strains of parainfluenza virus type 1, Sendai. *Vop. Virusol.* 6: 691 - 697.
- Ho Yun-de, (1962a). Study of antigenic variants of parainfluenza Sendai virus. *J. Hyg. Epidem. (Praha)* 6: 154 - 157.
- Ho Yun-de, (1962b). Comparative investigations into the haemolytic activity of different variants of parainfluenza 1 virus. *Acta virol.* 6: 114 - 121.
- Hsiung, G. D. (1961). The use of agar overlay cultures for detection of new virus isolates. *Virology* 9: 717 - 719.
- Hsiung, G. D., P. Isacson, and R. W. McCollum (1962). Studies of a myxovirus isolated from human blood.
I. Isolation and properties. *J. Immunol* 88: 284 - 290.
- Hsiung, G. D., (1963a). Conference on newer respiratory disease viruses. *The Am Rev. Resp. Dis.* 88: 366.
- Hsiung, G. D., (1963b). Conference on newer respiratory disease viruses. *The Am Rev. Respt. Dis.* 88: 189 - 192.
- Hsiung G. D., (1963). Studies on parainfluenza virus. Serologic interrelationship in humans. *Yale J. Biol. and Med.* 35: 534.
- Hsiung, G. D., Pinheiro, F. and Gabrielson, Mary, O., (1963). The virus diagnostic laboratory: functions and problems based on 3 years experience. *Yale J. Biol. and Med.* 36: 104 - 123.

- Hsiung, G. C., Chang, P. W., Cuadroado, R. R. and Isacson, O., (1965).
Studies of parainfluenza viruses III. Antibody responses
of different animal species after immunization.
J. Immunol. 94: (1). 67 - 73.
- Hull, R. N., Minner, J. R. and Smith, J. W., (1956). New viral
agents recovered from tissue cultures of monkey
kidney cells: origin and properties of cyto-
pathogenic agents SV1, SV2, SV4, SV5, SV6, SV11,
SV12 and SV15. Am. J. Hyg. 63: 204 - 215.
- Isacson, P. and Holden, D. M., (1962). Human blood group activity
associated with a myxovirus. Virology 17: (3)
495 - 496.
- Isacson, P. and Koch, A. E., (1965). Association of host antigens
with a parainfluenza virus. Virology 27: (2) 129.
- Ishida, N., Hinuma, Y., Numazaki, Y. and Homma, M., (1959). Common
antigens shared by Sendai, mumps, newcastle disease
viruses. Fed. Proc. 18: 575.
- Ishida, N. and Homma, M., (1960). A variant Sendai virus, infectious
to egg embryos but not to L cells. Third report.
Growth characteristics of myxoviruses in tissue
culture. Tohoku J. Exptl. Med., 73: 56 - 69.
- Jensen, K. E., Minuse, E. and Ackermann W. W., (1955). Serologic
evidence of American experience with new born
pneumonitis virus (Type Sendai). J. Immunol. 75:
71 - 77.
- Jensen, K. E., (1957). The nature of serological relationships among
influenza viruses. Adv. Virus Res. 4: 279.
- Jensen, K. E., Peeler, B. E., and Dulworth, W. G., (1962).
Immunization against parainfluenza infections.
J. Immunol. 89: 216 - 226.
- John, T. J. and V. A. Fulginiti (1966). Parainfluenza 2 virus,
increase in haemagglutinin titre on treatment
with Tween-80 and ether. Proc. Soc, Exp. Biol.
Med. 121: 109.
- Johnson, K. M., Chanock, R. M., Cook, M. K., and Huebner, R. J.,
(1960). Studies of a new human haemadsorption
virus. I. Isolation, properties and character-
ization. Am. J. Hyg. 71: 81 - 92.

- Johnson, K. M., (1963). Am. Rev. of Resp. Disease 88 (3): 195.
