Studies on the Assembly of Newcastle Disease Virus: Incorporation of Structural Proteins into Virus Particles

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SUMMARY

The mechanism of assembly of Newcastle disease virus in chick embryo cells was investigated in two series of experiments. When protein synthesis was inhibited by addition of puromycin, cycloheximide or fluorophenylalanine at any time during the course of infection, subsequent virus production was soon inhibited. These drugs were inhibitory even when they were added to the cultures as late as 10 hr after infection, when large amounts of virus precursor proteins were present within the cell. In the second series of experiments, the kinetics and efficiency of incorporation of radioactive amino acid into virus particles were examined by the pulse-labelling technique. A 30 min. labelling period at the 3rd or 6th hr of infection resulted in the release of highly radioactive virus during the period of 1½ hr immediately after the pulse. However, when pulse-labelling was performed at the 9th hr, the maximally labelled virus was found in the yield obtained 3 hr after the pulse, and the specific radioactivity of virus was less than 1% of that of the virus harvested from cultures labelled earlier. On the basis of these findings, possible mechanics of virus assembly are discussed.

INTRODUCTION

At present, little is known about the morphogenesis of paramyxoviruses. Several ultrastructural and immunological studies have been made, which presented evidence for the assembly and release of virus by budding at the cell surface. These studies suggested that the maturation of virus occurs in several characteristic stages: (a) migration of virus structural components to the cell periphery; (b) incorporation of virus proteins (haemagglutinin and neuraminidase) into cellular membrane and its conversion into virus envelope; (c) enclosure of virus nucleocapsid within the envelope; and (d) release of virus particles. Many details of the process are not yet known.

During studies on the effect of metabolic inhibitors on the multiplication of Newcastle disease virus in chick embryo cells, we have found that continuous protein synthesis is required for virus maturation. When protein synthesis was inhibited by addition of puromycin, cyclohexamide or fluorophenylalanine to infected cultures, the rate of maturation of virus soon decreased, even though large quantities of virus structural proteins had accumulated within the cell by the time of addition of the drug. In order to elucidate the mechanism of this phenomenon we examined the kinetics and efficiency of incorporation of isotopically labelled amino acids into virus particles. In this communication we describe in detail the results of these experiments.
METHODS

Virus, virus assay and cell cultures. The MIYADERA strain of Newcastle disease virus (NDV) was used throughout this study. The method of preparation of chick embryo cell cultures and the plaque assay of NDV using chick embryo cell monolayers were described by Maeno et al. (1966).

Haemagglutinin titrations. Serial twofold dilutions of virus in 0.25 ml. volumes of phosphate-buffered saline (PBS) were made in plastic trays. Chicken erythrocytes were added as measured drop (0.025 ml.) of a 5% suspension. Titres were read by the pattern method after 35 min. at room temperature.

Complement fixation tests. Complement fixation tests were done for detection and titration of intracellular S-antigen according to a modification of Kolmer's method. Briefly, 0.1 ml. of twofold serial dilutions of antigen, 0.1 ml. of 4 units of antisera and 0.2 ml. of 2 units of complement were mixed and allowed to stand for 1 hr in a water bath at 37°. Then 0.2 ml. of 2.5% sheep erythrocytes, sensitized with 3 units of haemolysin, was added and allowed to stand for a further 30 min. at 37°. The complement fixing titre was expressed as the reciprocal of the highest dilution of antigen exhibiting more than 75% fixation of complement. The anti-S serum was obtained from rabbits immunized with extracts of HeLa cells infected with NDV. Before use, the serum was absorbed with virus purified by sucrose density-gradient centrifugation to remove antibodies against virus envelope antigens. Anti-S titre of the serum after absorption was 1/32.

Neuraminidase assay. Neuraminidase assays were performed with a fetuin substrate by a modification of Warren's thiobarbituric acid method (Warren, 1959). A 0.2 ml. volume of
test materials diluted with saline was added to 0.1 ml. of substrate and adjusted to pH 5.0 (optimum pH for NDV enzyme) with 0.1 ml. of 0.4 M-sodium acetate buffer. After 30 min. incubation in a water bath at 37°, 0.2 ml. of the reaction mixture was used for the assay of free N-acetylneuraminic acid (NANA). Extinction was read at 549 nm. ($E_{549}$) against a blank tube containing fetuin plus saline. Enzyme activity was measured by extinction readings taken from the part of the slope where they vary linearly with the enzyme concentration. Extracts of uninfected cells did not contain measurable quantities of free NANA or other colour-forming substances.

