STUDIES ON DEFECTIVE STRAINS OF NEWCASTLE DISEASE VIRUS

By

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ABSTRACT OF THESIS

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Title of Thesis Studies on Defective Strains of Newcastle Disease Virus

The properties of strains of NDV occurring in persistently infected cultures of ox (BK pi), pig (PK pi) and sheep (OK pi) kidney cells were compared with those of lentogenic and virulent strains of NDV, grown in fertile hens' eggs and cell cultures.

The results obtained indicate that a number of changes have occurred in the carrier cell-lines during prolonged serial sub-culture. The most important of these include, a greater release of infectious virus and an increased percentage of cells capable of haemadsorption, despite lower titres of released haemagglutinins.

Detailed studies of the biological activities and infectivity of the persistent virus show these to be generally reduced compared with 'wild-type' strains of NDV grown in fertile hens' eggs. However, the characteristics of 'wild-type' strains of NDV released from mammalian cell-lines were also defective. In addition the enzyme phosphodiesterase which has not been previously described in NDV, was characterised and shown to be associated with the small viral glycoprotein. It was found to be of reduced activity in the virus released from the carrier cell-lines.

Polyacrylamide gel electrophoresis (PAGE) of purified virions revealed that virus released from persistently infected cells or from control cell cultures infected with'wild-type'strains of NDV, contained a large amount of a protein of 70,000 daltons which was not found in virus grown in fertile hens' eggs. This protein was not seen when electrophoresis was carried out under non-reduced conditions and its disappearance was apparently related to an increase in the quantities of the nucleocapsid and haemagglutinin proteins observed. An abnormal protein of 62,000 daltons was also found when virus released from cell cultures were examined by PAGE, and this was shown tobe an inactive form of the small glycoprotein. Haemolysin activity and normal molecular weight were restored to this protein by treatment with 4 p.p.m. of trypsin. The large glycoprotein of NDV was not present in the virus released from the persistently infected BK pi cells and its absence was associated with an extremely low haemagglutinin activity in this strain of virus.

The synthesis of viral proteins was studied in the BK pi cell-line and in a healthy bovine kidney cell-line (MDBK) infected with the B1 strain of NDV. Virus-associated proteins were identified with the following molecular weights: 180,000; 75,000; 70,000; 62,000; 55,000; 53,000; 49,000; 42,000 and 38,000 daltons. All but three of these corresponded to the structural proteins of wild-type strains of NDV, grown in fertile hens' eggs. The exceptions were the 70,000 and 62,000 dalton proteins which are found in virus released from mammalian cells and also the 38,000

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38,000 dalton protein which is thought to be a precursor of one of the structural proteins.

Growth of the carrier cell-lines under conditions that slowed the rate of cellular metabolism, including low levels of nutrition and low temperatures of incubation, allowed the release of greater quantities of virus, without increasing its infectivity.

Virus from all three persistently infected cell-lines was shown to be incapable of replication at 41°C but was able to persist without synthesising viral antigen for periods of over two months at this non-permissive temperature. Viral protein synthesis was also inhibited by actinomycin D but the infectivity of the released virus was increased. These two phenomena may be related to the ability of the persistent virus to utilise a replicative pathway involving DNA.

The carrier cell-lines were capable of forming 'colonies' in semi-solid agar. This is characteristic of cellular transformation and may be related to the fact that BK pi cells were able to adsorb erythrocytes to areas of the cell membrane from which budding virus was absent.

Infection of fertile hens' eggs and 5-week-old chickens with aliquots of concentrated virus obtained from the carrier cell-lines resulted in the release of highly virulent strains of NDV.

There was also evidence that:

1) the replicative cycle of NDV in the healthy mammlian cell-lines is generally abnormal and defective virus particles are formed with low infectivity. Reduction in their biological activities is probably due to abnormalities in the synthesis of viral proteins and in the assembly of mature virions.

2) persistence of virus in a non-permissive cell-system requires the mutation of the virus, giving rise to a form that is capable of employing the host cell machinery for RNA synthesis by means of a DNA transcription of the viral genome.

3) this mutation was accompanied by other changes in the virus, including increased temperature-sensitivity and decreased biological activities.

4) cells persistently infected with NDV undergo partial transformation because of the presence of the viral proteins.

The work presented in this thesis is my own; and some aspects of the experimental findings have been published in co-authorship with my supervisor, Dr. G. Fraser.

Jon M.S. Ruben

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SUMMARY

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INTRODUCTION

AND

REVIEW OF THE LITERATURE

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In this section, an outline is given of the known properties of Newcastle disease virus (NDV) and their relationship to virulence, with reference to the pathological effects observed in infected cells. Previous reports of persistent infections with paramyxoviruses are then examined and a summary is given of the abnormalities of the virus associated with them.

A / HISTORY

Newcastle disease was first described as a virulent infection of poultry by Kraneveld in 1926 (1). The causative agent was identified as a virus by Doyle, following an outbreak of the disease on a poultry farm near Newcastle-upon-Tyne in 1926 (2).

The classical velogenic (or virulent) form of the disease has an incubation period of 4 - 11 days followed by an acute illness. There are severe respiratory and neurological signs with up to 100% mortality (2). Further outbreaks in the U.K., due to virulent strains of NDV, have occurred in 1933, 1947 and 1970 (3).

Newcastle disease virus is now known to cause disease in many parts of the world but was not recognised in the U.S.A. until 1944. In that year, Beach (4) showed that an endemic disease of poultry flocks in California, characterised by low mortality (15%) and mild respiratory signs, was due to infection by a mesogenic strain of NDV.

NDV. Since 1947, strains of this type have been endemic in England.

More recently, McFerran (5) and Simmons (6) have described symptomless outbreaks of the disease in Ulster and Queensland, respectively. These avirulent (lentogenic) strains can be detected by inhibition of haemagglutination by convalescent sera and by isolation of the virus in fertile hens' eggs.

Newcastle disease virus can be readily adapted to culture in developing chicken eggs and in vitro growth in cell monolayers. Several avirulent strains, derived in this manner, are presently being used for the immunisation of poultry.

B / CLASSIFICATION

The myxovirus particle is characterised by a central core of RNA, exhibiting helical symmetry, and is surrounded by a glycoprotein-lipid envelope (7).

Three subgroups are proposed (8):

A) ORTHOMYXOVIRUSES (the influenza viruses including fowl plague virus). These have a nucleocapsid diameter of 9 - 10 nm, with haemagglutinin and neuraminidase activity in separate envelope proteins (9).

B) PARAMYXOVIRUSES (including NDV, mumps and parainfluenza viruses). These have a nucleocapsid diameter of 17 - 18 nm, with haemagglutinin and neuraminidase activity in the same envelope protein (11) and possess a haemolysin (164) associated with the envelope proteins.

C) PSEUDOMYXOVIRUSES (the so-called measles, rinderpest and distemper virus triad). These have a nucleocapsid diameter of 17 - 18 nm but have not been shown to possess neuraminidase activity. However, haemagglutinin, haemolysin and fusion activities of a similar nature to those of paramyxoviruses have been located on the equivalent envelope proteins (163).

A fourth group, the METAMYXOVIRUSES, has been proposed to incorporate such agents as the respiratory syncytial virus, which exhibit a similar helical symmetry to other myxoviruses but have a nucleocapsid diameter of about 12 - 15 nm and have not, as yet, been demonstrated to possess haemagglutinin (209).

In the following sections, a brief outline is given of current knowledge concerning the normal morphology,

morphology, structural components, replicative mechanisms in the host cell and the detectable biological activities of paramyxoviruses. Wherever possible these characteristics are related to their role in the life-cycle of NDV.

C / MORPHOLOGY

When viewed by electron microscopy, NDV is pleomorphic (12). There is an outer envelope, bearing spikes, containing an irregularly coiled helical core of nucleocapsid. The virus particle may exceed 200 nm in diameter. The nucleocapsid (consisting of ribonucleoprotein) is 17 - 18 nm wide and is wound round a hollow central core 5 nm across (12), with a helical periodicity of 5 nm and a length of up to 1.06 μm (13). In contrast to the orthomyxoviruses, the RNA appears to be of a single species, sedimenting at 57 Svedburg Units (as measured in 0.1 M NaCl, 0.01 M Tris/HCl and 0.001 M versene) (14) and makes up 9.3% of the total ribonucleoprotein (RNP) (16). The spikes, borne on the outer surface of the envelope, are 8 nm long and 1.5 nm wide. They are radially arranged on the surface of the virion. When virions are treated with ether, separation of the envelope and the core occurs. The spikes then aggregate into rosette-like sub-units (17).

D / THE STRUCTURAL PROTEINS OF NDV

Three major proteins are found by most workers when purified NDV is treated with sodium lauryl sulphate (SDS) and run in a reduced state on polyacrylamide gels (18, 19, 20 & 21). Between three (19) and seven (21) minor proteins have also been described. Moore and Burke (21) have shown that there are strain differences between the relative proportions of the proteins, as well as their amino acid composition. When the viral proteins were electrophoresed in non-reduced conditions, aggregation of several of the proteins occurred but the relative proportions of aggregated to non-aggregated protein varied from strain to strain (21). They further identified two glycoproteins and nine polypeptides, which did not contain carbohydrate, as detailed below:-

Three major polypeptides of molecular weights 75,000 (a glycoprotein), 55,000 and 42,000 daltons, described respectively as VGP 75, VP 55 and VP 42.

Eight minor polypeptides of molecular weights 180,000 (usually a glycoprotein), 110,000, 55,000 (a glycoprotein), 53,000, 52,000, 51,000 and 49,000 daltons, known respectively as VGP 180, VP 110, VGP 55, VP 53, VP 52, VP 51 and VP 49.

Although the location of all of the polypeptides within the virion is not known, VGP 75 and VGP 55 have been identified with the envelope spikes and sub-virionic rosette structures (17, 18 and 21), while VP 55 has been related to the RNP of the nucleocapsid (17, 18 and 21). VP 42 is believed to be associated with the viral envelope

envelope but not with the envelope spikes (18 and 19).

Recently Iinuma and Simpson (180) have isolated a small polypeptide of 26,000 daltons, which is immunologically distinct from the proteins described by Moore and Burke (21).

E / REPLICATION

The normal replicative cycle of NDV can be divided into four stages, as follows:- entry, RNA synthesis, protein synthesis and release.

a) Entry

i) Adsorption.... Paramyxoviruses attach themselves to the surface of susceptible host cells by binding of the haemagglutinin (which is contained within the spikes on the viral envelope) at specific sites, or receptors, on the surface of the cell.

If these sites are occupied or destroyed, adsorption does not occur and virus infection is unlikely to succeed (23, 24).

ii) Penetration.... In 1962, Mussgay and Weibel demonstrated that intact virions appeared within the cell, less than half an hour after infection of chick embryo fibroblasts (25). However, more recent work (26, 27) has shown that this is not the normal method of entry for paramyxoviruses. In 1972, Morgan (26) showed that, as the virus adsorbs to the cell surface it becomes distorted and fusion between the viral and host membranes takes place. Filaments of the viral nucleocapsid then pass into the host cell cytoplasm. Morgan (26) has also shown that phagocytosis of the intact virion may take place (as described by Mussgay (25)) either before or after fusion with the cell membrane but that this is a relatively unimportant mechanism for the entry of virus.

b) Viral RNA Synthesis in the host cell

In paramyxovirus infection, virus-specific RNA synthesis begins within two hours of adsorption of the virus particle (28, 30).

Although it is generally believed that the site of synthesis is probably in the polyribosomes in the cytoplasm (30), it is interesting to note that Bukrinskaya (31) detected nucleolar synthesis, three to four hours after infection by means of very brief pulse labelling techniques and autoradiography.

Most of the novel viral RNA is complementary (+) to viral RNA (-) (30). The complementary RNA is of four main species; some being of the same size as viral genome RNA (57S), while the majority has a Svedburg value of 18 S. However, complementary 22 S and 35 S RNA are also found (31).

The large amount of (+) RNA formed, supports the present model of viral RNA synthesis in paramyxoviruses. Portner and Kingsbury (36) postulate that the infecting viral genome ((-)RNA) is 'anti-message' and messenger RNA is formed from this, in a double stranded RNA transcription complex.

The messenger RNA (+) then forms a second transcription complex, which results in the formation of new genome (-) RNA. This hypothesis is supported by the presence of two transcription complexes described in Sendai virus replication (36) and the stability of 18 S RNA (+) during NDV replication (37), (suggesting that it has a messenger RNA function). ~~However, since Bratt and Robinson (32)~~

~~(32) found only one transcription complex in NDV infection, this suggests that NDV is able to employ infecting genome RNA as messenger RNA like other single stranded RNA viruses (33,37).~~

A virus-specific RNA-dependent-RNA-polymerase has been described in both Sendai (41,42) and NDV virions (38, 44) and also in cells infected with either of these viruses (39, 40, 42, 43) where it has been associated with the transcriptive complex (42, 46).

Actinomycin D treatment of cells infected by NDV, has only a slight inhibitory effect on viral RNA synthesis, while greatly inhibiting cellular RNA synthesis (30). Since actinomycin D acts by preventing the transcription of DNA molecules into RNA, this evidence supports the hypothesis that paramyxovirus RNA replication does not involve DNA transcription and shows that NDV replication is largely independent of host cell RNA synthesis.

However, recent work by Hallum and Furman (45) has suggested that a pathway involving a DNA intermediate may exist. They have shown that an RNA-dependent-DNA-polymerase is produced in cells persistently infected with NDV and is present in virus released from these cultures. Nevertheless, these workers could not detect this enzyme in cells infected with 'wild-type' NDV or in the virions of these strains.

c) Protein synthesis by NDV in the host cell.

Synthesis of viral protein begins within two hours of infection, and may be demonstrated by various techniques, including radioactive labelling (51, 135, 48),

(51, 135, 48), immunofluorescence (29) and electron microscopy (141). Although early protein synthesis is cytoplasmic (29, 141), fluorescent antibody staining (FAS) has revealed that viral antigen is present in the nucleus within sixteen hours of infection with NDV (29, 92), while Zaides has reported that nucleocapsid is found in the nucleoli of cells infected by Sendai virus (46).

Alexander and Reeve have demonstrated no fewer than six virus-induced proteins by polyacrylamide gel electrophoresis (PAGE) of radioactively labelled cells, infected with NDV (48). Four of these correspond to the structural proteins, VP 180, VGP 75, VP 55 and VP 42, described by Moore and Burke (21), but the remaining two proteins were presumed to be non-structural. By similar methods, Hecht and Summers (49) and Samson and Fox (50) have also shown that the major structural polypeptides, VGP 75, VP 55, VP 42 are synthesised in infected cells. In addition, the latter workers described a non-structural protein of 64,800 daltons which they believed to be a possible precursor of either the nucleocapsid (VP 55) or the small envelope glycoprotein (VGP 55) (50). However, neither of the non-structural proteins described by Alexander and Reeve (48) correspond to that found by Samson and Fox (50). Thus, at present, three non-structural proteins are thought to be synthesised in cells infected with NDV.

A precursor protein to the small glycoprotein of Sendai virus has been demonstrated in cells infected

infected with Sendai virus (142, 11). In a normal replicative cycle, this undergoes cleavage to form the active protein, but in some abortive infections, cleavage does not occur and the released virions contain the larger precursor protein, in place of the small glycoprotein.

Fluorescent antibody staining has shown that the site of VGP 75 (haemagglutinin) synthesis is diffuse throughout the cytoplasm, while that of the nucleoprotein is perinuclear (29), or possibly nucleolar (52). There is also evidence that more of these proteins are formed in infected cells than are released in mature virions.

Fluorescent antibody studies (29) also indicate that nucleoprotein antigen appears three hours after infection, while sufficient accumulations of haemagglutinin capable of being stained by fluorescent antibody do not occur until five hours after infection (29, 61). However, both proteins are synthesised within two hours of infection and may be detected by radioactive labelling techniques (48). None of the other structural proteins of NDV has been located within the host cell, with the exception of that described by Iinuma and Simpson (180), although their synthesis has been demonstrated (48).

d) Maturation and release of NDV.

Mature virions are detectable 4 - 10 hours after infection with NDV (51). As in other paramyxoviruses (141), nucleocapsid is formed from the newly synthesised RNA and nucleoprotein (46), and is aligned under areas of the cell membrane that appear thickened when viewed

viewed by electron microscopy (141, 53, 54). The cell membrane in these areas is deficient in neuraminic acid (55) and contains haemagglutinin, which is associated with the haemadsorption phenomenon (57, 58).

The virus particle is formed by a bud of nucleocapsid surrounded by the thickened cell membrane, which has been infiltrated with viral envelope proteins.

The affected portion of the cell membrane constricts at the base of the bud and the mature virion is released. Production of NDV continues for 12 - 72 hours depending on the virulence of the infecting strain.

In orthomyxoviruses, neuraminidase has been implicated in virus release and there is evidence that this enzyme removes the neuraminic acid-containing sites from the cell surface, thereby preventing the newly-budded virion from re-attaching to the host cell by means of the haemagglutinin in its envelope (59, 67). The neuraminidase of paramyxoviruses probably has a similar function.

Inhibition of viral protein synthesis with cycloheximide or other inhibitors of protein synthesis, results in the rapid cessation of the release of NDV, although large quantities of VGP 75 and VP 55 may be present (51). This suggests that another of the viral proteins is more slowly synthesised and that its production is closely related to virus release.

F / BIOLOGICAL ACTIVITIES OF NDV

The following biological activities have been reported in paramyxoviruses or cells infected by paramyxoviruses :-

a) haemagglutinin (HA)

b) neuraminidase (NA)

c) RNA polymerase (Rp)

d) DNA polymerase (RdDp)

e) exo- and endonucleases

f) adenosine triphosphatase (ATPase)

g) protein kinase (PK)

h) fusion of cell membranes

(i) fusion from within (f.f.w.i.)

(ii) fusion from without (f.f.w.o.)

i) haemolysin (HY).

a) Haemagglutinin (HA)

Haemagglutinin is contained in the envelope spikes (VGP 75) of NDV (17, 18). When a virus particle comes into contact with a cell, the haemagglutinin forms a linkage with certain radicals found in the glycoproteins of the cell membrane. These glycoprotein receptor sites are found on many types and species of cell (including red blood cells) and are the means by which myxovirus particles attach themselves to potential host cells (10) prior to infection.

If a suspension of red blood cells is treated with a suspension of NDV particles, agglutination between the red blood cells and the virus particles occurs and clumps

clumps of red blood cells can be seen. This phenomenon of haemagglutination is used as a basis for the assay of the quantity of virus particles present in a suspension.

Haemagglutination by NDV takes place at all temperatures from 4°C to 37°C.

Neuraminidase, vide infra, destroys the glycoprotein of the cell receptor sites, so preventing the attachment of virus to treated cells and allowing previously adsorbed virus to elute (64).

The haemagglutinin that is synthesised within the cell may be readily detected by immunofluorescence techniques or by haemagglutination assays of disrupted cellular material.

The ability of erythrocytes to adhere to the surface of infected cells (haemadsorption) has been shown, in the case of NDV (57), to be due to the presence of mature NDV budding from the cell membranes. However, in mumps virus infection (60) two types of haemadsorption have been described :

(i) The red blood cells are separated by 10 - 20 nm from the cell membrane which is not thickened or differentiated, but the infected cells can be shown by immunological methods to contain viral haemagglutinin,

(ii) The red blood cells are attached directly to the cell membrane, which has been differentiated into a viral membrane, and which bears spikes, aligned above viral nucleocapsid.

Thus, the latter will occur only when virus is being released, while the former will occur in any infection

infection where haemagglutinin is being synthesised.

Therefore, haemadsorption may be due either to the presence of haemagglutinin spikes in the cell membrane or to the adherence of erythrocytes to a plasmalemma, which has been physiologically altered, consequent to the formation of viral proteins in a cell infected with a paramyxovirus (vide infra)Section G, II (c) and (d)).

b) Neuraminidase (NA)

Neuraminidase activity is a characteristic property of all "typical" strains of myxovirus (8). Most work on the biological function of this enzyme has been carried out with influenza and other orthomyxoviruses and there is general agreement that it is associated with a different structural protein from that of haemagglutinin. Despite earlier reports to the contrary, it has now been shown that neuraminidase activity is not associated with viral entry (10), but rather is involved in the mechanism of viral release (59, 66). Recent work by Compans et al (67) demonstrated that lack of neuraminidase activity allowed viral haemagglutinin in budding virions to bind to cell membrane glycoproteins, thereby preventing the release of virus particles.

Neuraminidase acts on neuraminic acid linkages in glycoproteins, so destroying the ability of these sites to act as receptors for haemagglutinin binding. Thus, infected cell membranes lack neuraminic acid, and sites of virus maturation can be located by the absence of colloidal iron hydroxide staining (55).

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Unlike orthomyxoviruses, the neuraminidase and haemagglutinin activities in paramyxoviruses are borne on the same viral glycoprotein - VGP 75 (11, 18, 62), which is found in the viral envelope spikes (17).

Neuraminidase activity may be assayed quantitatively using an artificial substrate (34) and its presence permits the elution of red blood cells following haemagglutination assays.

c) RNA polymerase activity. (Rp)

RNA-dependent RNA polymerase activity is found in cells infected by NDV (39). In the case of Sendai virus infections this enzyme has been associated with RNA transcription complexes which contain viral RNA (both (+) and (-)) and two structural proteins, the nucleocapsid protein and the largest of the Sendai virus polypeptides (46, 43). These structures appear similar to the viral nucleocapsid, when viewed under the electron microscope.

RNA polymerase activity is also found in purified NDV (38) and Sendai virus preparations (41). When the proteins obtained from purified Sendai virus are separated, the fraction containing the nucleocapsid protein and the largest Sendai virus protein, retain a high level of RNA polymerase activity (42). However, RNA polymerase activity is absent from preparations of purified nucleocapsid derived from NDV (44).

It is likely, therefore, that RNA polymerase activity in NDV is associated with a viral protein that is equivalent

equivalent to the large Sendai virus protein as well as the nucleocapsid. In both Sendai virus and NDV, RNA polymerase activity is virus-specific and is probably associated with the synthesis of viral messenger RNA, as well as viral genome RNA.

d) RNA-dependent DNA polymerase (RDDP).

RNA-dependent DNA activity was isolated by Temin et al. from ~~avian myeloblastosis~~ Rous sarcoma virus (167) and has since been described in a variety of other RNA viruses including avian and murine tumour viruses (22) and Visna/Maedi virus (a 'slow' or latent virus infection of sheep)(168). Recently Hallum and Furman (45) demonstrated that virus-specific RDDP is found in cells persistently infected with NDV and in virions released from these cells, while Zdhanov et al. (150) have found DNA transcripts of measles virus RNA in cells persistently infected with this virus. However, RDDP has not been described in cells infected with wild-type NDV.

It is of interest that, so far, RDDP has only been found in association with viruses that are oncogenic, 'slow' (i.e. with a long incubation period followed by progressive, prolonged illness, which usually terminates in death), or capable of causing persistent infection in cell cultures.

Transcription of an RNA virus genome would allow the DNA copy to be incorporated into the cellular genome and thus provide a mechanism for latent or persistent infection. The transformation of cells associated with viral oncogenesis may also depend on the incorporation

incorporation of viral DNA transcripts into the host genome.

e) Exonuclease and endonuclease activity.

Phosphodiesterase activity associated with Sendai virus has been reported by Pristasova and Rosenbergova (70). The same workers have also reported the presence of RNA and DNA endonucleases (71) in Sendai virus.

These enzymes have not been associated with any of the viral structural proteins and no function has been ascribed to them. However, a report by Huo and Wilson (79) that breakdown of host RNA follows infection of cells with NDV, suggests that the viral nucleases may have a role in this phenomenon.

f) and g) ATPase and protein kinase.

Two activities necessary for the synthesis of paramyxoviruses have been demonstrated in cells infected by Sendai virus. Although these enzymes have been described in uninfected cells, their presence in infected cells, when other cell mechanisms have been inhibited, implies a function in viral replication. There is also the possibility that these enzymes are coded for by the infecting virus.

The first of these activities, adenosine triphosphatase, was described by Neurath (69) in cells infected by Sendai virus and has recently been identified in purified parainfluenza 3 virions (181). Degradation of ATP is necessary to provide the requisite energy for RNA breakdown and synthesis.

The second, protein kinase activity, has also been

been demonstrated in cells infected by Sendai virus. This is necessary for the process of viral protein synthesis and promotes the phosphorylation of proteins (68).

It is not known if these activities are associated with any of the structural proteins of Sendai virus and there are no reports, so far, that they are present in NDV virions or cells infected by NDV.

Lamb (169) has shown that protein kinase is found in Sendai virions and that although activity is not demonstrated by purified envelope or nucleocapsid proteins, it is still associated with the virion, after the removal of the surface glycoproteins. This worker also suggests that, by promoting the phosphorylation of the RNA polymerase protein(s) and thus causing their activation, PK may regulate the transcription of the viral genome by RNA polymerase.

h) Fusion of cell membranes.

The ability to induce fusion of cell membranes is a property of many enveloped viruses. It can take place either between uninfected cells in the presence of virus, i.e. fusion from without (f.f.w.o.) or between cells infected with virus, i.e. fusion from within (f.f.w.i.).

Fusion from without requires adsorption of the causative virus to the cell surface but the virus need not be fully infectious.

Following adsorption of NDV, a change in the cell membrane in the region of the virus takes place (72) due to the action of the large and small glycoproteins found in paramyxoviruses (73). (It should be noted that the

the large glycoprotein is necessary for adsorption of virus to the cell). Fusion between adjacent cells then takes place. There is usually direct fusion between the modified cell membranes in the region of the virus but the process does not usually involve fusion of the cell membranes to the virus envelope.

Fusion from within is probably due to a similar mechanism brought about by the accumulation of virus envelope proteins and lipids in the infected cell membrane which, in a like manner to that described for haemadsorption, brings about a change in the cell membrane, thereby allowing cell fusion to take place (74).

It is emphasised that f.f.w.o., where no virus multiplication has taken place requires a far higher input of virus (as measured in multiplicities of infection) than does fusion from within.

Differences in the cell membrane composition affect the degree of fusion possible in a particular type of cell and may also have some bearing on the capacity of mature virus to be released from such cells (75, 76 77).

Alexander et al. (74) employed the Herts strain of NDV to induce fusion from without, using four mammalian cell lines as well as chick embryo fibroblasts. Within three hours of infection, (employing 1000 p.f.u. of virus per cell), fusion occurred in all the cell lines. However, when the same cell lines were infected by a lower multiplicity of infection (ten p.f.u. per cell) and incubated for 24 hours, fusion from within took

took place in three of the mammalian cell lines, baby hamster kidney (BHK21), human epithelial (HEp-2) and mouse L929 cells, and the chick embryo fibroblasts but not in the bovine kidney (MDBK) cells. It is further notable that the degree of haemadsorption and cell-associated haemagglutinin were much less in MDBK than in the other cell lines.

The function of fusion in viral pathogenesis is not understood at present. It is likely that fusion is a consequence of the alteration in cell membrane physiology, necessary for virus-to-cell fusion, during the process of viral entry. The infiltration of viral proteins into the cell membrane, during release of virus, results in a similar change in the physiology of the cell membrane; thus inducing fusion of adjacent cells. This modification of the cell membrane physiology may aid in release of virus.

i) Haemolysin (HY)

Unlike orthomyxoviruses, paramyxoviruses produce a haemolysin which destroys red blood cells and this activity may be used in a quantitative assay for the presence of virus.

Klemperer (78) found that NDV haemolysin acts by altering the permeability of the cell membrane to potassium ions, while Apostolov and Almeida (172) have shown that integration of the viral membrane with that of the cell is required before haemolysis takes place. Moreover, Neurath et al. (171) have demonstrated that some cleavage of the erythrocyte polypeptides occurs prior to haemolysis.

haemolysis. Therefore, since the lysis of cells infected with NDV is probably brought about by the release of cellular lysozymes, the physiological effect on the cell membrane, which results in the destruction of red blood cells, probably contributes to other surface-related phenomena associated with paramyxovirus infection.

Indeed, Hosaka (73) has reported that haemolysin activity is closely associated with fusion, which occurs in the presence of both viral glycoproteins. However, haemolysis requires a greater proportion of the smaller glycoprotein than cell fusion, which is enhanced when the proportion of the small glycoprotein is lower (73). In addition, the presence of phospholipid has been shown to be essential for both phenomena (171) and, recently, Seto et al (170) have confirmed the inter-relationship of cell fusion and haemolysis for NDV. In their experiments they showed that antibody prepared to the large glycoprotein of NDV inhibits haemagglutinin and neuraminidase activities, and to a lesser extent the fusion and haemolysin functions of NDV. Antibody to the small glycoprotein of NDV (VGP55) inhibits the haemolysin and fusion functions but has no inhibitory effect on the haemagglutinin and neuraminidase activities of NDV.

However, the function of the haemolysin in viral replication or pathogenesis remains unknown.

The biological activities of NDV, discussed above, fall into two main categories:

i) those related to the synthesis of viral proteins and

and RNA. These include RNA polymerase, the exo- and endonucleases, ATPase and protein kinase. It is interesting to note that some of these enzymes are similar to those produced by the host cell and it may be that further 'duplicates' are coded for by the virus. The evidence available at present suggests that these enzymes are not concerned with viral entry and are either associated with the internal components of the virion or with non-structural proteins manufactured during viral infection,

(ii) those related to entry and release of virus, including haemagglutinating, neuraminidase, cell-fusing and haemolysing activities. These properties are not found in normal cells and are associated with the envelope glycoproteins of the virion. The haemagglutinin and neuraminidase are concerned respectively with the attachment and release of virions. It is likely that cell fusion is a consequence of the changes in cellular physiology necessary for the fusion of the viral envelope to the plasmalemma, during the entry and liberation of virions. Haemolysis, which is very closely related to f.f.w.o. by immunology, may merely be the consequence of the fusion of virus to the fragile erythrocyte membrane.

On the other hand, it was once believed that two of the phenomena, haemadsorption and cell fusion, associated with the surface of infected cells, only took place due to the presence of specific viral structures incorporated into the cell membrane. However, these processes, together with several properties of infected

infected cells discussed in the following section, can also occur as soon as synthesis of viral protein has commenced.