- Jordan, W. S. and A. E. Feller (1950). The relationship of complement-fixing and antihaemagglutinating factors against the viruses of mumps and Newcastle disease. J. Lab. Clin. Med. 36: 369.
- Jungherr, E., Luginbuhl, R. E. and L. Kilham (1949). Serological relationships of mumps and Newcastle disease. Science 110: 333.
- Kallinikova, O. N., and Morozenko, M. A., (1961). Uchastic paragrippoznykh virusov V. vzniknovenuzabolevanu dykhatel nykh putei u detei do 2 let. The role of parainfluenza viruses in the appearance of respiratory disease in children under 2 years. 40 children, 12 strains. Peditria, Moskva 40: 29.34.
- Kapikian, A. Z., Bell, J. A., Mastrotta, F. M., Huebner, R. J., Wong, D. C. and R. M. Chanock (1963). An outbreak of parainfluenza 2 (Croup-Associated) Virus Infection A.M.M. 183: 324 - 330.
- Kates, M., Allison, A. C., Tyrrell, D. A. J., and James, A. T. (1961). Lipids of influenza virus and their relation to those of the host cell. Blochem. Biophys. Acta. 52: 455 - 466.
- Ketler, A., Hamparian, V. V. and Hilleman, M. R. (1961). Laboratory and field investigations of bovine myxovirus parainfluenza 3 virus and vaccine. I. Properties of the SF-4 (Shipping Fever) strain of virus. J. Immunol. 87: 126 - 133.
- Kilham, L., Jungherr, E., and R. E. Lunginbuhl (1949). Antihaemagglutinating and neutralizing factor against Newcastle Disease Virus (NDV) occurring in sera of patients convalescent from mumps. J. Immunol. 63: 37
- Kim, H. W., Vargosko, A. J., Chanock, R. M. and R. H. Parrott (1961). Para-influenza 2 (CAO virus - etiologic association with croup. Pediatrics 28: (4) 614 - 621.
- Klemperer, H. G., (1960). H molysin and the release of potassium from cells by Newcastle Disease Virus (NDV). Virology 12: 540 - 552.
- Knight, C. A., (1944). A sedimentable component of allantoic fluid and its relationship to influenza viruses. J. Exptl. Med. 80: 83 - 99.

- Knight, C. A., (1946). Precipitin reactions of highly purified influenza viruses and related materials. *J. Exptl. Med.* 83: 281 - 294.
- Koprowski, H., (1958). Immunity and Virus infection. Willey and Sons Inc., New York, p. 45.
- Kuroya, M., Ishida, N. and Shiratori, T., (1953). Newborn virus pneumonitis (Type Sendai) II. Report: The isolation of a new virus possessing haemagglutinin activity. *Yokohama Med. Bull* 4: 217 - 233.
- LaPlaca, M. and C. Moscovici (1962). Distribution of parainfluenza antibodies in different groups of population. *J. Immunol.* 88: (1) 72 - 77
- Laxdal, O. E., Robertson, H. E., Braaten, V., Walker, W. A. (1963). Acute respiratory infections in children. 1. An intensive study of etiology in an open community. *Can. Med. Assoc. J.* 88: 1049 - 1054.
- LeLong, M., Vialette, J., Cotlenko, M., Chany, C. and Nodot, A., (1959). Isolation of a virus producing syncytia in cellular culture (myxovirus parainfluenza type 3) on the occasion of a small epidemic among infants with benign acute respiratory manifestations. *Archives Francaise de Pediatrie* 15: 113.
- Lennette, E. H., Jensen, F. W., Guenther, R. W., Magnoffin, R. L., (1963). Serologic responses to para-influenza viruses in patients with mumps virus infection. *J. Lab. Clin. Med.* 61: 780 - 8.
- Lepine, P., Chany, C., Droz, B., Robbe-Fossat, F., (1959). Cytopathogenic effect of two newly recognized myxovirus strains: Mechanism of syncytial formation. *Ann. N. Y. Acad. Sci.* 81: 62 - 72.
