Studies on the Cytopathic Effects of Newcastle Disease Virus: the Cytopathogenicity of Strain Herts 33 in five Cell Types

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(Accepted 20 June 1973)

SUMMARY

The cytopathogenicity and production of Newcastle disease virus (NDV) strain Herts cultivated in chick embryo (CE), baby hamster kidney (BHK-21), HEp-2, MDBK and L929 cells was investigated. Infection at high multiplicities (1000 p.f.u./cell) induced cell fusion in cultures of all cell types within 3 h after infection. Infection at low multiplicities (10 to 20 p.f.u./cell) produced extensive cell fusion in CE, BHK-21, HEp-2 and L929 cultures within 24 h, but MDBK cells failed to fuse. The latter cells failed to show high levels of virus haemagglutinin at the cell surface or accumulation of virus products, but infective virus was released to significantly higher titres than from the cell types which fused. However, infected MDBK showed similar levels of virus-induced cell damage to the plasma membrane, as measured by the release of lactate dehydrogenase, to HEp-2 and L929 cells which were susceptible to fusion. The morphology of the c.p.e. produced by strain Herts in the different cell types is described. The relationship of virus accumulation and virus release to the process of cell fusion is discussed.

INTRODUCTION

Previous studies with Newcastle disease virus (NDV) strains that differ in the c.p.e. induced in chick embryo (CE) cells and baby hamster kidney (BHK) cells have suggested that c.p.e. in these cells is caused by an accumulation of virus products (Reeve & Alexander, 1970; Reeve, Rosenblum & Alexander, 1970; Reeve & Poste, 1971; Reeve et al. 1971; Reeve et al. 1972). Further, c.p.e. may occur when the rate of production of virus material is greater than the rate of release of virus from the cell (Alexander, Reeve & Poste, 1973). Work with other viruses supports this mechanism of cytopathogenicity (Plowright, 1962; Compans et al. 1966; Mallucci & Skehel, 1971; Bablanian, 1972).

Holmes & Choppin (1966) and Compans et al. (1966) using simian virus 5 (SV5) demonstrated that the infected cell is as important as the virus itself in determining the degree of c.p.e. In infections of monkey kidney (MK) cells, SV5 was non-cytopathogenic, was released
to high titres and virus products did not accumulate, but in BHK 21-F cells SV5 was highly
cytopathogenic, little virus was released and accumulation of virus products took place.

NDV also appears to show degrees of cytopathogenicity depending on the cells infected:
our previous studies suggest that c.p.e. occurs earlier and is more dramatic in BHK than in
chick cells (unpublished observations).

Johnson & Scott (1964) suggested that NDV grown in HEp-2 cells has an intranuclear
phase and produces two morphologically distinct types of polykaryocyte formation.

Growth of NDV in mouse L cells is reported as abortive (Reda, Rott & Schafer, 1964;
Thacore & Youngner, 1969) in that increases in released haemagglutinin (H.A.) are not
associated with comparable increases in released infectivity, although some infective particles
are released. Thacore & Youngner (1969) also reported that no c.p.e. occurred if L cells
were infected at an input multiplicity of 0.1 p.f.u./cell. Bader & Morgan (1961) showed that
within 48 h of infection, one ID$_{50}$ of NDV per culture induced more than 10 % cellular lysis
in HeLa cells, while a similar level of c.p.e. was induced in L cells only if multiplicities of
0.5 to 0.7 ID$_{50}$/cell were used. However, the study of macroscopic c.p.e. after infection at low
multiplicity gives little guide to the effect of virus on the single infected cell, particularly
when there is little released infectivity.