G / THE EFFECTS ON CELLS OF INFECTION WITH NDV

The main effects of NDV on infected cells can be summarised as follows:-

I) METABOLIC EFFECTS

a) RNA synthesis
b) Protein synthesis
c) DNA synthesis
d) Lipid synthesis

II) PHYSIOLOGICAL EFFECTS

a) The effect of the virus on cell membrane physiology:
   i) Haemadsorption
   ii) Fusion
b) The effect of the virus on cell lysosomes
c) Agglutination of infected cells that are treated with lectins
d) Thinning of the cell membrane of infected cells.

III) CELL DEATH CAUSED BY NDV, AND ITS EFFECT ON THE CELL POPULATION (PLAQUE FORMATION)

IV) THE RESPONSE OF INFECTED CELLS TO NDV:

Interferon induction

V) MISCELLANEOUS EFFECTS OF INFECTION OF CELLS WITH NDV

a) Cellular transformation
b) Inclusions: storage of viral antigens and virus induced material.

I) METABOLIC EFFECTS

a) and b) RNA and protein synthesis in infected cells.

Ortho- and paramyxovirus infections usually result in a diminution of cellular RNA and protein synthesis.

Therefore, if the synthesis of cellular RNA is inhibited, protein synthesis will eventually be prevented due to the lack of messenger RNA. However, there is evidence (85) that some strains of NDV may have an independent, inhibitory effect on the synthesis of protein by infected cells.

Early work (79, 83, 84) showed that inhibition of cellular synthesis did occur following infection by NDV but that the degree of inhibition varied, depending on the strain of NDV that was used. No link could be clearly established between virulence and inhibition of RNA and protein synthesis in infected cells.

Reeve et al. (81) tested a number of virulent, mesogenic and avirulent strains and the results they obtained suggested a correlation between virulence and the inhibition of cellular RNA and protein synthesis.

However, Moore et al. (80) showed recently that, while many strains of NDV inhibit cellular RNA and protein synthesis, some lentogenic strains cause as much inhibition as do velogenic strains. They also found that two strains of NDV, namely Beaudette C (mesogenic) and Asplin F (mesogenic/lentogenic), induce an overall increase in cellular RNA synthesis. This is probably a cumulative effect due to both viral and cellular RNA synthesis. In contrast, some strains

strains produce complete inhibition of cellular RNA and protein synthesis within 15 hours of infection and the effect can usually be distinguished within four hours of infection.

According to Wilson (85) cellular RNA shut-down requires previous synthesis of viral RNA whereas cellular protein shut-down requires the previous synthesis of both viral RNA and protein.

I.c) DNA synthesis in infected cells.

Although inhibition of cellular DNA synthesis is generally considered to be secondary to that of cellular protein synthesis (82, 86), Huo and Wilson (79) did not observe inhibition of cellular DNA synthesis following infection of cells with NDV. Fuchs and Kohn (87) found inhibition of cellular DNA formation following infection of cells with NDV, and showed that the immediate cause is the inhibition of thymidine synthesis, which occurs in the early stages of viral replication.

I.d) Lipid synthesis in cells infected by NDV.

Blair (88) demonstrated an increase in lipid metabolism during an abortive infection of chick fibroblasts by Sendai virus, which is probably due to the extra lipid required for viral envelope synthesis. It is not clear whether any inhibition of lipid synthesis for cellular requirements occurs.

The metabolic effects of infection of cells with NDV can be summarised as follows:

Most strains of NDV directly inhibit cellular RNA and protein synthesis and, consequently, cellular DNA

DNA synthesis, but these properties are not related to the virulence of the infecting strain.

II) PHYSIOLOGICAL EFFECTS

a) The effect of the virus on the cell membranes.

i) Haemadsorption.

The phenomenon of haemadsorption has been described above (Section F(a)). It may be, that the haemadsorption seen prior to the differentiation of the cell membrane (60) is due to a physiological change in the cell membrane akin to that induced in red blood cells prior to haemolysis (78), or that it is associated with the processes described below (Sections G, II(c) & (d)). In the case of cells infected with NDV, haemadsorption can be observed five hours after infection and increases in a linear relationship with viral synthesis up to eleven hours after infection (15).

ii) Fusion.

Fusion from within leads to syncytial formation which can be seen from eight hours after infection (94). As described previously, (Section F, (h)), the phenomenon of cell fusion, although dependent on the presence of viral proteins, is due to a change in the character of the cell and is possibly mediated by lysozymes released from destroyed lysosomes (107). Alternatively, the fusion effect may be related to the closely associated phenomenon of haemolysis (172).

II b) The destruction of cellular lysosomes, associated with the infection of cells with NDV.

Lysosomes are cellular sub-units, containing lysozyme, an enzyme which is released when the lysosome is ruptured. Lysozyme hastens the process of autolysis which occurs on

on the death of a cell. Although lysozyme has been shown to have been released in the course of infection of cells with NDV (95), there is no evidence that this is the means by which a velogenic strain of NDV causes the death of an infected cell.

II c) Agglutination of cells that have been infected with NDV following treatment of the cells with lectins.

Following infection of cells with virulent strains of NDV, an alteration (which requires the synthesis of proteins induced by NDV) takes place in the cell membrane. This change results in the agglutination of the infected cells when they are treated with a lectin and is accompanied by a decrease in the thickness of the cell coat material (96). Agglutination by lectins is normally associated with cellular transformation (173).

II d) Thinning of the cell membrane following infection of cells with NDV.

Thinning of the cell membrane has been reported to occur following infection of cells with several species of virus, including NDV.

Poste (107) has postulated that the effect is due to the release of lysozyme, consequent on the destruction of lysosomes by the infecting virus. He further proposed that the thinning of the cell membrane would facilitate fusion from within. However, if thinning of the cell membrane is due to an enzyme activity, it is possible that the enzyme concerned is associated with the viral envelope proteins that infiltrate the cell membrane prior

prior to the process of fusion from within (72).

Further evidence of damage to the cell membrane has been provided by Katzman and Wilson (97). Lactic dehydrogenase (LDH), which is synthesised within normal cells is found to leak across the plasmalemma, following the formation of the NDV glycoproteins and their incorporation into the cell membrane.

III) CELL DEATH AND THE FORMATION OF PLAQUES IN TISSUE CULTURE MONOLAYERS

Autolysis (95), damage to the cell membrane (96, 97) and the inhibition of cellular RNA and protein synthesis (80), all of which may follow infection of a cell by NDV, will result in cell death. Synthesis of both viral protein and viral RNA is apparently necessary for the death of the cell to occur.

When low doses of NDV are used to infect a confluent cell monolayer, areas that are free of cells are seen 24 to 48 hours after infection. The plaque size is larger when velogenic strains of NDV are used and is smaller with lentogenic strains, providing the assay is carried out in chick embryo cell monolayers (106).

IV) INDUCTION OF INTERFERON AND THE RELATIONSHIP OF INTERFERON TO OTHER VIRAL MECHANISMS

Viral reproduction can be inhibited or prevented in several ways and will be discussed under the headings "intrinsic interference", "homologous interference" and "interferon mediated interference".

a) Intrinsic interference: Often two unrelated species of virus can be shown to replicate within the same cell; thus simultaneous infection by fowlpox virus and herpes simplex virus has been demonstrated in chick embryo cells (63). Intrinsic interference, which functions independently of interferon, is due to the synthesis of a short-lived protein induced by the initial virus infection, which prevents the second virus from replicating within that cell. Thus, human fibroblast cells which have been infected with cytomegalovirus will not support replication by a subsequent infection of NDV (65).

b) Homologous interference (that is interference between virus populations of the same species), can be due to several different interfering mechanisms:

i) Interferon (see below), may be induced by the initial infection .

ii) A large infecting dose of NDV will remove all the available adsorption sites and so prevent a secondary infection by NDV (100).

iii) Portner and Kingsbury (153) showed that Sendai virus with incomplete RNA was capable of infecting cells and undergoing limited replication without releasing progeny virus. However, while the cells were still susceptible

susceptible to infection by NDV, they were refractory to infection by Sendai virus, although both Sendai virus and NDV are paramyxoviruses. When the incomplete Sendai virus was inactivated by ultra-violet light, superinfection with normal Sendai virus was possible. The progeny virions contained both complete and incomplete RNA. Thus, homologous interference may be due to competition for a virus specific enzyme necessary for the synthesis of viral protein.

Interferon is produced by the host cell in response to viral infection, as well as a variety of other inducing substances and organisms. It is now clear that interferon induction is due to the presence in the cell of a foreign double stranded nucleic acid and that RNA is a better inducer than DNA (138). However, Shu (98) has suggested that lipid may also be involved.

In brief, the induction of interferon synthesis is believed to take the following course. The infecting virus enters the host cell and proceeds to the replicative intermediate stage which involves a double stranded RNA complex (99). Moreover, it has been shown that viral RNA polymerase activity is necessary for induction of interferon following infection by active or inactivated NDV (145). An inducer protein produced by the host cell is stimulated by the presence of double stranded RNA and this protein derepresses the host cell DNA sequence involved in the translation of interferon messenger RNA, allowing the synthesis of interferon to proceed.

proceed.

Interferon is believed to prevent viral replication by promoting the synthesis of a Translation Inhibition Protein (TIP), which in turn derepresses the appropriate host cell DNA cistron. Interferon acts in this way on any cells of the same species. Thus, TIP is induced in neighbouring cells to those in which the interferon has been produced, and in cultures which have been overlaid with the nutrient medium from monolayers producing interferon (127). TIP attaches to host cell ribosomes and prevents foreign messenger RNA combining with the ribosomes.

The species-specific nature of interferon may be related to its ability to derepress DNA translation. It is possible that the recognition of foreign messenger RNA by a ribosome that has been activated by TIP is graded. By this hypothesis, host cell messenger RNA is recognised, other cellular messenger RNAs are partially recognised while some virus messenger RNAs 'appear more foreign' than others. Thus some viruses will be more susceptible to interferon than others (147, 165).

NDV is a good inducer of interferon but it has been shown that tolerance to interferon induction occurs in repeatedly infected cells (104). There are a number of reports that certain viruses may prevent the synthesis of interferon when infecting one cell line, but may have no effect on others. In this way infection of calf kidney cells with parainfluenza 3 virus prevents the

the induction of interferon by U-V inactivated NDV and so allows subsequent infection of these cells by active NDV to take place (132). Some types of persistent infection produced by NDV have also shown to be maintained by interferon (see Section I(b)) (118).

The chemical structure of interferon has not yet been fully elucidated; it is believed to vary considerably from species to species and molecular weights ranging from 12000 to 110000 daltons have been proposed (166). Interferon produced by any species of cell is highly resistant to low pH and cannot be pelleted by centrifugation at 200,000 g.

V) MISCELLANEOUS EFFECTS

a) Transformation of cells.

Transformation is a feature of neoplastic cells. Normal cells are prevented from overlying each other when growing *in vitro* on glass, and so form a monolayer. This is due to the process of contact inhibition. Contact inhibition probably depends on the emission and reception of chemical signals from neighbouring cells. Dulbecco states that, in normal cells, receptors on the cell surface register overcrowding of cells or exhaustion of nutrients in the media and "switch off" cell growth but in transformed cells the "switch" is stuck in the "on" position (174).

In a normal cell, the cell growth "switch" is only "on" during mitosis and hence, only during mitosis will a cell show the lectin agglutination effect. Transformed cells are agglutinable by lectins at all times (173), as they are in a perpetual state of incipient mitosis. Thus the features of a normal cell in mitosis are present at all times in transformed cells. Transformation may be viewed as a gradient in which the final stage is the loss of contact inhibition. Most continuous cell lines have undergone the initial step of transformation in that their life is infinite. Associated with the infinite life of a continuous cell line, are changes in the chromosomes, such as heteroploidy and the loss of sex chromatin, as well as the change to an epithelioid morphology. Loss of contact inhibition may be induced in continuous cell lines (and also in totally untransformed

untransformed primary tissue culture cells) by various carcinogenic agents including oncogenic viruses (101, 102). Loss of contact inhibition (and hence the occurrence of transformation) is shown by the piling up of cells grown in monolayers and the formation of 'colonies' in semi-solid agar. (Colony formation is due to the cells growing both vertically and horizontally in close proximity.)

MacPherson and Montagnier (102) demonstrated this latter property of transformed cells in certain clones of BHK cells, which had been infected with polyoma virus. Cellular transformation has not been reported following paramyxovirus infection, although incorporation of viral genetic material into host cell genomes (in a similar manner to that found following leukovirus (oncornavirus) infection) has been observed in persistently infected cells (45, 150). Furthermore, chromosomal defects have been detected within 24 hours of infection of cells by Sendai virus (103); significantly, in this case, the Sendai virus infection is persistent in these cells. Moreover, a change in the cell surface constitution following NDV infection, but before budding of viral particles occurs, results in the infected cells agglutinating in the presence of lectins (96).

b) Formation of inclusion bodies.

Inclusion bodies containing viral material are detectable by both light and electron microscopy. By staining with haematoxylin and eosin (89, 90) or with Giemsa (91, 141), acidophilic inclusions can be seen

seen which correspond to inclusions detected by phase contrast (89, 90). Using the fluorescent antibody staining technique, two types of inclusions have been found; those due to VP 55 (nucleoprotein) and those due to VGP 75 (haemagglutinin). The VGP 75 inclusions are scattered through the cytoplasm of the cells, while those due to VP 55 are perinuclear or even nuclear (92). The nucleoprotein inclusions can be stained with acridine orange for their RNA content (141), and nucleocapsid material is seen in infected cells when they are examined by electron microscopy (141, 53). It is probable that other viral proteins may also be found within these inclusions.

Inclusion body formation is best demonstrated in host cells which permit prolonged infection by NDV (90, 91).

H / STRAIN DIFFERENCES

Differences between strains of NDV are observed when the following characteristics are studied.

a) Virulence

b) Serology

c) Morphology

d) Structural proteins

e) Replication

f) Biological activities

- i) haemagglutinin
- ii) neuraminidase
- iii) induction of cell fusion
- iv) haemolysin

g) The effects of NDV on infected cells

- i) inhibition of cellular RNA and protein synthesis
- ii) haemadsorption
- iii) induction of cell fusion
- iv) destruction of cell lysosomes
- v) alteration of cell membrane physiology
- vi) mortality of chick cells infected by NDV
- vii) induction of interferon
- viii) cytopathic effect of NDV in mammalian cells.

a) Virulence

Many strains of NDV have been isolated which differ, not only in their degree of virulence for chickens, but also in many of their other properties. For example, there is a close relationship between some properties of NDV strains grown in chickens or chick cells and the virulence of NDV strains for chickens. However, other features of NDV activity that vary with the strain are not related to its virulence for chickens. The virulence of a strain of NDV for other host systems bears no relationship to its virulence for chickens, chick embryos or chick embryo cells in vitro. Moreover, the characteristics of strains that are virulent to other host systems, are not necessarily the characteristics associated with strains of NDV that are virulent to chicken-based systems.

Virulence for chickens has been related to the following effects and activities of NDV.

i) cytopathic effect (CPE) on the developing chick embryo (105)

ii) replication rate in the developing chick embryo (113)

iii) cytopathic effect on chick embryo fibroblast monolayers (106)

iv) lysosome damage (107)

v) neuraminidase activity of virus grown in embryonated hens' eggs (108)

vi) cell-associated neuraminidase in the chorio-allantoic membrane of developing chicken embryos (112)

vii) haemadsorption in infected chick embryo fibroblasts (113)

viii) presence of temperature stability in both infectivity and haemagglutinin (35)

ix) agglutination of infected BHK cells by lectins (96)

x) interferon induction (99)

xi) inhibition of antibody-complement haemolysis (172)

Virulence is often associated with the following characteristics of NDV, but some workers dispute this.

xii) induction of the formation of syncytia (90, 74) and

xiii) inhibition of cellular protein and RNA synthesis (80, 81)

It will be noticed that most of these characteristics (i, ii, iii, x, xii) are the effects rather than the causes of virulence. Waterson (152) has proposed that virulence may be associated with differences in the synthesis of non-structural proteins, which may themselves be concerned with lysosome damage, and the alteration in the cell membrane demonstrated by the characteristics (vi), (vii) and (ix) above. These factors and the inhibition of cellular synthesis of RNA and protein (xiii) may lead to cell destruction. Other features such as (vi) and (viii) are generally associated with a virulent strain of NDV but are probably not directly concerned in the expression of virulence.

b) <u>Serology</u>

Although species of paramyxovirus are distinguished

distinguished from one another by the specificity of antibody to their haemagglutinin or nucleocapsid, no variation between strains of NDV has been found in either of these respects or in the neutralisation of infectivity by antibody (109).

c) Morphology

The pleomorphism of NDV makes comparison difficult, but there appears to be no variation in range of either size or shape between strains of NDV (110).

d) Structural proteins

Moore and Burke (21) demonstrated that the polypeptides of all strains of NDV examined were of similar molecular weight but that under non-reduced conditions some strains showed the formation of a polymer of two (or more) of the proteins, while others did not. Analysis of the amino acid composition of the three major polypeptides showed that this also varied with the strain examined. No connection between structural protein variation and virulence was found.

e) Replication

i) Infectivity.

Lomniczi (35) has shown that the temperature stability of NDV infectivity varies between strains, as does the temperature stability of haemagglutinin.

In the strains so far examined, no velogenic or mesogenic strain has been found to be temperature labile for both infectivity and haemagglutinin. Moreover, although it may not necessarily be temperature

temperature-stable for both infectivity and haemagglutinin, a virulent strain must be temperature-stable for one of these factors. On the other hand, lentogenic strains may be temperature-stable for one or both of these factors but may also be temperature-labile for both infectivity and haemagglutinin.

ii) Replication rate.

Virulent strains of NDV replicate rapidly in chick embryos, with the accumulation of cell-associated antigen, while lentogenic strains replicate more slowly, cause less cell death and less accumulation of viral antigen in infected cells (113).

iii) Non-structural protein formation.

Insufficient work has been done on the rate of synthesis and the quantity of non-structural proteins synthesized in different strains of NDV.

iv) Appearance of budding virus.

When cells which have been infected with strains of NDV that vary widely in pathogenicity, are examined under the electron microscope, no differences can be seen in the morphology of budding virus of any strain (151).

f) Biological activities.

i) Haemagglutinin.

As mentioned above, there is variation between strains in haemagglutinin temperature stability (111), which may be associated with virulence if considered with the temperature stability of infectivity (35) but not, if considered as haemagglutinin per infectious virus

virus unit (111).

ii) Neuraminidase.

A high neuraminidase activity per microgram of purified virus has been associated with virulence by McNulty et al. (108), as has a high neuraminidase activity per cell in chick embryo chorio-allantoic membrane (112). However, Alexander et al. (112) have previously reported (in 1970) that strain variation in the neuraminidase activity of purified NDV does not occur and is not related to the virulence of the strain.

iii) Induction of cell fusion.

The ability of NDV to induce cell fusion varies between strains (111, 47). 'Fusion from within', in chick fibroblast monolayers, has been associated with virulence by Poste et al (47) but not by Fuchs and Kohn (111).

iv) Haemolysin.

The ability of a strain of NDV to cause haemolysis varies with the strain but is unrelated to virulence (111), nor is it related to the ability of that strain to induce cell fusion (111).

Another phenomenon related to haemolysin has been described (172), and is associated only with Sendai virus or lentogenic strains of NDV that have been grown in embryonated chicken eggs. When a suspension of red blood cells is treated with an amount of virus insufficient to lyse all the erythrocytes, further haemolysis occurs if the mixture is then incubated in

in the presence of complement and antibody to either the virus, chicken chorio-allantoic membrane or the red blood cells. Nevertheless, this effect does not take place with virulent strains of NDV grown in fertile hens' eggs or with avirulent strains cultured in duck eggs. Since haemolysis is consequent on the fusion of virions with the erythrocyte membrane, this result suggests that avirulent strains incorporate more host cell antigen than virulent strains. In addition, Apostolov (172) has shown that the Forssman hapten (a glycolipid which is not found in duck eggs) is probably the essential antigenic component

g) The effects of NDV on infected cells.

i) Inhibition of cellular RNA and protein synthesis.

In chick embryo fibroblasts, strain variation has been observed in the degree of inhibition of cellular synthesis of RNA and protein, when these cells are infected by NDV (80, 81). Reeve (81) found this variation to be related to virulence while Moore did not (80).

ii) Haemadsorption.

Alexander (113) has shown that in chick embryo fibroblasts, virulent strains induce haemadsorption faster than lentogenic strains and cause the adsorption of a greater number of red blood cells per infected cell.

iii) Induction of cell fusion from within.

Fusion from within, in chick embryo fibroblasts, has been described above. In other host cells, variation

variation also occurs (90) and is unrelated to the virulence of the infecting strain but may be related to the composition of the cell membrane (74, 107).

iv) Destruction of cell lysosomes.

Lysosome destruction has been related to the virulence of the infecting strain of NDV (107), (pp. 29 and 30. Section G (II) (b) & (d)) and thus may be one of the immediate causes of cell death following infection by a velogenic strain.

v) Alteration of cell membrane physiology.

When cells are infected by avirulent strains of NDV, the composition of the cell membranes is unaltered and agglutination of infected cells by lectins does not occur in either chick fibroblasts or BHK cells (96).

vi) Mortality of chick cells infected by NDV.

Cell death in chick embryos and chick embryo fibroblasts varies between strains and is closely associated with the virulence of NDV. Intracerebral pathogenicity (ICP) in one-day-old chick embryos is used as the index of virulence (111). Embryonic death, following inoculation of virus into the allantoic cavity of 10-day-old chick embryos, is closely related to ICP (105), as are the size and the number of plaques formed on chick embryo fibroblast monolayers infected with NDV (106).

(vii) Induction of interferon production.

Interferon production and pyrexia during NDV infection have been ascribed to the presence of an

an inducing lipoprotein (98). The formation of interferon is less in chicken cells during infection by a lentogenic strain of NDV (99).

Lomniczi (176) confirmed these observations in chick embryos but his in vitro experiments suggested that the ability of a strain of NDV to induce interferon production was not related to its virulence. He postulated that the greater quantity of interferon produced from chick embryos, infected by virulent strains of NDV, is due to the rapid cell death which occurs. By this hypothesis, two distinct proteins are produced by NDV in infected cells. The first is formed mainly by virulent strains and causes cell death, possibly by inhibiting the synthesis of cellular proteins. The second is produced by all strains of NDV, and inhibits the production of interferon in the infected cell. When cell death is rapid, the effect of this second protein is slight. However, when infection by a lentogenic strain occurs, viral proteins may be synthesised for up to 72 hours and interferon production is inhibited.

viii) Cytopathic effect of NDV in mammalian cells.

Brandt (89, 90) and Butler (91) showed that there is considerable variation in cytopathic effect (inclusions, syncytial formation and cell death) between several NDV strains. This variability is also dependent on the type of host cell that is infected.

I / PERSISTENT INFECTION

a) Classification.

Viruses of many different families are capable of initiating and establishing persistent infections in a variety of continuous cell cultures.

In the 'normal' infection of an animal or human patient by a virus, replication of the virus occurs in the host cells and generally concludes with the destruction of the infected cells and the release of infectious progeny.

In certain virus infections, such as Visna of sheep, development of the pathological signs of infection may be gradual and may take place over a time-scale measured in years rather than days. The infecting virus may be termed 'slow' in character. In other virus infections, such as rabies, development of pathological illness may not take place for months or, as in the case of sub-acute sclerosing panencephalitis (SSPE) caused by measles virus, for several years after infection. The pathological effect of the virus in this latter case may be described as 'latent'.

Oncogenic viruses, such as that causing lymphatic leukaemia in cats, may be both 'slow' and 'latent' in development, while the consequence of infection of the host cells by these viruses is transformation rather than cell death.

In all these instances of '*in vivo*' infection, the virus is 'persistent' for long periods in the host,

host, often without replicating itself and killing very few host cells.

Apart from persistent infections, *in vitro* infection of cell monolayers by a virus may deviate from the 'normal' in several other ways; the mechanisms of which may also play a part in the development of a persistent infection, as will be described below.

A viral infection may be described as abortive, if the replicative cycle of the virus is incomplete and thus little or no infectious virus is released. There are many causes of abortive infection, varying from an insusceptible host cell system to the non-synthesis of an essential viral protein.

An incomplete virus particle may be released as the consequence of an abortive infection or it may be the result of interference between virus particles following a high multiplicity of infection. Incomplete virus may also be released as the result of damage to the genetic material of a virus, by such factors as ultra-violet radiation. The released virus particle may be defective, in that its nucleic acid is incomplete; and thus, although the virus may still be infective, normal virions will not be released from cells infected by incomplete virus.

Latent viral infection of bacterial cells occurs when the infecting virus enters a prophage stage in which the viral genome is carried within the bacterial DNA.

Oncogenic viruses cause transformation of infected

infected cells *in vitro* and a DNA replicate of the viral genome is incorporated into the cell chromosomes.

In a persistent infection, synthesis of virus or viral components continues without affecting the survival of the host cell system. The relationship established can vary from a latent bacteriophage infection (in which the virus genome is incorporated into that of the bacterium) to an antibody controlled infection (in which the majority of cells are protected by antibody, while a minority are infected and destroyed by the fully virulent virus). An alteration of the environment, such as incubation of the infected cells at a different temperature, can affect the equilibrium between the cell and the virus, resulting in either cellular or viral death.

Several features of '*in vitro*' persistent infections may be related to the 'slow' and 'latent' *in vivo* infections described above (152). While measles virus normally causes an acute, epitheliotropic disease *in vivo*, *in vitro* infections are characterised by typical paramyxovirus cytopathology and cell death. However, SSPE occurs as a latent infection *in vivo* and persistent infections are relatively easily induced *in vitro*. Moreover, DNA transcriptions of the measles virus RNA genome have been found in persistently infected cells (150), suggesting that the virus genome may be incorporated in the cellular DNA. The presence of an RNA-dependent DNA transcriptase has been reported in several viruses with an RNA genome which cause slow

slow, latent or oncogenic infections *in vivo* such as avian leukosis virus and Visna virus (122, 123); as well as in NDV associated with persistent infections of cell cultures (45).

In 1964, Walker (115) attempted to classify persistent *in vitro* infections into four classes, as follows:-

Class A: The majority of cells are genetically resistant but the susceptible minority support replication of the virus and are destroyed. The released virus infects the susceptible minority of daughter cells and may be prevented from doing so, by the addition of homologous antiserum, by which means the infection may be cured (116).

Class B: All the infected cells are susceptible but, as most of the released virus is neutralised by antiserum which is always present in the medium, only a small proportion of the cells are infected. Alteration of the antibody concentration will either cure the culture of the viral infection or permit its destruction by virus that has not been neutralised (117).

Class C: The majority of cells are not infected; and are protected from virus released from the infected minority of cells by the production of endogenous interferon. The virus that is produced is a mutated form and the infection may be cured by prolonged treatment with antiserum (118).

Class D: The majority of cells are infected but are not necessarily capable of producing infectious virus.

virus. The virus produced is often of reduced infectivity. Interferon is not detectable and the cells may not be cured by treatment with antiserum (119).

Persistent infections may involve features of all these classes (115).

In both class C and D infections, there is mutation of the virus, as well as inhibition of the virus by the host cell. One expression of the viral mutation is the development of temperature-sensitivity, which has been found in many persistent infections.

Two major forms of temperature-sensitivity can be described.

i) The virus is unable to manufacture certain viral proteins at the non-permissive temperature. This may result in the failure of the virus to be released (67, 121) or the release of virus that is not infectious (128, 129, 146).

ii) There is a decrease in the production of viral RNA at the non-permissive temperature, which leads to a diminished yield of infectious virus (124).

b) Persistent infections in paramyxoviruses.

Amongst reports of persistent infections, those featuring paramyxoviruses are prominent.

Wilcox (130) and Henle (131) described persistent infections of NDV in L cells. Only small number of the cells were infected and Henle (118) and Thacore (120) showed that this form of persistence was related to interferon induction and production. Similar systems have been described involving NDV infection

infection of calf kidney cells (132), parainfluenza 3 virus infection of KB cells (133) and measles virus infection of chick embryo fibroblast cells (150). These infections correspond to Walker's class 'C' (115).

Colobert and others (140, 141) investigated a persistent infection of Sendai virus in KB cells, in which the majority of cells were infected but very little infectious virus was released. Interferon was not involved in this system. When the cells were treated with trypsin there was an increase in the yield of infectious virus.

The types of persistent infection, described by Colobert (141) and Walker (128), probably correspond to Class D or 'regulated' persistent infection as described by Walker (115) and have been shown to be unrelated to cellular interferon activity.

Investigation of persistent infections maintained due to the presence of interferon, has shown the following characteristics to be present.

Interferon causes an abortive infection by inhibiting the formation of 18S RNA (137). Infection of L cells by wild-type NDV is also abortive but is unrelated to the above effect, as normal quantities of viral messenger (18S) RNA are formed, viral synthesis is normal and the abortive defect occurs just prior to virus release (49). A further effect of interferon may be the inhibition of virus specific RNA polymerase at non-permissive temperatures (123) and the resulting reduction in the release of infectious virus at these

these temperatures (136). Temperature-sensitivity was found to be a prerequisite of persistence (124). However, temperature-sensitive mutants derived from alternative sources, did not share all the other characteristics of virus released from persistent infections. The virus produced from persistent infections was of reduced virulence in chick embryos and caused smaller plaques in chick embryo fibroblasts (120). The virus was more infectious for L cells and, in contrast to wild-type NDV, infectious virus was released from L cells (135). However, neuraminidase and haemagglutinin activity were less stable that that of wild-type virus. Following serial passage in chick embryos, the characteristics of virus obtained from persistently infected L cells largely reverted to that of the wild-type virus (134). A virus - specific RNA-dependent DNA polymerase has been reported from this type of persistent infection (45) by NDV, and virus-specific DNA sequences in the host cell DNA have been shown to anneal to viral RNA in a persistent infection due to measles virus (150).

Thus, persistence in this instance, appears to be dependent on the evolution of a temperature-sensitive RNA polymerase and an 18S RNA that is susceptible to interferon but the virus, unlike wild-type virus, is released from and is fully infectious for L cells.

Reversion of the virus towards the wild-type occurs on passage through the natural host. Although the infection may be cured by prolonged treatment with

with antiserum (118), there is evidence that viral genetic material may be incorporated in the DNA of the host cells (45).