- Leibhaber, H., Krugman, S., McGregor, D., and Giles, J. P., (1965). Studies of a myxovirus recovered from patients with infectious hepatitis. *J. Exptl. Med.* 122: (6) 1135 - 1150.
- Lim, K. A., and Pong, W. S., (1964). Agglutination by antibody of erythrocytes sensitized by virus haemagglutinin. *J. Immunol.* 92 (4): 638 - 647.

- Lotte, V. D. and Kirillova, F. M., (1961). Use of the fluorescent antibody method in studies on the localization of influenza and parainfluenza virus antigens during the process of their multiplication in cells. *Vop. virusol.* 6: 656 - 664.
- Marine, William M., (1964). Antigenic relations between mumps and the parainfluenza myxoviruses. *Fed. Proc.* 23 (2), 580. And, personal communication 1965.
- Marston, R. Q., (1958). Cytopathogenic effect of haemadsorption virus type 1. *Proc. Soc. Exptl. Biol. Med.* 98: 853 - 856.
- Marston, R. Q., and E. R. Vaughan (1960). Parainfluenza 3 - Assay and Growth in Tissue Culture *Proc. Soc. Exp. Biol. and Med.* 104: (10) 56 - 60.
- Maassab, H. F., and P. C. Loh (1961). Fluorescent antibody, studies in tissue culture of parainfluenza 3 infection. *Excerpta Med. Sect. 1*: 15 (7) 567 - 568.
- Massah, Hussein, F., and Loh, Philip C., (1962). Fluorescent antibody studies on tissue cultures of parainfluenza 3 infection. *Proc. Soc. Exptl. Biol. and Med.* 109 (4): 897 - 900.
- Matsumoto, T., and Maeno, K., (1962). A host induced modification of haemagglutinating virus of Japan (HVJ, Sendai virus) in its hemolytic and cytopathic activity. *Virology*, 17: 563 - 570.
- McHugh, N. F., Andersen, O. F., Harellick, S. and J. W. Frankel (1963). Effect of antibody on parainfluenza 3 virus in hamsters. *Bacteriol. Proc.* 156.
- Marcus, P. I. (1962). Dynamics of Surface Modification in Myxovirus infected Cells. *Symposium on Quantitative Biology* 27: 351 - 365.
- McKinney, R. W., England, B. L. and Froede, S., (1959). Studies with haemadsorption virus type I. Recovery from two cases of influenza-like disease in military personnel and related investigations *Am J. Hyg.* 70: 280 - 296.

- McLean, D. M., Roy, T. G., O'Brien, J. M., Wyllie, J. C. and McQueen, E. J., (1961). Parainfluenza viruses in association with acute laryngotracheobronchitis, Toronto, 1960 - 61. *Can. Med. Assoc. J.* 85: 290 - 294.
- McLean, D. M., Edward, H. E., McQueen, E. J. and Petite, H. E., (1962). Myxovirus infections in acute laryngotracheobronchitis, Toronto 1961 - 62. *Can. Med. Assoc. J.* 87: 998 - 1001.
- Miller, F. A. and Brackett, R. G., (1963). Parainfluenza 3 (HA-1). Virus plaques in the KB cell line. *Bact. Proc.* 1963, p. 155.
- Misao, T., (1953). Cited by Kuroya *et al.* (1953). Newborn virus pneumonitis (type Sendai) 11. Report: the isolation of a new virus possessing haemagglutinin activity *Yokohama Med. Bull.* 4: 217 - 233.
- Mogabgab, W. J., Dick, E. C., and Holmes, B., (1961). Parainfluenza 2 (CA) virus in young adults. *J. Hyg.* 74: 304 - 310.
- Morbery, M. L., Marrinett, G. V., Witter, R. F. and Morgan, H. R., (1958). Studies of the hemolysis of red blood cells by mumps virus II. Alterations on lipoproteins of the red blood cell wall. *J. Eptl. Med.* 107: 87 - 94.
