Influenza Type A Virus Neuraminidase Does Not Play a Role in Viral Entry, Replication, Assembly, or Budding

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We have used a neuraminidase-deficient influenza virus, NWS-Mvi, which was selected by supplying bacterial neuraminidase in the medium (C. Liu and G. M. Air, Virology 194:403–407, 1993), to define the role of neuraminidase in influenza virus replication. Electron microscopy showed that virions of the NWS-Mvi mutant assembled normally and formed large aggregates associated with cell surfaces. The NWS-Mvi virus grown in the absence of neuraminidase was able to carry out a second round of replication in MDCK cells without added neuraminidase, indicating that the virus particles contained in these aggregates were infectious. Aggregates of virus were also found in cytoplasmic vacuoles. When virus-infected cells were incubated in the presence of ferritin, such aggregates were found to be labeled with ferritin, indicating that they are derived from uptake at the cell surface. When the neuraminidase-deficient virus was administered intranasally to C57BL/6 mice, low titers of virus were recovered from the lungs and major histocompatibility complex class I-restricted cytotoxic T cells were generated: evidence that cells were infected in vivo. In C57BL/6 nu/nu mice, the low level of virus persisted for at least 28 days but never increased. These results suggest that neuraminidase is not required for influenza virus entry, replication, or assembly in cell culture or in mice.

Influenza type A and B viruses contain two major surface glycoproteins embedded in the viral membrane, hemagglutinin (HA) and neuraminidase (NA). The HA mediates binding of influenza virus to its receptor, sialic acid, on the cell surface and a fusion process between viral and endosomal membranes. The NA has a receptor-destroying activity to cleave the α-ketosidic linkage between terminal sialic acid and an adjacent sugar residue. Influenza virus HA and NA are both glycosylated proteins, but the terminal sialic acids are cleaved away by the enzymatic activity of NA (reviewed in references 1 and 12).

Results from early studies with antisera that inhibited NA activity suggested that influenza virus NA may function in the final stage of infection (11, 21). When the activity of NA was abrogated by antibody (5), inhibitors (15), or temperature-sensitive mutation at nonpermissive temperature (16, 18), sialic acids were not cleaved and remained linked to the complex carbohydrate of the HA and NA. Under these conditions, viral particles attached to each other or to the cell surface to form large aggregates. Formation of these aggregates could be prevented by addition of bacterial NA into the medium (16). From these results, Palese and Compans (16) proposed that the function of NA in influenza virus replication is to prevent formation of virus aggregates by removing sialic acids from the virus envelope. Another proposed role for the NA is to release newly assembled virus particles from the cell surface (16, 19), although concentration effects have to be invoked to explain why the receptor-destroying activity of the NA does not preclude attachment of the virus to cellular receptors. A third hypothesized role for the NA was to remove sialic acids in mucin to lower the viscosity and allow the virus to reach the epithelial cells (3).

It has been shown for the neurovirulent variant, A/WSN/33, that the growth properties of this virus conferred by the NA gene are the result of increased cleavability of the HA and that this can be modified by glycosylation of the NA (13, 17). In some influenza viruses, NA may also play a role in virus-mediated membrane fusion under certain conditions (8, 9). The experiments used fowl plague virus (H7N5) in a system in which fusion was observed between liposomes containing defined proteins and erythrocytes. It was reported that in addi-