The small quantities of virus released during persistent infections of MDBK, HeLa and L cells by Sendai virus, were shown to be related to inhibition of cleavage of the non-active precursor of the small glycoprotein of the viral envelope (11, 42). Treatment with trypsin increased the release of infectious virus (141, 56), as well as restoring normal haemolysin (114), fusion (139) and infectivity (56) activities to the virus, by splitting the precursor protein. Fusion with monkey kidney cells also produced infectious virus (143, 144). Measles virus in HeLa (148) and BHK cells (122) is also released by co-cultivation with other cell lines. There are no reports of 'trypsin dependent' persistent infections that are temperature-sensitive; although measles virus (122) and Sendai virus (125) infections in BHK cells do exhibit temperature-sensitivity and appear to be closely related to 'trypsin dependent' persistent infections. A temperature-sensitive effect was also noted in the mumps virus infection of C-M cells (146). In this case, virus production was found to be independent of cellular metabolism, and an increase in infectious virus resulted from treatment of the cells with Actinomycin D (126). Actinomycin D inhibits the translation of RNA from DNA and so inhibits cellular but not paramyxovirus RNA synthesis.

synthesis.

A comparison of virus-specific RNA formed during infection of HeLa cells by persistent measles virus and by 'wild-type' measles virus (148), showed no qualitative difference in the species of RNA that were produced, but there was a diminuition (149) in the quantity of low molecular weight RNA similar to that found in persistent infections of NDV in L cells, where the effect has been shown to be mediated by interferon. Abortive infection of L cells by Sendai virus, gave rise to two forms of viral ribonucleoprotein, both of which contained normal RNA but neither of which was capable of being incorporated into infectious virus (61).

Measles virus, which was released at the permissive temperature from BHK cells (122), was found to be less infectious than that released during co-cultivation. However, on further passage, the virus became less temperature-sensitive and the virus released at the original permissive temperature was as virulent as that released during co-cultivation. The incorporation of haemagglutinin into the cell membrane of BHK cells infected by Sendai virus, was found to be a temperature-sensitive step (125). Portner (121) demonstrated that abortive infections of chick embryo lung cells by Sendai virus exhibited this form of temperature-sensitivity, while in other cases, RNA synthesis was found to be temperature-sensitive.

Class D "regulated" infections as described by

by Walker (115), are varied in their characteristics and possible pathogenesis but the following conclusions may be drawn.

i) The majority of cells are infected (125, 128, 140, 142, 143).

ii) Interferon plays no part in the maintenance of the infection (128, 141, 143, 144).

iii) In some cases, there is a clear relationship to abortive infections (11, 142), involving the non-cleavage of a trypsin-sensitive precursor to the small viral glycoprotein. This factor may be related to the structure of the host plasmalemma (74, 49).

iv) In other cases, a temperature-sensitive mutation has been related to persistence (121, 122, 125, 146); at the non-permissive temperature, the incorporation of active haemagglutinin into the cell membrane is limited (125) or viral synthesis is inhibited (149). Similar temperature sensitive phenomena have been found in abortive infections (121, 61).

v) In both of the above (iii and iv) co-cultivation with permissive cells increases the amount of virus released (122, 144, 148).

vi) In one case, the effect of inhibition of cellular protein and RNA synthesis was to increase the yield of infectious virus (126). This suggests that both in this instance, and in the examples of the other 'regulated' infections discussed

discussed above, inhibition of one or more steps in virus replication may occur due to the action of a cellular product that is not related to interferon.

The relationship of the virulence of a strain of virus to its pathogenic effect during and after a persistent infection is not clear. Although the temperature-sensitive mutants isolated from L cells that are persistently infected by NDV are less pathogenic to chick embryos, restoration of virulence occurred following passage through chick embryos (134). Similarly, the trypsin-sensitive Sendai virus released from HeLa or L cells regained normal infectivity on treatment with trypsin or passage through chick embryos (142). Thus it appears that any diminuition in virulence is 'phenotypic' rather than 'genotypic' when paramyxoviruses are involved is persistent infections.

c) Persistent infections with NDV in ox kidney (BKpi), pig kidney (PKpi) and sheep kidney (OKpi).

The present work is a continuation of the investigations by Edwards (93) into persistent infections by NDV of continuous lines of ox, pig and sheep kidney cells, which took place from 1967 to 1971.

Three cell lines were brought into this laboratory in 1959 and at that time were presumed to be healthy, but three years later, all three cell lines

lines were shown to be persistently infected by NDV. The origin of the infection is unknown and the cultures have been maintained routinely since that time, without obvious signs of cell destruction.

The three persistently infected cell lines that were studied by Edwards were:

(1) A bovine kidney cell culture, originally MDBK (155); it is now designated BK pi and the virus obtained from it, is described as NDV-BK pi;

(2) an ovine kidney culture, originally MDOK (155); it is now designated OK pi and the virus obtained from it, is described as NDV-OK pi;

(3) a pig kidney culture, originally the PK 2a Stice line (156) and now referred to as PK pi and the virus produced from it, is denominated NDV-PK pi.

In summary, these cell lines present the following features.

i) Antibody is not necessary for the persistence of infection nor are the cultures cured by antiserum prepared against NDV.

ii) There is resistance to superinfection by NDV but not to other related or unrelated strains of viruses.

iii) The majority of cells are infected and there is a close correlation between the number of cells containing inclusions following staining with eosin, acridine orange or fluorescent antibody. Viral structures can also be detected by electron microscopy. Cell growth is relatively unaffected (as compared with that

that of controls), although there may be some enhancement.

iv) The persistently infected cells show colony formation in semi-solid agar - unlike the uninfected control cells - which is an indication of possible cellular transformation.

v) Haemagglutinin incorporation into the cell membrane is temperature-sensitive and thus at the non-permissive temperature of 41°C, haemadsorption disappears. However, even after three months at this temperature, haemadsorption re-appears when the temperature of incubation is returned to 37°C.

vi) Release of infectious virus was not observed at this time. Co-cultivation, growth in serum-free medium, ultraviolet irradiation, treatment of released virus with neuraminidase, superinfection of persistently infected cells with unrelated viruses and concentration of the virus by passage through Sephadex G-200 columns, were all unsuccessful in demonstrating infectious virus that produced pathogenic effects in chick embryo fibroblast monolayers or in embryonated hens' eggs or one-day-old chicks.

vii) Virus particles are seen by electron microscopy both as mature virions budding from persistently infected cells, and also, in concentrations of exhaust media from the carrier cell monolayers. Haemagglutinin is associated with these particles but the titres are lower than those obtained from wild-type NDV infections. However, no neuraminidase activity is detectable and the haemagglutinin released from BK pi cells is less heat stable than that of Herts strain NDV that has

has been grown in the allantoic cavity of 10-day-old embryonated hens' eggs.

viii) When the persistently infected cells are co-cultivated with uninfected chick embryo fibroblast cells, fusion occurs. Heterokaryons stained with acridine orange show intranuclear and cytoplasmic inclusions, similar to those found in paramyxovirus infections. However, viral antigen was not detectable in the nuclei by staining with fluorescent antibody and the release of infectious virus did not occur following co-cultivation of the carrier cells with either chick embryo fibroblasts or chick macrophages.

ix) There is a positive correlation between the number of cells showing haemadsorption and inclusions stained by fluorescent antibody, in each of the persistently infected cell lines, but the proportion (30-60%) of cells that are infected in PK pi is lower than that in BK pi or OK pi (80-85%). The haemagglutinin titres of the exhaust media are generally higher in PK pi and BK pi than in OK pi. Titres above 32 HAU per millilitre are not found in the exhaust medium of any of the persistently infected cell lines.

x) The proportion of infected cells in PK pi (and to a lesser extent BK pi and OK pi) increased over the five years period up to 1971 - in the case of PK pi rising from 30% to 60%. In this connection it may be relevant that instability in a persistent infection has been reported by Haspel (122) but not by Thacore (120) and

and Henle (118).

The systems of persistent infection, as described by Edwards (93), correspond in many ways to those classified as 'regulated' - (Class D) - by Walker (115).

MATERIALS AND METHODS

MATERIALS AND METHODS

A / REAGENTS

I) RADIOACTIVE MATERIAL

The following chemicals were obtained from the Radiochemical Centre, Amersham, Buckinghamshire:-

($5,6 - {}^{3}H$) - uridine, 40 Ci/mMol

($U - {}^{14}C$) - protein hydrolysate, 58 mCi/mAtom carbon

(${}^{3}H$) - amino acid mixture, same proportion by radioactivity as ($U - {}^{14}C$) protein hydrolysate.

II) BUFFERS

Dulbecco's phosphate buffered saline (PBS) pH 7.2 was purchased as PBSA from Oxoid, London.

Phosphate buffer pH 7.2 (PB) 0.01M sodium dihydrogen phosphate brought to pH 7.2 with 1.0M sodium hydroxide.

Saline Trypsin Versene (STV) 0.01% trypsin (1:250 trypsin, Difco, Detroit, U.S.A.)
0.01% versene (ethylene diaminetetra-acetic acid) in PBS

TKB
0.05 M tris-HCl
0.05 M potassium chloride
0.0015 M magnesium chloride
in water, pH 7.4

TRB
0.15 M sodium chloride
10mM sodium citrate
25mM sodium phosphate
0.1% triton X-100
in water brought to pH5.5 with
0.1 M sodium hydroxide

III) TISSUE CULTURE MEDIA

(a) Minimum essential medium (MEM), Eagle's/Dulbecco Glasgow modification.

(b) Basal medium, Eagle's (BME), both were purchased as 10 x concentrates from Gibco-Biocult, Paisley, Renfrewshire.

(c) Calf serum, pooled, sterilised by positive filtration through a membrane (APD 0.2 μm) and inactivated by heat at 56°C for 30 minutes.

IV) OTHER REAGENTS

Nuclear Chicago Solubiliser (NCS) was purchased from Hopkin and Williams, Romford.

Folin and Ciocalteu's phenol reagent was purchased from BDH, Poole, Dorset.

Schiff's Reagents (208) 0.8% potassium metabisulphite
1.14% 12N hydrochloric acid
0.4% basic fuchsin
in water
filtered after treatment with activated charcoal.

V) ERYTHROCYTES

Fowl red blood cells were obtained by venipuncture from healthy, unvaccinated adult hens and were treated with sterile 3.8% sodium citrate to prevent coagulation. After three washes with PBS, stock suspensions of one and two per cent were made up in PBS.

Guinea-pig erythrocytes were obtained by heart puncture of anaesthetised, healthy animals and were

were treated in the same manner as fowl red blood cells. Stock suspensions were made of 0.4% erythrocytes in PBS and stored for 3-4 days at 4°C.

VI) WATER

Deionized water was used in the preparation of all materials other than tissue culture media, for which autoclaved double deionised water was employed.

All other chemicals were normal laboratory reagents of the 'Analar' grade, unless otherwise specified.

B / VIRUS TECHNIQUES

I) STRAINS OF VIRUS EMPLOYED

Six 'wild-type' strains of NDV were kindly supplied by Dr. J.B. McFerran, Veterinary Research Laboratory, Stormont, Belfast. Three of these, Herts 33, Italien and Lurgan, are velogenic having mean death times (MDT, measured in embryonated chicken eggs) of around 50 hours and intracerebral pathogenicity indices (ICP, estimated in one-day-old chicks) of approximately 1.9 (109, 112). On the other hand, the remaining three strains are lentogenic and their MDT and ICP are as follows (109, 112):

STRAIN	MDT (Hours)	ICP
B1	120	0.025
F	168	0.025
Ulster	∞	0.00

Three carrier cell-lines maintained in this laboratory (See Introduction) produce defective NDV (93) and virus was collected from the supernatant fluid of the persistently infected monolayers.

M6 virus, which is believed to be a strain of mucosal disease virus (162) was kindly provided by Mr. R. Huck, Central Veterinary Laboratories, Weybridge.

II) GROWTH OF VIRUS

Observing aseptic precautions, 10-day-old fertile hens' eggs were inoculated by the allantoic route with 0.2 ml of a suspension of NDV, containing eight haemagglutinating units (HAU) per ml. After incubation for two (in the case of virulent strains) or occasionally

occasionally three days (in the case of avirulent strains) at 37°C, the eggs were chilled at 4°C for two hours and the allantoic fluid was harvested. The virus was stored overnight at 4°C or, for periods of up to one year, at -70°C.

All the strains of NDV and M6 virus, were also grown in primary and continuous cell-lines, as described in Section C III.

C / TECHNIQUES RELATED TO THE CULTURE OF HEALTHY AND VIRUS-INFECTED CELLS

I) CELL-LINES EMPLOYED

a) Primary Cell culture.

Chick embryo fibroblasts (CF) were prepared from 10-day-old chicken embryos. Using aseptic techniques, the head, limbs and viscera were removed and the remaining tissue rinsed in PBS. The tissue was coarsely chopped and placed in 0.25% trypsin (1:250 Trypsin, Difco.) in PBS for 10 minutes at 37°C. The digest was shaken every three minutes. After allowing the undigested tissue to settle, the supernatant was removed by pipette and fresh trypsin was added. The process was repeated four times and the first and second trypsin digests were discarded. The supernatant fluids from the third and fourth treatments were pooled, centrifuged at 300 g for ten minutes and resuspended in BME (containing 10% serum) at a dilution of 500,000 cells/ml. Cultures were seeded at this dilution. However, in experimental work, secondary cultures were usually employed and these were obtained by STV treatment of the primary monolayers.

b) Continuous Cell Cultures.

MDBK (a bovine kidney cell-line), HeLa and BHK (baby hamster kidney) cells were obtained by normal purchase (Flow Laboratories, Irvine, Ayrshire.). PK(W)K6 (porcine kidney) cell-line was provided by the Moredun Animal Research Institute, Gilmerton Road,

Road, Edinburgh and was derived from the fifteenth clone of PK2a Stice (156), treated six times with Kanamycin to remove mycoplasmal contamination.

c) Carrier Cell Cultures.

The origins and maintenance in this laboratory of the three cell-lines persistently infected with NDV, BK pi, PK pi and OK pi, are fully described in the introduction to Section 1 of the Results.

II) TECHNIQUES OF CELL CULTIVATION

Cells were grown in 6" x $\frac{5}{8}$" test-tubes, 2 oz and 4 oz medicinal flats, one litre Roux flasks, Brockway Saniglass tissue culture bottles or Winchester bottles in roller culture. The volume of medium used in each case was 1, 5, 10, 100, 50 and 200 ml respectively and the cells were seeded at 100,000 cells per ml, unless otherwise stated. For some experiments, monolayers were cultured in sterile microtitre tissue culture plates (Nunc-Jobling, Stone, Staffordshire) (MTCP) and, for this purpose, 10,000 cells were seeded in 0.05 mls per well. Generally the cells were grown in MEM containing 10% calf serum, 0.16% sodium bicarbonate, 0.2 mM glutamine, 100 units per ml, penicillin (Sodium benzyl penicillin - Glaxo, Greenford, Middlesex) and 100 µg/ml streptomycin (Streptomycin sulphate - Glaxo), but for maintenance the percentage of serum was dropped to between 2 - 5%. Occasionally BME was used in place of MEM but calf serum, glutamine, bicarbonate and antibiotics were added in the same

same manner. Unless otherwise stated, the monolayers were incubated at 37°C and refed on maintenance medium every 3 - 4 days. The cultures were reseeded every 7 - 10 days at which time the cells were detached from the glass with STV, pelleted at 300 g, resuspended in growth medium and counted in a Neubauer chamber, before reseeding.

III) INFECTION OF MONOLAYERS

Cells were grown overnight and the supernatant fluid was decanted. Eight HAU of NDV per ml were adsorbed to the monolayer in a quantity of medium (containing 3% calf serum) just sufficient to cover the cells (approximately 20% of the normal volume of nutrient medium). After 2 hours' incubation at 37°C, the viral suspension was removed and the culture refed with the usual amount of maintenance medium. The virus, released into the supernatant fluids, was harvested after 2 - 3 days incubation at 37°C.

In the case of M 6 virus, infection was carried out in a similar manner but 2.0 $\log_{10}$ $TCID_{50}$ (10^2 x the dose sufficient to infect 50% of the cell cultures) of virus was adsorbed to the monolayer.

IV) EXAMINATION OF MONOLAYERS

a) Light Microscopy

Unfixed monolayers were examined at 32x and 80x magnification, by direct light, for the presence of syncytia and evidence of healthy growth, while occasionally phase contrast was used to detect viral inclusion bodies.

bodies.

Fixed monolayers were stained with haematoxylin and eosin (HES) or by Giemsa and examined for cytopathic effects (CPE) by direct illumination.

i) HES method.

Monolayers of cells, grown on coverslips in 6" x $\frac{5}{8}$" tubes (flying coverslips), were removed at suitable intervals, fixed in either methyl alcohol or Bouin's solution and then rehydrated through a graduated series of ethanol-water mixtures. After washing in distilled water, they were stained with 4% Harris' haematoxylin (Gurr, High Wycombe, Buckinghamshire) in 25% ethanol, briefly rinsed in water, decolourised in acid alcohol, differentiated with saturated lithium carbonate solution and counterstained with 1.0% yellowish eosin (Gurr, High Wycombe, Buckinghamshire) in 25% ethanol. The coverslips were then dehydrated through a series of alcohol-water mixtures to absolute alcohol and finally acetone. After clearing in xylol, the stained monolayers were mounted in DePeX (Gurr).

ii) Giemsa staining method.

Flying coverslips were fixed in methanol, stained in Giemsa (Hopkin and Williams) water (1:5) for 15 minutes and then differentiated in pH 6.8 buffer for a further 15 minutes. The monolayers were rehydrated and mounted as above.

b) U-V light microscopy.

i) Fluorescent antibody staining (FAS).

Flying coverslips were removed at the times

times indicated, washed gently in PBS, lightly blotted and then, after two rinses to remove water, fixed in acetone for exactly ten minutes. Following fixation, the coverslips were dried at room temperature for one hour and then, were either immediately stained or stored, in the presence of silica gel, at -20°C for up to two years.

Unless otherwise stated, the indirect method of staining (159) was employed: a drop of an appropriate dilution of anti-NDV rabbit serum (approximately 1:5 in PBS, for serum with a haemagglutination inhibition titre of 1/256) was spread over the coverslip and incubated in a water saturated atmosphere at 37°C for 40 minutes. The monolayer was then washed twice in PBS, with continuous stirring, and restained with a similar dilution of anti-rabbit globulin serum, which has been conjugated with fluorescein isothiocyanate (Sigma, Kingston-upon-Thames, Surrey). The coverslip was incubated and washed as before, rinsed in distilled water and finally mounted in glycerol-PBS (8:2). In some experiments, the monolayer was counter-stained with a 1:1,000 dilution of Evans' blue (Gurr) at this stage. Antiserum against the envelope fraction of NDV was occasionally used in place of the antiserum prepared against whole virus. In one experiment, chicken serum containing antibody produced in response to infection with virus released from PK pi monolayers was employed together with anti-chicken-globulin serum, conjugated with fluorescein isothiocyanate (Flow laboratories).

ii) Acridine orange staining method.

Monolayers were fixed by immersion in acid-alcohol for five minutes, rinsed in citrate-phosphate buffer (pH 3.8; 0.0646 M citric acid, 0.0708 M sodium hydrogen phosphate), stained with freshly prepared 0.01% acridine orange (Gurr) for six minutes and washed twice in the buffer, being mounted in the same solution.

c) Electron microscopy.

i) Ultrathin sections.

Monolayers, grown in 4 oz medicinal flats for the periods indicated, were then washed in PBS, pre-fixed in 3% glutaraldehyde, removed from the glass with a 'rubber policeman', post-fixed in 2% osmium tetroxide, dehydrated and embedded in Araldite. After sectioning in an ultramicrotome, the cells were stained first with saturated uranyl acetate (in 50% ethanol) and then with lead acetate (158). In the case of monolayers to which erythrocytes were adsorbed, the cultures were fixed successively in chrome-osmium mixture and 0.5% aqueous uranyl acetate (60). Following the removal of the cells from the tissue culture flask, they were sectioned and stained as described above.

ii) Negative staining.

A drop of the material to be examined was placed on a carbon-coated copper grid and stained with 4% potassium phosphotungstate, pH 6.5.

D / IMMUNOLOGICAL METHODS

I) ANTIBODY

a) Preparation of antibody.

i) Antibody to whole virions and envelope proteins of NDV.

Inocula of 0.2 ml, containing either the Herts strain of NDV in allantoic fluid or purified preparations of this virus, were injected intramuscularly into one-year -old rabbits. Two weeks later, a similar amount was administered intravenously. These routes were used alternately, for eight weeks. The rabbits were re-injected at the 16th and 20th weeks and were bled at weekly intervals up till the 24th week after the first inoculation. The blood was allowed to clot and the serum was removed by pipette. Antiserum to the envelope proteins, purified by Scheid and Choppin's method (11), was obtained in a similar manner. Sera against NDV were also produced in chickens, during experiments on the infectivity of B1 strain of NDV and with virus released from PK pi and BK pi cells.

ii) Antibody to rabbit globulin.

Anti-rabbit globulin, that had been prepared in sheep, was provided through the courtesy of Dr. P. Wells, Moredun Research Institute, Gilmerton Road, Edinburgh.

b) Preparation of antibody for use in immunofluorescence.

i) Absorption of sera.

In order to reduce non-specific fluorescence, sera were absorbed against liver homogenates of mouse, calf sheep or pig, as well as extracts of the species of cell

cell that was to be stained.

Absorption was for 24 hours at 4°C and one hour at 37°C, using one ml of homogenate per ml of serum.

Preparation of homogenates:

The liver was finely chopped and suspended in PBS. After two washes in this buffer, the cell suspension was treated for ten minutes with acetone, washed a further five times with PBS and then stored, until use, at -20°C.

ii) Conjugation of antisera.

The method used was based on that described by Nairn (159). Two ml of antiserum were mixed with 4 ml of freshly prepared sodium bicarbonate buffer, pH 9.1, in an ice-water bath. Seventy mg of fluorescein isothiocyanate-celite (Celite '545', Hopkin & Williams) powder (1:10) were added and the mixture was stirred for five minutes. The celite granules were removed by pelleting at 1000 g for 10 minutes and 4 volumes of ice-cold 50% saturated ammonium sulphate were added to the fluorescein-serum conjugate. After 30 minutes at 4°C, the precipitated globulins were pelleted at 3,000 g for 20 minutes and washed twice with fresh ice-cold 40% saturated ammonium sulphate. The final pellet was dissolved in 0.5 ml of distilled water and passed through a 20 ml column of Sephadex G 75 (Pharmacia, Uppsala, Sweden), which had been previously equilibrated with 0.01 M phosphate buffer. The faster moving of the two bands containing the conjugated globulins, was collected. All sera

sera were stored, in small aliquots, at -20°C.

c) Haemagglutination inhibition tests (HIT).

Assays for the ability of sera to inhibit the agglutination of fowl erythrocytes were performed, employing antibody prepared in rabbits against the Herts strain of NDV and also with antibody produced by chickens, in response to infection with virus released from PK pi monolayers.

Non-specific inhibitors were removed by treating the sera with periodate (Section L (III)) and holding them at 56°C for 30 minutes before use. A standard volume of 4 HAU of virus was added to doubling dilutions of the serum in WHO or microtitre plates. The serum-virus mixture was allowed to react for 10 minutes at room temperature, an equivalent volume of 1% fowl red blood cells was added and the plates incubated at room temperature for a further 45 minutes. The wells were then examined for the presence or absence of haemagglutination.

d) Serum neutralisation test (SNT).

Mixtures of doubling dilutions of antiserum and a standard quantity of 4 HAU (10^4 $TCID_{50}$) of Herts virus, were held at 4°C for 2 hours and were then overlaid on to CF monolayers, in the manner described for other assays of infectivity (Section F (III)).

The cultures were incubated at 37°C for 2 days and the dilutions of antiserum which prevented infection of cells with NDV were ascertained by the absence of

of haemadsorption and immunofluorescence from such monolayers.

(II) INTERFERON

a) Preparation of interferon.

Monolayers of MDBK cells were grown overnight in 4 oz medicinal flats and were overlaid with 14 HAU of Herts or B1 strains of NDV. After 2 hours' incubation at 37°C, the cells were refed on MEM containing 5% calf serum and cultured for a further 3 days at this temperature. The supernatant was then decanted, centrifuged at 95,000 g for 2½ hours and passed through a Millipore filter (0.45 μm APD). This preparation was shown to be free of both infectious virus and haemagglutinin.

b) Assay of interferon activity.

The procedure used was based on that described by Sellars and Fitzpatrick (154).

Overnight cultures of MDBK cells were prepared in 6" x $\frac{5}{8}$" tubes and treated with 0.3 ml of undiluted interferon for 5 hours. Medium MEM containing 5% calf serum was then added to give a total volume of 1.0 ml per tube, and the period of incubation was continued at 30°C for a further 16 hours.

Ten-fold dilutions of M 6 virus were added in 0.2 ml aliquots to the supernatant fluids of (i) the cultures that had been treated with interferon, (ii) two-day-old monolayers of untreated MDBK cells and (iii) untreated BK pi cells. The tubes were then incubated at 37°C for 4 days and examined for cytopathic effect. Controls

Controls consisted of (i) untreated, uninfected monolayers of MDBK and (ii) BK pi cells treated with interferon but not infected with M 6 virus.

c) Homologous interference.

i) Superinfection of persistently infected cells with NDV.

Overnight cultures of control and persistently infected cells grown in 6" x $\frac{5}{8}$" tubes were treated with strains of NDV as follows:

INFECTING STRAIN OF NDV.	INFECTING DOSES Ten-fold dilutions of $\log_{10}$ $TCID_{50}$ (measured in PK(W)K6)	CELL-LINE
Herts	4.0 - $\overline{5}$.0	PK(W)K6,MDBK,PKpi, BKpi.
	4.0	OK pi
Lurgan	6.5 - $\overline{2}$.5	PK(W)K6, PK pi
	6.5	BK pi
Bl	4.5 - $\overline{4}$.5	PK(W)K6, PK pi
F	3.0 - $\overline{6}$.0	PK(W)K6, PK pi

The monolayers were overlaid for two hours at 37°C with 0.2 ml of the infecting dilution. The virus suspension was decanted and the culture washed twice in 5 volumes of PBS and the same amount of MEM. The monolayers were then treated with NDV antiserum (neutralising titre (SNT): 5.0 $\log_{10}$ $TCID_{50}$ of Herts strain) for one hour at 37°C. The cultures were rewashed in the manner described above, refed on MEM with 5% calf serum and incubated at 37°C for at least two days. At the times

times stated, the monolayers were examined for haemadsorption and loss of cells. Flying coverslips were stained by appropriate methods (HES and FAS) and the presence of inclusions and syncytia was determined. In each case, the supernatant fluid was assayed for haemagglutinin and infectious virus particles released from the monolayers.

ii) Superinfection of persistently infected cells with M 6 virus.

Aliquots of M 6 virus (0.2 ml) were inoculated on to one-day-old monolayers of the carrier cell-lines, BK pi and PK pi, as well as uninfected MDBK cells. After 2 hours at 37°C, the cultures were washed and refed with MEM containing 6% calf serum.

The cytopathic effects (i.e. loss of cells from the monolayer) due to M 6 virus were examined after 2 days incubation at 37°C.

E / EXAMINATION OF THE PROPERTIES OF INFECTED CELLS

I) CALCULATION OF THE PERCENTAGE OF CELLS CONTAINING INCLUSIONS.

Monolayers, stained with Giemsa, haematoxylin and eosin or acridine orange were viewed under a light or U-V light microscope, employing magnifications varying from 80x to 320x. Four hundred cells were examined in each monolayer and the percentage containing inclusions was calculated.

In the case of monolayers stained by immunofluorescence techniques, the same method was used but, in order to count the cells not containing fluorescent material, the total number of cells in a chosen field was assessed by phase contrast illumination.

II) THE ABILITY OF INFECTED CELLS TO FORM SYNCYTIA.

a) The formation of homokaryons.

The percentage of syncytia in monolayers of the three carrier cell-lines, BK pi, PK pi and OK pi, and in cultures infected or superinfected with NDV, was ascertained in the following manner:

Stained and unstained monolayers were examined under the low power of the microscope (less than 80x), the number of syncytia in 1,000 cells was counted and the percentage calculated from these figures.

b) The formation of heterokaryons.

Three ml of MEM, containing 10% serum and 0.68×10^6 cells of one of the persistently infected cell-lines, were mixed with a suspension of 4.0×10^6 healthy chick

chick embryo fibroblasts in 2 ml of medium. The mixture was seeded on to coverslips contained in a glass Petri dish (60 mm diameter) and incubated for 5 days at 37°C, in an atmosphere of 5% CO_2. The monolayers were then examined in the manner described above, for homokaryons, and the percentage of heterokaryons was calculated.

(III) EXAMINATION OF MONOLAYERS BY HAEMADSORPTION

The method employed for the assessment of the haemadsorbing ability of cells was adapted from that of Vogel and Shelokov (157).

Monolayers, suspected of infection with NDV, were washed with PBS and then overlaid with 0.4% guinea-pig erythrocytes for 15 minutes at 37°C. The volume of red blood cells used was equivalent to that of the discarded nutrient medium. The cell suspension was then decanted and the monolayer washed twice with PBS. Usually, between 2000 and 4000 cells were examined under the low power of the microscope and the percentage with adherent erythrocytes was calculated. An estimate was also made of the average number of red blood cells attached to each haemadsorbing tissue cell.

Occasionally, two other methods were employed to discover the ability of infected cells to adsorb erythrocytes:

i) after the haemadsorbing monolayer had been rinsed for the second time with PBS, it was treated with 1.0 ml of water for 30 seconds at room temperature in order to disrupt the erythrocytes (203). The lysate was then

then decanted, held at 37°C for 10 minutes, spun at 1,500 g for 10 minutes to remove cell debris and the absorption of the supernatant was read at 440 nm in a Unicam SP500 spectrophotometer. Calibration was made according to the results of lysates of known numbers of erythrocytes and hence the number of red blood cells adsorbing to the monolayer could be calculated. The assay was performed in triplicate.

ii) Cells, previously treated with STV, were pelleted, resuspended in an equal volume of 0.2% guinea-pig cells, incubated at 37°C for 15 minutes and examined in a Neubauer cell counting chamber. In this manner, the number of tissue cells per ml, the percentage of these that were haemadsorbing and the number of erythrocytes attached to each cell was estimated. Up to 200 cells were counted on each occasion.

(IV) ESTIMATION OF THE BIOLOGICAL ACTIVITIES OF INFECTED CELLS.

a) Cell-associated haemagglutinin.

Cells were removed from the tissue culture vessels with STV, pelleted at 400 g and resuspended in a known volume of PBS. The number of cells per ml was counted and the cell suspension was sonicated for 2 minutes in an MSE 3000 ultrasonic disintegrator. The haemagglutinin content was assayed in the manner described in Section F (IV).

b) Cell-associated protein, neuraminidase and phosphodiesterase.