- Morozenko, M. A., (1961). Uchastie paragrippoznykh virusov v vozniknoveni, dychatel mych putei u detei. The role of parainfluenza viruses in the pathogenesis of diseases of respiratory tract in children. *Vop. virus.* 7: 51 - 59.
- Neurath, A. R., and Sokol, F., (1962). Subunits of myxoviruses. IV. Haemolysin of parainfluenza I (Sendai) virus. *Acta virol. (Praha)* 6: 66 - 76.
- Neurath, A. R., and Sokol, F., (1962a). Interference by non-haemolytic myxovirus with haemolysis by Sendai virus. Arrangement of viruses and haemagglutinating subunits into a receptor gradient, their estimation and titration of specific antisera. *Acta Virol.* 6: 531 - 539.
- Neurath, A. R., (1965). The interaction of Sendai virus with red blood cells I. Absorption, elution and their relationship to haemolysis. *Acta virol.* 9: 34 - 46.

- Neurath, A. R., (1965a). Interaction of Sendai virus with red blood cells. II. Colloid-osmotic haemolysis. *Acta virol.* 9: 119 - 129.
- Nishikawa, F. and Fukumi, H., (1954). Shape and size of haemagglutinating virus of mice. (HVM). *Jap. J. Med. Sci. Biol.* 7: 513 - 522.
- Numazaki, Y., (1960). Common antigens shared by Sendai and mumps viruses III. Common antigens proved in experimental animals. *Virus.* 10: 366.
- Oda, M., (1963). Vaccinia Virus - HeLa cell interaction. I. The effect of immune serum on the production of infective virus, complement-fixing antigen and haemagglutinin. *Virology, N.Y.* 20 (4): 552 - 558.
- Ohashi, K. (1953). Cited by Kuroya *et al.* (1953) Newborn virus pneumonitis (type Sendai) II. Report: The isolation of a new virus possessing haemagglutinin activity. *Yokohama Med. Bull.* 4: 217 - 233.
- Okada, Y., Suzuki, T., and Hosaka, Y., (1957). Interaction between influenza virus and Ehrlich's tumor cells. III. Fusion phenomenon of Ehrlich's tumor cells by the action of HVJ F strain. *Med. J. Osaka Univ.* 7: 709 - 717.
- Ondera, Tokio, Hinuma, Yorio, Ishida, Nakao, (1964). Ineffectiveness of antibody on the intracellular development of influenza virus WS strain in Ehrlich Ascites cells in mice. *J. Immunol.* 92 (4): 648 - 656.
- Osato, T., and Ishida, N., (1961). The development of Sendai virus in Earle's L cells as revealed by fluorescent antibody staining. Growth characteristics of myxoviruses in tissue culture. 4th report. *Tohoku J. Exptl. Med.* 73: 201 - 214.
- Parrott, R. H., Vargosko, A. J., Kim, H. W., Bell, J. A. and Chanock, R. M., (1962). Acute respiratory disease of viral etiology III. Myxovirus, parainfluenza. *Am. J. Pub. Hith.* 52: 907 - 917.
- Parrott, R. H., (1963). Conference on newer respiratory disease viruses. *Am. Rev. Resp. Dis.* 88: (3) 196.
- Pereira, M. S. and Fisher, O. D., (1960). Outbreak of acute laryngotracheobronchitis associated with parainfluenza 2 virus. *Lancet*, 2: 790 - 791.

- Petersen, K. B., and Magnus, P. von., (1958). Isolation of a new virus from a child with an influenza-like disease. Danish Med. Bull., 5: 157 - 159.
- Petersen, K. B., (1959). Studies on the properties of a new cytopathogenic and haemagglutinating virus (Copenhagen-222). Acta Path. et Microb. Scandinavica. 45 (2): 213 - 224.