FIG. 1. Productive infection by the NWS-Mvi mutant virus that has not been exposed to NA. The medium and cell debris from a first round of NA-free infection with NWS-Mvi mutant virus were sonicated and used to infect MDCK cells in the absence of NA. At 1, 3, 5, and 7 h postinfection, viral proteins were radiolabeled, immunoprecipitated, and separated as described in Materials and Methods. An autoradiograph of the SDS-gel of the precipitated viral proteins from MDCK cells infected with NWS/G70c (wild type) or NWS-Mvi (NA deficient) is shown. Influenza viral proteins HA, NA, NP, and M1 are indicated. The sizes of molecular mass markers (in kilodaltons) are indicated.
FIG. 2. Assembly of the NA-deficient NWS-Mvi virus particles. MDCK cells were infected with either wild-type NWS/G70k virus or NWS-Mvi mutant virus. At 10 h postinfection, the cells were processed for electron microscopy as described in Materials and Methods. (A) Low-magnification view of a cell showing aggregates of the NA-deficient NWS-Mvi mutant virus (arrows) at the cell surface. Bar, 5 μm. (B) Higher magnification of a large aggregate associated with the cell surface. Bar, 1 μm. (C) NWS-Mvi virus in the presence of NA. Bar, 0.5 μm. (D) NWS/G70k virus. Bar, 1 μm.
FIG. 2—Continued.
The NWS-Mvi mutant virus grown in MDCK cells was centrifuged. The initial concentration was omitted and the sucrose gradient was centrifuged to collect the purified virus. The NWS-Mvi mutant virus was grown in MDCK cells in a 35-mm plate were infected with 200 \( \mu l \) of PBS containing 0.9 mM CaCl\(_2\) and 0.5 mM MgCl\(_2\) (CaMg-PBS). Then 2 ml of infection medium (Dulbecco's modified Eagle's medium-Ham's F12 [1:1] with ITS series in CaMg-PBS, and 50 \( \mu l \) of the dilution was used to infect MDCK cells in quadruplicate wellsof 96-well plates. Viruses were allowed to attach to and infect cells by incubation for 1 h at 37\(^\circ\)C. Then, an equal volume of minimal essential medium containing 2% bovineserum albumin, 0.5 mg of trypsin per ml, and gentamicin was added, and the plates were incubated for 72 h at 37\(^\circ\)C. The infection medium and cell debris were collected, sonicated twice for 2 min each, and briefly centrifuged. The supernatant was then used to infect MDCK cells.

For the second round of NA-free infection, 400 \( \mu l \) of the supernatant de- posited on the plate at room temperature for 30 min. The infection was repeated with another 400 \( \mu l \) of inoculum.

The cells were washed once with CaMg-PBS to remove unattached virus and incubated at 37\(^\circ\)C in infection medium without addition of trypsin or bacterial NA. At the indicated time points, the cells were starved for 1 h before being labeled with transfectants or labeled with meth (NEN/DuPont) protein labeling mix for 1 h. At the end of the labeling, the cells were lysed on the plate, and immunoprecipitation was performed. The antibody used for the immunoprecipitation was affinity-purified rabbit sera made against A/PR/8/34, NWS HA (H1), and NWS/G70c NA (N9) in a ratio of 2:1:1. The anti-A/PR/8 core serum recognized influenza virus A polymerase proteins and nucleocapsid protein; it also bound to the matrix protein to a lesser extent (23). The precipitated viral proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

**Electron microscopy.** The infected cells were washed three times with PBS, fixed with 1% glutaraldehyde in PBS for 30 min at room temperature, and postfixed with 1% osmium tetroxide. The fixed cells were dehydrated with increasing concentrations of ethanol from 50% to 100% and embedded in an epoxy resin mixture. Polymerization was done at 60\(^\circ\)C for 72 h. The ultrathin sections were stained with uranyl acetate and lead citrate. The sections were viewed and photographed with a Philips transmission electron microscope. Infection of mice with influenza virus. Female C57BL/6J (B6) mice were purchased from Jackson Laboratories, Bar Harbor, Maine. B6 nu/nu mice were bred in the Animal Resource Center, St. Jude Children's Research Hospital. All mice were maintained under specific-pathogen-free conditions and first exposed to virus at 6 to 9 weeks of age. Groups of mice were anesthetized and exposed intranasally to 30 \( \mu l \) of PBS containing virus (2) at a dilution indicated in the text. The infected mice were sacrificed at 4 days postinfection or at the time indicated in the text. Lungs were homogenized, and virus titers were determined to obtain the 50% tissue culture infective dose (TCID\(_{50}\)) in MDCK cells.