Cells were disintegrated, in the manner described

described above, and protein concentration, neuraminidase and phosphodiesterase activities were estimated, employing the methods described in Sections F(II), (V) and (VII) respectively.

(V) ESTIMATION OF THE RATE OF GROWTH OF CELLS.

Unless otherwise stated, the rate of growth of monolayers was estimated by the increase in the number of cells observed at appropriate time intervals after seeding. Cultures were seeded in 4 oz bottles at 100,000 cells per ml. in MEM containing 5% serum inducated at 37°C for the times indicated, then detached from the glass with STV. The cells were stained with 0.1% trypan blue and the number of viable cells per ml was estimated in a Neubauer chamber.

(VI) ESTIMATION OF CELL SIZE.

At suitable times after seeding, the size of cells was calculated by means of a calibrated eye-piece inserted into the optics of a Watson's steroscopic microscope. It was found that at 32x magnification 1.00mm was represented by 4.2 large divisions of the scale. By this means the diameter of unstained cells could be examined either in the intact monolayer or suspended in PBS, following treatment with STV. At least 100 cells were examined in each case and the average diameter was calculated.

(VII) ESTIMATION OF THE DEGREE OF TRANSFORMATION OF CELLS.

Transformation of the persistently infected cells was estimated by their ability to form 'colonies' in semi-solid agar, according to the method described by

by MacPherson and Montagnier (102).

Seven ml of MEM, containing 0.5% Noble agar (Difco) and 10% calf serum, were pipetted into plastic Petri dishes (60 mm diameter) and allowed to set.

Cells from the cell-lines indicated in the text were prepared at a concentration of 200,000 - 250,000 cells per ml in 3 ml of medium, incorporating 0.3% Noble agar with 10% calf serum and were added to the solidified base. The 'colonies' formed were examined after 6 days' incubation at 37°C in an atmosphere of 5% CO_2.

F / EXAMINATION OF THE PROPERTIES OF LIBERATED VIRUS

I) ELECTRON MICROSCOPY (VIRAL MORPHOLOGY).

Suspensions of viral material were negatively stained by the method described in Section C (IV) c ii.

II) ESTIMATION OF PROTEIN CONCENTRATION.

The amount of protein in a sample of virus was determined by the method of Lowry et al. (201). The absorption was read at 750nm, following treatment with copper sulphate (buffered with sodium carbonate and sodium potassium tartrate) in combination with Folin and Ciocalteu's phenol reagent. Occasionally, the approximate absorption of the untreated samples was read at 280nm and, in both cases, bovine serum albumin was employed for the preparation of standards.

III) ESTIMATION OF INFECTIVITY OF VIRUS

Assays were made of the ability of NDV to infect monolayers of mammalian cell-lines or chick fibroblasts, and infectivity was estimated either by the number of $TCID_{50}$ per ml (doses sufficient to infect 50% of the cells) or of PFU (plaque forming units). On occasion, infectivity was also assessed by the number of EID_{50} per ml (50% egg infectious doses) in 10-day-old fertile hens' eggs or by the capability of the virus to infect 5-week-old chicks.

a) Infectivity of virus in cell culture.

i) The measurement of $TCID_{50}$.

Monolayers were grown overnight in 6" x $\frac{5}{8}$" tubes and overlaid with 0.2 ml of ten-fold (or doubling)

doubling) dilutions of the virus suspension in medium containing 3% serum. After 2 hours' incubation at 37°C, unadsorbed virus was removed by thoroughly washing the cultures twice with PBS and medium, and the monolayers were then refed with 1.0 ml of maintenance medium.

After 2 days of incubation at 37°C, the monolayers were examined for the presence of infection by haemadsorption, cytopathic effects (cell loss and formation of syncytia) and fluorescent antibody staining. The supernatant fluid was tested for the presence of released haemagglutinin and infectious virus.

The $TCID_{50}$ of the virus was calculated by a modification of Karber's method (161) and expressed as the log to the base 10 (log_{10}). Since haemadsorption and FAS were found to be the most sensitive methods of determining the presence of infection, the $TCID_{50}$ was generally estimated by these methods.

The $TCID_{50}$ of NDV was also assayed in microtitre plates (MTCP), and the following procedure was employed. The monolayers in 8 wells were overlaid with 0.02 ml of each dilution of the virus and then incubated and washed in a similar manner to infected tubes. The infected cells were refed with 0.05 ml of maintenance medium and infectivity was determined after 2 days' incubation as described above.

ii) The estimation of PFU.

One-day-old monolayers of healthy cells were grown in Petri dishes and infected in the manner delineated

delineated for the estimation of $TCID_{50}$. After unadsorbed virus had been washed off, the monolayers were overlaid with maintenance medium containing either 1.0% Noble agar or 1.5% methyl cellulose (Koch-Light, Colnbrook, Buckinghamshire). The cultures were incubated at 37°C in an atmosphere of 5% CO_2 for 3 days and the overlay medium was then removed. The monolayers were either stained with Giemsa or treated with 0.4% guinea-pig red blood cells to determine the location of destroyed or infected cells. The number of plaques was counted at the highest dilutions, in which infected cells were seen and hence the PFU per ml were calculated.

iii) The infectivity of M 6 virus was determined by its $TCID_{50}$ in MDBK cells, grown in tubes.

b) Infectivity of NDV in embryonated chicken eggs.

Ten-fold dilutions of the test virus were made in PBS and inoculated in quadruplicate into 10-day-old developing hens' eggs by the allantoic route. The presence of haemagglutinins in the allantoic fluid after 2 days' incubation at 37°C was used as an indication of infection and the EID_{50} was calculated by Karber's method (161).

c) Infectivity of NDV in 6-week-old chicks.

Pools of pelleted virus, released from BK pi and PK pi cells respectively, were adjusted to the same protein concentration and phosphodiesterase activity as the B1 strain of NDV grown in HeLa cells (B1/HeLa). The $\log_{10}$ EID_{50} of the pelleted B1/HeLa virus was

was shown to be 9.75. Twelve chicks were infected with each strain of virus; half of each group received 0.2 ml intracardially and the other six birds were inoculated with 0.4 ml intramuscularly. A control bird was left uninoculated in each flock. Each group of chicks was kept in a separate room with an individual source of ventilation, water and food.

Blood was withdrawn by venipuncture from every chicken on the day before infection and on the 4th, 7th, 13th and 17th days thereafter. Serum was prepared from these samples and HITs performed by the methods described in Sections D(I)a and D(I)c respectively. The birds were examined daily for clinical signs of infection and following death due to disease or sacrifice.

IV) EXAMINATION OF VIRAL HAEMAGGLUTININ

a) Haemagglutinin assays.

These were performed in WHO perspex plates using the techniques described by the WHO Expert Committee on Influenza (160). Doubling dilutions of the test material were prepared in PBS and an equal volume (0.25 ml) of an 1.0% suspension of fowl red blood cells was added to each well. The results were read after 45 minutes' incubation at room temperature or, in the case of rapidly eluting virus, at 4°C. Assays were also performed using one-tenth of these volumes in microtitre plates (Flow Laboratories).

b) Estimation of the temperature stability of haemagglutinin.

i) Purified virus at 56°C.

i) Purified virus at 56°C.

Purified virus was incubated at 56°C for the periods indicated and, after rapidly chilling in an ice-water bath, the haemagglutinin titre was found by the usual method.

ii) Supernatant fluids from BK pi and OK pi monolayers (at several temperatures).

Samples of supernatant fluids were held at 56°C, 41°C, 37°C, 31°C, 20°C and 4°C for 7 days and assayed for haemagglutinin activity at hourly intervals, for the first 12 hours, and daily thereafter.

V) ESTIMATION OF NEURAMINIDASE ACTIVITY.

The technique employed is that described by McNulty et al. (108). Orosomucoid (α_1 glycoprotein concentrated from human plasma), obtained from the Scottish National Blood Transfusion Association, was used as a substrate at a concentration of 8 mg/ml. Triplicate samples of the virus in 0.2 M sodium acetate buffer (pH 5.1) were incubated with 0.25 ml of orosomucoid at 37°C for 15 minutes. The reaction was halted by the addition of periodic acid and the excess of this oxidising agent was removed by shaking the reaction mixture with sodium arsenite. The oxidised free N-acetyl neuraminic acid (NANA) was then combined with thiobarbituric acid, following the procedure described by Aminoff (202), and the chromogen was developed by 7½ minutes' incubation at 100°C. The colour was extracted into butanol/hydrochloric acid (19 : 1) and the absorption read at 549 nm. Controls

Controls containing known quantities of NANA (Sigma, Kingston-upon-Thames, Surrey) were employed and specific activity was expressed as nmoles of NANA released per minute per µg protein.

VI) ESTIMATION OF THE HAEMOLYSING ACTIVITY OF NDV.

The method employed was based on that described by Kohn (23). A virus suspension of 0.1 ml was added to 1.0 ml of 2.0% fowl red blood cells, incubated for one hour and then centrifuged at 1,500 g for 10 minutes to remove cellular debris. The absorption of the released haemoglobin was read at 550 nm.

VII) ESTIMATION OF PHOSPHODIESTERASE ACTIVITY.

The activity of this enzyme was measured by the method of Sinsheimer and Koermer (178), employing the chromogenic substrate bis-p-nitrophenyl phosphate, which is split to yield phosphate and yellow p-nitrophenol. The reaction mixture contained 0.2 ml virus, 0.3 ml 0.1 M glycine buffer (pH 8.8), 0.1 ml 0.3 M magnesium acetate and 0.5 ml 0.001 M sodium bis-p-nitrophenyl phosphate (Sigma, Kingston-upon-Thames, Surrey). After 3 hours' incubation at 37°C, the reaction was halted by plunging the tubes into an ice-water bath and, following centrifugation at 10,000 g for 20 minutes, the absorbance was read at 405 nm. Specific activity per µg of protein was calculated by calibration with the spectrophotometric absorbance of known concentrations of the substrate which had been totally hydrolysed. In order to assess the optima of pH and temperature

temperature for different strains of virus, these factors were varied, as indicated, in certain experiments.

G / VIRUS PURIFICATION

Virus was grown in the allantois of fertile hens' eggs or on infected monolayers and was purified in the manner described by McNulty *et al*. (108). All the following processes were carried out, as far as possible, at 4°C. The infected allantoic or supernatant fluids (designated Al.Fl. and SN, respectively) were centrifuged at 5,000 *g* for 20 minutes to remove cellular debris and the virus contained in the clarified supernatant (Cl.Al.Fl or Cl.SN) was pelleted at 45,000 *g* for 1½ hours. The deposit was resuspended in 2-3 ml of PBS (Pellet), employing a glass homogeniser and thoroughly mixed for one minute with an equal quantity of trichlorotrifluoroethane (BDH). After the mixture had been centrifuged at 5,000 *g* for 20 minutes, the upper aqueous phase containing the virus (designated TCTF) was removed and carefully layered on to 10 ml of 15-40% (w/w) potassium tartrate in PB, which had been formed into a linear gradient with a Buchler auto-DensiFlow apparatus. Following 2½ hours' centrifugation at 284,000 *g*, the opaque band of virus was collected and dialysed overnight against PB. (This stage of purification is referred to as TG 1). The sample was banded once again in a similar potassium tartrate gradient and redialysed (TG 2). On occasion, this process was repeated a third time (TG 3).

H / DISRUPTION OF VIRUS

The disruption of virus was necessary in several experiments and one of the following three procedures was generally employed. All of these methods involved detergent treatment, since it was found that the use of a proteolytic enzyme (trypsin) produced unwanted results (see Section L(I) and Results, Section 4, C).

(I) TREATMENT OF VIRUS WITH SODIUM DODECYL SULPHATE (SDS)

Prior to polyacrylamide gel electrophoresis, purified virus was disrupted with SDS (BDH), with or without the reducing agent 2-mercaptoethanol.

Fifty µl of the solubilising solution, containing 1.0% SDS, 6.0 M urea, 0.002% bromophenol blue, 10% sucrose and usually 0.1% 2-mercaptoethanol in PB, was added to between one and five volumes (depending on the protein concentration) of the viral sample. After incubation of 30 minutes at 60°C, the suspension was placed in a boiling water-bath for one minute.

(II) TREATMENT OF VIRUS WITH TRITON X-100 (T X 100)

In order to isolate the proteins of purified virions of NDV by Scheid and Choppins' method (11, 18), one volume of 20% Triton X-100 (BDH) in PB, containing 1.0 M potassium chloride, was added to nine volumes of a suspension of virus in the same buffer. After being held for one minute at 30°C, the viral suspension cleared but, during a further 19 minutes of incubation at this temperature, cloudiness returned due to the precipitation of viral nucleocapsid proteins, in

in solutions of high molarity.

(III) TREATMENT OF NDV WITH NONIDET P 40 (NP 40)

Virus was disrupted with NP 40 (BDH), to expose the internal components of the virion. The procedure used was based on that employed by Huang et al. (38) for the enhancement of RNA polymerase activity associated with nucleocapsid. In some experiments in the present work, one volume of NP 40 was mixed with four volumes of virus in glycine buffer before carrying out assays for phosphodiesterase activity.

I / ISOLATION OF VIRAL PROTEINS

(I) SEPARATION OF VIRAL PROTEINS ON THE BASIS OF SOLUBILITY IN SOLUTIONS OF DIFFERENT MOLARITIES.

Following the procedure laid down by Scheid and Choppin (11, 18), the virus was disrupted with Triton X-100 (see Section H (II)) and the nucleocapsid pelleted at 10,000 g for 20 minutes (Pellet 1). The remaining undisrupted virus and nucleocapsid fragments were removed from the supernatant by centrifugation at 200,000 g for one hour and the supernatant obtained in this manner (SN 2) was dialysed overnight against PB. However, in contrast to the findings of the original workers, the reduction in molarity did not result in the precipitation of VP 42 and only a small deposit was seen after the dialysate had been centrifuged at 10,000 g for 20 minutes (Pellet 3). The final supernatant (SN 3) was further fractionated by affinity chromatography and the column of fetuin-sepharose used for this purpose, was prepared in the following fashion.

Employing the simplified method of March et al. (204), Sepharose 4B (Pharmacia) was mixed with cyanogen bromide dissolved in acetonitrile and 5 ml of the activated Sepharose was then coupled with 200 mg of fetuin (Fetuin (Spiro), Gibco-Biocult, Paisley, Renfrewshire) by rotating the mixture for 24 hours at 4°C. Unreacted groups were masked by the addition of 1.0 M glycine for a further 4 hours. A column,

column, 22 mm long and 7 mm in diameter was poured and washed with TRB before use.

A mixture of 0.9 ml SN 3 and 0.1 ml of a ten-fold concentration of TRB was then applied to the column and run at 4°C for 90minutes at a flow rate of 8 ml per hour. The column was then transferred to an environment at 25°C and run for a further 2½ hours at a flow-rate of 4 ml per hour. The size of the fraction collected was normally 0.66 ml, although occasionally 1.0 ml aliquots were taken. Scheid and Choppin (11) reported that the envelope protein bearing the haemagglutinin and neuraminidase activities bound preferentially to the fetuin and thus eluted off the column following the increase in ambient temperature.

Before any of the proteins prepared from virus treated with Triton X-100 were assayed for their haemagglutinating capacity or electrophoresed on polyacrylamide gels, they were precipitated with ten volumes of ice-cold butanol, held at 0°C for 30 minutes and pelleted at 10,000 g for a further 30 minutes. This process removed the Triton X-100 and was repeated employing diethyl ether to remove traces of butanol. The ether was driven off with a jet of carbon dioxide gas and the pellet was resuspended in PBS.

(II) SEPARATION OF VIRAL PROTEINS BY POLYACRYLAMIDE GEL ELECTROPHORESIS.

(This process was also employed for analysis of any other mixture of proteins.)

a) Formation and running of gels.

a) Formation and running of gels.

Mixtures of proteins were treated with SDS, in the manner previously described (Section H (I)) and migrated through the polyacrylamide gel largely on the basis of their molecular weights. However, the migration of glycopeptides is anomalous (205).

Gels of 5 mm diameter and approximately 60mm in length were formed by the polymerisation of a solution containing 10% (w/v) acrylamide, 0.3% N,N'-methylenebisacrylamide, 0.375 M tris-hydrochloric acid buffer (pH 8.9), 5.0 M urea and 0.075% N,N,N',N'- tetramethylethylenediamine, employing 0.15% ammonium persulphate as a catalyst. Approximately 30 - 50 µg of the solubilised proteins were electrophoresed at 2mA per gel for 2-3 hours and the run was completed when the tracker dye (bromophenol blue) had reached a suitable point. A discontinuous buffer system was used with 0.05 M tris-glycine (pH 8.9) containing 0.1% SDS in the upper (cathode) tray and 0.1 M tris-hydrochloric acid (pH 8.1), also containing 0.1% SDS, in the lower (anode) tray.

b) Location of polypeptides in electrophoresed gels.

i) Staining with Coomassie blue.

Generally the gels were stained for 2-3 hours with 0.25% Coomassie brilliant blue (R250, Serva, Heidelburg, Germany) in methanol-acetic acid-water (5:1:5), employing the same solvent, without the dye, to destain overnight. The gels were scanned at 580 nm employing a Unicam SP500 spectrophotometer with a

a Gilford Linear Transporter 2410-S attachment. Densitometer tracings were made, with the origin to the left in all gel profiles. The method used to estimate molecular weight was as follows: chymotrypsinogen (Sigma) and bovine serum albumin (Sigma) were employed as markers and the approximate molecular weights for the three major proteins of NDV, determined in this manner, were 78,000, 60,000 and 40,000 (108). However, Moore and Burke (21) obtained figures of 75,000, 55,000 and 42,000 daltons respectively (21) and, in order to clarify the nomenclature of the viral proteins, the latter values were assigned to the three major polypeptides of NDV isolated on our gelsystem. Therefore, in the present work, the molecular weights of other proteins were determined by employing as standards the relative migration of the VGP 75, VP 55 and VP 42 of NDV.

ii) Staining by periodic acid-Schiff's (PAS).

Occasionally, gels were stained for glycoprotein by PAS. The following procedure was usually followed and was based on the technique described by Kobylka et al. (205).

The gels were fixed in 12.5% trichloracetic acid for 75 minutes, washed overnight in 15% acetic acid, with five changes of the solution, oxidised with 1.0% periodic acid for 2 hours, rewashed in 15% acetic acid for one hour, stained in Schiff's reagent (208) for one hour and destained in 7% acetic acid for 42 hours. The stained areas were differentiated by reduction with

with sodium metabisulphite (1.0% in 0.1 N hydrochloric acid) and the gels were given a final rinse in 7% acetic acid for 5 minutes. The gels were scanned at 560 nm in the densitometer.

iii) Radioactive labelling with ^{3}H-amino acids or ^{14}C protein hydrolysate.

In the case of radioactively labelled polypeptides, PAGE was carried out in duplicate. On completion of electrophoresis, one gel was not stained but was frozen with solid carbon dioxide and 0.75 mm sections were made with a Yeda GTS hand macrotome. The slices were prepared for scintillation spectrophotometry by an adaptation of the procedure of Scheid et al. (62). The sections were placed in scintillation vials, rehydrated by the addition of 0.05 ml of water, solubilised by incubation at room temperature with 0.2 ml of NCS for 2 hours, followed by a further 0.3 ml of the reagent and, after holding at 31°C overnight, 10 ml of scintillation fluid (126) were added to the solubilised gel.

Radioactivity was estimated by reading counts per minute (cpm) of ^{14}C and ^{3}H in an ICN liquid scintillation spectrometer. The other gel was stained in the normal way and the peaks obtained by the two methods were compared. In this manner, stained bands of protein could be correlated with the radioactivity present in the appropriate slice of gel.

(III) EXTRACTION OF RIBONUCLEOPROTEIN (RNP) FROM INFECTED CELLS.

The method of Compans and Choppin (182) was followed

followed throughout. Approximately 12 x 10^7 cells in intact monolayers were thoroughly washed twice in serum-free nutrient medium, followed by two rinses in PBS. The cells were detached with 0.07% versene in water, pelleted at 6,000 g, resuspended in 15 ml of water and disrupted with a Dounce homogeniser. Seven ml of the homogenate were layered on to a discontinuous gradient of caesium chloride ($CsCl_2$), composed as follows:

2.0 ml, 25% $CsCl_2$ (w/w) in water, 2.5 ml, 30% $CsCl_2$ and 1.25 ml 40% $CsCl_2$. The tube was then spun at 65,000 g for 3 hours. A band was removed, made up to 5 ml with 30% $CsCl_2$ and centrifuged to equilibrium at 10,000 g for 12 hours. A band of buoyant density 1.29 was dialysed overnight against distilled water and for a further 2 days with PB. The preparation was then examined by electron microscopy for nucleocapsid structures and by PAGE for a polypeptide of the appropriate molecular weight.

J / EXPERIMENTS PERFORMED WITH RADIOACTIVE ISOTOPES

(I) LABELLING OF CELLS WITH RADIOACTIVE ISOTOPES

a) Labelling of cellular (and viral) RNA with ^{3}H-uridine.

One-day-old monolayers grown in 6" x $\frac{5}{8}$" tubes, were washed twice with MEM (without serum) and refed with 1.0 ml of MEM containing 7% calf serum and 5 µ Ci/ml of ^{3}H-uridine. The tubes were incubated at 37°C for the times indicated, and the monolayer was then washed twice with MEM and twice with TKB. After blotting thoroughly, 0.5 ml of TKB containing 10% SDS were added to each tube and the cells were solubilised by heating to 60°C for 30 seconds.

Samples of 30 µl were pipetted on to 15 mm discs of chromatography paper (3MM, Whatman) and treated with ice-cold 5% trichloroacetic acid (TCA) for 10 minutes in order to precipitate acid-insoluble cell contents, including RNA (206). Soluble material containing ^{3}H-uridine was removed by washing the discs twice with 5% TCA and rinsing once with 15% ethanol (206). The impregnated papers were then dried at 37°C for one hour.

Each disc was placed in a vial of 20 ml capacity, containing 10 ml of scintillation fluid (0.3 g phenyl-oxazol phenyl-oxazol phenyl (POPOP) and 4.0 g 2-5-diphenyloxazole (PPO) in one litre of toluene (126). The counts per minute were read in an ICN liquid scintillation spectrometer.

b) Labelling of proteins, synthesised in infected cells with ^{3}H-amino acids and ^{14}C-protein hydrolysate.

Measurement of the amount of radioactively labelled amino acids incorporated into the proteins of infected cells and controls was achieved by the techniques outlined above (Section J(I)a).

The concentrations of the ^{3}H- and ^{14}C labels in the overlay media were 10.0 μCi and 2.5 μCi per ml respectively.

The following variation in procedure, when such cells were to be analysed by PAGE, is based on that described by Samson and Fox (50). After incubation in the presence of the label for one hour, monolayers, grown in 4 oz medicinal flats, were washed twice with MEM and once with PBS. They were then treated with 1.0 ml of PB, containing 5.0% SDS and 4.0% 2-mercaptoethanol. The lysate was heated to 100°C for 2 minutes and dialysed for 38 hours against PB buffer, containing 0.1% SDS and 0.1% 2-mercaptoethanol. The material was then analysed by PAGE, by the methods described above (Section I(II)b(iii)).

(II) DOUBLE LABELLING TECHNIQUES

The procedure followed here is similar to that described by Alexander and Reeve (48).

One-day-old monolayers of MDBK cells, grown in 4 oz medicinal flats, were infected with 4 HAU per ml of the B1 strain of NDV and, after two hours' incubation at 37°C, the unadsorbed virus was removed by three washes with maintenance medium. The cell cultures were refed with MEM, containing 5% calf serum and treated 2½ hours later with actinomycin D (7 μg per ml). Monolayers

Monolayers of healthy MDBK and BK pi cells were similarly treated with actinomycin D. (However, in certain experiments this antibiotic was not added to any of the cultures.) After a further period of 2 hours, the monolayers were washed, refed with maintenance medium and reincubated until $8\frac{1}{2}$ hours had elapsed since the beginning of the experiment. The cultures of MDBK cells infected with B1 virus, healthy MDBK monolayers and BK pi cells were now overlaid for one hour with serum-free MEM, containing 10.0 μCi per ml of ^{3}H-amino acids or 5.0 μCi per ml of ^{14}C protein lysate. The cells were then solubilised by the standard method outlined in Section J(I). Lysate from either infected MDBK cells or BK pi cells, labelled with ^{3}H-amino acids, was mixed in equal quantities with the solubilised proteins obtained from control MDBK monolayers, which had been labelled with ^{14}C. However, in certain experiments, ^{14}C-amino acids were incorporated into infected cells and ^{3}H-amino acids into the controls. The mixtures were run on polyacrylamide gels and the radioactivity in the sliced gels measured in the usual fashion (Section I(II)b(iii)).

The ratio of counts per minute of ^{3}H to ^{14}C was measured and was calibrated by comparing the ratio obtained for this level of radioactivity in gels run with a mixture of equal amounts of ^{3}H and ^{14}C labelled proteins from control monolayers of uninfected MDBK cells. The ratios were further adjusted by dividing

dividing them by the ratio of $^{3}H:^{14}C$ in the non-variable portions of the gel, so that the background ratio of $^{3}H:^{14}C$ was brought to unity. In this manner, an increase in the amount of ^{3}H-amino acids incorporated into infected cells (because of viral protein synthesis), was expressed as a rise in the ratio of $^{3}H:^{14}C$, since these proteins were not formed in the control cells labelled with ^{14}C-amino acids.

K / ATTEMPTS TO CHANGE THE CHARACTER OF THE PERSISTENT INFECTION BY ALTERING THE ENVIRONMENT OF THE CELLS

(I) ALTERATION OF THE PERCENTAGE OF SERUM IN THE MEDIUM

In these experiments, BK pi cells were sub-cultured in medium containing the appropriate concentration of calf serum and incubated for one week at 37°C to allow stabilisation to occur.

The cells were then reseeded at concentrations of 100,000 cells per ml in 4 oz medicinal flats and refed with MEM, containing 0, 5, 10, 20% calf serum respectively. The cells were refed with these media at 3 day intervals and examined on the 3rd, 7th, 10th and 14th days of incubation for released haemagglutinin in the supernatant fluids and for cell-associated haemagglutinin. The number of cells per ml was also estimated.

(II) COMPARISON OF GROWTH IN MEM AND BME

After two weeks' incubation in the appropriate medium, OK pi, BK pi and PK pi cells were reseeded in either MEM or BME, containing 10% calf serum and were maintained for a further 7 days, by refeeding them daily with MEM or BME, containing 5% calf serum. The cultures were examined daily for free haemagglutinin, their capacity to haemadsorb, the formation of syncytia, the number of cells per ml and the average cell diameter.

(III) INCUBATION AT TEMPERATURES OTHER THAN 37°C.

a) Incubation of cells at 41°C.

Parallel cultures of each of the carrier cell-lines

cell-lines (OK pi, BK pi and PK pi) were set up at 41°C and 37°C. The cells were refed daily on MEM, containing 20% calf serum and reseeded every 3-7 days. The monolayers were examined for haemadsorption, immunofluorescence, the number of cells per ml and, after preparing ultra-thin sections, by electron microscopy. These procedures were carried out daily for the first week and at weekly intervals thereafter. At the times stated, the cultures were returned to 37°C and examined for evidence of infection, in the manner described.

b) Incubation of cells at 37°C.

The persistently infected cell-lines (OK pi, BK pi and PK pi) were grown at 31°C for periods of up to 10 weeks. The monolayers were refed every 3 days with MEM, containing 10% calf serum, and reseeded weekly. The amount of haemagglutinin released into the supernatant was assayed at three-day intervals and compared with the HA titres produced by similar cultures grown at 37°C. The infectivity of pooled virus released at 31°C was also examined.

(IV) TREATMENT OF CULTURES WITH ANTISERUM TO NDV

Monolayers of OK pi, BK pi and PK pi were cultivated in MEM, containing 5% calf serum and also antiserum, prepared in rabbits against the Herts strain of NDV. The titre of the antiserum was sufficient to neutralise the infectivity and haemagglutinating capacity of 512 HAU of NDV per ml.The cells were assayed daily for haemadsorption, immunofluorescence and the presence of

of active haemagglutinin in the supernatant.

(V) TREATMENT OF CULTURES WITH INHIBITORS OF PROTEIN AND RNA SYNTHESIS

One-day-old monolayers of BK pi and MDBK cells, grown in 6" x $\frac{5}{8}$" tubes, were refed with MEM, containing 5% calf serum and the appropriate amount of inhibitor. Actinomycin D (Sigma) was added to give final concentrations of 1.0, 2 or 5 µg per ml, cycloheximide (Sigma) at 25 or 75 µg per ml and, occasionally, monolayers were treated with a mixture of 1.0 µg per ml of actinomycin D and 25 µg per ml of cycloheximide for the times indicated. The incorporation of ^{14}C protein hydrolysate and ^{3}H-uridine into protein and RNA respectively, was measured by the technique described in Section J(I)a. The amount of haemagglutinin and infectious virus released was assayed, and the monolayers were examined at the times stated for haemadsorption, immunofluorescence, the quantity of cell-associated haemagglutinin and the number of cells per ml. In certain experiments, neuraminidase and phosphodiesterase activities of the liberated virus were also measured.

(VI) EXAMINATION OF CARRIER CELLS SEEDED AT LOW CONCENTRATIONS.

Cloning of single cells was attempted by several techniques.

a) The method of Wildy and Stoker (207).

Single cells were grown in drops of 'conditioned' MEM containing 10% serum, under liquid paraffin.

paraffin. Although cells were observed to attach, employing this or other related techniques (195), mitosis was not seen unless three or four cells were inoculated in the drop.

b) Low density seeding.

The lowest density of seeding at which cell division took place in 60 mm Petri dishes was 10 cells per ml and required a total of at least 50 cells. MEM, containing 10% calf serum was employed. On the other hand, in microtitre plate cultures, mitosis occurred when only 10 cells were attached to the base of each well in 0.05 ml of medium and, therefore, required cell concentrations of greater than 200 cells per ml. Employing either of these methods, 'colonies' of cells could be seen within 2-3 days.

c) Seeding on to complete monolayers of chick embryo fibroblasts.