- Prose, P. H., Balk, S. D., Liebhaber, H., Krugman, S., (1965). Studies of a myxovirus recovered from patients with infectious hepatitis. J. Exptl. Med. 122 (6): 1151 - 1160.
- Reichelderfer, T. E., Chanock, R. M., Craighead, J. E. Huebner, R. J. Turner, H. C., James, W., and Ward T. G. (1958). Infection of human volunteers with type 2 haemadsorption virus. Sci. 128: 779 - 780.
- Riesinger, R., Heddleston, K. L., and Manthei, C., (1959). A myxovirus (SF-4) associated with shipping fever of cattle. J. Am. Vet. Med. Assoc. 135: 147 - 152.
- Sassahara, J. Hayashi, S., Kumagai, T., Yamamoto, Y., Hirasawa, N., Mumekata, K., Okaniwa, A. and K. Kato, (1954). On a swine virus disease newly discovered in Japan. Its characteristic traits of pneumonia. I. Isolation of the virus. 2. Some properties of the virus. Virus, 4: 131.
- Sawicki, L., (1962). Studies on experimental sendai virus infection in laboratory mice. Acta. virol. 6: 347 - 351.
- Schultz, E. W., and Habel K., (1959). J. Immunol. 82: 274.
- Shelokov, A., Vogel, J. and Chi, L. (1958). Haemadsorption (adsorption-haemagglutination). Test for viral agents in tissue culture, with special reference to influenza. Proc. Soc. Exptl. Biol. Med. 97: 802 - 809.
- Shindarov, L., (1962). Propagation of parainfluenza I virus in tortoise kidney cell cultures. Acta Virolog. (Praha) 6 (6): 540 - 543.
- Shirnizu, T., Ishizaki, R., Konni, Y., Kumagai, T., Arai, S., Gasahara, J., Ishii, S. and Matumoto, M., (1955). J. Exptl. Med. 25: 211.

- Smith, W., Belyavin, G. and Sheffield, F. W., (1955). The host tissue component of influenza viruses. *Proc. Roy. Soc. Biol.* 43: 504 - 522.
- Smorodintsev, A. A., (1961). New data on live vaccines against poliomyelitis, mumps and measles. *Prog. Med. Virol.* 3: 245 - 310.
- Smorodintsev, A. A., Jr., (1962). Experiences with the isolation and propagation of parainfluenza viruses. *Acta. Virol.* 6: 338 - 346.
- Sokol, F., Blaskovic, D. and Krizamova-Lancikova, (1961). Haemagglutinating subunit released from Newcastle disease, parainfluenza I and mumps viruses by treatment with ether. *Nature (London)* 190: 834 - 835.
- Soul, D. W., Marinette, G. V. and Morgan, H. R. (1959). Studies of the hemolysis of red blood cells by mumps virus. IV. Quantitative study of changes on red blood cell lipids and of virus lipids. *J. Exptl. Med.* 110: 93 - 102.
- Spurrier, Elmer, R., and Robinson, R. Q., (1965). Antigenic relationships among human and animal strains of parainfluenza viruses. *Hlth. Lab. Sci.* 2: (4) 203 - 207.
- Stark, J. E. Heath, R. B. and Peto, S., (1964). A study of the antibodies against parainfluenza viruses in childrens sera. *Arch. ges. Virusforsch.* 14: 160 - 168.
- Stenback, W. A., and Durand, D. P., (1963). Host influenza on the density of Newcastle disease virus (NDV). *Virology* 20: 545 - 551.
- Strandli, O. K., Mortensson-Egmund, K., and Harboe, A., (1964). Purification of the normal allantoic antigen which reacts with influenza virus HI-Antibody to host material. *Acta. Path. et Microbiol. Scand.* 60: 265 - 270.
