Infection with influenza virus is commonly associated with polymorphonuclear neutrophil (PMNL) dysfunction and consequent secondary bacterial pneumonia. A recently isolated human-derived protein that inhibits PMNL chemotaxis and oxidant production shows a striking homology to the influenza A nucleoprotein. In the present study, the effects of purified influenza A nucleoprotein on PMNL chemotaxis, oxidant production, degranulation, and calcium homeostasis were studied. Results of the study demonstrate that purified nucleoprotein inhibits PMNL chemotaxis as well as superoxide production. In addition, purified nucleoprotein induces a rise in PMNL cytosolic calcium concentration in a manner similar to that demonstrated for crude influenza A lysates. In contrast, no difference in FMLP-stimulated PMNL elastase or β-glucuronidase release was noted after exposure to nucleoprotein. These studies suggest that the influenza A nucleoprotein may account for some of the neutrophil defect associated with cellular infection by this virus.

Methods

Purification of influenza A nucleoprotein. Influenza A/N4S Tokyo strain lysates that had been depleted of coat proteins, including neuraminidase and hemagglutinin, by treating with pronase were a gift from W. Graeme Laver (John Curtin School of Medical Research, Canberra, Australia). These lysates demonstrated two major bands on SDS-PAGE: 52 kDa, representing viral nucleoprotein, and 24 kDa, representing viral matrix protein. The 52-kDa viral nucleoprotein was further purified by either size-exclusion high-performance liquid chromatography (HPLC) or immunoaffinity chromatography.

HPLC was done as previously described [16] using a silica-based size-exclusion column (Zorbax; DuPont, Wilmington, DE) and a 0.1 M potassium phosphate buffer. Approximately 100 μg of pronase-treated viral core lysates was injected into the column and eluted at a flow rate of 0.25 mL/min. Material absorbing at 280 nm at the elution time appropriate for the size of the nucleoprotein (52 kDa) was collected and used for studies. Western blotting showed this purified fraction to be recognized by antiserum to whole influenza A nucleoprotein (provided by R. Webster, St. Jude Children’s Research Hospital, Memphis) and by commercially prepared antiserum produced in rabbits to amino acids 483–492 of the nucleoprotein (Research Genetics, Huntsville, AL).

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Human experimentation guidelines of the US Department of Health and Human Services and those of authors’ institutions were followed in the conduct of clinical research.

Financial support: Birmingham VA Medical Center Research Service and Career Investigator Award from American Lung Association (both to J.A.D.C.).

Received 6 March 1995; revised 30 August 1995.


The Journal of Infectious Diseases 1996; 173:279–84 © 1996 by The University of Chicago. All rights reserved.
Immunoadfinity chromatography was done using the antiserum produced in rabbits to amino acids 483–492, which are conserved in nucleoprotein from all strains of human influenza A virus tested to date [17]. The affinity column was constructed using the Affinica antibody orientation kit (Schleicher & Schuell, Keene, NH), which uses protein A–agarose to bind immunoglobulin and dimethyl suberimidate to cross-link the immunoglobulin to the protein A with proper orientation. Pronase-treated influenza A core lysates were first treated with deoxycholic acid (final concentration in PBS, 0.2%) for 5 min at 37°C, and then ~1 mg of protein was loaded onto the column, and nonbinding material was washed off with PBS. Bound material was then eluted with 0.2 M glycine HCl (pH 2.3) into TRIS buffer (pH 8.0) to immediately neutralize the pH.

**PMNL chemotaxis.** The degree of inhibition of PMNL chemotaxis by natural purified nucleoprotein was determined as previously described [14, 15] using 10 nM human recombinant C5a (Sigma, St. Louis) or 10 nM interleukin-8 (R and D Systems, Minneapolis) as agonists and multiwell chemotaxis chambers (Neuroprobe, Bethesda, MD) using nitrocellulose membranes. PMNL purified as previously described by ficoll–hypaque and dextran sedimentation [14] were suspended at 4 X 10^6 cells/mL in Hanks’ balanced salt solution (HBSS) with 0.1% bovine serum albumin (BSA) and directly exposed to various concentrations of purified nucleoprotein or BSA (control) for 15 min at room temperature.