One of the cytopathic effects of NDV in CE cells is the formation of polykaryocytes,
which occurs several hours after the infection of cells with moderate or low multiplicities of
cytopathic NDV strains. This type of cell fusion is related to the intracellular growth of the
virus and requires virus-specific macromolecular synthesis (Reeve and Poste, 1971; Reeve
et al. 1971; Poste et al. 1972c). Polykaryocytes may also be formed after treatment with very high
multiplicities of either infective or non-infective virus; this type of fusion is a laboratory
phenomenon which occurs within 3 h and does not require host-specific or virus-specific
macromolecular synthesis. Bratt & Gallaher (1969) designated these types of cell fusion as
‘fusion from within (FFWI)’ and ‘fusion from without (FFWO)’, respectively. In a recent
study Bratt & Gallaher (1972) showed that NDV strains which induce polykaryocytes in CE
cells by FFWO are equally capable of fusing BHK and MDBK cells by this method. How-
ever, although polykaryocytes may be formed in CE and BHK cells by low multiplicities of
infective virus, none of the NDV strains that they have tested induced FFWI in MDBK cells
even though these cells were infected productively.

We report various aspects of NDV cytopathogenicity and growth in CE, BHK HEp-2,
MDBK and L cells with a view to demonstrating relationships that may exist between virus
growth, accumulation, release and cytopathogenicity.

**METHODS**

**Newcastle disease virus.** The highly virulent cytopathic strain Herts 33 (Herts) was grown
in eggs (Reeve et al. 1970).

**Cell cultures.** Primary chick embryo (CE) cells were prepared from 10 to 12-day-old
embryos (Alexander, 1971).

CE cells, BHK-21 clone 13 (BHK) cells (original seed culture provided by Dr J. Best,
St Thomas’s Hospital Medical School, London S.E.1.), Madin-Darby bovine kidney
(MDBK) cells (original seed cultures provided by Mr J. Lukey, Central Veterinary Labora-
tory, New Haw, Weybridge, Surrey), mouse L-929 cells (original seed cultures provided by
Dr L. W. Greenham, University of Bristol) and HEp-2 cells were all grown in Eagle's BHK
medium supplemented with 10 % tryptose phosphate broth and 10 % foetal calf serum.

Cells were infected by virus in Eagle's BHK medium, which contained only 2 % foetal calf
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serum (maintenance medium). After adsorption for 1 h at 37 °C the virus was removed, the cells washed once with warm phosphate-buffered saline (PBS), pH 7.2, overlaid with maintenance medium and incubated at 37 °C.

**Haemadsorption assay.** Coverslip cultures were infected with 10 to 20 p.f.u./cell of the Herts strain of NDV. At the times specified, coverslip cultures were washed three times in PBS and treated with 0.5 ml 0.5% (v/v) chicken red blood cells (RBC) at 4 °C for 20 min. Coverslips were then washed three times in ice cold PBS and fixed in methanol for at least 30 min before staining with May–Grünwald–Giemsa (Reeve & Poste, 1971).

After staining, the RBC adsorbed to cells were counted and expressed as the number of RBC per nucleus.

**Estimation of fusion as % polykaryocytosis.** Fusion from within (FFWI). Cell coverslip cultures were infected with 10 to 20 p.f.u./cell of strain Herts. At the specified times cell cultures were fixed in methanol and stained with May–Grünwald–Giemsa (Reeve & Poste, 1971).

Fusion from without (FFWO). Cell coverslip cultures were treated with 1000 p.f.u./cell for 3 h then fixed and stained as above (Poste et al. 1972c).

The extent of NDV-induced cell fusion, expressed as % polykaryocytosis, was estimated by counting the number of nuclei present in polykaryocytes and expressing this as a percentage of the total number of nuclei present in the same microscope field (Reeve & Poste, 1971).

**Estimations of virus growth.** Confluent monolayers in 9 cm plastic Petri dishes were infected with 10 to 20 p.f.u./cell of strain Herts. After virus adsorption the washed monolayers were covered with 3 ml maintenance medium. At the specified times after infection the medium was removed and released haemagglutinin activity (H.A.) and infectivity in eggs (EID$_{50}$) were determined as described previously (Alexander, Reeve & Allan, 1970), except that the EID$_{50}$ end-point was estimated from tables (Meynell & Meynell, 1965). The monolayer was washed twice with PBS and scraped from the dish into 1 ml of ice-cold PBS. The cells were disrupted by sonication, the debris pelleted at 2000 g for 10 min and the H.A. titre of the supernatant fluids estimated. The protein content of the supernatant fluids was estimated by the method of Lowry et al. (1951) and the cell-associated H.A. activity expressed as units (H.A.U.)/mg protein.