- Sugaira, A., Enomoto, Cl, and Shunojo, H., (1962). A serological survey of antibodies against sendai, mumps and HA-2 viruses in a rural community. *Bull. Inst. Pub. Hlth. Tokyo* 11: (2), 49 - 55.

- Sun, W. and Fang, C., (1960). Studies on two strains of influenza D virus isolated from lung tissues of guinea pigs. I. Isolation and identification of the virus strains. *Acta Microbiol. Sinica* 8: 129 - 136.
- Sun, W., Fang, C., and Wong, T-K., (1960). Studies on two strains of influenza D virus isolated from lung tissues of guinea pigs. II. Biological characteristics of virus and epidemiological observations. *Acta Microbiol. Sinica* 8: 137 - 149.
- Sutton, R. N. P., Clarke, S. and Tyrrell, P. A. J., (1959). Newly recognized myxoviruses in Europe. *Lancet* 1: p. 395.
- Tadokoro, Jun., (1958). Modified virus particles in undiluted passages of HVJ. The production of modified particles. *Biken's Jrnl.* 1: 111 - 117.
- Tadokoro, J., Suzuki, T., and Fukazawa, R., (1958). On the heat stability of haemagglutinating and hemolytic activities of HVJ (F) virus. *Med. J. of Osaka Univ.* 8: 699 - 709.
- Takokodo, J., Suzuki, T., and Fukazawa, (1958). On the heat stability of HVJ (F) virus. *Med. J. Osaka Univ.* 8: 699.
- Tanaka, H., Moor, D. H., (1966). Immune electron microscopy of mouse mammary tumour virus. *Fed. Prod.* 25 (2): 375.
- Taylor-Robinson, D. (1963). Conference on newer respiratory disease viruses. *The Am. Rev. Resp. Dis.* 88: (3), 195.
- Thomas, E. C., (1962). An investigation of the Morphological changes in Ultrastructure in Cultured Human Amnion Cells Infected with Adenovirus. A thesis, Department of Bacteriology and Immunology, University of Manitoba.
- Tong, M. J. and Fong, Jacob., (1964). Immunologic studies of purified viral haemagglutinin. *J. Immunol.* 93 (1): 35 - 44.
- Traver, Myrna, I., Northrop, R. L. and Walker, K. L., (1960). Site of intracellular antigen production by myxoviruses. *Proc. Soc. Exptl. Biol. Med.* 104 (2): 268 - 273.

- Trombetta, N. and Vitali-Mazza, L., (1961). Osservazioni sull'effetto cito putico indotto dai virus parainfluenzali in culture di tessuto. *Am Oclavo*. 3 (6): 692 - 702.
- Tyrrell, D. A. J., Bynoe, M. L., Birkum, Petersen, Sutton, R. N. P., and Pereira, M. S., (1959). Inoculation of human volunteers with parainfluenza viruses type 1 and 3 (HA-2 and HA-1). *Brit. Med. J.* 2: 909 - 911.
- Tyrrell, D. A. J., and Hoorn, B., (1965). The growth of some myxovirus in organ culture. *Brit. J. Exptl. Path.* XLVI (5), 514.
- Ugoleva, N. A., Beskina, S. R., Cheburkina, W. V., Nozacheva, A. D., Slako, T. D., (1964). Infectivity of RNA (K) isolated from tissues infected with sendai virus. *Vop. Virusol.* 2 (2): 184 - 188.
- Ugoleva, N. A., (1965). Infectivity of RNA isolated from tissues infected with sendai virus. *Fed. Proc. (Transl. Suppl)* 24: 481 - 483.
- Vargosko, A. J., Chanock, R. M., Huebner, R. J., Luckey, A. H., Kim, H. W., Cumming, C. and Parrott, R. H. (1959). Association of type 2 haemadsorption (parainfluenza 1) virus and Asian influenza A virus with infectious croup. *New England J. Med.*, 261: 1 - 9.
