Newcastle Disease Virus Infection of L Cells

TOBY T. HECHT¹ AND DONALD F. SUMMERS

Departments of Microbiology and Immunology and Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461

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Newcastle disease virus (NDV) California strain reportedly grows poorly in L cells but replicates very well in chicken embryo cells. NDV-infected L cell cultures show a characteristic virus growth curve with respect to uridine incorporation, but plaque assays of the virus produced 24 h postinfection (PI) show no infectious particles when assayed on L cell monolayers and only a very low titer on chick cell monolayers. Plasma membranes isolated and purified from infected L cells 8 h PI contain all of the major virion proteins. In addition, NDV-infected L cells show a 50% loss of H-2 antigenic activity, a phenomenon previously observed in cells productively infected with vesicular stomatitis virus. These results suggest that at least part of the normal process of NDV maturation occurs in NDV-infected L cells. Sodium dodecyl sulfate-polyacrylamide gel patterns of supernatant virus purified from cells radiolabeled with amino acids from 3 to 24 h PI in the presence of actinomycin D show that all the major NDV structural proteins are present. Electron micrographs of NDV-infected L cells show extensive virus maturation at cell membranes. It can be concluded that infection of L cells with NDV results in a normal production of virus-specific RNA, synthesis of all the major structural proteins, association of the viral envelope proteins with the L cell plasma membrane, and the loss of cell surface H-2 antigenic activity. However, most of the virus particles produced are noninfectious.

Newcastle disease virus (NDV) is an enveloped RNA-containing virus of the paramyxovirus group that matures at host cell membrane surfaces by a budding process (5, 6). In 1959, Wilcox (34) observed that large amounts of NDV added to L cell monolayers caused the cells to undergo a cytopathic effect but that this effect was not transmissible to other cells. It was also demonstrated that in these cells only a small amount of infectious NDV was produced, although the synthesis of virus-specific hemagglutinin was seemingly unaffected. However, NDV replicates to high titers of infectious virus in the allantoic sac of the chicken embryo as well as in chicken embryo fibroblast tissue culture cells (3, 15). It has been suggested that the defect in the replicative cycle of NDV in L cells is in the maturation of the virus at the cell membrane because only 1% of the low level of infectious NDV produced in these cells is released into the culture medium, whereas the remainder of the virus accumulates intracellularly and is destroyed without being released (29, 30). Previous studies have shown that the

production of interferon in L cells plays no role in the limited infection of these cells by NDV (35), but the basis of this abortive replication has not yet been established.

We therefore have studied the intracellular events of NDV-infected L cells in an attempt to understand more fully the block in NDV replication in these cells.

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MATERIALS AND METHODS

Cell culture and virus stock. Spinner cultures of L cells were grown in Joklik-modified Eagle medium (MEM) (Schwartz/Mann) plus 6% fetal calf serum (Grand Island Biological Co.) at a concentration of 5×10^5 to 10×10^5 cells/ml.

Primary chicken embryo cells were grown in roller bottles in MEM (Schwartz/Mann) supplemented with 8% fetal calf serum.

The California strain of NDV (kindly provided by Philip I. Marcus) was grown in the allantoic cavity of 10-day-old embryonated eggs and titered on confluent monolayers of secondary chicken embryo cells as previously described (15) and stored at -70°C . Plaque

¹ Present address: Department of Biology, Yale University, New Haven, Connecticut 06520.

assays performed on L cell monolayers were carried out by the method of Jimenez and co-workers (12).