**Cell leakage and lysis** was measured by the release of lactate dehydrogenase (LDH) activity into the supernatant fluids of cell cultures. Infected cell cultures in 5 cm plastic Petri dishes overlaid with 1 ml of maintenance medium were taken at the specified times and the supernatant fluids removed. The cells were then scraped from the dish (microscopic examination showed that only a few isolated cells remained), suspended in 1 ml of maintenance medium and sonicated to disrupt all the cells. Both the supernatant fluids and the sonicated cell suspension were then assayed for LDH activity by the method of Kornberg (1955). Released LDH activity was expressed as % total activity.

**RESULTS**

**Virus growth**

The activity of haemagglutinin released from the cells after infection by NDV strain Herts differed widely according to the cell type (Fig. 1).

Up to 8 h after infection none of the supernatant fluids contained detectable H.A. L cell cultures released no detectable H.A. until 20 h after infection and at 24 h had only reached 10 H.A.U./ml. H.A. release from HEp-2 cell cultures was similar, being first detectable at
12 h and reaching 20 H.A.U./ml by 24 h. BHK cells released H.A. from 10 h until 20 h after infection when a maximum level of 80 H.A.U./ml was reached. Cultures of CE and MDBK cells showed much greater H.A. release: CE cells producing 510 H.A.U./ml by 24 h, while MDBK cells reached 1280 H.A.U./ml at 24 h which increased to 5120 H.A.U./ml at 32 h after infection.

Released H.A. and released infectivity were approximately proportional for each cell type with the exception of L cells (Table 1). The EID_{50}/H.A.U. ratio of virus released from L cells was 0.7 x 10^6 compared with an average of 6.0 x 10^6 for the other cell types.

Cell-associated H.A., expressed as H.A.U./mg protein, was detected at 8 h after infection in MDBK cells and at 4 h after infection with the other four cell types (Fig. 2). The production of cell associated H.A. followed a common pattern but there were large differences in the rates of production and amounts produced in the different cells.

Table 1. The release of infective virus from different cell types and its relationship to activity of released haemagglutinin

<table>
<thead>
<tr>
<th>Cell type</th>
<th>EID_{50}/cell</th>
<th>EID_{50}/H.A.U.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE</td>
<td>450</td>
<td>5 x 10^6</td>
</tr>
<tr>
<td>BHK</td>
<td>200*</td>
<td>9 x 10^6</td>
</tr>
<tr>
<td>HEP-2</td>
<td>50</td>
<td>7 x 10^6</td>
</tr>
<tr>
<td>L</td>
<td>3</td>
<td>0.7 x 10^6</td>
</tr>
<tr>
<td>MDBK</td>
<td>1170</td>
<td>3 x 10^6</td>
</tr>
</tbody>
</table>

* Released infectivity was estimated 24 h after infection of CE, HEP-2, L and MDBK cells but 20 h after infection of BHK cells.
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Fig. 2. Activity of cell-associated haemagglutinin in cell monolayers. ○--○, BHK; △--△, CE; ▲--▲, HEp-2; △--△, L; ■--■, MDBK cells infected with NDV strain Herts.

Haemadsorption is also a measure of virus production, basically measuring the degree of cell surface modification. The results (Fig. 3), predictably, followed the pattern of production of cell-associated H.A.

It is not practicable to count cultures with more than 30 red blood cells adsorbed per nucleus, for this reason no points past 12 h after infection were included for BHK cells.

The degree of haemadsorption to infected L cells tended to fluctuate after the onset of c.p.e. and cell fusion and the average number of red blood cells/nucleus at 20 h was considerably lower than expected (Fig. 3).