Purified nucleoprotein or BSA was also added to the agonist solution. Agonist solution (22 μL) was placed in bottom wells of the chemotaxis chamber, and a nitrocellulose membrane with 8-μm pores was overlaid, followed by a rubber gasket. The top plate of the chamber was secured, and the entire chamber was warmed to 37°C for 15 min. The PMNL suspension (50 μL) was then added to the top wells, and the plates were incubated for an additional 1–1.5 h. Chemotaxis was assessed by comparing the maximal distance migrated (leading-front technique) to agonist after the various treatments.

**PMNL superoxide production.** PMNL superoxide production in the presence of nucleoprotein or BSA control was measured using an assay using the reduction of ferricytochrome C by superoxide as previously described [18]. PMNL were suspended at 4 X 10^6 cells/mL in HBSS. Cells (50 μL) were added to wells of a 96-well microtiter plate. Purified nucleoprotein suspension (10 μL; final concentration, 1 μM) was then added, and the mixtures were incubated at room temperature for 15 min. HBSS (50 μL) or superoxide dismutase (final concentration, 12.5 μg/mL; Sigma) was then placed in appropriate wells, and cytochrome C (final concentration, 80 μM) was added, followed by FMLP (final concentration, 8 μM). Plates were incubated for 1 h at 37°C, and absorbance was determined at 550 nm on a microplate reader. Superoxide concentration was calculated by determining the degree of superoxide dismutase–inhibitable cytochrome C reduction and multiplying by the known extinction coefficient. Results are reported as (nmol/10^6 cells) – buffer control.

**PMNL degranulation.** Effects of nucleoprotein on PMNL degranulation were determined by measuring elastase and β-glucuronidase concentrations in supernatants of PMNL stimulated with FMLP after exposure to nucleoprotein or BSA control. PMNL (10 X 10^6 cells/mL) were suspended in HBSS with 0.1% BSA. Cells (300 μL) were added to microcentrifuge tubes, and nucleoprotein or BSA was added (final concentration, 1 μM). Tubes were incubated for 15 min at room temperature. Cytochalasin B (final concentration, 10 μg/mL) was then added, and the plates were incubated at 37°C for 5 min. FMLP (final concentration, 10 μM) was added and the tubes were incubated 20 min more at 37°C. Supernatant was then separated from cells by centrifugation and frozen prior to use in assays.

**Elastase measurement.** Elastase was measured by a sandwich ELISA developed using commercially available antibodies to neutrophil elastase. Microtiter plates were coated overnight at 4°C with 100 μL of anti-neutrophil elastase antiserum (Calbiochem, San Diego, CA) diluted 1:5000. The next day, plates were washed with PBS containing 0.05% Tween and 0.1% BSA. Known concentrations of neutrophil elastase (Calbiochem) or dilutions of supernatants were added to wells, and the plates were incubated for 1.5 h at 37°C. Plates were washed, and 100 μL of a 1:4000 dilution of another anti-elastase antiserum (ICN, Costa Mesa, CA), prepared in sheep, were added. Plates were incubated 1.5 h more at 37°C then washed, and a 1:2000 dilution of a peroxidase-labeled antibody prepared in rabbits against sheep IgG was added. The plates were incubated for 1.5 h at 37°C and washed, and peroxidase substrate was added for enzyme development. Concentrations of elastase in the unknown samples were determined by comparing results against the standard curve produced for each assay. Results are reported as micrograms per milliliter.

**Measurement of β-glucuronidase.** A commercially available kit (Sigma) was used, with modification of the protocol, to measure β-glucuronidase. Modifications included doing all incubations in a microtiter plate and a 10-fold reduction in the volume of the various reagents used to allow for incubation in microtiter plates. Results are reported as phenathaline units per milliliter.

**Measurement of cytosolic calcium concentrations.** Effects of purified influenza A nucleoprotein on cytosolic calcium concentrations were determined using PMNL loaded with the fluorescent calcium indicator indo 1-AM (Molecular Probes, Eugene, OR) as previously described [15]. PMNL (10 X 10^6/mL) in HBSS with 0.1% BSA were incubated with 1 μM indo 1-AM for 1 h at room temperature, washed twice at 400 g for 10 min, and resuspended at 1 X 10^6 cells/mL in sterile HBSS. PMNL suspension (2.4 mL) was equilibrated at 37°C in a cuvette, and 24 μL of purified nucleoprotein or BSA (control) was added to a final concentration of 1 μM. The ratio of emission fluorescence alternating every 6 s between 405 nm (calcium-bound dye) and 485 nm (calcium-free dye) was recorded with a scanning spectrophotofluorometer (Shimadzu Instruments, Columbia, MD; excitation wavelength, 355 nm). After ~10 min, FMLP (final concentration, 1 μM) was added, and fluorescence monitoring was continued.