NDV infection of L cells and purification of extracellular virus. L cells, at a concentration of 10^7 cells/ml, were infected at 37°C with 5 PFU of NDV per cell in MEM without serum in the presence of 14 mM N-2-hydroxyethyl-piperazine-*N'*-2'-ethanesulfonic acid buffer (Nutritional Biochemicals Corp.). At 30 min postinfection (PI), actinomycin D (a gift of Merck Institute, Rahway, N.J.) was added to a concentration of 10 µg/ml and at 1.5 to 2 h PI, the infected cell culture was diluted to 10^6 cells/ml with MEM containing 6% fetal calf serum. At 22 to 24 h PI cells were removed from the culture medium by centrifugation for 15 min at $700 \times g$. The supernatant fluid was subjected to centrifugation in a Spinco SW 27 rotor at 27,000 rpm for 2 h at 4°C. The virus-containing pellet was suspended in ET (1 mM Tris-hydrochloride; 1 mM EDTA, pH 7.6) buffer yielding a total volume of 2 ml, layered onto a 5 to 50% (wt/wt) sucrose gradient prepared in ET buffer, and centrifuged for 16 h in a Spinco SW 27 rotor at 24,000 rpm. Fractions of the gradient were collected and analyzed for absorbance at 260 nm in a continuous-flow cell in a Gilford recording spectrophotometer, and the density of selected fractions was determined either by measurements with a Bausch & Lomb, Inc., refractometer or by weighing 100-µl samples of the gradient fractions at room temperature. Virus-containing fractions were pooled and further analyzed or stored at -70°C.

Plasma membrane preparations from NDV-infected and uninfected L cells. Infected and noninfected L cells were radiolabeled with L- ^{14}C -labeled amino acid (U) mixture (New England Nuclear) from 3 to 8 h PI. Plasma membranes were then isolated from these cells by the procedure of Atkinson and Summers (2) except that the cells were swollen before homogenization in a saturated solution of fluorescein mercuric acetate (Nutritional Biochemical Corp.) made up in 10 mM Tris-hydrochloride, pH 8.0 (32). Radiolabeled purified membrane samples were solubilized and subjected to electrophoresis on 18-cm 7.5% polyacrylamide-sodium dodecyl sulfate (SDS) gels as previously reported (27).

Assay for H-2 alloantigen and the production of alloantisera. The production of anti-H-2^k antisera and the detection of H-2^k activity on the surface of virus-infected and uninfected L cells by the method of inhibition of immune cytotoxicity measured by the release of ^{51}Cr from target lymph node cells has been described in detail previously (9, 19). Antigenic activity is defined as units of antigen per milligram of protein. Total protein was determined by the method of Lowry with bovine serum albumin (Calbiochem) as standard.

RESULTS

Incorporation of radiolabeled uridine into NDV-infected L cells. L cells were infected with NDV in the presence of actinomycin D as described above. At 2 h PI, the infected cells were diluted 10-fold in MEM containing serum

and 0.2 µCi of [^{14}C]uridine (50 mCi/mMole) (New England Nuclear Corp.) per ml. Samples were removed at intervals, added to cold 5% trichloroacetic acid, and assayed for acid-insoluble radioactivity as previously detailed (8). After a 2-h lag, the cumulative incorporation of radioactive uridine was linear until approximately 10 h PI, with the maximal incorporation at 11 h PI (Fig. 1).

Synthesis of virus-specific proteins and the association of NDV structural proteins with the host cell plasma membrane. To test whether virus-specific proteins were synthesized in infected L cells and whether the envelope and nucleocapsid proteins were assembled at the plasma membrane of L cells as during the normal maturation of NDV particles in permissive hosts (16, 22), plasma membranes were isolated from infected L cells radiolabeled with amino acids from 3 to 8 h PI in the presence of actinomycin D. Surface membranes were partially purified on discontinuous sucrose gradients (2) and subjected to electrophoresis on 7.5% polyacrylamide-SDS gels. The three major virus-specific structural protein peaks and the several minor protein peaks resolvable by this gel electrophoresis system (Fig. 2A) are identi-

FIG. 1. The incorporation of [^{14}C]uridine in NDV-infected L cells. Cell samples were removed at indicated times and assayed for trichloroacetic acid-insoluble radioactivity.

cal to those described by other workers using various strains of NDV in chick cells and bovine kidney cells (7, 11, 18). Uninfected L cell plasma membranes showed gel patterns as seen in Fig. 2B. Therefore, NDV-specific protein synthesis occurs in L cells and at least the major structural protein products associate with the plasma membrane of these cells.