The very limited haemadsorption to MDBK cells, compared with that for other cells, suggests that the relatively large amount of virus released was cleared very quickly from the cell surface or that the virus was released from foci which were limited both in size and number.

The cytopathic effects

The earliest c.p.e. in any of the cells following infection by 10 to 20 p.f.u./cell of NDV strain Herts was the low level of cell fusion in CE, BHK and HEp-2 cells at 4 h after infection (Fig. 4).

In monolayers of BHK cells fairly large polykaryocytes were formed by 8 h (Fig. 5b). Under the conditions of this study these polykaryocytes increased in size by fusion of more cells until the polykaryocytes themselves apparently fused to form enormous polykaryocytes
consisting of almost the entire monolayer by 16 h after infection (Figs. 4 and 5c.) These 'massive' polykaryocytes eventually degenerated and peeled from the coverslip.

Monolayers of CE cells followed the same course as BHK cells and formed 'massive' polykaryocytes before cell death (Fig. 6a–d), however, polykaryocyte formation occurred at a slower rate in CE than in BHK cells (Fig. 4).

The fusion of HEp-2 cells by NDV strain Herts never induced 'massive' polykaryocytes, although large discrete polykaryocytes were formed (Fig. 7). The progression of fusion in HEp-2 cultures was at a slower rate than in CE cells and about 20% of the HEp-2 cells did not become involved in syncytia within 24 h of infection (Fig. 4). The polykaryocytes which were formed rarely had more than 30 nuclei and appeared to round up and lyse or detach from the glass before fusion into larger polykaryocytes could take place (Fig. 7a–d). In some polykaryocytes the nuclei were grouped towards the centre, while in others, particularly in the earliest hours after infection they were more peripheral.

The onset of fusion in L cells was quite dramatic (Fig. 4). Until 12 h after infection little or no syncytial formation could be seen (Fig. 8b). However, between 12 to 16 h of infection of L cells 60% of the nuclei became involved in polykaryocytes and by 20 h after infection fusion of the whole monolayer had taken place (Fig. 8d). In infections of the other cell types no gross changes were noted in the nuclei, although during later stages of infection HEp-2 nuclei took up more stain and became less distinct. The nuclei of infected L cells showed marked morphological changes and by 20 h after infection were considerably damaged. Monolayers of MDBK cells infected with 10 to 20 p.f.u./cell of strain Herts did not form polykaryocytes, although by 24 h after infection the cells appeared less healthy and stained less distinctly (Figs. 4 and 9a, b). Cell death in infected MDBK cells did occur and was apparently due to lysis of individual cells without rounding-up (Fig. 9c).
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Fig. 4. Polykaryocyte formation in BHK (●), HEp-2 (▲), L (△), CE (○) and MDBK (■) cells infected with 10 to 20 p.f.u./cell of NDV strain Herts.

Fusion from without

Infection of all five cell types with 1000 p.f.u./cell of NDV strain Herts induced cell fusion within 3 h (Fig. 10).

Compared with FFWI, monolayers of BHK and CE cells showed much lower levels of polykaryocytosis (47 and 46 % of nuclei involved, respectively). The polykaryocytes were also relatively small although they often contained 15 to 20 nuclei (Fig. 10a, b).

About 40 % of the nuclei in L cell cultures were involved in polykaryocytes after FFWO. The polykaryocytes were small, usually involving three to ten nuclei although some of ten or more nuclei could be seen (Fig. 10c). Polykaryocytes in HEp-2 cells contained 3 to 10 nuclei and involved about 48 % of the total nuclei. The polykaryocytes formed were quite distinctive in that the nuclei were found almost exclusively at the periphery of the polykaryocytes (Fig. 10d).