BK pi cells were dispersed in growth medium and diluted to contain between 20 and 50 cells per ml. The cells were then added to complete one-day-old monolayers of secondary chick embryo fibroblasts in microtitre plates, employing 0.05 ml of MEM, incorporating 10% calf serum. Examination showed that over 35% of the wells contained only one BK pi cell and, that in most cases, these had undergone repeated division by the 5th day (BK pi cells were distinguished from CF cells by their epithelial morphology in stained and unstained monolayers).

Cells grown by methods (b) or (c) were examined

examined daily for haemadsorption and, in the latter case for heterokaryon formation. Although BK pi cells were normally detached from the glass of the stock culture bottle with STV, in some experiments, they were dispersed in 0.05% trypsin, while in others, 0.007% versene was employed.

L / ATTEMPTS TO ALTER THE CHARACTER OF VIRUS RELEASED FROM BK PI CARRIER CELLS

(I) TREATMENT WITH TRYPSIN

Purified particles of NDV were treated with trypsin, employing the method described by Homma and Ohuchi (142). Usually, 0.3 ml of the virus suspension, containing 500 - 1,000 HAU per ml of virus, was mixed with 0.1 ml of trypsin, to give a final concentration of the enzyme of 4 ppm. After 8 minutes' incubation at 37°C, the process was halted by the addition of 0.1 ml of an equimolar concentration of soya bean trypsin inhibitor (Sigma). The virus was then assayed for infectivity, haemagglutinating and haemolysing activity, compared with that of controls, to which 0.1 ml of PBS and 0.1 ml of trypsin inhibitor had been added. In some experiments different concentrations of trypsin were used in order to assess their effects on the properties of the virus.

(II) TREATMENT OF VIRUS WITH NEURAMINIDASE (RDE)

This experiment follows the procedure used by Palese *et al.* (67), to activate the haemagglutinin of defective influenza virus.

Two hundred units of RDE (Receptor destroying enzyme) derived from *Vibrio cholerae* (BDH), were incubated for 15 minutes with 0.1 ml of pelleted BK pi virus (HA titre 1/128). Before assaying for haemagglutinin, N-acetyl neuraminic acid (NANA) was added to a concentration of 5 µg per ml, in order to prevent the neuraminidase causing premature elution.

elution. Control experiments were also carried out to test the haemagglutinin activity of BK pi virus,

(i) in the presence of NANA without neuraminidase treatment, and

(ii) following exposure to RDE but in the absence of the artificial substrate.

(III) TREATMENT OF VIRUS WITH PERIODATE

Prior to their employment in haemagglutination or haemagglutination inhibition assays, samples of serum or virus in allantoic fluid or nutrient medium, were treated with 0.2 volumes of 0.1 M sodium periodate in order to remove non-specific inhibitors. After 30 minutes incubation at room temperature, the oxidising agent was neutralised by the addition of 0.2 volumes of 40% glucose solution (199).

In an attempt to remove serum glycoproteins from the surface of BK pi virions, samples were treated with periodate before being purified by the fluorocarbon and tartrate density gradient procedure (Section G).

RESULTS

SECTION 1

THE CHARACTERISTICS OF CELL-LINES PERSISTENTLY INFECTED WITH NDV

RESULTS

SECTION 1

THE CHARACTERISTICS OF CELL-LINES PERSISTENTLY INFECTED WITH NDV.

A / INTRODUCTION

Cell-lines of pig kidney (Stice-2a), ox kidney (MDBK) and sheep kidney (MDOK) were brought into this laboratory in 1959 by the courtesy of Dr. Madin, Berkeley, California. They were received in good condition and formed confluent monolayers without visible abnormalities. Subcultures were made at weekly intervals by inoculating 2 x 10^5 cells in Earle's saline containing 0.5% lactalbumin hydrolysate, 0.1% yeast extract and 5-10% heat-inactivated calf serum. For maintaining the cells, the same medium was used but with the calf serum reduced to 2%. Two hundred units/ml of penicillin, 100 µg/ml of streptomycin and 25 units/ml of nystatin or fungazole were incorporated in all media. Cells were removed from the glass with 0.05% bovine trypsin in 0.02% versene (STV) at 37°C. In 1962, the three cell-lines were found to be infected with NDV and have carried the infection to the present time. The general characteristics of these persistently infected cultures, prior to 1972, were described by Edwards (93) and have been summarised in the Introduction (vide supra).

In the present work, the persistently infected

infected cultures of pig, ox and sheep kidney cells are designated PK pi, BK pi and OK pi respectively. The three carrier cell-lines have now been maintained in this laboratory for over 15 years but the precise origin of the infection is not known.

There are now many reports in the literature of cells infected or persistently infected with NDV, (or related paramyxoviruses). These systems exhibit features including haemadsorption, syncytial formation and decreased cellular RNA and protein synthesis, that are not found in uninfected cells. In this Section an attempt has been made to quantify these effects in the manner in which they occur in the persistently infected cultures maintained in this laboratory, and to compare them with the results obtained from parallel infections of control lines by 'wild-type' NDV. Moreover, since it was observed that some of the characteristics ascribed to the persistently infected cell-lines by Edwards (93) had altered since the completion of her Thesis, a comparison is made, where appropriate, in respect of the features of the carrier cells prior to 1972.

Because Madin's sheep kidney cell line (MDOK) is no longer available and control cultures cannot be obtained for OK pi cells, the majority of the results reported below were obtained using cultures of BK pi and PK pi only.

B / REPLICATION OF PERSISTENTLY INFECTED CELL-LINES

I) GROWTH RATE

Introduction

Because inhibition of cellular RNA and protein synthesis is known to occur following infection of cells by most 'wild-type' strains of NDV (80), it is reasonable to suppose that mitosis would be reduced in infected cells. On the other hand, since certain of the changes induced in cells infected with NDV, including agglutination by lectins (96) are reminiscent of cellular transformation, it might be anticipated that the rate of replication would increase. Of these two effects, the latter is the more probable in carrier cultures and, indeed, there is evidence that transformation of BHK cells carrying polyoma virus is due to a form of persistent infection (102).

a) Degree of transformation of carrier cell lines.

In view of this, it was decided to examine our control and carrier cell-lines with regard to transformation. It will be seen from the results in Table I that there is little difference in the rate of growth between the persistently infected cells and their controls. Since a confluent monolayer is usually formed within 24 hours of seeding at 100,000 cells/ml, the increase in the number of cells/ml (which is up to eightfold after seven days of incubation) suggests that both the persistently infected cultures and their controls are relatively unaffected by contact

T A B L E I

INCREASE IN NUMBER OF CELLS/ML OF PERSISTENTLY INFECTED AND CONTROL CULTURES

DAY	CELLS PER ML IN CELL-LINES				
	PK(W)K6	PK pi	MDBK	BK pi	OK pi
1	140,000	160,000	295,000	310,000	90,000
3	400,000	330,000	400,000	330,000	225,000
7	900,000	700,000	800,000	600,000	450,000
7*	940,000	1020,000	320,000	390,000	450,000

All monolayers were seeded at 100,000 cells/ml on Day 0 and refed on Days 1 and 4 with MEM containing 5% calf serum.

*The results obtained by Edwards (93).

Figure 1. MDBK cells, grown in semi-solid agar, 6 days after seeding, showing absence of 'colony' formation. (x 150)

Figure 2. BK pi cells, grown in semi-solid agar, 6 days after seeding, showing the presence of piled-up cells ('colonies'). (x 150)

contact inhibition. Moreover, BK pi and healthy MDBK cells were capable of satisfactory growth in MEM medium without added serum or other complex biological materials. The rate of mitosis and growth in the absence of serum are both suggestive of cellular transformation and thus it is not surprising that 'colony' formation occurred in semi-solid agar. This technique was devised for the detection of transformation in BHK cells infected with polyoma virus (102) and all three of our persistently infected cell-lines formed three dimensional aggregations of cells, similar to that shown in Fig. 2. The largest colonies were formed by PK pi and BK pi cells, followed by OK pi, while the control line PK(W)K6, produced small and infrequent colonies. 'Colony' formation was not observed in MDBK cells (Fig. 1).

Discussion.

It is apparent, therefore, that the control cell-lines exhibit some of the features of transformation, which might be expected because all continuous cell lines acquire some of these characteristics in order to adapt to *in vitro* culture. However, although the persistently infected cells show a greater degree of transformation, this is not necessarily an indication of oncogenicity. All the characteristics of 'transformation' are found in 'normal' cells during the process of mitosis (174). Carcinogenic agents may cause changes in cell surface receptors such that

that the cells remain perpetually in this stage and may also alter the expression of the cellular genome (173). It appears that following infection by NDV, modifications occur in cell membrane physiology which bring about some features of transformation (96). However, the effect of NDV on cellular genetic material is likely to be minimal, although the fact that an RNA-dependent DNA polymerase has been found in one case of persistent infection, implies that there may be some involvement.

The results of the present work suggest that persistent infection by NDV causes transformation, which may be due to an alteration in cell membrane physiology.

b) Rate of mitosis and cell size of carrier cell-lines.

It will be noted from the results shown in Table I that there is considerable variation in the multiplication rates between the three species of kidney cells. In the case of OK pi the relatively low increase in the number of cells/ml at seven days (4.5×10^5 as opposed to 7×10^5 in PK pi) and the smaller colonies formed in semi-solid agar, might imply that the degree of transformation in the persistently infected sheep kidney cell line was less than in the other carrier cultures. However, it will be seen from Table II that the optimum cell diameter of OK pi cells is much greater than that of the other cell lines and that, in general, the cells with the fastest rate of multiplication have the smallest cell diameter. Thus,

T A B L E II a

THE SIZE OF CELLS OF VARIOUS CELL-LINES FOLLOWING TREATMENT WITH STV

CELL LINE	CELL SIZE (Diameter in μm)
PK pi	17.0
PK(W)K6	17.0
BK pi	19.0
MDBK	19.0
OK pi	25.0

Cells seeded at 75,000/ml and grown for two days were fed daily with MEM containing 5% serum. The monolayers were then treated with STV as described.

the rate of growth in OK pi is at least as rapid as that of the remaining types of cell but cell division does not take place until the cell is larger. (See Section 3). Indeed, because sheep kidney cell lines are difficult to maintain in continuous culture, it is possible that the transformation mediated by the persistent NDV infection is necessary for the adaptation of these cells to *in vitro* cultivation.

Discussion.

Although the medium employed for the nutrition of these cells by Edwards (93) was different from that used in the present work, her results for rates of cell growth are similar to those obtained during the past three years (1972-1975) with the exception of MDBK and BK pi cells. The increase in the rate of multiplication since 1972 is similar in both the persistently infected bovine kidney cells and their controls, and it is possible that this type of cell is better suited to culture in MEM.

II) RNA AND PROTEIN SYNTHESIS IN PERSISTENTLY INFECTED CELLS (BK pi).

Introduction.

Since it has been shown above that the rate of cellular multiplication is undiminished by persistent infection with NDV, it is probable that the total RNA and protein synthesis in the carrier cells is similar to that of uninfected controls.

a) RNA synthesis.

T A B L E II b

SIZE OF CULTURED CELLS IN INTACT MONOLAYERS (UNSTAINED)

CELL LINE	CELL SIZE (Diameter in µm)
PK pi	20.0
PK(W)K6	19.5
BK pi	16.0
MDBK	16.5
OK pi	32.0

Cells were grown as described under Table II a.

a) RNA synthesis.

The incorporation of ^{3}H-uridine into acid insoluble components is similar for both BK pi and its control line MDBK (Fig. 3). The cells were seeded at a lower concentration than normally used, in order that cell division and growth would be optimum when the label was added one day after seeding.

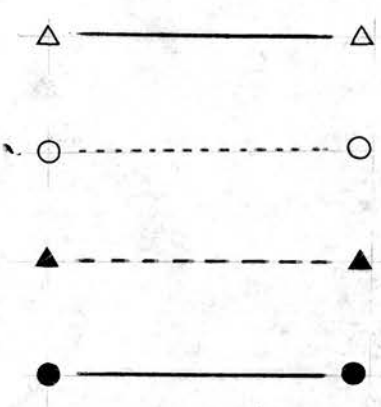
b) Protein synthesis.

The incorporation of amino acid into protein was indicated by the presence of ^{14}C amino acid label in acid insoluble cellular material. The initial rate of incorporation is similar for BK pi and MDBK cells but it declines after 8 hours in the persistently infected cells. This difference may best be interpreted as being due to a higher rate of turnover of polypeptides in the BK pi cell-lines.

Discussion.

It appears, therefore, that RNA and protein synthesis in BK pi cells is slightly increased compared with the control. This contrasts with the inhibition of these components in cells infected with most wild-type strains of NDV (80, 81). In the present case, the lack of effect on cellular metabolism may be associated with the diminished virulence of BK pi virus. However, although some workers (81) have related virulence to inhibition of cellular RNA synthesis, others have shown that lentogenic strains may prevent the formation of RNA in infected cells (80). Thus, inhibition of

Figure 3. The incorporation of ^{3}H-uridine and ^{14}C-protein hydrolysate into MDBK and BK pi cells, shown as c.p.m. of acid-insoluble material found at various intervals after addition of the labelled substances.



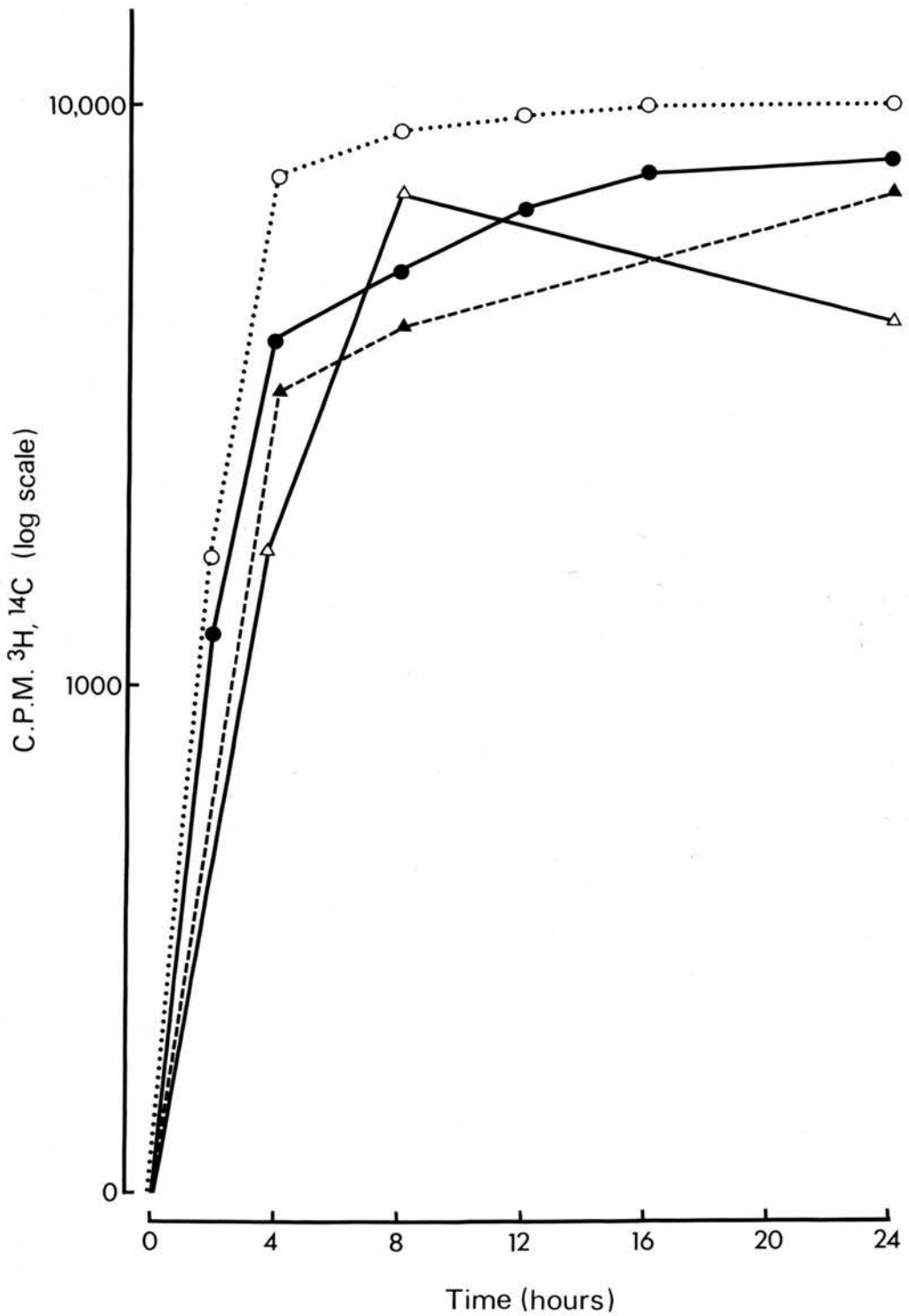
Incorporation of ^{14}C-protein hydrolysate into BK pi cells

incorporation of ^{3}H-uridine into BK pi cells

incorporation of ^{14}C-protein hydrolysate into MDBK cells

incorporation of ^{3}H-uridine into MDBK cells

Fig.3



of cellular RNA synthesis may be a normal consequence of infection with a virulent strain of NDV. Although the lack of this facility may be a contributory cause of avirulence in some strains, it is possible that reduced cytopathogenicity may have a different origin in others.

C / VIRUS- INDUCED PHENOMENA IN THE CARRIER CELL LINES

I) THE FORMATION OF INCLUSION BODIES IN PERSISTENTLY INFECTED CELLS.

Introduction.

Following infection of cells with NDV, cellular RNA and protein synthesis may or may not cease (as discussed above). However, the formation of viral proteins begins and, in productive infections, proceeds unabated. One of the consequences of the synthesis of progeny virus protein is the accumulation of viral antigen within the infected cell and these aggregations (which are not found in uninfected cells) are generally present in the cytoplasm and/or nucleus of most cells in which myxoviruses are replicating. These aggregations of material are detectable by light as well as by electron microscopy, although their size and shape varies with the method used. The presence of inclusion bodies provides a simple method of assaying the number of cells infected in a monolayer, and a study of the differences in quantity, location, time of appearance and nature of the type of material within them may be helpful in characterising the virus responsible. Cells infected with 'wild-type' strains of NDV contain acidophilic inclusions within 48 hours of infection (90), which may be seen after staining with haematoxylin and eosin (HES), although the inclusions are largest and most easily detectable

detectable from three to five days after infection. Staining with Giemsa or the use of phase contrast light microscopy delineates similar areas. By electron microscopy (E-M), viral material is seen within eight hours of infection but large inclusions are not generally seen until later. Fluorescent antibody staining (FAS) (using virus-specific antisera) will show the presence of viral antigens from about 6 hours after infection (92), that is, within 2 hours of the commencement of protein synthesis (51). Although inclusions are an indication of the accumulation of products induced during virus infection, plaques of fluorescing material do not necessarily correspond to inclusions shown by simple staining or E-M procedures. They may correspond to viral components (i.e. the nucleocapsid-like structures seen under the electron microscope), or to the sites of synthesis and accumulation of specific viral protein (e.g. the location of haemagglutinin by fluorescent antibody prepared against this viral component). Alternatively, as in the case of acidophilic inclusions, they may indicate aggregations of viral material of an unknown nature.

a) Inclusion bodies detected by staining with HES or Giemsa.

In our persistently infected cells, the percentage of cells shown to contain viral antigen by FAS was similar (approximately 90%) in all three cell-lines

lines (Tables III a,b,c) and did not appear to increase with the length of time from seeding. A similar percentage of cells in monolayers of OK pi contained cytoplasmic inclusions, which were stained with haematoxylin and eosin (HES), and the number remained constant throughout the period of incubation (Table IIIa). On the other hand, only about 30% of PK pi cells contained cytoplasmic inclusions that could be distinguished with HES (Table IIIc). It is thus of interest that on the day after seeding, HES revealed that 25% of the cells in a monolayer of BK pi contained acidophilic inclusions but that the number increased to 80%, five days later. An increase in the percentage of cells containing cytoplasmic inclusions is found in all three carrier cultures when the monolayers are stained with Giemsa. Thus in OK pi the number rises from 54% on the second day to 70% on the fifth day, while in BK pi the equivalent increase is from 25% to 62% and in PK pi from 31% to less than 42% (Tables IIIa,b,c).

Discussion.

Thus, it is possible that the inclusion bodies stained by these two acidophilic dyes may consist of two distinct 'components'. One component stains with HES and is found in all infected OK pi cells (i.e. those positive to FAS), and in a third of PK pi cells. The number of cells containing this component remains static throughout the period of incubation in both of

T A B L E S III a, b, c

FORMATION OF CYTOPLASMIC INCLUSIONS IN CULTURES OF OK pi, BK pi and PK pi CARRIER CELL-LINES

a)

OK pi

DAY	METHOD OF STAINING				
	FAS	HES	Giemsa	A.O.	P.C.
1	89 (93)‡	89	54	85	26
3	90	92***	65	N.D.	N.D.
5	90	N.D.	70	N.D.	N.D.
7	91**	90	N.D.	N.D.	N.D.

b)

BK pi

DAY	FAS	HES	Giemsa
1	86 (94)‡	25	15
3	83	55***	35
5	90	80	62
7	91**	N.D.	N.D.

c)

PK pi

DAY	FAS	HES	Giemsa
1	90 (86)‡	16*	31
3	90	30***	30
5	91	N.D.	42
7	92**	N.D.	N.D.

Figures show percentage of cells containing cytoplasmic inclusions.

FAS Fluorescent antibody staining, using antibody prepared against Herts strain of NDV ..... indirect method.

HES Haematoxylin and Eosin stain

A.O. Acridine Orange staining, viewed by ultra violet illumination

P.C. Phase contrast microscopy of unstained monolayers.

N.D. Not Done.

‡ Figures in brackets are the results obtained by Edwards (93)

* No inclusions were detected by this method from 1962-68 (210)

** 42%, 7% and 0% of respectively PK pi, BK pi and Ok pi contained perinuclear inclusions on Day 7 by FAS. The inclusions were larger in all 3 lines on Day 7.

*** 3%, 1% and 0% of respectively OK pi, BK pi and PK pi contained intranuclear inclusions by HES on Day 3.

of these cell-lines. However. in the case of BK pi cultures, the number of cells containing accumulations of this material increases during this time. On the other hand, the second component which stains with Giemsa, is present in an increasing percentage of cells in all three cell-lines but generally is never found in more than three-quarters of the number showing inclusions when stained with HES. These two 'components' may, in fact, be the same and the above results merely indicate differences in the affinity for Giemsa and HES depending on how recently the inclusion material was synthesised. It is evident, however, that the amount of material formed varies between cell-lines and it is probably significant that the greatest accumulations occur in the cell-line with the slowest rate of mitosis, namely OK pi. This observation is confirmed by the fact that the inclusions are largest in OK pi monolayers (Fig. 7) and smallest in PK pi (Fig. 9) with BK pi in between (Fig. 8).

b) Viral material detected by F.A.S.

A comparison of the pattern of intracytoplasmic aggregates seen by FAS (Figs. 4,5,6) with that of cells stained by HES, (Figs. 7,8,9), reveals that the cell-line (OK pi) with most eosinophilic inclusion material contains the least antigen. This suggests that the contents of the inclusions made visible by HES consist of either abnormal viral components or are of cellular origin. It may further be postulated that this material accumulates because it is not incorporated

Figure 4. OK pi cells, 3 days after seeding, stained with rabbit anti-NDV serum and counter-stained with anti-rabbit-globulin serum conjugated with fluorescein isothiocyanate, showing discrete aggregates of viral antigen in the cytoplasm (x 640)

Figure 5. Three-day-old BK pi cells, stained with fluorescent antibody in the manner described for Fig. 4, showing discrete but large cytoplasmic 'plaques' (x 640)

Figure 6. Three-day-old PK pi cells, stained with fluorescent antibody in the manner described for Fig. 4, showing large perinuclear 'plaques' as well as more diffuse, small, cytoplasmic aggregates (x 640)

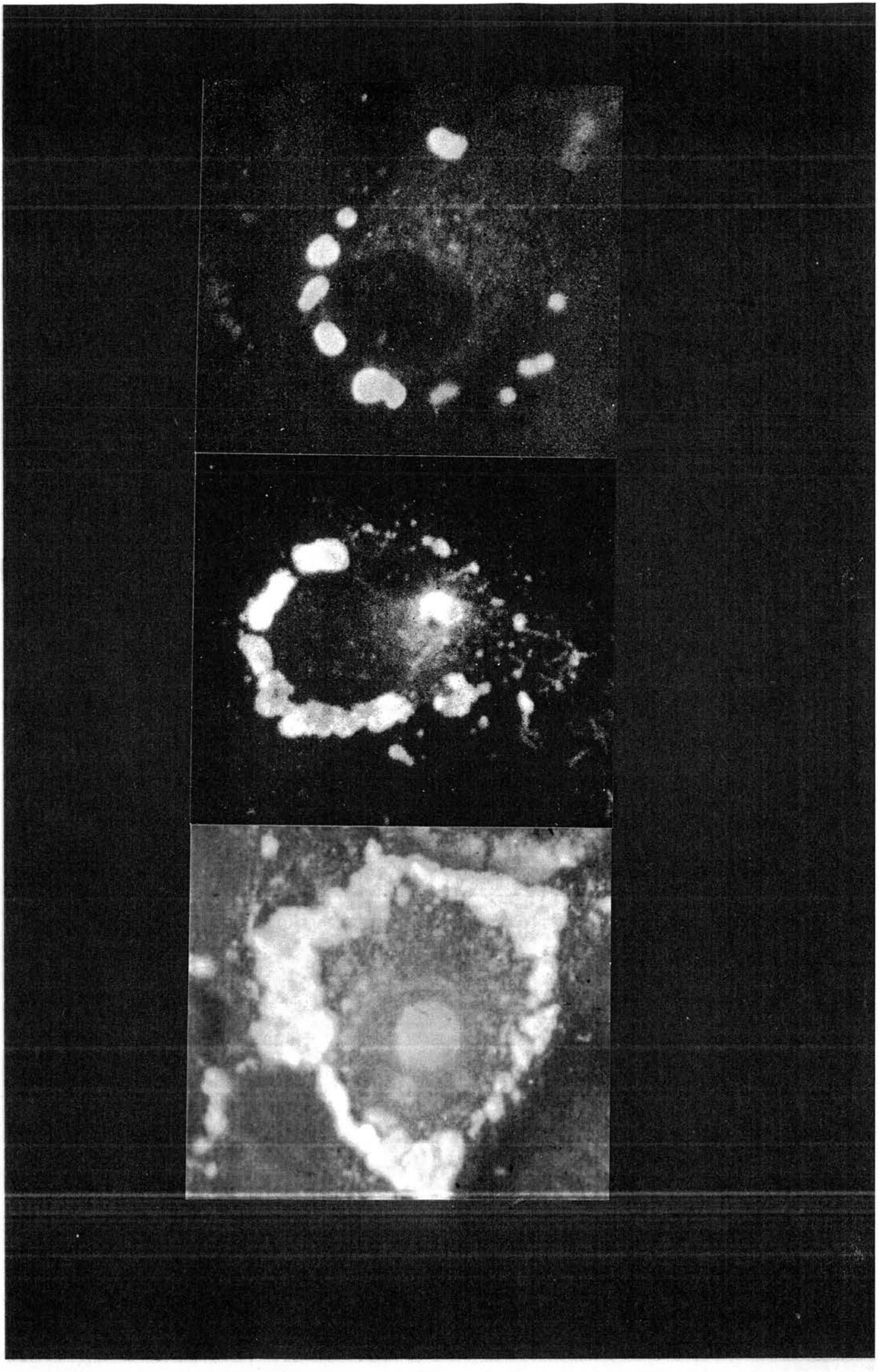
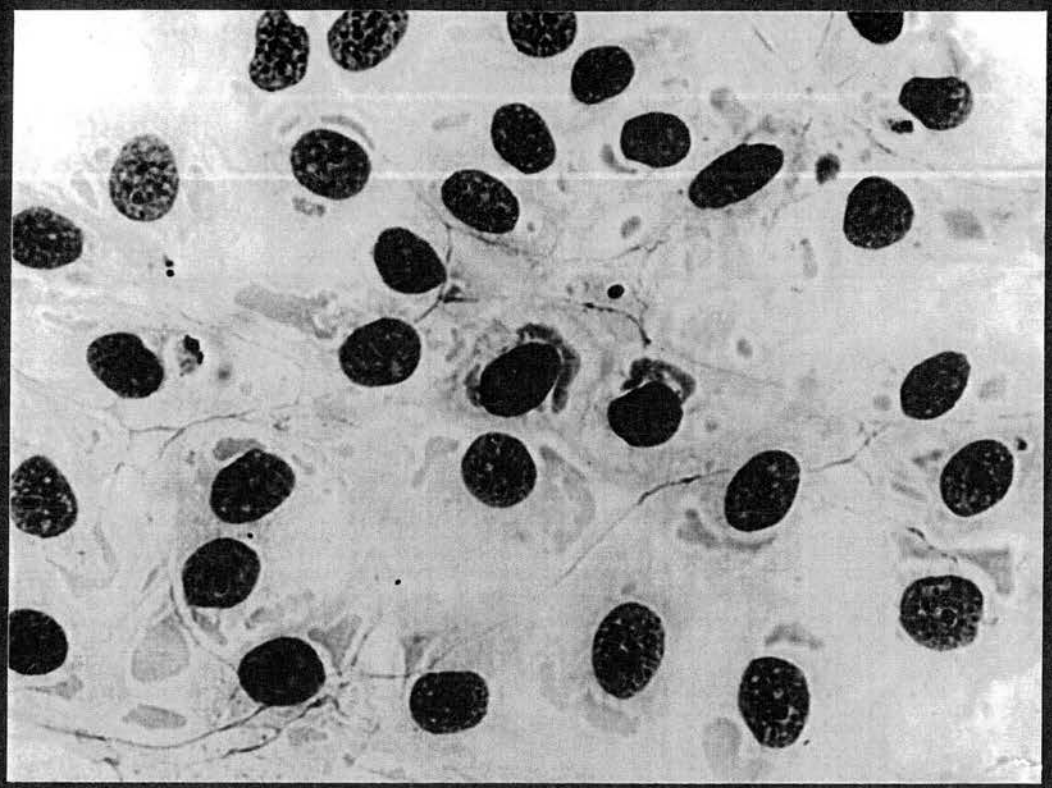


Figure 7. Three-day-old OK pi monolayer, stained with Giemsa, showing the presence of syncytia and numerous, prominent intracytoplasmic acidophilic inclusions (x 310)