**Isotopic labelling of virus.** Chick embryo cell monolayers were infected with NDV at an input multiplicity of 10 p.f.u./cell. They were washed 3 times with Eagle’s minimum essential medium (MEM) lacking phenylalanine, then incubated with the same medium containing actinomycin D at a concentration of 0.8 µg./ml. At various times after infection the medium was removed and fresh medium containing both [14C]phenylalanine (1.0 µC/ml.) and actinomycin D was added. After 30 min. exposure to the isotope the cultures were washed 3 times with Eagle’s medium supplemented with L-phenylalanine at a concentration of 100 µg./ml. Any further incubation was performed in this medium.

**Purification of isotopically labelled virus.** Culture fluids were harvested at various times after the pulse-labelling, and isotopically labelled virus was purified from these fluids by a combination of differential centrifugation and sedimentation through a linear 10 to 40 % sucrose gradient in PBS at 17,000 rev./min. for 45 min. Fractions were collected by puncturing the bottom of the tube and assayed for their biological activity and radioactivity. Purified unlabelled NDV grown in chick embryo cells was added to the test material as a marker for haemagglutinin. Infectivity, haemagglutinin and radioactivity sedimented homogeneously in the same peak (Fig. 1). This method of purification was therefore used throughout this study. The peak of infectivity always coincided with that of the radioactivity. The specific radioactivity of virus (counts/min./p.f.u.) was calculated from the infectivity and radioactivity of the peak fraction. Counting of radioactivity was done in an Aloka scintillation spectrometer.

**RESULTS**

**Intracellular development of virus components**

The time course of development of virus nucleoprotein antigen (S), haemagglutinin (HA), and neuraminidase (E) was investigated in chick embryo cells infected with NDV. Haslam, Cheyne & White (1969) reported that these three proteins are the major protein components of the virus and may account for at least 84 % of the total virus protein. Monolayer cultures of chick embryo cells grown in 50 ml. bottles with Eagle’s MEM containing 2 % calf serum were washed and infected with NDV at an input multiplicity of 8 p.f.u./cell. After washing and addition of maintenance medium (Eagle’s MEM without serum), they were further incubated at 36°. At various intervals after infection, cultures were harvested by scraping off the cells and washing in PBS, and finally resuspending the cells in 1 ml. of the buffer. The cell suspensions obtained from three bottles were pooled and subjected to three cycles of freezing and thawing and centrifugation at low speed. The resulting supernatant fluids were assayed for biological activity (Fig. 2). Intracellular S antigen and HA activity were first detected 4 and 5 hr after infection respectively, and reached maximum levels 5 and 10 hr after infection, respectively. The intracellular enzyme activity first appeared 5 hr after infection continued to increase exponentially up to 10 hr and then reached a plateau. The S antigen appeared 1 hr earlier than either E or HA. The E and HA appeared at almost the same time and increased in parallel thereafter.
Effect of inhibition of protein synthesis on virus maturation

The effect of inhibition of protein synthesis on virus maturation was examined by addition of puromycin or cycloheximide to infected cells during the phase of active virus production. Bottle cultures of chick embryo cells were infected with NDV under the experimental conditions described above. At intervals after infection, culture fluids were harvested for virus titration to examine one-step growth of the virus, and at the same time puromycin (200 μg./ml.) or cycloheximide (20 μg./ml.) was added to duplicate cultures. The drug-treated cultures were further incubated and their culture fluids were harvested at various times. The collected fluids were then assayed for virus infectivity by the plaque method on monolayers of chick embryo cells (Fig. 3). A similar experiment was made using fluorophenylalanine as an inhibitor of protein synthesis. In this experiment cultures of chick embyro cells were washed and incubated for 1 hr with MEM lacking phenylalanine. They were then infected with NDV and incubated in phenylalanine-deficient medium. At various times fluorophenylalanine (100 μg./ml.) was added and after further incubation culture fluids were harvested and assayed as described above (Fig. 4). Thus, when puromycin, cycloheximide or fluorophenylalanine was added at any time during the phase of active virus production, subsequent virus release was markedly inhibited. After addition of these drugs there was little or no increase in virus infectivity. To investigate this phenomenon more
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precisely, differential yields of virus obtained from cultures after treatment with cycloheximide were compared with those from untreated cultures. At various times of infection, cycloheximide was added to infected cultures at a concentration of 20 μg./ml., and thereafter the culture medium was replaced every ½ hr with fresh medium containing the drug. The virus in each serial sample of medium was titrated. There was scarcely any virus production in the drug-treated cultures (Table I). Clearly the release of virus was soon inhibited by treatment with inhibitors of protein synthesis. These findings raised the question of whether the inhibition of virus release was due to inhibition of virus assembly. If virus assembly could occur in the presence of an inhibitor of protein synthesis in the cell in which virus precursor proteins had already been synthesized, and if the drug inhibited the step of virus release selectively, a significant increase in virus titre should be detectable within such cells after treatment with these drugs. To test this possibility, monolayer cultures of chick embryo cells were infected with NDV as described above, and puromycin (200 μg./ml.) or cycloheximide (20 μg./ml.) was added at 5, 7 or 10 hr after infection. At the 15th hr after infection cells were harvested and disrupted by freezing and thawing, then assayed for cell-associated virus by the plaque method (Table 2). Infectivity did not increase but remained unchanged or even decreased slightly after addition of these drugs.