- Veem, Van Der, J., and Smeur, F. A. M., (1961). Infections with parainfluenza viruses in children with respiratory illnesses in Holland. *Am J. Hyg.* 74 (3): 326 - 331.
- Veen, Van Der, J., and Sonderkamp, H.J.A., (1965). Secondary antibody response of guinea pigs to parainfluenza and mumps viruses. *Archiv. ges. Virusforschung* 15 (5): 721 - 734.
- Vogel, J., and A. Shelokov, (1957). Adsorption Hemagglutination Test for Influenza Virus in Monkey Kidney Tissue Culture. *Science* 126: 358.
- Von Euler, L., Kantor, F. S., and G. D. Hsiung. (1963). Studies of parainfluenza virus 1. Clinical, pathological and virological observation. *Yale J. Biol. and Med.* 35: 523 - 533.
- Warren, J., Jensen, K. and R. Mason (1962). The Syncytial Viruses. *N. Y. Acad. Sci.* 101: 520 - 525.

- Waterson, A. P. and J. G. Cruickshank (1963). The effect of ether on Newcastle disease virus - a morphological study of eight strains. *Z Naturforsch* 18: 114 - 8.
- Wenner, H. A., Jenson, M. H., and A. Monley (1952). A study of infection caused by mumps and Newcastle disease viruses 1. Specific and non-specific serologic relations. *J. Immunol.* 68: 343.
- Wilt, J. C., Hildes, J. A., Parker, W. L., Delaat, A., (1961). Sendai virus antibodies in the Arctic. *Can. Med. Assoc. J.* 84 319 - 321.
- Wong, F. C., (1965). Unpublished data.
- Yorio, Himuna, (1964). Studies on a carrier state of HA-2 virus in HeLa cell culture by means of immunofluorescent procedure. *Jap. J. Med. Sci. Biol.* 17: (5), 281.
- Zhdanov, V. M., Ritova, V. V., and Golygina, L. A., (1957). Influenza D in infants. *Vop, Visusol.* 4: 243 - 247. *Acta Virol. Praha,* 1: (3/4) 216 - 219.
- Zhdanov, V., and Bukrinskaya, A., (1960). Further consideration on nomenclature of parainfluenza viruses. *Virology* 10: 146 - 149.
- Zhdanov, V. M., (1961). Personal communication cited in a manual of new viruses causing respiratory illness in man (1961), presents for commission on acute respiratory disease of the Armed Forces Epidemiological Board p. 39.
- Zhdanov, V. M., and Bukrinskaya, A. G., (1961). Mechanism of penetration of virus into the cell - Facts and Hypothesis. *Vop. virusol* 6: 416 - 418.
- Zhdanov, V. M., Bukrinskaya, G. A., and Azadova, N. B., (1961). Fluorescent microscopic study of sendai incomplete virus production in tissue culture cells. *J. Immunol.* 87: 647 - 653.
- Zhdanov, V. M., and Bukrinskaya, A. G. (1962). Studies on the initial stage of virus cell interaction. *Acta. Virol.* 6: 105 - 113.

- Zhdanov, V. M., Bukrinskaya, A. G. and Ramenskaya, G. P. (1963).
Autoradiographic study of the penetration of
sendai virus into the cell. III. Use of virus
preparations labelled with macii-C-14.
Acto. virol. 7 1 - 6.
- Zhdanov, V. M., and Smirnova, G. A., (1965). On the nature of
the inactivating effect of animal tissue extracts
against myxovirus haemagglutinins.
Acta. Viral 9: 137 - 143.
- Zhdanov, V. M., Azadova, N. B., and L. V. Uryvayev, (1965).
Topography of synthesis of S and V antigens of
Sendai virus. J. Immunol. 94 (5): 658 - 661.
- Zhdanov, V. M., Azadova, N. B., and A. Y. Kulberg, (1965a).
Synthesis and transport of protein components of
Sendai virus. Arch. Ges. Virusforsch 17 (5):
527 - 533.