Figure 8. Three-day-old BK pi monolayer, stained with haematoxylin and eosin, showing the presence of syncytia and a small number of cells with intracytoplasmic inclusions (x 310)



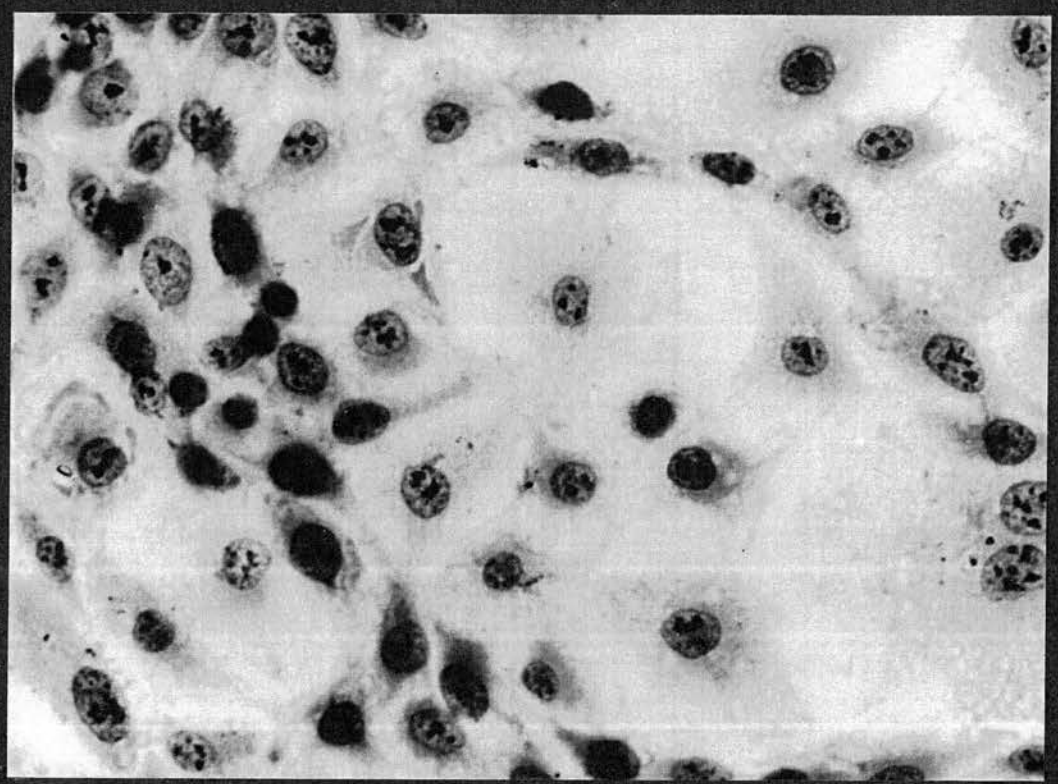
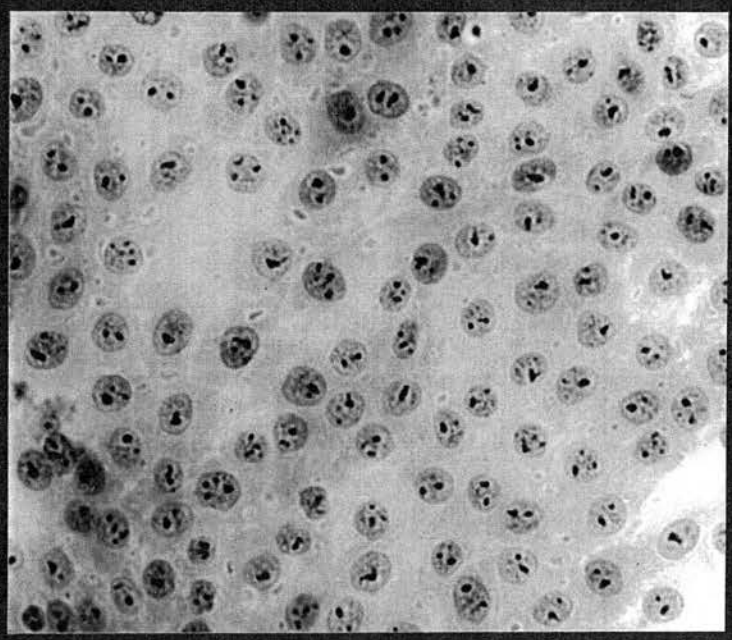
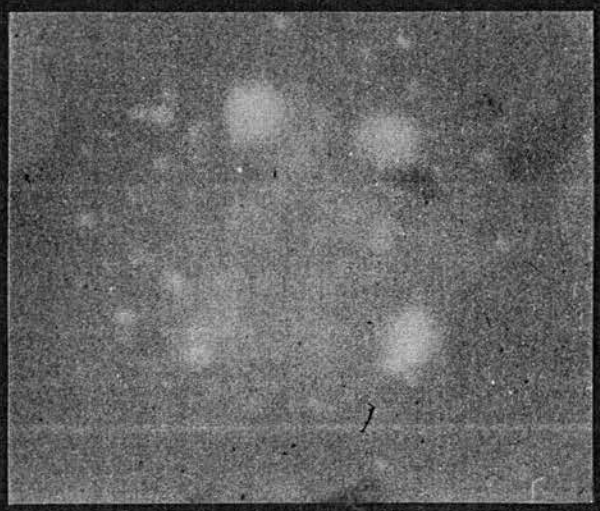


Figure 9. Three-day-old PK pi monolayers, stained with haematoxylin and eosin, showing numerous small intra-cytoplasmic inclusions (x 310)

Figure 10. Three-day-old BK pi cells, stained with fluorescent antibody in a similar manner to that described for Fig. 4 but employing antibody to the envelope proteins of NDV. Small cytoplasmic inclusions are seen in place of the large 'plaques' shown in Fig. 5. (Staining was faint and therefore the contrast obtained in this photograph is poor.) (x 640)





incorporated into mature virions.

c) Inclusion material detected by electron microscopy

It is also possible that a relationship exists between the development of inclusions and the nucleocapsid-like structures seen under the electron microscope (Fig. 13). Electron microscopy of all three cell-lines suggested that the large plaques of nucleocapsid-like material did not have so well defined a structure as that found in cells infected by 'wild-type' strains of NDV (Fig. 14). This is especially true of OK pi cells, although some 'normal nucleocapsid' may be present, particularly in PK pi cells.

Discussion.

It may thus be postulated that the inclusions seen in HES preparations consist of abnormal nucleocapsid which also stains with Giemsa, although in the latter case the stain is insensitive to constituents that have not recently been formed. The areas stained by FAS probably contain normal antigenic components whereas degenerate nucleocapsid does not and will not fluoresce. Haemagglutinin and other structural proteins of NDV will, of course, stain normally. It may be further deduced that much of the abnormal viral component will be found in both OK pi and BK pi cells but that it accumulates more slowly in the latter. On the other hand, synthesis of viral material in PK pi cells, more closely resembles that found in 'normal' infections with NDV.

II) FORMATION OF SYNCYTIA IN PERSISTENTLY INFECTED CELLS

Introduction.

Although several abnormalities can be observed within cells infected with NDV, as has been described on the previous pages, most of the readily discernible changes occur at the cell membrane. These may be divided into two groups:

a) those which take place because the physiology of the plasmalemma is altered, following viral infection. These include the closely related events of thinning of cell membrane (107), lectin-mediated agglutination of infected cells (96, 173) and cellular transformation.

b) those which occur following the incorporation of viral components into the plasmalemma giving rise to thickening of the cell membrane at these points. This category includes haemadsorption and cellular fusion. However, there is some evidence that the latter phenomenon may also be due to effects akin to those described in (a).

One of the predominant characteristics of paramyxovirus infections is the induction of cell fusion, both from the same clone and from widely differing species. The process may be initiated by virus located 'within' or 'without' the cell. The mechanism is probably the same in each case and requires the presence of the two envelope glycoproteins (73). Fusion from 'without', requires large numbers of virus particles,

particles, capable of being adsorbed to the cell membrane but which are not necessarily able to complete a full replicative cycle. Fusion from 'within' requires synthesis of the two glycoproteins and their accumulation in the plasmalemma, but the release of virus particles is not necessary (94). It will be shown in Section 2 that release of virus particles from any of the three persistently infected cell-lines is insufficient to account for fusion from 'without', for which concentrations containing 10,000 haemagglutinating units/ml are required (72). Thus, in the present work, only syncytial formation from within has been determined.

Fusion from within in persistently infected cells.

The percentage of homokaryons found in OK pi varies between 0.5 and 3.3 (Table IVa) and reaches a maximum 3-4 days after seeding. Heterokaryons are readily produced when this cell-line is grown in mixed culture with uninfected chick embryo fibroblasts and are larger, containing more nuclei per syncytium. The number of homokaryons formed in BK pi and PK pi monolayers (1.3%) is less than that found in OK pi, although it appears that the percentage of syncytia increases during the period of incubation. In all three cell-lines 8.0 - 12.0% of the cells are heterokaryons, when seeded together with chick embryo fibroblasts (Table IV a,b,c). It should be noted that neither heterokaryons nor homokaryons are formed

T A B L E IV a

CELL FUSION IN OK pi CELLS

DAY	HOMOKARYON FORMATION*			
	OCTOBER 72	MARCH 73	OCTOBER 73	APRIL 74
1	0.5	N.D.	N.D.	N.D.
2	1.6	N.D.	1.25	N.D.
3	1.8	1.4	N.D.	3.3**
4	2.1	N.D.	2.0	N.D.
5	1.9	N.D.	N.D.	N.D.
6	N.D.	N.D.	1.6	N.D.
7	N.D.	N.D.	N.D.	N.D.
8	N.D.	N.D.	1.0	N.D.

* Showing 3-5 nuclei per synkaryon.

** Associated with high haemagglutinin and haemadsorption levels.

Mixed cultures were made with chick fibroblasts, and heterokaryons, containing 10-20 nuclei were found by Day 3.

Figures indicate the percentage of cells forming synkaryons, observed in monolayers stained with HES, as described.

T A B L E IV b

CELL FUSION IN BK pi CELLS

DAY	HOMOKARYON FORMATION* IN	
	MARCH 1973	NOVEMBER 1973
3	0.5	N.D.
5	N.D.	1.3

*showing 2-3 nuclei per synkaryon

By day 5 in mixed culture with chick fibroblasts 8.0% of the cells were heterokaryons, each of which contained 10-20 nuclei.

Figures indicate the percentage of cells forming synkaryons, observed in monolayers stained with HES, as described.

T A B L E IV c

CELL FUSION IN PK pi CELLS

DAY	HOMOKARYON FORMATION* IN MARCH / APRIL 1973
1	1.2
3	1.3

*showing 2-3 nuclei per synkaryon

By day 5 in mixed culture with chick fibroblasts 12.0% of the cells were heterokaryons, each of which contained 10-20 nuclei.

Figures indicate the percentage of cells forming synkaryons, observed in monolayers stained with HES, as described.

formed by uninfected controls of chick fibroblasts or mammalian kidney cells. However, studies of the capacities of control cell-lines to undergo fusion when infected with 'wild-type' strains of NDV (Section 2), showed that syncytia were formed far more readily in chick embryo fibroblasts than PK(W)K6 monolayers and that MDBK cells were unlikely to develop into synkaryons.

Discussion.

Other workers have demonstrated similar differences in the ability of various cell-lines to fuse and have shown that the membrane of MDBK cells appears to be too thick to readily permit the formation of syncytia (77),(74). It may be significant that these workers also found that infection of MDBK cells was less likely to induce lectin agglutination than others, and the present results indicate a similar lack of ability to form 'colonies' in semi-solid agar. In conclusion, cellular fusion induced by viral infection requires not only the presence of the two envelope glycoproteins (73) but also an alteration in the physiology of the cell membrane, similar to that believed to be responsible for the transformation (and related phenomena) of infected cells. Indeed, in the case of 'fusion from without', it has been shown that generally fusion occurs in areas adjacent to the point of virus attachment and does not usually involve the virus membrane (72).

However, certain types of cell (notably MDBK) are unlikely to fuse because of the characteristics of their cell membranes. Thus, the percentage of syncytia found in persistently infected monolayers may depend on the features of the host cell membrane as well as the presence of normal viral protein. The ability of the persistent virus in our carrier cell-lines to induce cell fusion is discussed further in Section 2 (E) IV.

III) HAEMADSORPTION

Haemadsorption, which is another phenomenon associated with the activity of the virus in the persistently infected cell-lines, has been examined in relation to the synthesis of haemagglutinin and is discussed in Section 1 (E) I b (iii).

D / VIRUS SYNTHESIS IN PERSISTENTLY INFECTED CELLS

I) SYNTHESIS OF STRUCTURAL AND NON-STRUCTURAL VIRAL PROTEINS

Introduction.

All the phenomena so far discussed apart from fusion from without - which is not of relevance to the systems examined here, take place only after the synthesis of viral proteins has commenced. These changes are: the alteration in the physiology of the cell membrane necessary for transformation or lectin-mediated agglutination (96), the inhibition of formation of cellular proteins (80), the incorporation of viral proteins into the cell membrane prior to fusion from within (77) and the aggregation of viral products into inclusions.

By labelling with radioactive isotopes, synthesis of viral proteins has been detected within 2 hours of infection with NDV, and the three major structural components together with several possible non-structural polypeptides have been demonstrated in chick embryo fibroblasts (48,50).

Evidence of the synthesis of virus-specific proteins.

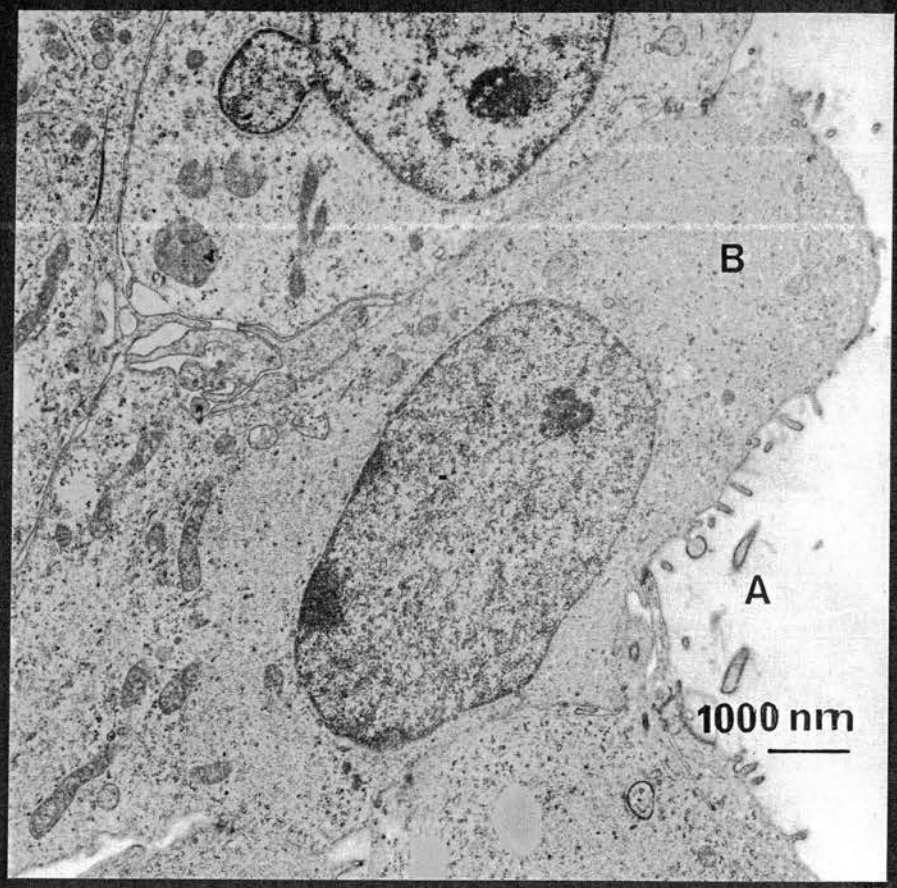
In this current work, formation of several non-cellular proteins has been shown to take place in BK pi cells and the detailed results are presented in Section 2. Moreover, because 90% of cells in all three persistently infected cell-lines contain antigen which can be stained with fluorescent antibody (Table III),

(Table III), it may be assumed that synthesis of viral polypeptides takes place within these cells. Furthermore, as the size of these inclusions (and those seen in HES) increases during incubation, it is likely that the viral proteins are being continuously formed.

It is generally agreed that about ten polypeptides are found in NDV released from infected chicken embryos (21) and, of these, six were found in BK pi cells. These were the two envelope glycoproteins (VGP 75, VGP 55), the nucleocapsid (VP 55), and three others of molecular weights 110,000, 49,000 and 42,000. In addition five more polypeptides were identified, some of which were believed to be precursors of structural proteins (Figs. 41, 42, Section 2). The presence of the haemagglutinin (VGP 75) was confirmed by FAS using antibody prepared against this protein (Fig. 10). Although 91% of BK pi cells showed specific fluorescence, the type of inclusion differed from that obtained by FAS, when antibody to whole virus was used (Fig. 5). In most preparations examined under the electron microscope, nucleocapsid could be seen within cells of BK pi, PK pi and OK pi, although it was sometimes degenerate compared with that seen in cells infected with 'wild-type' NDV (Figs. 11, 12, 13, 14), and nucleocapsid-like protein was also extracted from monolayers of BK pi (Section 2, G(I). Furthermore, 'differentiation' of areas of cell membrane takes place in all three persistently infected lines, and involves a typical alignment of nucleocapsid and haemagglutinin showing that at least

Figure 11. Ultrathin section of BK pi cells showing the presence of cytoplasmic aggregations of nucleocapsid-like structures (B) and areas of differentiated cell membrane with budding virus particles (A). Stained with uranyl acetate and lead citrate (x 10,000)

Figure 12. Ultrathin section of BHK cells one day after infection with the B1 strain of NDV showing similar (A) budding virus particles to those observed in Fig. 11. Stained with uranyl acetate and lead citrate (x 10,000)


B
A
1000 nm

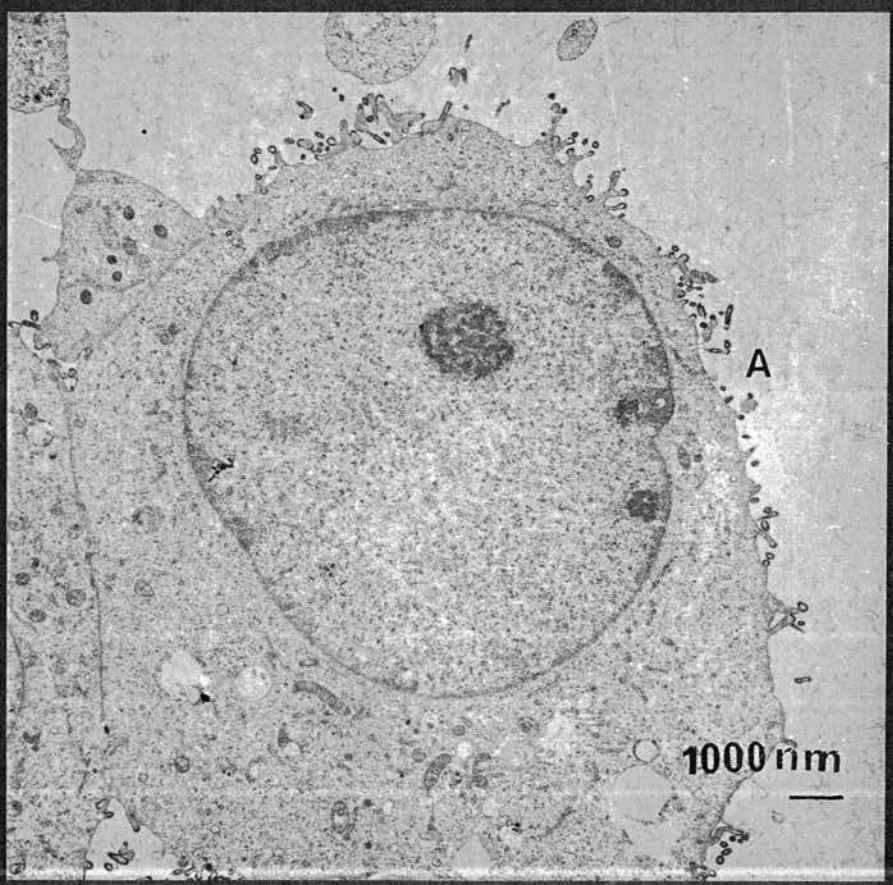
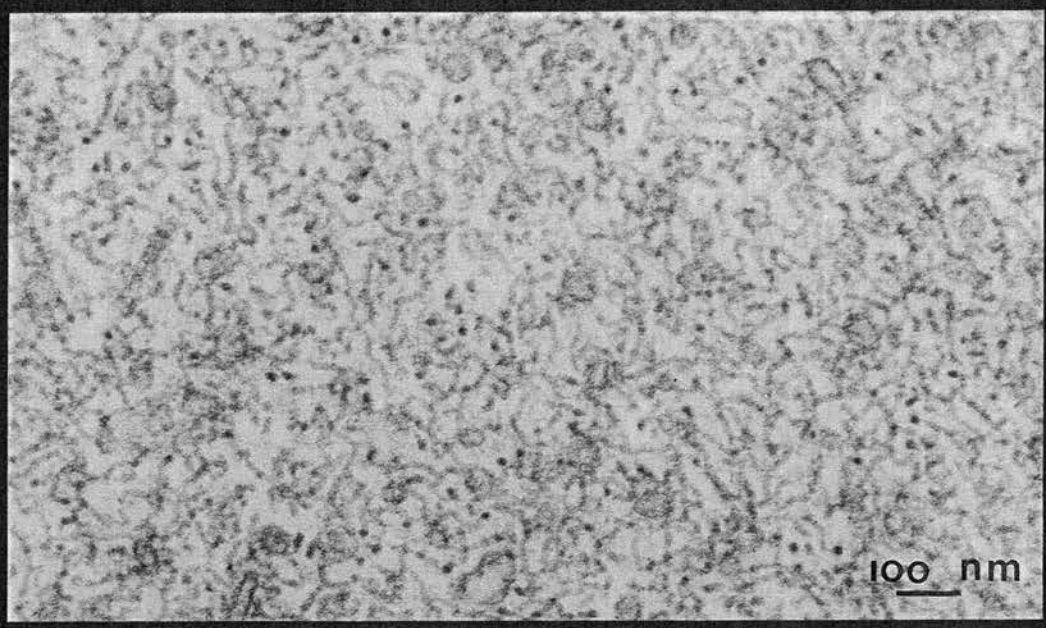

A
1000 nm

Figure 13. Nucleocapsid-like material in a BK pi cell at a higher magnification than in Fig. 11. Stained with uranyl acetate and lead citrate (x 80,000)

Figure 14. Nucleocapsid material in a BHK cell infected with the B1 strain of NDV at a higher magnification than that in Fig. 12. Note the greater density of the nucleocapsid strands compared with those seen in Fig. 13. Stained with uranyl acetate and lead citrate (x 80,000)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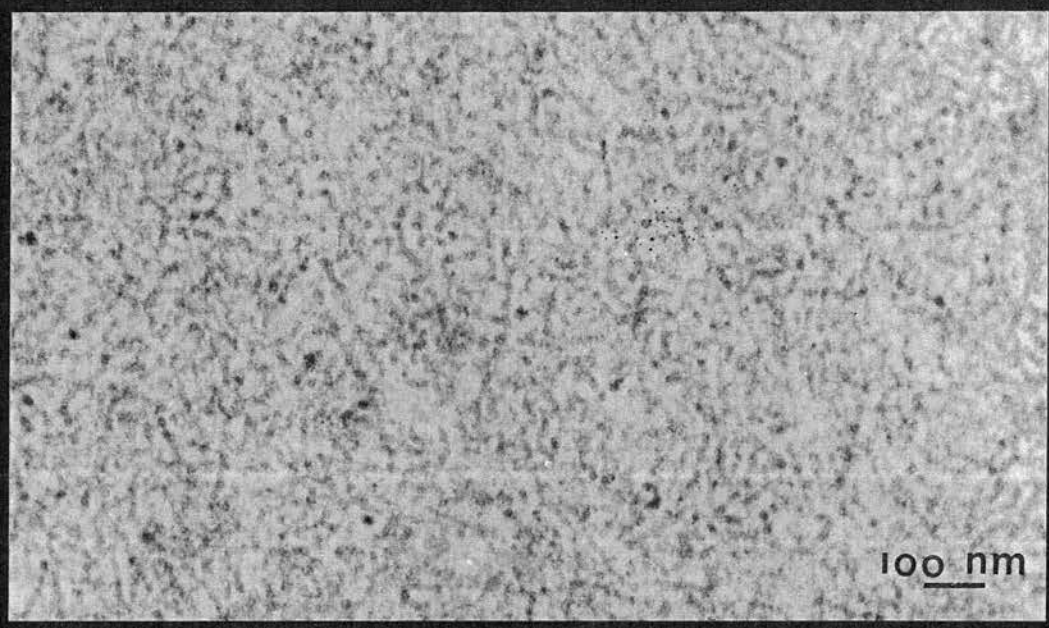

100 nm

least the majority of proteins necessary for the release of mature virus must be synthesised within the cells (Figs. 15, 16). Budding virus was seen in electron micrographs prepared from PK pi, BK pi and OK pi monolayers on the seventh day after seeding, as well as at earlier times. Large inclusions of nucleocapsid are present in rapidly dividing cells, within the first day of incubation, implying that they are carried in the cytoplasm of dividing cells. Thus the presence of large amounts of nucleocapsid material does not indicate a high rate of synthesis and this is borne out by the small size of the nucleocapsid peak shown in polyacrylamide gel electrophoretograms of BK pi cells which had been labelled with radioactive amino acids (Fig. 42). Moreover, it would appear that the formation of viral proteins in BK pi cells is more closely linked with that of the host cell than that is the case with 'wild-type' NDV, because actinomycin D treatment has a greater inhibitory effect on the former than on the latter, (Section 3(IV)).

Discussion.

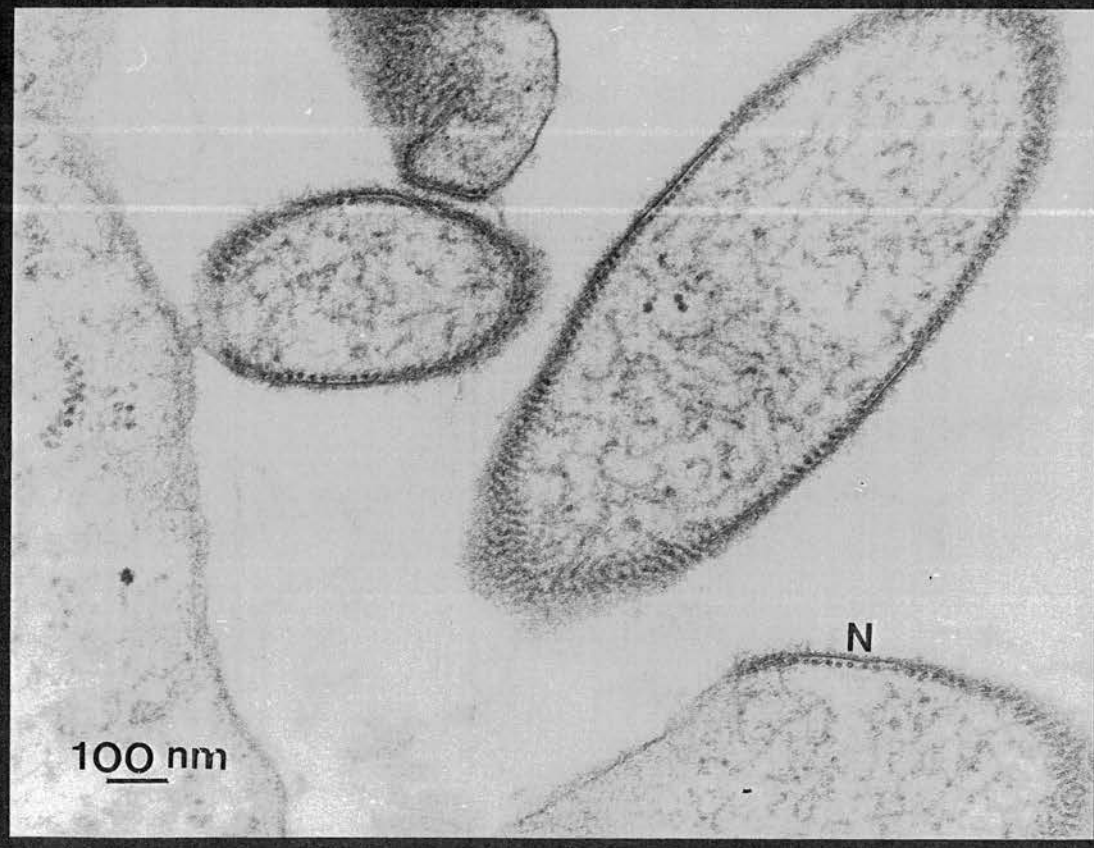
In conclusion, the majority of viral proteins appear to be replicated in the persistently infected cells and their presence can be demonstrated by a variety of methods. However, the rate of synthesis is slow and it is probable that some of the proteins are incompletely formed (Section 2).