The relationship Ca^{2+} = (K_d [S] [R - [R]])/[R_o - [R]] was used to calculate [Ca^{2+}] by determining R, the ratio of fluorescence intensity of indo 1-AM–loaded cells at 405 or 485 nm; [R_o] the ratio of fluorescence intensity of loaded cells in a calcium-free environment using 2 mM EDTA; and [R], the ratio of fluorescence intensity of loaded cells in a 1 mM CaCl_2 environment. R_o and R were determined by lysing cells with 10 μg/mL digitonin in EDTA or CaCl_2 buffer. The published K_d (250 nM at physiologic pH) was used for these calculations.

**Determination of endotoxin concentration.** Concentrations of endotoxin in purified protein preparations were determined by the limulus lysate test (Associates of Cape Cod, Woods Hole, MA).
as previously described [18]. Sensitivity of the test was 4.0 pg/mL.

Statistics. Data were stored and analyzed using a software package (PCSTAT) and an Insight computer (both from Human Systems Dynamics, Northridge, CA). Differences in means were assessed by Student’s t or Wilcoxon signed rank tests. When multiple groups were involved, differences were assessed by analysis of variance and individual group differences were assessed by the Newman-Keuls test. All data represented are mean ± SE. P ≤ .05 was considered significant [19].

Results

Purification of influenza A nucleoprotein. Core lysates of influenza contained only two bands of protein on SDS-PAGE (figure 1). The molecular mass of these two proteins were consistent with those of the two main core proteins of influenza A, the nucleoprotein (52 kDa) and the matrix protein (24 kDa). After affinity chromatography or HPLC processing of the core lysates was done, only the 52-kDa protein remained. This protein was recognized on Western blotting by antiserum to whole influenza A nucleoprotein.

Effects of purified nucleoprotein on PMNL chemotaxis. PMNL exposed to various concentrations of nucleoprotein purified by immunoaffinity chromatography exhibited attenuated chemotaxis to C5a and interleukin-8 (IL-8). The IC50 for this effect was < 1 μM. Nucleoprotein purified by size-exclusion HPLC also inhibited PMNL chemotaxis to C5a, with >50% reduction after exposure to 1 μM nucleoprotein compared with 1 μM BSA (figure 3a). When PMNL were exposed to 10 nM to 1 μM concentrations of purified nucleoprotein, no significant effects on random migration to buffer alone were noted (data not shown).

Purified nucleoprotein (1 μM) inhibited C5a-induced chemotaxis whether it was added directly to PMNL (figure 4, “Above”) or mixed with C5a in the lower well of the chemotaxis chamber (figure 4, “Below”). The effects of purified

![Figure 1](link)  
**Figure 1.** SDS-PAGE of influenza A core lysates used as source of purified nucleoprotein. Lanes contains low–molecular mass standards (Bio-Rad, Richmond, CA) (A) and core lysates (B). Bands represent nucleoprotein (NP) and matrix protein (M).

![Figure 2](link)  
**Figure 2.** Effects of influenza A nucleoprotein, purified by immunoaffinity chromatography, on PMNL chemotaxis to C5a or interleukin-8 (IL-8). Control cells (C) were exposed to 1 μM bovine serum albumin. P < .05 vs. control; n = 5.

![Figure 3](link)  
**Figure 3.** Effects of influenza A nucleoprotein purified by high-performance liquid chromatography on PMNL chemotaxis to C5a (A) and superoxide production after FMLP stimulation (B). * P < .05 vs. bovine serum albumin (BSA) control; n = 5.
nucleoprotein on PMNL chemotaxis to C5a increased as the concentration of agonist was reduced (figure 5). Nucleoprotein (1 \( \mu M \)) inhibited PMNL chemotaxis to 10 nM C5a and 5 nM C5a by \( \sim 32\% \). However, chemotaxis to 1 nM C5a was inhibited \( \sim 75\% \) by a similar concentration of purified nucleoprotein.