H-2 antigenic activity of NDV-infected L cells. It has previously been reported that mouse cells productively infected with enveloped viruses such as vesicular stomatitis virus and murine leukemia viruses show a loss of cell surface histocompatibility (H-2) antigenic activity (1, 9). Because it is likely that it is the process of maturation of these enveloped viruses that results in this phenomenon (9), we

have studied the fate of H-2 antigenic activity in L cells infected with NDV.

Infected and noninfected L cells were harvested at 22 h PI by centrifugation, washed twice with cold Earle solution, and assayed for H-2 activity. Compared with uninfected cells, NDV-infected L cells showed a 50% loss of H-2 antigenic activity (Fig. 3). In other experiments in the absence of actinomycin D, NDV-infected cells showed a similar loss of activity compared with control cells (T. T. Hecht, unpublished observations). These results suggest that at least part of the normal process of NDV maturation occurs in NDV-infected L cells.

Composition and infectivity of L cell-grown extracellular NDV. L cells were infected with NDV and radiolabeled with amino acids from 3 to 24 h PI, at which time the cells were removed by centrifugation. Extracellular NDV was collected from the supernatant fluid and purified on sucrose gradients. Fractions from these gradients had an extremely high background of radioactivity as well as a low absorbance at 260 nm in the presumed virus-containing region. The fractions of density 1.16 through 1.18 g/cm³ yielded no plaques on L cell monolayers, but the same preparation assayed on chicken embryo cells yielded a total of 1.5×10^7 PFU of extracellular NDV from 10^8 L cells or 0.15

FIG. 2. Plasma membranes isolated from NDV-infected and uninfected L cells. A total of 2×10^8 L cells in 20 ml were infected with NDV or left uninfected in the presence of actinomycin D. At 90 min PI, cell cultures were diluted ten-fold, and at 3 h PI, 5 μ Ci of ¹⁴C-labeled amino acids per ml were added to the cultures. At 8 h PI, cells were washed twice with cold Earle solution and plasma membranes were prepared through a discontinuous density gradient step (2). The membranes were then resuspended in 0.1 ml of 0.01 M phosphate buffer containing 2% SDS and 1% 2-mercaptoethanol and subjected to 7.5% polyacrylamide-SDS gel electrophoresis for 14 h. The anode in this figure is to the right. I, II, and III represent the major NDV structural protein peaks previously described (11). (A) Virus-infected plasma membranes; (B) uninfected plasma membranes.

FIG. 3. H-2 antigenic activity of NDV-infected L cells. Cell suspensions (antigen) were serially diluted 1:2 and reacted with appropriately diluted anti-H-2^k antiserum, 1:10 dilution of guinea pig complement (Grand Island Biological Co.), and ⁵¹Cr-labeled lymph node cells from a B10.Br mouse (Jackson Laboratories). The percentage of lysis, measured by the release of ⁵¹Cr, was calculated for each dilution by using the release of radioactivity from a sample containing undiluted antiserum, 1:10 complement, and radiolabeled lymph node cells as the 100% lysis level. The reciprocal of 10 times the dilution that caused 50% lysis was taken as H-2 units per milliliter. When the full release control containing diluted antiserum but no inhibiting antigen was not 80% of the total radioactivity released by undiluted serum, the 50% lysis level for determining H-2 units per milliliter was adjusted. Symbols: ●, NDV-infected L cells; ○, uninfected L cells. The horizontal line is the adjusted 50% lysis level.

PFU/cell. Because the original inoculum was not removed from the culture before progeny virus appeared, it was not clear whether the low titer of infectious virus detected was actually newly synthesized virus or whether it was input NDV. That some new progeny particles were produced was ascertained by polyacrylamide gel electrophoresis of the purified extracellular virus. Figure 4 shows that all of the major NDV structural proteins were present in the purified virus and were radiolabeled, although there was proportionally less radioactivity in structural protein peaks I and III compared with the proteins present in NDV-infected plasma membranes at 8 h PI (Fig. 2a).