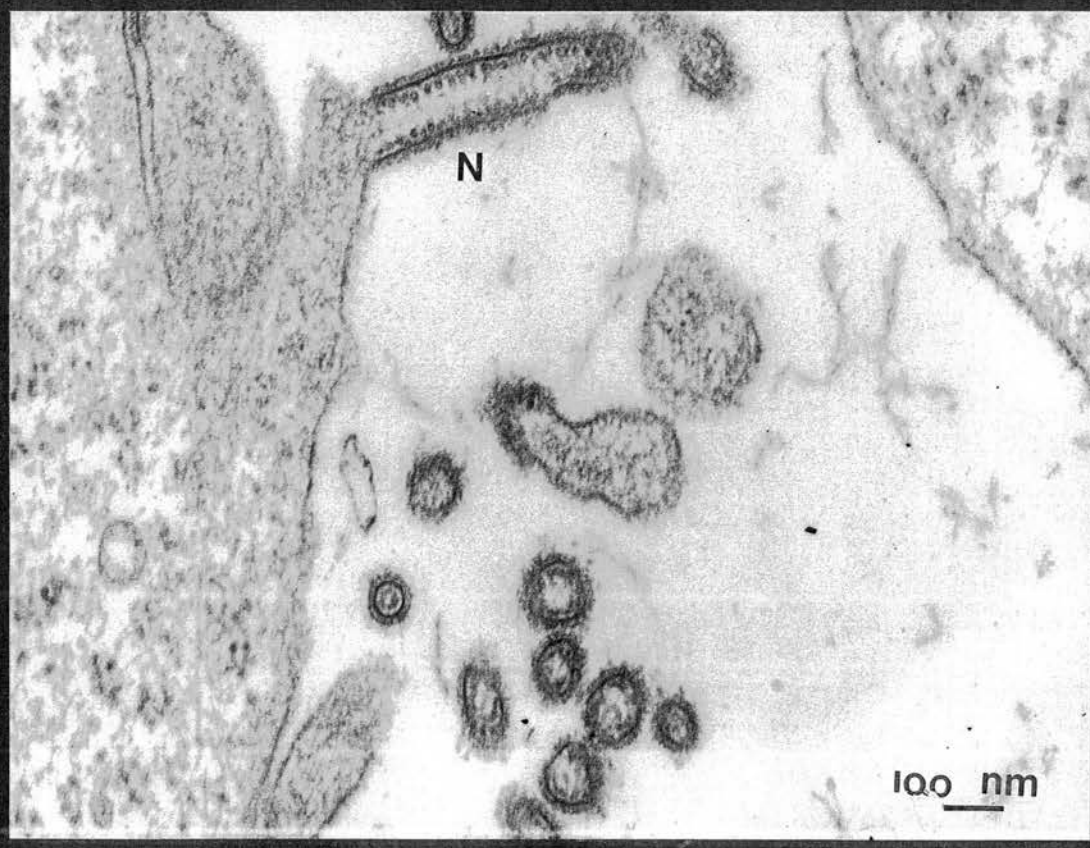
II) SYNTHESIS OF VIRAL RNA IN PERSISTENTLY INFECTED CELLS

Introduction.

Figure 15. The membrane of a BK pi cell at a higher magnification than in Fig. 11, showing nucleocapsid aligned beneath haemagglutinin-neuraminidase spikes (N). Stained with uranyl acetate and lead citrate (x 80,000)

Figure 16. The membrane of a BHK cell infected with the B1 strain of NDV, at a higher magnification than in Fig. 12, showing nucleocapsid aligned below a differentiated region in a manner similar to that observed in Fig. 15. Note, however, the smaller diameter of the budding virions and the greater density of the nucleocapsid material. Stained with uranyl acetate and lead citrate (x 80,000)


N
100 nm


N
100 nm

Introduction.

Since it has been shown above that viral protein synthesis occurs in all three persistently infected cell-lines, viral RNA synthesis must also be presumed to take place.

Staining of carrier cell-lines with acridine orange.

The formation of viral RNA is supported by the fact that staining of all three cell-lines with acridine orange, revealed orange cytoplasmic inclusions, typical of accumulations of single stranded RNA (141), which were not present in uninfected controls. Similar inclusions were observed in HeLa cell monolayers that had been experimentally infected with the Herts strain of NDV. It should be noted that in OK pi cultures, the percentage of cells showing inclusions is of the same order as those showing fluorescence when stained by FAS (Table III a).

Discussion of the normality of the RNA formed in the carrier cell cultures.

The RNA incorporated into the viral genome is synthesised by means of at least one replicative intermediate (36). It is therefore possible, in the infections under examination here, that, although normal messenger RNA is formed, the virion RNA is incomplete. Nevertheless, evidence presented in Section 2 (D) IV suggests that passage through chicken cells of virus released from PK pi or BK pi monolayers, results in the resumption of normal properties in the progeny,

progeny, which suggests that the RNA of the viral genome is normal.

On the other hand it is probable that the rate of synthesis in the persistently infected cells is lower than that found in wild-type cultures in which wild-type NDV is present. It is known that actinomycin D, which prevents the transcription of DNA into RNA, will inhibit cellular RNA synthesis without affecting the replication of RNA by NDV, because this does not involve DNA. However, when BK pi and MDBK cells were treated with actinomycin D (Section 3 (IV) the total inhibition of synthesis of RNA was only slightly less in the persistently infected cells. This suggests either that viral RNA synthesis was largely mediated through host or viral DNA transcription or that the rate of synthesis of viral RNA was always low. In this connection it should be noted that there is recent evidence that viral RNA-dependent DNA transcriptase is present in other cell systems persistently infected with NDV (45).

E / BIOLOGICAL ACTIVITIES OF CARRIER CELLS

I) VIRAL ACTIVITIES ASSOCIATED WITH PERSISTENTLY INFECTED CELLS.

Introduction.

Apart from the alterations in the character of the cells described above, and the detection of virus-specific proteins, other biological activities may be used to detect infection with NDV.

a) Infectivity

The release of infectious virus from the three lines of persistently infected cell was extremely low and detailed results are given in Section 2. However, it was apparent that this was due to liberation of virus of reduced infectivity rather than to small numbers of mature virions being produced. Thus the infectivity per cell was low (Table XVII) as was infectivity per haemagglutinating unit (HAU) and infectivity per μg of purified viral protein (Table XV, XVI). It is generally found that for a particular strain of NDV the amount of infectious virus in a sample varies directly with the number of HAU present. Thus, infectivity per HAU remains constant whatever the haemagglutinin (HA) titre of the material. In this way, assay of the HA released from persistently infected monolayers provides an estimate of production of infectious virus, so long as the infectivity/HAU ratio is known for the particular strain under examination. It was found that the amount of HA (and thus infectious

infectious virus) released, varied considerably from day to day and month to month, in the same cell-line, and indeed, it was shown that even when a negative HA titre was recorded, concentration of the exhaust cell culture medium revealed the presence of both haemagglutinin and infectious virus.

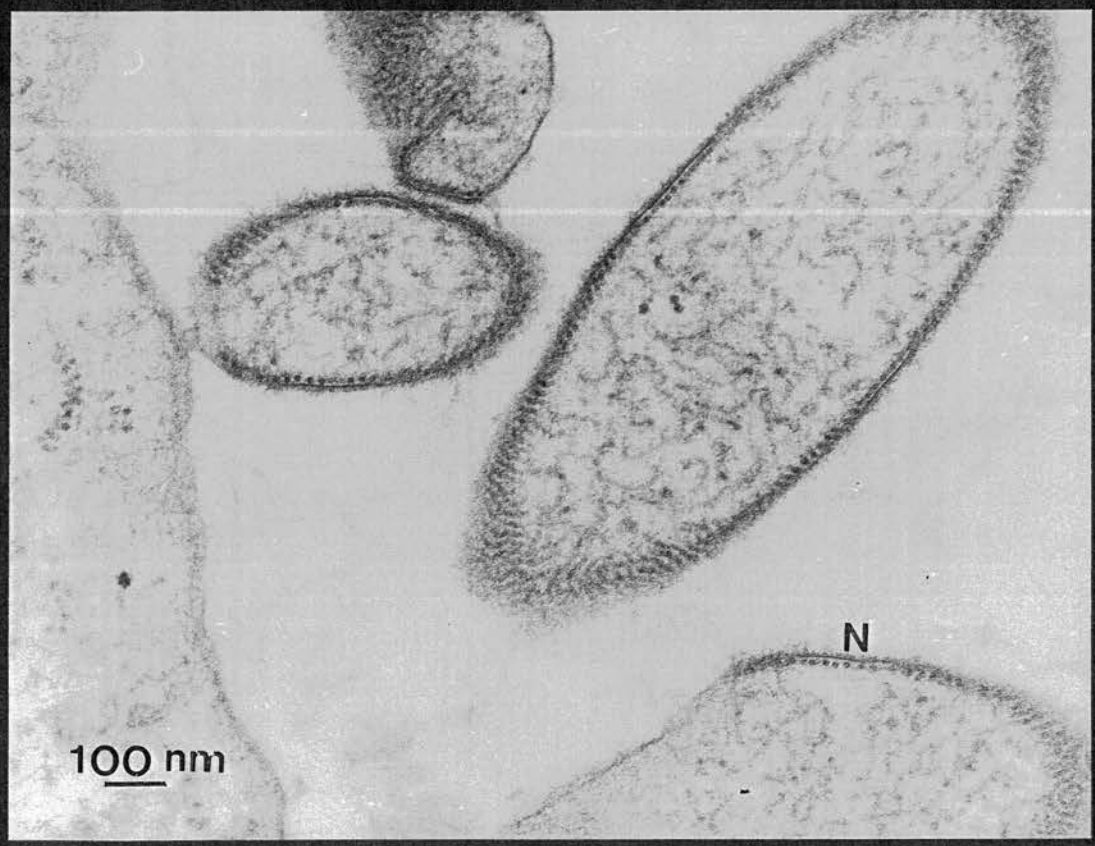
The hypothesis that the amount of virus released in these cultures was variable and that the virions were of low infectivity, was confirmed by examination of the persistently infected monolayers under the electron microscope. The areas from which virus is budding is 'differentiated' and appear thickened with nucleocapsid aligned below a spike-bearing membrane. Such regions were seen in ultra-thin sections of all three persistently infected cell-lines, within 24 hours of seeding and until at least 7 days thereafter. However, on some occasions, budding virus was more difficult to find and these usually corresponded to periods of reduced release of haemagglutinin. The appearance of the budding virus was very similar to that seen in cells infected by 'wild-type' strains of NDV (Figs. 15,16). However, the size of the released virions observed by this method or by examination of negatively stained preparations of pelleted virus was generally larger in the case of BK pi virus than the B1 strain of NDV virus released from BHK cells (Figs. 17, 18).

b) Haemagglutination

Introduction.

Figure 17. Negatively stained BK pi virion, obtained after tartrate density gradient purification. The particle is very large and pleomorphic. It is surrounded by a membrane with radially arranged spikes and contains nucleocapsid. Stained with phosphotungstic acid (PTA) (x 80,000)

Figure 18. Negatively stained B1 virion, showing typical NDV morphology, similar to that observed in virus released from BK pi cells (Fig. 17) but smaller in size. Stained with PTA (x 80,000)


N
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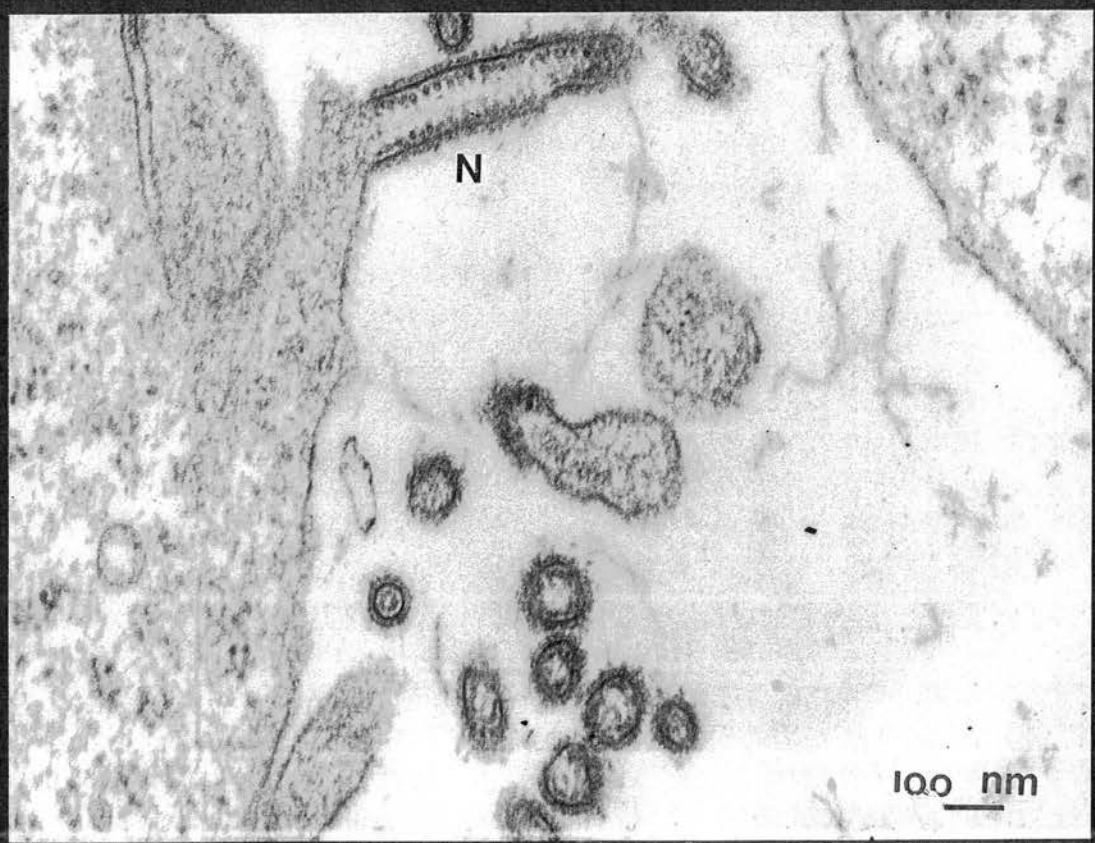
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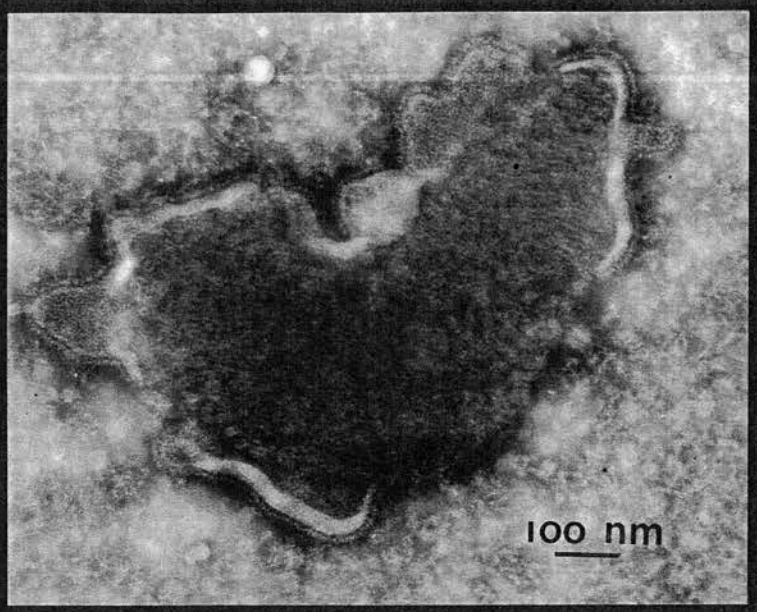
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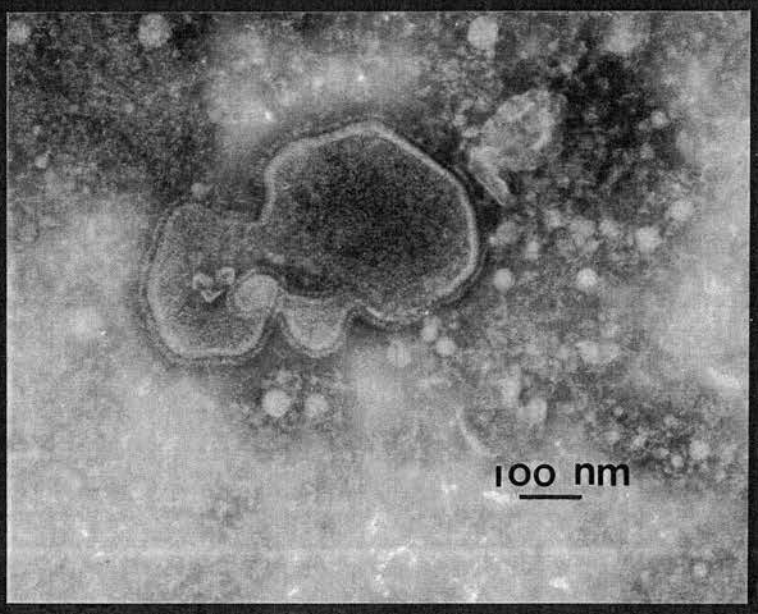

100 nm

Introduction.

Viral haemagglutinin (HA), which is a biological activity of the large envelope glycoprotein (VGP 75) of NDV (21), can be measured in the released virion (Tables V and VI, and section 2) or the infected cell (Table VII). The presence of haemagglutinin in the cell membrane may be assayed by haemadsorption.

i) Released haemagglutinin (RHA).

The release of haemagglutinin from infected cells is usually related to the budding off of mature NDV. However, it may also be associated with 'differentiated' membranes from dead cells, which may be floating in the nutrient medium, although it is unlikely that the latter source will be found in any but senescent or overcrowded monolayers. The amount of haemagglutinin present in the supernatant fluids of persistently infected cultures was variable, whether measured 'per ml' of medium or 'per cell' of the monolayer. It was shown that in purified virions of BK pi and PK pi virus, the HA/µg protein was lower than that of NDV grown in embryonated hens' eggs (Section 2) Table XXIII, and this clearly indicates that the virus produced by the persistent infections is defective in HA as well as infectivity. Despite this, it is still possible to assess the differences between the cell-lines on the basis of haemagglutinin released per cell or per ml. of supernatant fluid and this will be discussed below, in relation to the production of cell-associated HA and haemadsorption. It is notable that all the persistently

TABLE Va

THE RELEASE OF HAEMAGGLUTININ FROM OK pi CARRIER CELL CULTURES

DAY	HAEMAGGLUTININ RELEASED FROM OK pi CELLS (HAU/ml)							
	1972 Oct.	1973 Feb.	1973 Apr.	1973 Aug.	1973 Oct.	1973 Dec.	1974 June	1974 Dec.
1	0	0	N.D.	0	0	N.D.	N.D.	N.D.
2	0	N.D.	8	0	0	N.D.	8	N.D.
3*	0	4	N.D.	0	0	4	N.D.	8
4	0	N.D.	N.D.	4	0	N.D.	N.D.	N.D.
5	0	N.D.	N.D.	N.D.	0	N.D.	4	N.D.
6	0	4	16	4	0	8	4	N.D.
7*	0	N.D.	N.D.	N.D.	0	N.D.	N.D.	32
8	0	4	N.D.	N.D.	0	N.D.	N.D.	N.D.
9	N.D.	8						
10	N.D.	8						
11	N.D.	N.D.			N O T D O N E			
12	N.D.	8						
13	N.D.	N.D.						
14	0	N.D.						

*Edwards (93) gives figures of 1 and 3 for released HAU/ml on days 3 and 7.

The cultures were seeded in MEM at 100,000 cells/ml containing 10% calf serum and refed every three days with MEM plus 5% calf serum.

T A B L E V b

THE RELEASE OF HAEMAGGLUTININ FROM PK pi CARRIER CELL CULTURES

DAY	HAEMAGGLUTININ RELEASED FROM PK pi CELLS (HAU/ml)					
	1972	1973				1974
	Oct.	Feb.	April	Aug.	Oct.	Dec.
1	N.D.	N.D.	N.D.	N.D.	O	N.D.
2	8	N.D.	16	6	O	N.D.
3*	N.D.	6	N.D.	8	O	4
4	12	6	16	N.D.	O	N.D.
5	N.D.	4	N.D.	N.D.	O	N.D.
6	N.D.	6	N.D.	N.D.	4	N.D.
7	N.D.	4	16	8	4	24
8	16	8				
9	N.D.	8				
10	8	4				
11	16	N.D.		N O T D O N E		
12	N.D.	8				
14	32	N.D.				
17	64	N.D.				
25	N.D.	8				

*Edwards (93) gives figures of 4 and 24 for released HAU/ml on days 3 and 7.

The cultures were seeded in MEM at 100,000 cells/ml containing 10% calf serum and refed every three days with MEM plus 5% calf serum.

TABLE Vc

THE RELEASE OF HAEMAGGLUTININ FROM BK pi CARRIER CELL CULTURES

DAY	HAEMAGGLUTININ RELEASED FROM BK pi CELLS (HAU/ml)						
	1972	1973				1974	1974
	Oct.	Feb.	April	Aug.	Oct.	June	Dec.
1	N.D.	N.D.	N.D.	N.D.	O	N.D.	N.D.
2	N.D.	4	8	N.D.	O	N.D.	16
3*	8	4	16	N.D.	O	4	4
4	4	8	N.D.	N.D.	O	N.D.	N.D.
5	N.D.	4	N.D.	N.D.	O	4	N.D.
6	24	4	N.D.	4	6	N.D.	N.D.
7*	N.D.	4	4	4	N.D.	N.D.	N.D.
12	N.D.	8	64	16			
14	N.D.	N.D.	32	16			
15	16						
17	16			NOT DONE			
18	16						
24	16						
27	16						
30	16						

*Edwards (93) gives figures of 2 and 6 for HAU/ml released on days 3 and 7.

The cultures were seeded in MEM at 100,000 cells/ml containing 10% calf serum and refed every three days with MEM plus 5% calf serum.

T A B L E VI

THE RELEASE OF HAEMAGGLUTININ FROM OK pi, PK pi and BK pi CARRIER CELL LINES

(HAU/CELL x 10^6)

DAY	CELL LINE		
	PK pi*	BK pi*	OK pi**
1	9	N.D.	N.D.
3	17	19	25
7	33	35	35
14	N.D.	35	64
21	40	N.D.	N.D.
25	64	N.D.	N.D.

*) assayed in April 1973

**) assayed in December 1974

The cells were seeded in MEM containing 10% calf serum and refed every three days with MEM plus 5% calf serum.

N.D. Not Done

T A B L E VII

CELL-ASSOCIATED HAEMAGGLUTININ (CA HAU/CELL X 10^6) IN CARRIER CELL-LINES

DAY	CELL LINE		
	PK pi*	BK pi*	OK pi**
1	130	55	196
3	162	85	150
5	260	71	56
7	320	93	53
10	284	168	99
14	N.D.	127	100

*) assayed in February 1973

**) assayed in June 1974

The cells were seeded in MEM containing 10% calf serum and refed every three days with MEM plus 5% calf serum.

N.D. Not Done.

persistently infected monolayers apparently yield more HA, the longer the cells are held without reseeding (Table VI). This effect was associated with the presence of large amounts of cell debris and is considered to be due to the increased numbers of dead cells in older cultures (vide supra).

ii) Cell-associated haemagglutinin (CAHA)

Measurable haemagglutinin activity is found at all times, after seeding, in the three lines persistently infected with NDV. There is an increase in the number of HAU/cell in the case of PK pi and BK pi, which is especially marked in the latter, but during the first week of growth of OK pi cells, there is a decline in this ratio (Table VII). It thus appears that synthesis is slower in OK pi than in the other two cell lines.

Discussion.

The rate of accumulation of viral haemagglutinin in the carrier cells may be related to the extent of the formation of inclusions distinguished by HES. Thus, there is a large amount of inclusion material in OK pi cells but its rate of formation is slow and this may be associated with the reduced synthesis of haemagglutinin in this cell-line. In contrast, there is a gradual increase in the amount of inclusion material detectable in BK pi monolayers, which may be connected with the increase in CAHA titre recorded in these cultures. Indeed, in view of the possible relationship between CAHA titre and the percentage of cells with inclusions,

inclusions, it is likely that the initially low values for CAHAU/cell recorded in the case of BK pi cells (Table VII), are due to the small number of cells synthesising measurable quantities of haemagglutinin. Thus, the high levels of CAHA/cell found after several days of incubation may be related to the increase in the number of cells with inclusions (Table III b) and may therefore, be due to a greater number of cells synthesising haemagglutinin, rather than a raised rate of viral protein formation throughout the monolayer.

iii) Haemadsorption

Introduction.

Haemadsorption provides a useful method for assaying those cells which are synthesising haemagglutinin and hence may be employed to estimate the number of infected cells in a given population. However, although generally there is coincidence between the number of cells shown to be infected by the adherence of erythrocytes to their surface and the percentage of cells in a monolayer that contain inclusions when examined by FAS (see Section 2(D)), not all infected cells may be in the state in which haemadsorption can be demonstrated. Thus it is to be expected that the number of infected cells will always be greater than the quantity of cells seen to adsorb red blood cells. On the other hand, Duc-Ngyen has demonstrated that haemadsorption in a paramyxovirus infection is not always related to the incorporation of haemagglutinin into the cell membrane (60). He has shown by electron

electron microscopy that erythrocytes adhered to the surface of cells infected by mumps virus, many hours before mature virions were released and that the nature of haemadsorption was of two types:

α) the membrane of the cell was not 'differentiated' and so was unlikely to contain haemagglutinin. Nevertheless, red blood cells adhered to the cell surface but were separated from it by a distance of 10-20 nm.

β) Haemagglutinin spikes could be seen on the cell surface and the erythrocytes were attached directly to the differentiated plasmalemma. However, this phenomenon was not seen until shortly before the release of mumps virus began.

Electron microscope studies of BK pi cells.

In the present work, the nature of haemadsorption was studied under the electron microscope (E-M) at one day and seven days after seeding BK pi cells. (Figs. 20 & 21a,b). It was shown that in these cells, haemadsorption occurred both in areas of the cell membrane that were differentiated and in areas that were not (Fig. 21a). However, although gaps of up to 20 nm were seen on occasion, direct contact between the membranes of the erythrocytes and the infected cells apparently occurred, both in regions bearing spikes and in places where there was no evidence of viral structures (Fig. 21a).

Discussion.

Discussion.

The cause of haemadsorption to undifferentiated cell membranes may be associated with the changes in the physiology of the plasmalemma, that occur following infection with NDV and that are related to agglutination of cells by lectins, transformation and other events described above. However, these activities do not require the presence of viral components in the cell membrane, although synthesis of viral proteins is essential (96). Therefore, it is probable that haemadsorption exhibited by a BK pi cell is not necessarily indicative of haemagglutinin synthesis, although it does imply that the cell is infected and that viral proteins are being formed.

The effect of STV on haemadsorption.

A further factor may disguise the capacity of a cell to haemadsorb. It was shown that treatment with STV (or trypsin alone but at the same concentration as used in STV) reduced not only the percentage of cells showing haemadsorption but also the number of red blood cells per affected cell (Tables VIII a, b, c). However, within three days of treatment, 90% of monolayers of all three persistently infected lines usually haemadsorbed, thereby showing correspondence with assays of infectivity employing FAS.

Discussion.

This phenomenon was unrelated to the rate of mitosis and is probably related to removal of

T A B L E VIII a

HAEMADSORPTION IN OK pi CARRIER CELL CULTURES

DAY	PERCENTAGE OF CELLS SHOWING HAEMADSORPTION		NUMBER OF ERYTHROCYTES ADSORBING TO EACH INFECTED CELL	
	BY CELLS IN THE INTACT MONOLAYER	BY CELLS ISOLATED FOLLOWING TREATMENT WITH STV	WITH CELLS IN THE INTACT MONOLAYER	WITH CELLS ISOLATED FOLLOWING TREATMENT WITH STV
1	85±, 20*, 2**	2**	N.D.	N.D.
3	32*, 2**	N.D.	N.D.	N.D.
5	94*, 5**	1**	12.1**	2.0**
7	95*, 5**	2**	13.6**	7.1**

* in October 1972 ** in October 1973
± Quoted by Edwards (93), for the period 1968-1972.

T A B L E VIII b

HAEMADSORPTION IN PK pi CARRIER CELL CULTURES

DAY	PERCENTAGE OF CELLS SHOWING HAEMADSORPTION		NUMBER OF ERYTHROCYTES ADSORBING TO EACH INFECTED CELL	
	BY CELLS IN THE INTACT MONOLAYER	BY CELLS ISOLATED FOLLOWING TREATMENT WITH STV	WITH CELLS IN THE INTACT MONOLAYER	WITH CELLS ISOLATED FOLLOWING TREATMENT WITH STV
1	99±, 85***, 20**	20**	7.4**	1.6**
3	100***, 35**	33**	5.0**	2.3**
5	100***	N.D.	N.D.	N.D.
7	100***	N.D.	N.D.	N.D.

** in October 1973 *** in April 1973

± Edwards (93) 1968-1972.

T A B L E VIII c

HAEMADSORPTION IN BK pi CARRIER CELL CULTURES

DAY	PERCENTAGE OF CELLS SHOWING HAEMADSORPTION		NUMBER OF ERYTHROCYTES ADSORBING TO EACH INFECTED CELL	
	BY CELLS IN THE INTACT MONOLAYER	BY CELLS ISOLATED FOLLOWING TREATMENT WITH STV	WITH CELLS IN THE INTACT MONOLAYER	WITH CELLS ISOLATED FOLLOWING TREATMENT WITH STV
1	86±, 40*, 67**	39**	2.7**	2.6**
3	86*, 60**	54**	4.7**	4.8**
5	92*, 64**	45**	4.8**	4.0**
7	75**	75**	7.8**	4.5**

* in October 1972 ** in October 1973 ±Edwards (93) 1968-72

of haemagglutinin spikes from the cell surface. However, in view of the electron-microscope observations reported above, it is likely that the high percentage of cells that adsorb erythrocytes, while being correlated with the synthesis of viral proteins, is only in part due to the formation of haemagglutinin and its incorporation into the membrane of infected cells.

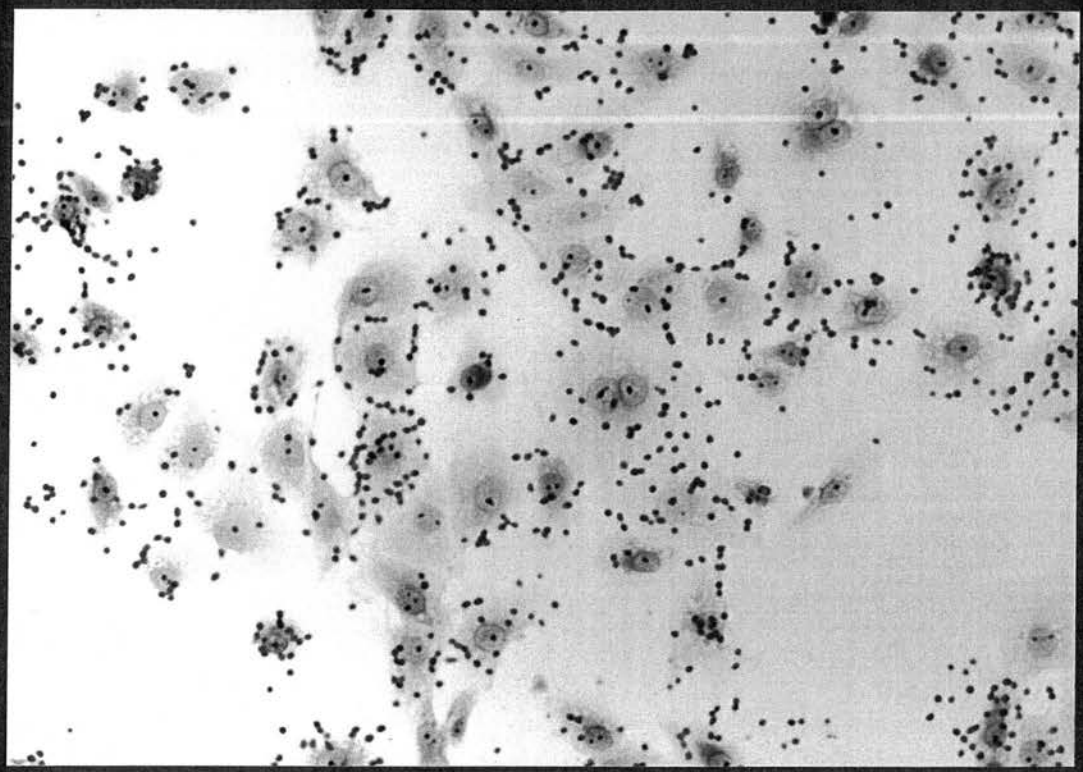
Fluctuations in the percentage of cells haemadsorbing in the carrier cell-lines.