The infected virus obtained from the lungs was characterized as the NWS-Mvi mutant virus by the hemagglutinin inhibition assay and determined to obtain growth on addition of NA.

**Virus titer determination to obtain TCID\(_{50}\).** Viruses were diluted in 10-fold series in CaMg-PBS, and 50 \( \mu l \) of the diluted virus was used to infect MDCK cells in quadruplicate wells of 96-well plates. Viruses were allowed to attach to and infect cells by incubation for 1 h at 37\(^\circ\)C. Then, an equal volume of minimal essential medium containing 2% bovine serum albumin, 0.5 mg of trypsin per ml, and gentamicin was added, and the plates were incubated for 72 h at 37\(^\circ\)C. For the NWS-Mvi mutant virus, 2 \( \mu l \) of NA per ml was added to the infection medium. Hemmagglutination was performed with 25 \( \mu l \) of the supernatant from the infected cells, 25 \( \mu l \) of PBS, 50 \( \mu l \) of 0.5% BSA, and 0.5% of the 0.1% final dilution. The end titration was read after 60 min at 4\(^\circ\)C. The TCID\(_{50}\) is the antilog of the dilution at which 50% of the cells were positive for virus.

**Immunofluorescence staining.** The lungs from infected mice were divided as follows. The right lobe was frozen for virus titer determination, and the left lobe was embedded in O.C.T. compound (Miles Inc., Elkhart, Ind.) to make sections for immunofluorescence staining. The sections were stained either with a polyclonal rabbit serum against H1N2 or a nonspecific rabbit antiserum, followed by fluorescein isothiocyanate-labeled secondary goat antiserum against rabbit immunoglobulin G.

**Cytotoxic T-cell response in mice infected with the NWS-Mvi mutant virus.** Cells were isolated from mediastinal lymph nodes and by bronchoalveolar lavage as described previously (2). The phenotype of cells was determined by labeling with appropriate antibodies and fluorescence-activated cell sorter analysis. Cells were cultured in the presence of syngeneic splenocytes which had been infected with an H3N2 influenza virus, X-31 (40), to stimulate the growth of any nucleoprotein-specific T cells. Cytotoxic activity of the cultured populations was measured on MC57G target cells that were infected with either X-31 or B/HK/8/73 virus. Effector-to-target cell ratios of 20:1 and 10:1 were used.

**RESULTS**

Infection of MDCK cells with NA-deficient influenza virus in the absence of NA. Growth of the NA-deficient NWS-Mvi virus depended on addition of NA. After purification through two rounds of sucrose gradient centrifugation, the NWS-Mvi virus was able to infect MDCK cells in the absence of added...
FIG. 3. Endocytosis of the NWS-Mvi virus into infected cells in the absence of NA. (A) Vesicles containing virus particles inside the cells infected with the NWS-Mvi mutant virus. Some vesicles appeared to be swollen or disrupted. Bar, 0.5 μm. (B and C) Ferritin clusters from surface labeling associated with virus particles inside the vesicles. Bars, 0.5 μm (for panel B) and 0.25 μm (for panel C).
When such an infection was at high multiplicity of infection, a cytopathic effect was observed but no virus could be detected by HA titration. To examine whether particles generated in the absence of NA were infectious, the medium with cell debris from the NA-free infection was sonicated. The supernatant from the sonication was used to infect MDCK cells in a second round of NA-free infection. The time course and extent of viral protein synthesis in the second infection with NWS-Mvi mutant virus was similar to that with wild-type NWS/G70c virus (Fig. 1). Because the virus used for the second round of infection had never been exposed to any NA, this result clearly demonstrated that infectious virus was produced in the absence of NA activity and that neither NA activity nor