Monolayers of MDBK cells showed FFWO, in contrast to the ability of the virus to induce FFWI in these cells, and up to 26 % of the nuclei were involved in polykaryocytes. This level of polykaryocytosis is comparable to our previous estimate of 32 % using 2000 EID₅₀/cell of inactivated strain Herts (Poste et al. 1972c). Few polykaryocytes in MDBK cultures contained more than six nuclei, but large numbers of small polykaryocytes containing 2 to 5 nuclei were present (Fig. 10e).
Figs 5 and 6. For legend see p. 332.
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Figs 7 and 8. For legend see p. 332.
Release of lactate dehydrogenase from infected cells

Increase in LDH activity in the supernatant fluid of cells infected with NDV has been used in this study as an indication of cell leakage and lysis.

After infection the leakage of LDH from the different cell types generally followed the pattern seen previously for cell associated H.A., haemadsorption and cell fusion (Fig. 11), although increases in LDH release occurred later after infection than increases in these other activities.

The LDH activity was not released at a high level in the supernatant fluids of L cell monolayers until 28 h after infection; this later release may have been due to cells detaching from the glass but not lysing until some time later.

Monolayers of MDBK cells showed relatively high levels of released LDH activity at 24 h after infection, when cells appeared comparatively healthy (Fig. 9b); this suggests that these cells were particularly 'leaky' prior to lysis and death.

Control cells run in parallel showed small rises in extracellular LDH activity but in no cell type did it exceed 10% of the total by 24 h.
Fig. 10. Cells infected with NDV strain Herts at 1000 p.f.u./cell and fixed 3 h after infection. (a) BHK cells; (b) CE cells; (c) L cells; (d), HEp-2 cells; (e) MDBK cells. (Magnification ×66).

**DISCUSSION**

The effects of NDV strain Herts on the different cell types reported in this study are similar to those reported previously in work with other cytopathic NDV strains.

Bader & Morgan (1961) showed that NDV was cytopathic in L cells when relatively high input multiplicities were used. Our results, showing the released and cell-associated virus in infections of L cells, are similar to those of Reda *et al.* (1964). The abortive cycle of NDV in L cells has been described (Reda *et al.* 1964; Thacore & Youngner, 1969).
In our study we report two morphologically distinct types of polykaryocyte similar to those reported by Johnson & Scott (1964) after infection of HEp-2 cells with NDV. These two forms consist of those with 'peripheral' or 'central' nuclei. The 'peripheral' nuclei-type polykaryocyte predominates earlier after fusion and is somewhat similar to syncytia caused by FFWO. It is likely that the different forms are 'young' and 'old' syncytia. Johnson & Scott (1964) used very low multiplicities of virus and were unable to confirm their suggestion that this was the case.

Degeneration of the nuclei in L cells and, to a much lesser extent, HEp-2 cells was more marked than in the other cells and may reflect the reported accumulation of antigen in the nuclei of these cells (Johnson & Scott, 1964; Reda et al. 1964).

Bratt & Gallaher (1972) showed that cytopathic strains of NDV that fuse CE or BHK21-F cells from within do not fuse MDBK cells, although these cells were both productively infected and at 20 h after infection were reported to show other cytopathic changes. Similarly, it has been shown that SV5-infected MK and MDBK cells release virus to high infectivity and do not fuse, but SV5-infected BHK21-F cells accumulate virus, fuse and die (Compans et al. 1966; Holmes & Choppin, 1966; Klenk & Choppin, 1970a).

In this study we have examined cell fusion and cell lysis and death as two measures of cytopathogenicity. Previously we considered these to occur by a similar mechanism, since all NDV strains that induced FFWI were cytopathic for CE and BHK cells and virulent for chickens and eggs (Reeve & Alexander, 1970; Reeve & Poste, 1971; Poste et al. 1972c).
The present results do not detract from the possibility that whatever mechanism brings about fusion also causes cell death. Thus infected BHK cells accumulate virus products at a greater rate than CE cells and fuse and die earlier than CE cells. However, the failure of infected MDBK cells to fuse from within, despite their ability to fuse from without, suggests the possibility that two distinct mechanisms may be involved, one causing fusion and the other death. This is particularly emphasized by the occurrence of cell leakage and death at similar rates in both fused and non-fused cell types following infection with NDV.