Electron microscopy of NDV-infected L cells. Because of the loss of H-2 antigenic activity from the surface of NDV-infected L cells and the presence of highly radiolabeled NDV-specific proteins in the culture medium, which suggested virus budding, and the low titer of NDV observed by plaque assays, electron micrographs of thin sections of infected cells were examined. NDV-infected L cells at 10 h PI in the presence of actinomycin D showed extensive maturation of virus particles at the plasma membrane (Fig. 5) as well as at intracellular membrane sites (Fig. 6). In addition to NDV particles of the usual round morphology, long filamentous virus particles also could be seen in these sections. These filamentous particles are typically found in paramyxovirus-infected cells and have been described previ-

ously in NDV-infected chick cells (6) and in SV₈-infected monkey kidney cells (4). It is not yet known whether these virus particles are infectious.

Therefore, it appears that although no appreciable infectious NDV particles were produced in L cells, maturation of the virus at the L cell membrane did occur.

DISCUSSION

NDV replicates to high titers of infectious virus in both the allantoic sac of the chicken embryo and chick fibroblast tissue culture cells (3, 15), to only a moderate extent in HeLa cells (33), and has been reported to undergo an abortive replicative cycle in L cells (34). By using a low multiplicity of infection, Thacore and Youngner demonstrated the establishment of a persistent infection of NDV in L cells under certain circumstances (29, 30). The virus produced, called NDVpi, in contrast to the input wild-type NDV, was no longer nonpermissive for L cells and replicated efficiently in these cells. These authors claimed that L cells are not nonpermissive for NDV replication but rather nonpermissive for NDV maturation at the cell surface membrane. Recently, it was demonstrated that NDVpi derived from various NDV strains was temperature-sensitive in chicken embryo cells at 43 C, and in all cases viral RNA was not synthesized (20, 21).

In light of these observations, we undertook a study of the intracellular events of NDV-infected L cells in order to elucidate the defect in NDV replication in this cell line.

A block in the penetration of virus into cells as well as in virus-specific RNA synthesis was ruled out because actinomycin D-treated NDV-infected L cells showed a seemingly normal virus growth curve with respect to uridine incorporation (Fig. 1).

In addition, synthesis of virus-specific polypeptides was not inhibited in L cells, and at least the major structural protein products became associated with the cell surface as they would during NDV maturation in permissive cells (Fig. 2a). L cells infected with NDV showed a 50% loss of the H-2 antigenic activity from the cell surface (Fig. 3). It is not yet clear whether this loss of H-2 activity is due to the incorporation of H-2 antigen into the virion envelope during its exit from the host cell or whether it is due to displacement of the antigen by virus-specific envelope proteins (9). In any case, these data indicate that part of the normal process of NDV maturation occurs in NDV-infected L cells.

FIG. 4. ¹⁴C-labeled amino acid radiolabeled proteins of extracellular virus from NDV-infected L cells. Infected L cells were radiolabeled from 3 to 24 h with 5 μ Ci of ¹⁴C-labeled amino acids per ml. Extracellular NDV was then purified on 5 to 50% (wt/wt) sucrose gradients. The virus-containing fractions were pooled and precipitated with an equal volume of cold 10% trichloroacetic acid. After two washings with cold 5% trichloroacetic acid and one washing with cold acetone, the precipitate was solubilized in 0.2 ml of 0.01 M phosphate buffer containing 2% SDS and 1% 2-mercaptoethanol and subjected to 7.5% polyacrylamide-SDS gel electrophoresis. The anode in this figure is to the right.

FIG. 5. *Maturation of virus particles at the plasma membrane of NDV-infected L cells. Thin sections of L cells infected with NDV for 10 h in the presence of actinomycin D were stained with uranyl acetate and lead citrate. Magnification, $\times 32,000$.*

Virus purified from the culture medium of NDV-infected L cells radiolabeled with amino acids contained all the major NDV structural

proteins, although there was proportionately less radioactivity in protein peaks I and III and more of the minor proteins compared with the

FIG. 6. Intracellular maturation in NDV-infected L cells. Thin sections of L cells infected with NDV in the presence of actinomycin D were stained with uranyl acetate and lead citrate. Note the filamentous viral particles in addition to spherical NDV. Magnification, $\times 66,000$.

proteins present in NDV-infected plasma membranes (Fig. 2 and 4). In these experiments, plasma membranes were isolated at 8 h PI,

whereas extracellular virus was collected at 22 h PI, and it is possible that the proportion of NDV-specific proteins synthesized in these cells

changes during the course of infection. Alternatively, the plasma membrane fraction may be enriched in proteins in peaks I and III compared with whole virions. This enrichment could have occurred either because the glycosylated and nonglycosylated viral envelope proteins (peaks I and III, respectively) (25) were synthesized and inserted into the plasma membrane in greater quantity than could be packaged into mature virions or because preferential sticking of viral envelope proteins to membrane occurred during the membrane isolation procedure.