NA (14). When such an infection was at high multiplicity of infection, a cytopathic effect was observed but no virus could be detected by HA titration. To examine whether particles generated in the absence of NA were infectious, the medium with cell debris from the NA-free infection was sonicated. The supernatant from the sonication was used to infect MDCK cells in a second round of NA-free infection. The time course and extent of viral protein synthesis in the second infection with NWS-Mvi mutant virus was similar to that with wild-type NWS/G70c virus (Fig. 1). Because the virus used for the second round of infection had never been exposed to any NA, this result clearly demonstrated that infectious virus was produced in the absence of NA activity and that neither NA activity nor

TABLE 2. Infection of C57BL/6 normal or nu/nu mice with NA-deficient NWS-Mvi or parental NWS/G70c influenza virus

<table>
<thead>
<tr>
<th>Virus and mouse</th>
<th>Log_{10} TCID_{50} in lungs of mice ona:</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 28</th>
<th>Day 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>NWS/G70c (C57BL/6 mice)</td>
<td>6.0 ± 0.4</td>
<td>6.1 ± 0.1</td>
<td>4.3 ± 0.8</td>
<td>&lt;3.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NWS-Mvi C57BL/6 mice</td>
<td>4.4 ± 0.1</td>
<td>3.3 ± 0.6</td>
<td>&lt;3.0</td>
<td>&lt;3.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NWS-Mvi nu/nu mice</td>
<td>5.6 ± 0.2</td>
<td>5.2 ± 0.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> The numbers are mean values for three mice ± standard deviations. The infecting dose was 1.2 × 10^{5} TCID_{50} per mouse for NWS-Mvi mutant virus and 6.7 × 10^{5} TCID_{50} per mouse for NWS/G70c virus.

<sup>b</sup> <3.0, undetectable.

FIG. 4. Infection and replication of NA-deficient NWS-Mvi mutant influenza virus in mice as detected by immunofluorescence. The C57BL/6 mice were infected with either the NA-deficient NWS-Mvi mutant or wild-type NWS/G70c virus. At 4 days postinfection, sections of lung and trachea were prepared from infected mice and stained with either a rabbit antiserum against the HA of NWS/G70c virus (A, C, E, and G) or a nonspecific rabbit serum (B, D, F, and H). The second antibody was fluorescein isothiocyanate-labeled goat anti-rabbit Ig antiserum. Panels A to D were trachea sections; panels E to H were lung sections. The tissues in panels A, B, E, and F were prepared from mice infected with NWS/G70c virus, and the tissues in panels C, D, G, and H were from mice infected with the NWS-Mvi mutant.
exposure to NA is required for virus binding to its receptor, entry into the infected cells, or subsequent fusion of the viral membrane with endosome to release replication-competent viral RNA into the cell.

Assembly of the NA deficient NWS-Mvi mutant influenza virus. In the absence of NA, virus particles of the NWS-Mvi mutant were indeed assembled and budded as seen by electron microscopy, although they formed large aggregates (Fig. 2A and B). In contrast, in cells incubated with Micromonospora viridis laciens NA in the medium, individual virions but no viral aggregates were observed on the cell surface (Fig. 2C). Similarly, in cells infected with wild-type virus (Fig. 2D), only individual virions were found associated with the cell surface. Morphologically, no difference was seen between the mutant and wild-type virus particles. The number of virus particles associated with surfaces of cells infected with the NWS-Mvi virus was somewhat larger than the number associated with cells infected with the wild-type virus (Table 1). Most of the virus particles appeared to exhibit an elongated morphology. Most of the contacts between the mutant virus particles were longitudinal. When sectioned perpendicularly to the longitudinal axis, the virus particles appeared in regular hexagonal arrays.

Reentry of viral aggregates into cells by endocytosis. In addition to viral aggregates at the cell surface, a number of large vesicles containing many viral particles in the MDCK cells infected with NWS-Mvi mutant virus were observed (Fig. 3A). Most of the viral particles within the vesicles were closely associated with the vesicle membrane. In some cases, the vesicle membrane appeared to be partially disrupted.