Effects of purified nucleoprotein on PMNL superoxide production. Nucleoprotein (1 \( \mu M \)) purified by size-exclusion HPLC attenuated FMLP-stimulated PMNL superoxide production (figure 3B). This was not due to nonspecific effects on cytochrome C, since addition of the nucleoprotein 30 min after FMLP stimulation of PMNL did not change cytochrome C absorbance.

Effects of purified nucleoprotein on PMNL degranulation. Preexposure of PMNL for 15 min to 1-\( \mu M \) size-exclusion HPLC-purified nucleoprotein did not significantly affect FMLP-stimulated release of elastase or \( \beta \) glucuronidase by these cells (table 1).

### Table 1. Effects of nucleoprotein (NP) on FMLP-stimulated elastase and \( \beta \) glucuronidase release.

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Assay</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Elastase (( n = 3 ))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>Buffer</td>
<td>3.1 ± 0.82</td>
<td>.073 ± .052</td>
</tr>
<tr>
<td>BSA</td>
<td>FMLP</td>
<td>49.6 ± 21</td>
<td>.481 ± .24</td>
</tr>
<tr>
<td>NP</td>
<td>FMLP</td>
<td>61.2 ± 24</td>
<td>.384 ± .085</td>
</tr>
</tbody>
</table>

NOTE. Data are mean ± SE. BSA = bovine serum albumin.

* \( \mu g/mL \).

Alteration of PMNL cytosolic calcium by purified nucleoprotein. Exposure of PMNL to 1 \( \mu M \) purified nucleoprotein resulted in a rise in cytosolic calcium concentration (table 2). This change in calcium concentration occurred within 30 s of exposure to the nucleoprotein (figure 6). When PMNL were subsequently exposed to 1 \( \mu M \) FMLP, the absolute maximal value of \( Ca^{2+} \) did not change, but the net change from baseline was significantly decreased (table 2).

Endotoxin content of protein preparations. One-micromolar concentrations of purified nucleoprotein and BSA control contained equal amounts of endotoxin, \( \sim 400 \mu g/mL \).

### Discussion

To our knowledge, this study is the first to demonstrate that purified influenza A nucleoprotein induces a neutrophil defect that mimics changes noted with crude influenza A lysates [6]. The investigation of this protein as a PMNL deactivating agent was prompted by our discovery that bronchial lavage of normal human subjects contains a peptide inhibitor of PMNL function with striking homology to the influenza A nucleoprotein [16]. In the current study, we documented that purified influenza A nucleoprotein inhibits PMNL chemotaxis to multiple agonists as well as FMLP-induced oxidant production, while FMLP-

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Baseline ( Ca^{2+} )</th>
<th>Peak ( Ca^{2+} )</th>
<th>Change in ( Ca^{2+} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>BSA</td>
<td>125 ± 11</td>
<td>127 ± 11</td>
</tr>
<tr>
<td>Buffer</td>
<td>NP</td>
<td>129 ± 14</td>
<td>273 ± 52*</td>
</tr>
<tr>
<td>BSA</td>
<td>FMLP</td>
<td>127 ± 11</td>
<td>280 ± 9.3</td>
</tr>
<tr>
<td>NP</td>
<td>FMLP</td>
<td>196 ± 25*</td>
<td>282 ± 22</td>
</tr>
</tbody>
</table>

NOTE. Data are mean ± SE; \( n = 5 \). BSA = bovine serum albumin.

* \( P < .05 \) vs. appropriate BSA control.

\( ^{*} \) Measured \( \sim 10 \text{ min} \) after NP exposure when signal had stabilized at value below peak fluorescence.

### Table 2. Effects of nucleoprotein (NP) on PMNL cytosolic calcium concentrations.

\( ^{*} \) Measured \( \sim 10 \text{ min} \) after NP exposure when signal had stabilized at value below peak fluorescence.
stimulated degranulation is unaffected. In addition, PMNL cytosolic calcium concentrations are elevated upon exposure to nucleoprotein, and the net change in cytosolic calcium concentration after FMLP exposure is blunted. We have previously demonstrated similar effects of the human-derived peptide inhibitor on PMNL chemotaxis, oxidant production, and calcium homeostasis [14–16].