Comparison of the results in Tables V and VIIIa,b,c, demonstrates that a low percentage of cells able to haemadsorb is usually associated with a reduced level of haemagglutinin release. Generally, OK pi releases less HA than BK pi and PK pi. Moreover, the amount of cell associated haemagglutinin (CAHA) per cell of the former cell-line (Table VII), is also lower, when it is considered that the diameter of the OK pi cell is about twice that of the other two lines. Furthermore, the low percentages of haemadsorption that are sometimes found in persistently infected sheep kidney monolayers (Table VIIIa) may be associated with the small amount of haemagglutinin synthesised (Table Va) and would suggest that, in contrast to the situation in BK pi cultures, only those OK pi cells to which erythrocytes adhered were producing HA. Therefore, the rate of haemagglutinin formation in these cells might be equivalent to that of BK pi or PK pi.

Discussion.

Figure 19. PK pi cells, stained with haematoxylin and eosin, showing haemadsorption of guinea-pig red blood cells (x 180)

Figure 20. An ultrathin section, showing a fowl erythrocyte adsorbing to a BK pi cell. Stained with uranyl acetate and lead citrate (x 20,000)



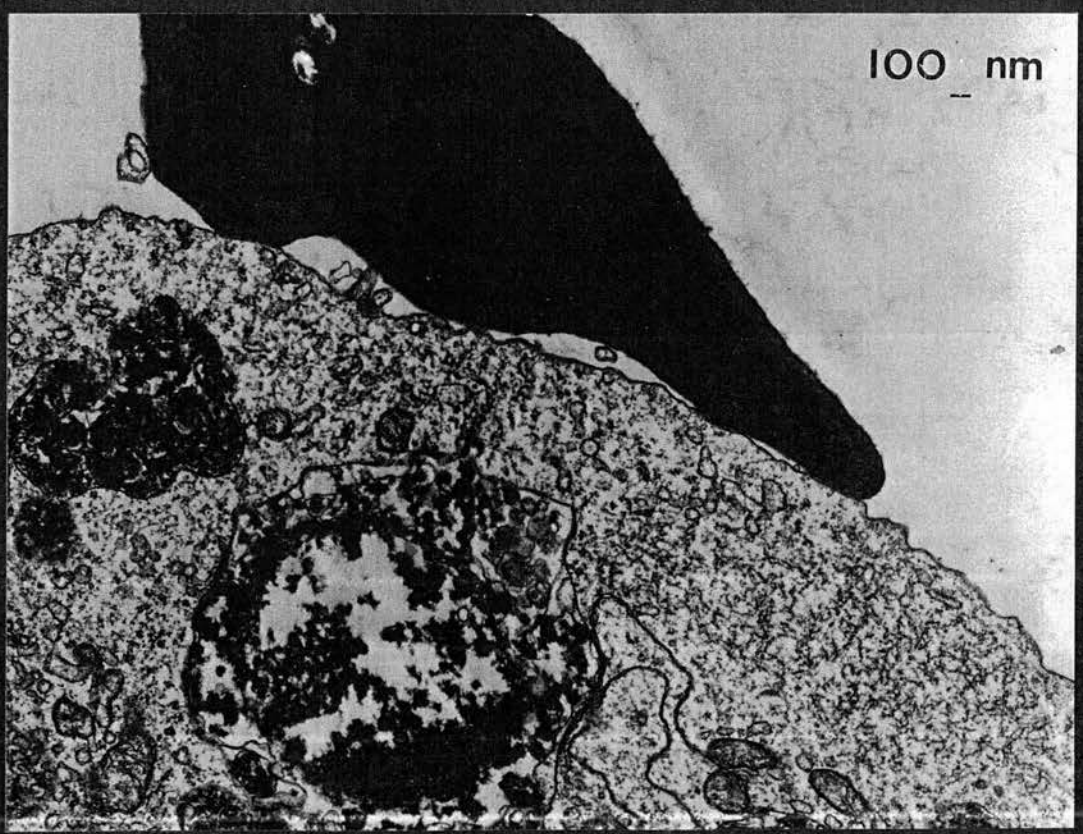
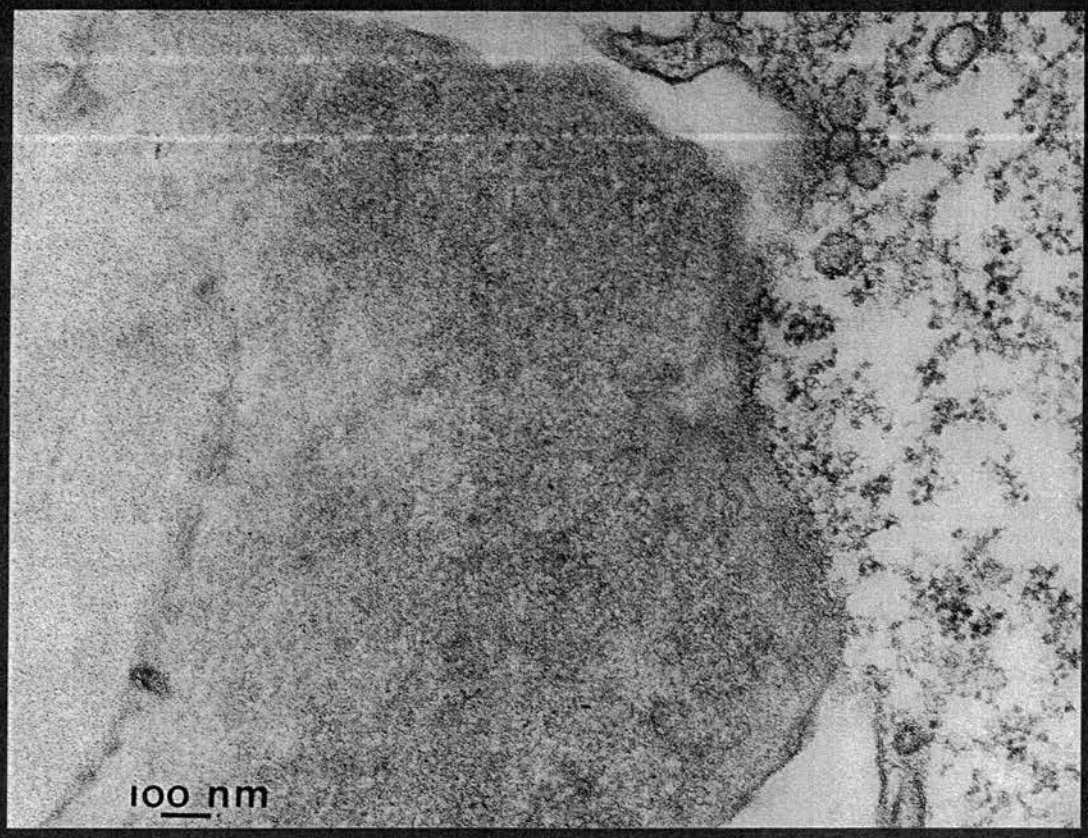
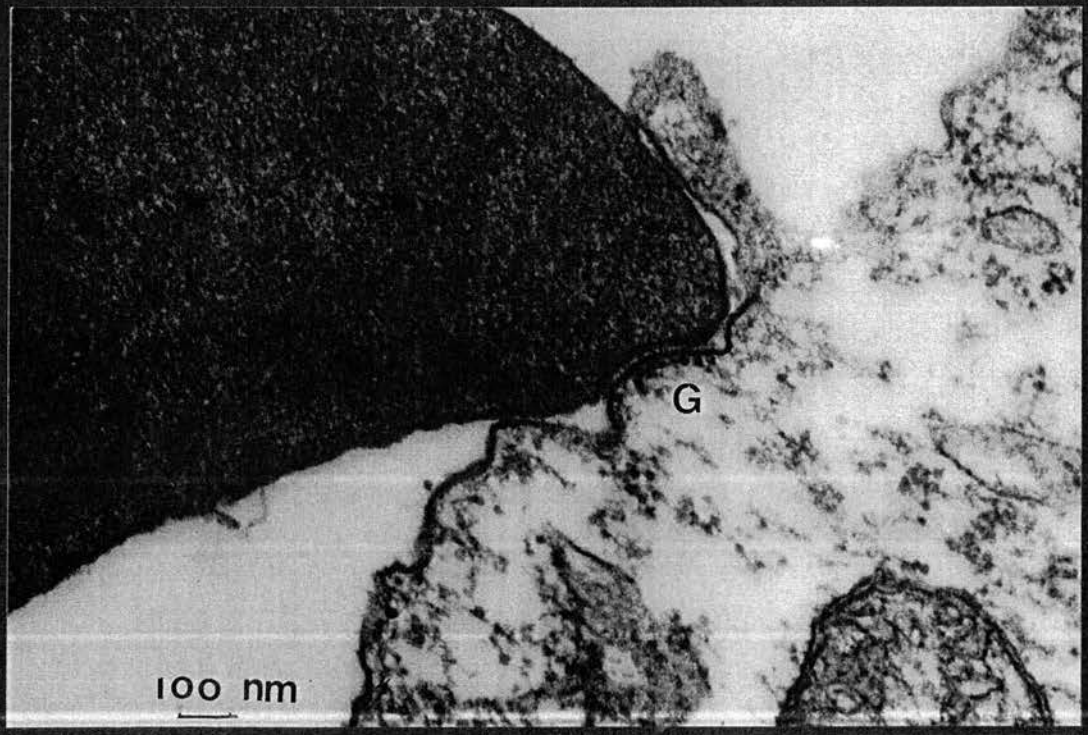

100 nm

Figure 21.

a: An ultrathin section, at a higher magnification than in Fig. 20, showing a fowl erythrocyte in extremely close proximity to an undifferentiated area of BK pi cell membrane. Stained with uranyl acetate and lead citrate (x 60,000)

b: An ultrathin section of a fowl erythrocyte adsorbed to a differentiated and thickened region of the membrane of a BK pi cell. Note the clear gap between the two membranes at most points (G). Stained with uranyl acetate and lead citrate (x 80,000)


100 nm


G
100 nm

Discussion.

The results for infectivity and haemagglutinin production differ from those obtained earlier by Edwards (93). In particular, haemadsorption appears to have been reduced in OK pi monolayers, while that of PK pi cells has increased compared with that recorded before 1968, when it was only 40-60% (210) (Fig.19).

In conclusion, it can be stated that in BK pi cells and possibly the other two persistently infected cell-lines, haemadsorption takes place with cells that are not releasing virus. Nevertheless, this phenomenon provides a method, comparable with immunofluorescence for identifying infected cells, which in all three cell-lines make up 90% of the total. It was also shown that there was a relationship between haemadsorption, CAHA and released haemagglutinin and that the amount of haemagglutinin synthesised in any particular carrier cell-line fluctuated from week to week and month to month. There was also evidence that the amount of haemagglutinin formed in OK pi monolayers was generally less than in the other two persistently infected cell-lines.

A further feature of OK pi cultures is that when haemagglutinin production falls to extremely low levels, this is probably due to restriction of synthesis to a small percentage of cells rather than reduced formation of haemagglutinin throughout the monolayer.

c) Neuraminidase

Introduction

Introduction.

Neuraminidase is the second biological activity associated with the large envelope glycoprotein of NDV (11). However, its activity is so low even in concentrated preparations of virus released from any of the persistently infected cell-lines, that although evidence has been presented above for the presence of this protein both in infected cells and in the virus released from them, the neuraminidase site must be non-functional (Section 2(E) III). Furthermore, the neuraminidase activity (expressed as n Moles NANA/min/ µg protein) of BK pi cells was 0.0007, which is approximately the same as uninfected MDBK cells (0.0009) and 1,640 times lower than the neuraminidase activity found in HeLa cells infected with the B1 strain of NDV (1.150). It is unfortunate that colloidal iron hydroxide treatment of ultra-thin sections, which demarcates areas of the cell membrane devoid of neuraminic acid due to the action of neuraminidase (55), was not attempted in the present work, since it is likely that this would have shown the absence of neuraminidase activity in 'differentiated' regions of the plasmalemma. These results substantiate the work of Edwards (93), who reported a deficient neuraminidase function in BK pi virus.

d) Phosphodiesterase

Phosphodiesterase activity has been isolated on the small glycoprotein of NDV (see Section 2) and has also been found to be associated with purified preparations

preparations of BK pi and PK pi. However, the presence of serum and cellular activity prevents assay of this viral property in infected cells. This is unfortunate because both haemolysin and the cell fusion factor, which are also connected with this protein, require the haemagglutinin to express their activity and thus cannot provide a direct measure of the synthesis of the smaller envelope polypeptide of NDV.

e) Cell fusion

It has been shown above that inter-cellular fusion is found in monolayers infected with the three persistent strains of NDV and therefore, it seems likely that both envelope proteins are synthesised and incorporated into the cell membrane.

f) Haemolysis

The haemolysin activity of NDV is closely related to the cell-fusion factor but its intracellular activity has not been examined in the present work.

II) INTERFERENCE AND THE INDUCTION OF INTERFERON IN THE PERSISTENTLY INFECTED CELLS

Introduction

In the previous pages, the behaviour of the three persistently infected cell-lines has been compared with that of 'normal' infections with NDV, in respect of the changes indicative of viral replication and the unusual events that ensue in the affected cells. A further phenomenon, seen in cells infected with NDV, is the

the interference with a secondary infection by the virus already present. Interference has three different mechanisms:

i) intrinsic interference, whereby a primary infection with virus will prevent superinfection with a second, unrelated virus. This activity is independent of

ii) interferon, which is manufactured by infected cells in response to foreign nucleic acids (especially double stranded RNA) and will prevent replication of both the same and unrelated strains of virus. The most sensitive are those whose synthesis wholly or partially involves RNA.

iii) A third mechanism, described as homologous interference, prevents secondary infection by the same strain of virus and is unconnected with either of the processes alluded to above. Homologous interference functions by blocking a pathway specific to the virus under examination: for example, receptor sites on the cell surface may be occupied or removed and enzymes essential for viral synthesis, may all be involved in replication of the primary infection.

In the present work, the last two mechanisms are considered: interferon because of the influence it may have on persistence of infection, a function which it has been shown to display in carrier cultures of L cells with NDV (138); and homologous interference, for the information it might provide on the completeness or otherwise of virus produced by the three persistent

persistent infections.

a) Interferon-mediated interference

Introduction

Interferon is usually induced by infection with NDV (104) and a number of workers have reported that persistent infection with NDV may be maintained by the action of interferon (138). On the other hand, it has been shown that repeated infection with NDV increases the tolerance of the cell and less interferon is produced in response to further stimulation (104). Furthermore, another paramyxovirus, parainfluenza 3 virus, prevents interferon synthesis in calf kidney cells.

The action of interferon in MDBK cells.

During the present study, it was shown that an interferon-like substance induced by Herts or B1 strains of NDV, in monolayers of MDBK, prevented infection with the M6 strain of mucosal disease virus, of other cultures of this cell-line. This confirmed the results obtained by other investigators who showed this virus to be sensitive to the action of interferon (154). However, since M6 virus had a higher infectivity titre in PK pi and BK pi monolayers than in untreated MDBK cells (Table X I), it is unlikely that interferon is produced in our persistently infected cells.

b) Homologous interference

Introduction

The evidence presented in Tables IX and X suggests

T A B L E IX a

SUPERINFECTION OF PK pi CARRIER CELL CULTURES WITH DIFFERENT STRAINS OF NDV

CELL-LINE	INFECTING STRAIN OF NDV	INFECTIVITY (Log $_{10}$ TCID$_{50}$*) assayed by:		
		FAS	CPE	IVR
PK pi	Lurgan	N.A.	none	1.0
	B 1	N.A.	none	4.5
	Herts	N.A.	none	5.5
	Asplin F	N.A.	none	4.0
PK(W)K6	Lurgan	6.5	3.0	7.5
	B 1	4.5	2.0	5.5
	Herts	4.0	1.0	4.5
	Asplin F	3.0	none	3.0

FAS Fluorescent antibody staining performed as described.

CPE Cytopathic effect determined by partial or complete destruction of the monolayer.

IVR The release of infectious progeny virus, as assayed in one-day-old monolayers of chick embryo fibroblasts.

N.A. Not applicable.

*$\log_{10}$ TCID$_{50}$ per HAU of infecting virus.

The infecting virus was overlaid on to one-day-old monolayers of the cell-lines, and infectivity was assayed after 2 days of incubation at 37°C in the manner described.

T A B L E IX b

INFECTIVITY OF PROGENY VIRUS RELEASED FROM PK pi CELLS SUPERINFECTED WITH DIFFERENT STRAINS OF NDV

VIRUS STRAIN	INFECTIVITY OF PROGENY VIRUS ($log_{10}TCID_{50}$) released from		
	PK pi monolayers	PK(W)K6 monolayers	allantois of developing chicken embryos
Lurgan	none	5.0	6.0
B1	1.5	2.5	2.5
Herts	5.5	5.5	7.5
Asplin F	3.0	2.0	4.0

One-day-old monolayers of the persistently infected cell-line, PK pi, and parallel cultures of healthy PK(W)K6 cells were infected with 4.0 $log_{10}TCID_{50}$ (assayed in PK(W)K6) of the strains of NDV, as described. A similar amount of virus was employed to infect 9-day-old developing chicken embryos. In all cases the infectious fluids were harvested after two days of incubation at 37°C, brought to the same HA titre and overlaid on one-day-old monolayers of healthy chick fibroblasts (CF), in ten-fold dilutions. The infectivity of these progeny viruses in CF was determined by the $TCID_{50}$ after two days of incubation at 37°C, employing the presence of haemadsorption to estimate the end-point.

T A B L E X

SUPERINFECTION OF THE BK pi CARRIER CELL-LINE WITH THE LURGAN STRAIN OF NDV

CELL-LINE	INFECTIVITY OF LURGAN VIRUS ($log_{10}TCID_{50}$) assayed by			
	HAds	CPE	HA	IVR
BK pi	N.A.	None	None	2.0
MDBK	6.0	4.75	2.0	6.0

There was no CPE, no haemagglutinin and no detectable infectious virus produced in BK pi cells which had not been superinfected with Lurgan virus.

One-day-old monolayers of persistently infected BK pi and healthy MDBK cells were overlaid with ten-fold dilutions of Lurgan virus and incubated for two days at 37°C. The cultures were then examined for evidence of infection as described.

HAds Adsorption to infected cells of guinea-pig erythrocytes.

CPE The cytopathic effect, determined by partial or complete destruction of the monolayer.

HA The presence of haemagglutinin in the supernatant fluid.

IVR The presence of infectious progeny virus in the supernatant fluid, as determined by its ability to infect one-day-old monolayers of HeLa cells.

suggests that there is a varying degree of homologous interference in the three carrier lines. Other workers (153) have demonstrated that infection with a defective strain of Sendai virus, which produced incomplete virus particles of low infectivity, prevented superinfection by 'normal' strains of this species. However,it was shown that inactivated Sendai virus, whose replication was totally abortive, did not inhibit further infection by other strains of this virus and moreover, in this instance, the progeny virus released following superinfection, was a mixture of the two parental types. Thus, if our persistent infections were completely abortive, it might be expected that superinfection with NDV would result in the release of the persistent strains of virus. On the other hand, if the progeny virus produced by these persistent infections were incomplete, it might be expected that varying degrees of homologous interference would be found.

Superinfection of carrier cells with strains of NDV.

NDV released following superinfections of the persistently infected cell-lines, shares some of the characteristics of the 'wild-type' strain that was employed. For example, it is known that dense haemadsorption is caused in monolayers of chicken fibroblasts, infected by virulent strains of NDV, such as Herts or Lurgan (113) but that in a monolayer infected by a lentogenic strain, the number of adsorbed erythrocytes per cell is less. These features

features are retained by the respective progeny released from carrier cells superinfected with velogenic or avirulent strains.

On the other hand, after passage through PK pi cultures, the Lurgan strain of NDV was less infectious to chick embryo fibroblasts than the two avirulent strains (Table IX b). Moreover, it may be seen from the results in Table IX a that the infectivity of the four strains, as measured by the production of progeny virus capable of infecting chick embryo fibroblasts, differs for PK pi monolayers from that found for PK(W)K6. Furthermore, the velogenic strains are not necessarily more infectious for PK(W)K6 than the lentogenic strains, although this is invariably the case when the infectivity of NDV is measured in chick fibroblasts. Nevertheless, the characteristics of a strain of NDV are not altered by passage through PK(W)K6 (Table IX b), although only some characteristics of the infecting strain are retained in the progeny virus released from PK pi cells. In spite of these complications, there appears to be a relationship between these results: for example, Asplin F is the strain that is least infectious for PK(W)K6 cells, but the progeny virus released from PK(W)K6 monolayers infected with this virus is of lower virulence for CF cells than that from PK pi cells superinfected with the Asplin F strain of NDV. On the other hand, Lurgan is the strain of greatest virulence for PK(W)K6 cultures and the

the virus released from PK(W)K6 cells infected with this strain of NDV is of greater infectivity for chick fibroblasts than the progeny virus produced from PK pi cells superinfected with Lurgan virus (Table IX b).

Thus, the less virulent a strain of NDV is for PK(W)K6 cells, the greater is the infectivity of the progeny virus released from superinfected PK pi monolayers, compared with that released from infected PK(W)K6 cultures.

Therefore, it appears that not only does the persistent virus in PK pi cells interfere with the replication of superinfecting strains which are virulent for PK(W)K6 cells, but it also enhances the infectivity of the progeny virus of an avirulent strain.

The persistent virus in BK pi cells may act in a similar manner to that in PK pi cells, since it is clear that there is interference with the replication of Lurgan virus in BK pi monolayers (Table X). Lurgan virus is a strain ofmoderate to high virulence for healthy MDBK cells (Table XIV) and in this experiment, 50% of all cultures tested produced infectious progeny virus following infection with a 10^6 dilution of Lurgan virus (Table X, column 4). However, the $TCID_{50}$ of this parameter in BK pi cells was 2.0 $\log_{10}$ (Table X, column 4). The results in Table X, together with those in Tables IXa, XIIa, XIIb and XIIc show that the cytopathic effect of most strains of NDV in the

T A B L E XI

SUPERINFECTION OF THE CARRIER CELL-LINES, BK pi AND PK pi, WITH THE M6 STRAIN OF MUCOSAL DISEASE VIRUS

CELL-LINE	INFECTIVITY OF M 6 VIRUS ($log_{10}TCID_{50}$)
MDBK	2.4
BK pi	3.5
PK pi	3.2

One-day-old monolayers of the carrier cell-lines, PK pi and BK pi, and of healthy MDBK cells were overlaid with ten-fold dilutions of M6 virus and incubated for two days at 37°C, as described. The presence of infectious virus was determined by its capacity to partially or completely destroy the monolayer.

Note: Interferon produced from MDBK cells infected with B1 or Herts strains of NDV protects MDBK cells from 2.4 $log_{10}TCID_{50}$ of M6 virus.

T A B L E XII a

THE EFFECTS OF SUPERINFECTION OF THE CARRIER CELL-LINE, PK pi, WITH DIFFERENT STRAINS OF NDV.

STRAIN OF INFECTING VIRUS	THE RELEASE OF HAEMAGGLUTININS FROM CELL-LINES TWO DAYS AFTER INFECTION (HAU/ml).	
	PK pi	PK(W)K6
Lurgan	0*	0*
Herts	8	16
B 1	0	16
F	0	0
None (Control)	0*	0*

*Nine days after infection, 16HAU/ml were found in the supernatant fluid from infected PK(W)K6 monolayers but not from the other cultures tested.

One-day-old monolayers of persistently infected PK pi and healthy PK(W)K6 cells were overlaid with 4.0 $\log_{10}$ $TCID_{50}$ (in PK(W)K6) of the infecting strain of NDV and incubated for two days (or occasionally nine days) at 37°C as described.

Note: i) With the exception of PK(W)K6 monolayers infected with Lurgan, Bl or Herts strains of NDV, no destruction of the monolayer occurred.

ii) In PK pi monolayers, superinfected with the Herts and Lurgan strains of NDV, 2.5% of the cells formed syncytia, compared with 1.3% of the cells in control PK pi monolayers.

iii) With the exception of PK(W)K6 control cells, over 90% of the cells haemadsorbed in all the infected, persistently infected and superfnfected cultures that were tested.

iv) No differences were observed between control and superinfected BK pi cells in regard to the percentage of cells containing inclusion bodies, stained with haematoxylin and eosin, or with aggregates of antigen, stain with fluorescent antibody.

T A B L E XII b

THE EFFECTS OF SUPERINFECTION OF THE CARRIER CELL-LINE, OK pi, WITH THE HERTS STRAIN OF NDV

CELL CULTURE	VIRUS-INDUCED PHENOMENA			
	HA	CPE	S.F.%	HAds%
SUPERINFECTED OK pi CELLS	none	none	15.0	45.0
CONTROL OK pi CELLS	none	none	2.0	10.0

There is no uninfected sheep kidney cell-line available for control experiments.

HA The supernatant fluids were assayed for the presence of haemagglutinin.

CPE Cytopathic effect, determined by partial or complete monolayer destruction.

S.F.% The percentage of cells forming syncytia.

HAds% The percentage of cells which adsorb guinea-pig erythrocytes.

One-day-old monolayers of persistently infected OK pi cells were infected with 4.0 $\log_{10}TCID_{50}$ (in PK(W)K6 cells) of the Herts strain of NDV and incubated for two days at 37°C, as described. Duplicate cultures of OK pi cells were left uninfected.

Note: Progeny virus, which was infectious to chick fibroblast, PK(W)K6 and HeLa cells was found in the unconcentrated supernatant fluids of superinfected OK pi cultures but was not detected in those of control OK pi monolayers.

TABLE XII c

THE EFFECT OF SUPERINFECTION OF THE CARRIER CELL-LINE, BK pi, WITH THE LURGAN AND HERTS STRAINS OF NDV

STRAIN OF INFECTING VIRUS	THE RELEASE OF HAEMAGGLUTININ FROM CELL CULTURES TWO DAYS AFTER INFECTION (HAU/ml)	
	BK pi	MDBK
LURGAN	0*	0*
HERTS	0	16
NONE (control)	0*	0*

*Nine days after infection, 8 HAU/ml were found in the supernatant fluid from BK pi monolayers, infected with Lurgan NDV but not from the other cultures tested.

One-day-old monolayers of persistently infected BK pi cells and healthy MDBK cells were overlaid with 4.0 $\log_{10}TCID_{50}$ (in PK(W)K6) of the infecting strain of NDV and incubated for two or nine days at 37°C as described.

Note: i) With the exception of MDBK cells infected with Herts virus, the monolayers were neither partially nor completely destroyed.

ii) In BK pi monolayers, superinfected with the Herts strain of NDV, 3.5% of the cells formed syncytia, compared with 1.3% of the cells in control BK pi monolayers.

iii) With the exception of MDBK control cells, between 80-90% of the cells haemadsorbed in all the infected, persistently infected and superinfected cultures that were tested.

iv) No differences were observed between control and superinfected BK pi cells, in regard to the percentage of cells containing inclusions, stained with haematoxylin and eosin.

the three persistently infected cell-lines, PK pi, BK pi and OK pi is generally much less than in the control PK(W)K6 cells.

It is emphasised, however, that when the features of viral infection (syncytial formation from within, haemadsorption, haemagglutinin release, and the production of infectious virus) (Table XII) in the superinfected cells are compared with that of persistently infected controls, there is usually evidence of increased viral activity.

It may therefore be concluded that there is usually a degree of homologous interference in the carrier cell-lines, and that strains of NDV that are most virulent for uninfected cells are the most affected by this phenomenon. Thus, the possibility exists that as the rate of viral synthesis is usually slower in infections with avirulent strains (80, 81), competitive inhibition at some point in the replicative pathway produces a smaller reduction in the total amount of complete virus produced, than would be the case when large quantities of viral material are involved.

F / SUMMARY

In this Section it has been shown that the cells persistently infected with NDV have altered membrane characteristics, although all of these are consistent with changes found in monolayers with a 'normal' productive cycle of infection with NDV. These include 'colony' formation in semi-solid agar, cell fusion and haemadsorption. The last two properties generally require the presence of viral proteins (as indicated morphologically by a 'differentiated' membrane) but apparently, can take place in the absence of these proteins from the cell membrane.

It has also been demonstrated that viral RNA and protein synthesis occurs in cells persistently infected with NDV, although the process appears to be slow and results in the formation of large quantities of abnormal nucleocapsid material which may be demarcated in electron micrographs or stained by HES. These inclusions are especially evident in OK pi cells. The manufacture of haemagglutinin protein is variable and evidence will be presented in Section 2, to show that it is extremely defective in BK pi cultures. The neuraminidase activity normally associated with this protein is almost totally lacking in BK pi and PK pi cells. However, there is evidence that virions with normal morphology were released although they were of extremely low infectivity. Interferon was not detected in the carrier cells and it appears that the persistence of infection is not due to this cellular product. On

On the other hand, there was evidence of homologous interference and it was noted that the strains of NDV which were most virulent for control cell cultures suffered, relatively, the greatest degree of inhibition in superinfected cells. There can be little doubt that certain of the characteristics of the persistently infected lines have altered since they were originally studied between 1962-70. However, direct comparisons are difficult because a different growth medium was employed at that time and the techniques available for the assay of viral activity were not as sensitive.