It is known that polykaryocyte formation does not lead to the death of fused cells as rapidly as they have died in this study, although this may vary depending on the cell type and, more importantly, on the size of the polykaryocyte (Harris, 1970; Poste, 1972). If two mechanisms of virus-induced cell death are involved it may be that the high rate of release by MDBK cells is responsible for a high level of membrane damage and eventual death. The fast clearance of virus from MDBK cells may also be related to the lack of polykaryocyte formation (see below).

Explanations of why one type of cell should fuse after infection but not another have been offered by other authors. Bratt & Gallaher (1972) discussed the possibility of a hypothetical 'fusion factor' which was missing in virus infections of MDBK cells. Klenk & Choppin (1970a, b) suggested that variations in the composition of the cell membrane may be responsible for the differences in the fusing ability of the paramyxovirus SV5 in different cell systems.

Fusion of cells from within following virus infection may be caused by modification of the cell surface membrane due to the accumulation of virus components (Ejercito, Kieff & Roizman, 1968; Keller, Spear & Roizman, 1970). Certainly NDV strains which accumulate virus products also bring about cell surface changes and fuse cells (Alexander et al. 1970; Reeve et al. 1970; Reeve & Poste, 1971; Poste et al. 1972b; Reeve et al. 1972; Alexander et al. 1973). The present results also add evidence that fusion of cells is brought about by accumulation of virus products. Monolayers of CE, BHK, HEp-2 and L cells infected by NDV reached high levels of cell-associated H.A. or haemadsorption and fused, whereas infected MDBK cells which showed little or no cell-associated H.A. or haemadsorption did not fuse.

The levels of cell-associated H.A. in HEp-2 or L cells were much higher than the levels of released H.A. but were low compared with cell-associated H.A. in CE and BHK cells. This suggests that, if fusion is related directly to accumulation of virus products, different cells may have different thresholds of accumulation above which they fuse. Conversely, the levels are different and may indicate that cell fusion occurs in the absence of accumulation of virus products.

The theory that differences in cell membrane composition are responsible for differences in cell fusing ability is particularly attractive since it can account for variations in accumulation of virus products as well as in fusion. Klenk & Choppin (1969, 1970a) showed that MDBK and primary rhesus monkey-kidney (MK) cells have a higher cholesterol/phospholipid ratio than BHK21-F cells or hamster kidney (HaK) cells and suggested that this may be responsible for differences in fusing ability. However, Poste et al. (1972a), using a wide range of viruses and cell types, failed to confirm this hypothesis.

It is not known if fusion from without and fusion from within are caused by the same mechanism. If FFWI is due to extensive modification of the cell membrane by accumulation of virus products then very high multiplicities of virus may bring about similar modification by virus attachment and fusion, as described by Apostolov & Almeida (1972) with Sendai virus. Haemadsorption studies suggest that, after infection with levels of virus that induce
FFWI in other cells, MDBK cells are modified only at isolated places on the cell surface. Under these conditions insufficient cell surface modification may occur to induce FFWI, but when high multiplicities of virus are used modification over a larger area is induced.

In this study not only were different amounts of virus accumulated and released in different cell types, but there were considerable variations in the total virus production. A further consideration in understanding the cytopathogenicity of a virus is that the effect of that virus on a cell may also be modified by the metabolism of that cell, as work with coxsackie B virus has demonstrated (Khesin & Amchenkova, 1972).

Some of this work was done while D. J. A. and G. H. were supported by the Agricultural Research Council (A.R.C.) as members of the Virology Department, Royal Postgraduate Medical School, London, and the Bacteriology Department, University College Hospital Medical School, London, respectively. P. R. was supported by the Wellcome Trust and G. P. by the Cancer Research Campaign.

This research was aided by a grant from the A.R.C. We thank Dr M. A. Bratt for pre-prints of his recent papers.

REFERENCES


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(Received 8 March 1973)