Previous studies have documented that influenza A–infected PMNL exhibit decreased chemotaxis and oxidant production [7]. Although the data vary, most studies suggest that PMNL phagocytosis of bacteria is unaffected [20] but bacterial killing may be depressed [21]. The molecular source of the influenza virus’ effects on these PMNL is unclear. Although inhibitory effects of viral neuraminidase through alteration of PMNL surface sialic acid have been proposed [11], studies have demonstrated that PMNL dysfunction occurs after exposure to neuraminidase-depleted influenza A virus [12]. It has also been suggested that viral hemagglutinin may be responsible for some of the PMNL deactivating properties of the virus [13]. It may be naïve to suggest only one protein component of the virus is the active mediator of PMNL dysfunction associated with influenza A virus infection.

Preparations provided as a source for the nucleoprotein used in our study were initially treated with pronase, an enzyme that degrades all coat proteins of the virus, including neuraminidase and hemagglutinin. SDS-PAGE confirmed that there were two major proteins in the pronase-treated preparations. The proteins had molecular masses consistent with the major core proteins of the influenza A virus, matrix protein and nucleoprotein. Although there might conceivably be some residual contaminating coat proteins in the initial preparation, it is doubtful there was any significant concentration of these proteins after further purification of the nucleoprotein by HPLC or affinity chromatography. Our recent finding that a human PMNL inhibitor protein contains a partial sequence that is homologous to the influenza A nucleoprotein also supports the concept that the viral nucleoprotein is an important inhibitor of PMNL activity.

Endotoxin is another potential contaminating molecule in our purified nucleoprotein preparations. Endotoxin at 10 ng/mL has been shown to inhibit PMNL chemotaxis [22]. However, two factors suggest this is not the molecular mediator of PMNL inhibition in our study. First, concentrations of endotoxin in preparations of both nucleoprotein and BSA control were low (<1 ng/mL) and equal. These concentrations are much lower than those documented to affect PMNL chemotaxis [22]. Second, studies examining effects of endotoxin on PMNL function have documented that this molecule primes PMNL for enhanced superoxide production. This is in contrast to the effects of nucleoprotein preparations that inhibited FMLP-induced PMNL superoxide production in our study.

Caldwell et al. [9] extensively studied potential mechanisms of PMNL deactivation by influenza A virus. In their study, PMNL were exposed to viral preparations that either did (deactivating) or did not (nondeactivating) inhibit PMNL chemotaxis and oxidant production. Deactivating preparations altered PMNL phosphorylation of proteins in response to FMLP or PMA and increased PMNL transmembrane potential; nondeactivating preparations had no such effects. In addition, exposure to deactivating preparations resulted in a rise in cytoplasmic calcium concentration. This concentration was not appreciable after exposure to nondeactivating preparations.

Hartshorn and Tauber [10] demonstrated that influenza A virus causes a rise in PMNL cytoplasmic calcium and an attenuated net increase in cytosolic calcium concentration after FMLP stimulation. This attenuation of the rise of cytosolic calcium concentration after FMLP exposure appears partly due to a triggering of cellular calcium efflux by the influenza virus [10]. These findings suggest that influenza A virus exerts subcellular effects mediated only by viral preparations that inhibit certain PMNL antibacterial processes. In the current study, we demonstrated that purified nucleoprotein is a source of some of these viral effects. Further studies using nucleoprotein purified from deactivating and nondeactivating preparations of influenza A would be of interest.
Others have demonstrated that release of lactoferrin and myeloperoxidase by PMNL is attenuated by exposure to preparations of influenza A virus [23]. In our study, although purified nucleoprotein inhibited PMNL chemotaxis and oxidant production, we were unable to demonstrate inhibition of PMNL degranulation as manifested by elastase and β-glucuronidase release. This finding suggests that the effects of the virus on PMNL degranulation, as demonstrated by others, might be important for a component(s) other than the nucleoprotein.

The present study documents that one PMNL deactivating component of the influenza A virus is the nucleoprotein. How this molecule inactivates PMNL is unclear but may be related to alteration in PMNL calcium homeostasis. Studies directed at inactivating the nucleoprotein, such as [24], may not only help reduce the incidence of viral infection but may also aid in overcoming the morbidity of secondary bacterial infections associated with influenza infection.

Acknowledgments

We thank W. Graeme Laver, Influenza Research Unit, John Curtin School of Medical Research, for providing influenza A core lysates and Robert G. Webster, Department of Virology and Molecular Biology, St. Jude Children’s Research Hospital, for nucleoprotein antiserum.

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