To differentiate whether these vesicles containing viral particles were the result of budding from internal cellular membranes or virus aggregates that had reentered the infected cells from the surface, the infected cells were exposed to ferritin, further incubated for 4 h, and examined by electron microscopy. As shown in Fig. 3B and C, ferritin particle clusters were associated with virions within the vesicles. These results indicate that such vesicles are formed by endocytosis of the virus associated with cell surfaces. Conceivably, such particles could be infectious, but there is no evidence to suggest that uncoating rather than degradation is occurring.

Infection of mice with the NA-deficient mutant virus. Groups of C57BL/6 mice were infected with either the NA-deficient NWS-Mvi mutant virus or the parental NWS/G70c virus. At 3 and 5 days postinfection, virus was present in the lungs of the mice infected with either the NWS-Mvi mutant virus or NWS/G70c virus (Table 2), but the yield of mutant virus (whose titer was determined in the presence of bacterial NA) was nearly 1,000 times less than that of wild-type virus at day 5 postinfection. Characterization of the virus from the lungs confirmed that it was the NA-deficient mutant virus as used for the infection (data not shown). At 7 days postinfection, infectious virus was undetectable in mice infected with the NWS-Mvi mutant virus. Viral infection and replication in mice infected with the NWS-Mvi mutant virus were also examined by indirect immunofluorescence staining of the lung and trachea from these mice (Fig. 4). The positively stained cells from mice infected with the NWS-Mvi mutant or NWS/G70c virus showed similar levels of fluorescence, indicating that the NWS-Mvi mutant virus infected and replicated in mice.

The dose response to the NWS-Mvi mutant and wild-type virus in mice is shown in Table 3. The NA-deficient NWS-Mvi virus replicated to a lower level that was independent of the infecting dose (Tables 2 and 3) and was cleared earlier than wild-type virus (Table 2). The parental strain NWS/G70c has not been adapted to mice; therefore, the output virus titers are not much above input levels. To demonstrate that the virus titers were the result of replication rather than persistence of infecting virus, the NWS-Mvi mutant or NWS/G70c virus was treated with polyclonal antisera against the HA before infection. No virus was detected in the mice infected with neutralized virus (results not shown).

Clearance of influenza virus infection is normally mediated by major histocompatibility complex class I-restricted CD8+ cytotoxic T cells (6). The lymphocyte subsets present in the lungs and mediastinal lymph nodes of mice infected with the NA-deficient virus were similar to those present in mice infected with wild-type virus (Table 4); these subset ratios are similar to those obtained with the X-31 virus (2). The influx of CD8+ T cells into the lung (Table 4) suggests that virus-specific cytolytic cells were present. The cytotoxic T-cell response against influenza virus in C57BL/6 mice is directed predominantly to a peptide of the NP protein (20). The generation of influenza virus-specific major histocompatibility complex class I-restricted T cells therefore indicates that cells which contain replicating virus are present. To determine whether the wild-type or mutant virus could generate such a response, cells from infected mice were stimulated with an H3N2 influenza virus and the cytotoxicity of the cultured cells was determined with a major histocompatibility complex class I+ II+ target cell and an effector/target ratio of 20:1. The cytolytic activity was measured as release of 51Cr from MC57G target cells infected with A/X-31 or B/HK/8/73. The percent release from target cells infected with A/X-31 is 15% for NWS/G70c and 10% for NWS-Mvi; the percent release for target cells infected with B/HK/8/73 is 3% for NWS/G70c and 0% for NWS-Mvi. Responses were seen following both wild-type and NWS-Mvi infections, further indicating that virus replication had occurred.