It may be significant that these drugs were inhibitory even when they were added to the culture as late as 10 hr after infection. The results shown in Fig. 2 suggested that the cells
then contained maximum amounts of virus structural components (S, HA and E). If so
the inhibition of virus production could not have been due to short supply of any of these
components.

**Kinetics of $^{14}$C[amino acid] incorporation into virus particles**

A possible explanation of our results may be that at the later stages of infection, virus
protein was made in excess of that needed for virus formation and not incorporated into
virus particles but accumulated within the cell. If only newly synthesized protein were
utilized for virus formation, the administration of inhibitors of protein synthesis would
inhibit virus production promptly.

Table 1. *Differential yields (per 1½ hr) of Newcastle disease virus after
treatment of infected chick embryo cells with cycloheximide*

<table>
<thead>
<tr>
<th>Time of treatment with cycloheximide (hr after infection)</th>
<th>Sampling interval (hr after infection)</th>
<th>Virus titre (p.f.u./cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Treated culture</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Untreated culture</td>
</tr>
<tr>
<td>3</td>
<td>3 to 4:5</td>
<td>0·0023</td>
</tr>
<tr>
<td></td>
<td>4:5 to 6</td>
<td>0·0012</td>
</tr>
<tr>
<td></td>
<td>6 to 7:5</td>
<td>0·0010</td>
</tr>
<tr>
<td></td>
<td>7:5 to 9</td>
<td>0·0004</td>
</tr>
<tr>
<td>6</td>
<td>6 to 7:5</td>
<td>0·0026</td>
</tr>
<tr>
<td></td>
<td>7:5 to 9</td>
<td>0·0021</td>
</tr>
<tr>
<td></td>
<td>9 to 10:5</td>
<td>0·0014</td>
</tr>
<tr>
<td></td>
<td>10:5 to 12</td>
<td>0·0005</td>
</tr>
<tr>
<td>9</td>
<td>9 to 10:5</td>
<td>0·0048</td>
</tr>
<tr>
<td></td>
<td>10:5 to 12</td>
<td>0·0030</td>
</tr>
<tr>
<td></td>
<td>12 to 13:5</td>
<td>0·0015</td>
</tr>
<tr>
<td></td>
<td>13:5 to 15</td>
<td>0·0013</td>
</tr>
</tbody>
</table>

Table 2. *Effect of cycloheximide and puromycin on the production of Newcastle disease virus
in chick embryo cells: development of cell-associated virus infectivity after drug treatment*

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Time of addition of the drug (hr after infection)</th>
<th>Cell-associated infectivity (p.f.u./bottle)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>at the time of drug treatment</td>
<td>15 hr after infection</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>5</td>
<td>5·67 x 10⁴</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1·60 x 10⁵</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>6·10 x 10⁵</td>
</tr>
<tr>
<td>Puromycin</td>
<td>5</td>
<td>5·67 x 10⁴</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1·60 x 10⁵</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>6·10 x 10⁵</td>
</tr>
<tr>
<td>Untreated control</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

To test this hypothesis the minimum time required for virus formation and the specific
radioactivity of virus were examined by the pulse-labelling technique. Cells infected with
NDV (m.o.i. = 10) and incubated with phenylalanine-deficient medium for 3 or 6 hr were
exposed to $[^{14}$C]phenylalanine for 30 min. and then incubated further in 'chase' medium
which was changed every 1½ hr. Actinomycin D was present in the medium throughout
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the incubation at a concentration of 0.8 μg/ml. The virus in each serial sample of medium was purified by our standard centrifugation in a sucrose density gradient and the specific radioactivity of the purified virus (counts/min./p.f.u.) was determined (Fig. 5a, b). Virus with the highest specific radioactivity was released in the period of 1½ hr immediately after the pulse. Virus released in the subsequent intervals was markedly less radioactive. A somewhat different pattern was observed in the cultures pulse-labelled later in the course of infection.