Extracellular NDV assayed for infectivity yielded no plaques on L cells and a titer of only 0.15 PFU/cell on chicken embryo cells. If all the virus particles that matured through the plasma membrane were infectious particles, this would imply approximately one NDV maturation site per seven cells, which is probably an overestimation because the inoculum virus was not removed during the course of infection. However, electron micrographs of thin sections of NDV-infected L cells showed large numbers of intracellular virus as well as extensive maturation of virus at the L cell plasma membrane (Fig. 5 and 6). These maturing viruses were NDV and not the endogenous C-type particles of L cells because "L cell virion" production is susceptible to actinomycin D used in these studies. Because a great many more than one NDV maturation site per L cell could be seen in these electron micrographs, it was concluded that although seemingly normal virus maturation did occur, most of the NDV particles produced at the L cell surface were noninfectious. Thus, the block in the production of infectious NDV in L cells likely resides not in the process of maturation but rather in the composition of the virion particle itself.

One possibility is that the virus particles from L cells lack the virion-associated transcriptase or that the transcriptase is inactive or unstable. Although it has been shown that the virus envelope proteins are not involved in transcriptase activity, the structural protein determining this activity has not yet been identified (17). It is possible that transcriptase activity is associated with a polypeptide not easily resolvable by electrophoresis and therefore its absence in NDV from L cells would not be detected.

A second possibility is that a protein other than RNA polymerase, either structural or non-structural, is absent or in less quantity in NDV grown in L cells compared with virulent NDV. A precursor of one of the viral envelope proteins has been described for another paramyxovirus, Sindbis virus, in chicken embryo cells (26) and

more recently, a similar precursor protein has been observed in NDV-infected chicken embryo cells (24). This NDV-specific precursor protein, P₁₁, has been shown to migrate on polyacrylamide gels between peaks I and II. NDV from L cells seems to contain at least two proteins between these two peaks, and one of these polypeptides may well correspond to P₁₁ (Fig. 4). These NDV-specific minor structural proteins are present in virions from L cells to a greater extent than are present in virions from permissive cell lines (7, 18, 25). It may be that this precursor protein is normally enzymatically cleaved by the host cell, but that this does not occur in L cells with defective progeny NDV resulting. Recent support for this possibility came from the work of Homma and Ohuchi (10), who demonstrated polypeptide differences between Sendai virus grown in L cells with low infectivity and Sendai virus grown in eggs with high infectivity. Trypsin treatment of the L cell-grown virus converted a large structural polypeptide to a smaller one seen in egg-grown Sendai virus, increasing the biological activity of the virion.

A final explanation for the lack of infectivity of L cell-grown NDV is that the virion envelope of this virus might be significantly different in lipid and carbohydrate composition from that of infectious NDV. It is well known that during the process of maturation of enveloped viruses, the virion envelope lipids and carbohydrates are derived from the plasma membrane of the host cell (13, 14, 23, 28). Thus, the lipids and/or carbohydrates of the L cell-grown NDV envelope might act either to limit or inhibit infectivity directly or to order the orientation of virus envelope proteins in the membrane in such a manner that infectivity is affected. Wainberg and Howe (31) suggested that free movement and rearrangement of plasma membrane lipids of the host cell as well as the virus-specific envelope proteins of Sendai virus promote virus-mediated cell fusion of cells. Perhaps, NDV grown in L cells no longer has the properties necessary to promote cell fusion and therefore can no longer fuse with the cell membrane itself in a manner that will allow entrance into a new host cell.

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