### Table 3. Dose response of mice infected with NA-deficient NWS-Mvi or parental NWS/G70c virus

<table>
<thead>
<tr>
<th>Virus</th>
<th>Infecting dose (log10 TCID50)</th>
<th>Virus yield in lungs (log10 TCID50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NWS-Mvi</td>
<td>3.0</td>
<td>3.9 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>4.8 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>4.6 ± 0.1</td>
</tr>
<tr>
<td>NWS/G70c</td>
<td>3.2</td>
<td>4.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>4.2</td>
<td>7.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>5.2</td>
<td>7.5 ± 0.3</td>
</tr>
</tbody>
</table>

* Average value for three mice ± standard deviation.

### Table 4. T-cell responses in mice infected with NWS/G70c or NWS-Mvi virus as shown by the percentage of cells staining for lymphocyte subset markers

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Marker</th>
<th>% of cells staining for markers in mice infected with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NWS/G70c</td>
<td>NWS-Mvi</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>Thy1</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>CD4</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>CD8</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>B220</td>
<td>45</td>
</tr>
<tr>
<td>Bronchial lavage</td>
<td>Thy1</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>CD4</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>CD8</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>B220</td>
<td>5</td>
</tr>
</tbody>
</table>
To confirm that the low levels of the NA-deficient NWS-Mvi virus were the result of replication and that clearance was by immune mechanisms rather than just inability to replicate, we determined whether NWS-Mvi virus could establish a persistent infection in the absence of an intact immune response. C57BL/6 nu/nu mice were infected, and the lungs were examined for titers of infectious particles at days 3 and 28 postinfection. In contrast to the results obtained with normal C57BL/6 mice, infectious virus was still present at 28 days postinfection (Table 2). The amount of virus found on day 28 was approximately the same as that on day 3. The prolonged infection with NWS-Mvi mutant virus in the nu/nu mice provided further evidence that the NA-deficient virus replicated in the lungs of infected mice.

**DISCUSSION**

The selection of a virus with a massive deletion in NA coding capacity has given us the opportunity to define the role of influenza virus NA in virus replication. In the absence of NA, the NWS-Mvi mutant virus carried out a complete round of infection in MDCK cells. The electron microscopy results showed that NWS-Mvi virions were assembled normally and formed virus aggregates without NA. When dispersed by sonication, virions contained in these aggregates were able to infect MDCK cells in a second round of NA-free infection (Fig. 1), demonstrating that the aggregated virions were infectious. Because the aggregated progeny virions had never contacted NA, this result indicated that exposure to NA was not required for virus binding to its receptor, entry into the infected cells, and subsequent fusion of the virus and endosome membrane to release viral RNA.

Influenza virus aggregates have been observed when the activity of NA was reduced by antibody (5), inhibitors (15), or temperature-sensitive mutation at nonpermissive temperature (7, 16, 18). The results presented in this paper show that formation of aggregates inhibits multiple-round replication of the NWS-Mvi mutant virus and that this inhibition can be overcome by addition of NA to the medium. In cell culture, the only defect of the NWS-Mvi mutant that we have been able to identify is formation of virus aggregates as a result of absence of NA activity. Virus replication is inhibited because progeny viruses cannot leave the infected cell to spread into new cells. The NWS-Mvi virus infected and replicated in mice but at a low level compared with wild-type virus. A cytopathic T-lymphocyte response was generated that apparently cleared the virus as in a normal influenza virus infection. In nu/nu mice, the NWS-Mvi mutant virus persisted for at least 28 days, confirming both the role of T cells in clearance and the notion that the low levels of virus detected are indeed the result of a limited infection. These results demonstrate that the NA is not required for influenza virus infection in the respiratory tract of an animal, suggesting that removal of sialic acid from mucin to lower the viscosity (4) is not a critical role of the enzyme. However, infection with an NA-deficient virus is much less productive, as would be expected if virus spread is reduced by the absence of NA.

In summary, the results presented in this paper show that NA activity is not required for influenza virus entry, replication, or assembly in cell culture or in mouse models. The block to multicyle infection in the absence of NA is only due to a shortage of released particles.

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