![Fig. 5. Kinetics of [14C]phenylalanine incorporation into Newcastle disease virus. The symbol ■ indicates the time of pulse-labelling. ○—○, p.f.u./ml.; •—•, counts/min./p.f.u.](image)

![Fig. 6. Comparison of specific radioactivity (counts/min./p.f.u.) of virus released after the pulse-labelling performed at the 3rd, 6th or 9th hr after infection.](image)
When cultures were labelled for 30 min. at the 9th hour of infection (Fig. 5c), the most radioactive virus was found in the yield obtained 3 hr after the pulse. The maximum specific radioactivity was less than 1% of that of the virus harvested from cultures pulse-labelled earlier.

The specific activity of virus declined steadily as the labelling was performed later and later (Fig. 6). If only newly synthesized protein could be incorporated into virus particles as postulated above, the virus released after the pulse should have had the same specific radioactivity, regardless of the time of labelling. The fact that the progressively later pulse yielded virus of lower specific activity might have been due to dilution of newly synthesized radioactive protein with preformed unlabelled protein. It seemed likely therefore that, at the late stage of infection, virus structural proteins which had accumulated within the cell by the time of labelling would also be incorporated into virus particles.

DISCUSSION

The results we have described suggest that maturation of Newcastle disease virus was inhibited by inhibitors of protein synthesis such as puromycin, cycloheximide or fluorophenylalanine. This conclusion is based upon the findings that these drugs could exhibit their inhibitory effect even when they were added to infected cultures as late as 10 hr after infection, when the cells contained a large pool of virus precursor proteins. Puromycin and fluorophenylalanine have proved to be very useful in the analysis of the early events in myxo- or paramyxovirus infection when they are added to infected cells at various times during the eclipse period of virus multiplication (Zimmermann & Schäfer, 1960; Scholtissek & Rott, 1961; Wheelock, 1962; Wilson & LoGerfo, 1964). Our results suggest that these drugs are useful also in the study of maturation process in virus infection.

Choppin & Holmes (1967) have reported that addition of puromycin to monkey kidney cells infected with SV5 resulted in a rapid inhibition of virus production. They suggested that, for maturation to occur, virus structural proteins had to be incorporated into virus particles soon after synthesis, and therefore continued protein synthesis was necessary for continued production of virus. In fact, incorporation of structural proteins of NDV into virus particles appeared to occur rapidly soon after synthesis. In our kinetic studies on the incorporation of isotopically labelled amino acid into virus particles we found that, when the pulse-labelling was performed at various times after infection, the highly radioactive virus was released during the period of 1½ or 3 hr immediately after the pulse. However, we also found that the specific radioactivity of virus declined steadily as the labelling was performed later and later. An explanation of this phenomenon would be that this was due to dilution of newly synthesized radioactive protein with pre-existing unlabelled protein which had accumulated within the cell by the time of addition of radioactive amino acid. In cells pulse-labelled at the early stage of infection, the size of the intracellular pool of unlabelled virus precursor protein would have been small and therefore virus would have been made predominantly from the newly synthesized radioactive protein. When the pulse-labelling was done at the late stage of infection, however, the existing large pool of virus precursor protein would have been utilized for virus formation and thus the specific activity of virus would have become lower. Thus, virus proteins synthesized later in the course of infection would have a very low probability of entering virus particles. As we have described, however, inhibitors of protein synthesis caused prompt cessation of virus production even when they were added at the late stage of infection.

There may be several possible explanations of this phenomenon. Maturation of virus
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might depend on the continuous synthesis of virus structural proteins. If so, when the synthesis of virus protein is blocked, further maturation of virus does not occur, regardless of the concentration of virus proteins within the cell. An alternative explanation might be to postulate a ‘maturation’ protein for this virus as has been shown for adenovirus (Russell & Becker, 1968). Although the RNA molecule of NDV can specify some thirty different polypeptides, the only proteins of which functional roles are known are those of haemagglutinin, neuraminidase, nucleoprotein antigen and RNA-polymerase (Scholtissek & Rott, 1969; Haslam et al. 1969). Recent experiments on the analysis of the structural proteins of NDV by acrylamide gel electrophoresis have revealed one major unidentified protein and perhaps some minor ones (Haslam et al. 1969; Evans & Kingsbury, 1969). It is possible to assume that some structural protein or a virus-specified protein involved in the assembly process may be labile and require constant synthesis. In this connexion, the serine-requiring step in the reproductive cycle of NDV reported by Ito et al. (1969) and the ‘R’ component of haemagglutinating virus of Japan described by Hosaka (1968) are of special interest.

Recently, inhibition of assembly of arboviruses by cycloheximide or puromycin has been reported by several workers (Scheele & Pfefferkorn, 1969; Friedman & Grimley, 1969). Their results are similar in many respects to our findings with NDV. Some common mechanisms might be involved in the assembly process of these enveloped viruses.

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REFERENCES


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