Characterization of the Effect of Influenza Virus on Polymorphonuclear Leukocyte Membrane Responses

By Jon S. Abramson, J. Wallace Parce, Jon C. Lewis, Douglas S. Lyles, Elaine L. Mills, Robert D. Nelson, and David A. Bass

Depressed chemotactic activity of polymorphonuclear leukocytes (PMNL) infected with influenza virus could be due to changes occurring at the plasma membrane. The present study examined the effect of unopsonized influenza virus on chemotaxis, adherence, receptor binding, shape change, membrane fluidity, and release of specific granules from PMNL. Chemotactic activity of PMNL under-agarose to the chemoattractants, zymosan-activated serum (ZAS) and N-formyl-methionyl-leucyl-phenylalanine (fMLP), and adherence of PMNL to a plastic surface were markedly decreased in virus-treated cells as compared to control cells. The binding of fMLP to the PMNL was increased in virus-treated cells compared with control cells. Exposure of cells to virus, ZAS, or fMLP caused 35%–50% of the cells to become bipolar in shape, whereas less than 5% of the cells exposed to buffer became bipolar. Influenza virus did not alter membrane fluidity as measured by electron spin resonance spectroscopy with the probe 5-doxyl stearate. Virus-treated PMNL stimulated with fMLP or Staphylococcus aureus exhibited a marked decrease in the amount of lactoferrin released into phagosomes, onto the cells' outer membrane, and into the extracellular medium as compared to control cells. The possible relationship between inhibition of lysosomal enzyme degranulation and decreased chemotactic activity and adherence of PMNL is discussed.

THE PHAGOCYTIC CELLS of patients infected with influenza virus have decreased chemotactic activity,1-5 and when polymorphonuclear leukocytes (PMNL) and monocytes are infected with influenza virus in vitro, chemotactic activity is depressed, as measured by migration through a filter.5-6 Recently, Gardner and Lawton7 have shown that human monocytes infected with influenza virus have decreased chemotactic activity as measured under agarose, suggesting that the defect in PMNL is not due to decreased deformability.

Changes that occur at the plasma membrane of PMNL upon stimulation of the cell have a profound effect on the chemotactic activity of the cell. When PMNL are exposed to a chemotactic stimulus, the stimulus attaches to membrane receptors and, subsequently, new receptors appear on the cell.10,11 In addition, the membrane ruffles, surface area increases, the cell changes shape from spherical to bipolar,12-14 and degranulation of specific granules onto the plasma membrane surface occurs in association with a decrease in net negative surface charge and increased adherence of cells to endothelial and plastic surfaces.14-16

We have recently reported that influenza A virus inhibits degranulation of azurophil granules.17 If the virus also inhibits release of secondary granules onto the PMNL membrane, this could alter the cell charge and decrease adherence.14,15 Other possible explanations for how the virus could decrease chemotaxis include decreasing the quantity or affinity of receptors for a chemoattractant, altering the cells' ability to become bipolar in response to a chemoattractant, and changing the membrane fluidity of the cell.

The present study was done to determine the effect that influenza virus had on various PMNL membrane properties when cells were treated with the virus alone or with the virus followed by a chemoattractant. When PMNL were exposed to influenza virus for 30 minutes, the following findings were noted: (1) chemotactic activity, as measured under agarose, in response to N-formyl-methionyl-leucyl-phenylalanine (fMLP) and zymosan-activated serum (ZAS) was decreased; (2) adherence of PMNL to a plastic surface was decreased; (3) the binding of the chemoattractant fMLP increased; (4) approximately 40% of the cells became bipolar; (5) membrane fluidity was unchanged; and (6) degranulation of lactoferrin-containing granules in response to subsequent stimuli was decreased.

MATERIALS AND METHODS

Preparation of Influenza Virus

The two naturally occurring strains of influenza A virus used in this study were an H3N2 A/Texas/77 virus (Texas 77) and an H1N1 A/PR/8/34 virus (PR8). Both viruses were harvested from allantoic fluid and further purified by sucrose density centrifugation.18 The hemagglutination titer of Texas 77 and PR8 were 1:10,240 and 1:5,120, respectively, and both viruses had a 50% egg infectivity dose of 103. Unopsonized virus was used in all assays.
Leukocyte Preparation

Purified populations of PMNL (≥97%) were obtained from heparinized whole blood as previously described. 17 The cells were resuspended in HBSS with gelatin (0.1% gelatin in Hanks' balanced salt solution with phenol red or NaHCO3, pH 7.4; GIBCO Laboratories, Grand Island, NY) to the desired concentration for the chemotaxis and lactoferrin release assays. For the adherence, membrane fluidity, and electron microscopy assays, PMNL were washed and resuspended in phosphate-buffered saline (PBS). Prior to doing each assay, PMNL were incubated with Texas 77, PR8, or buffer (100 μL virus/10^7 cells) for 30 minutes at 21°C unless otherwise noted. Viability of PMNL infected with influenza virus was greater than 95%, as measured by the cells' ability to exclude trypan blue. Uninfected allantoic fluid was purified in a manner identical to the virus and was used as an additional control in the various PMNL assays; the allantoic fluid had no effect on PMNL function. In the degranulation assays where cytochalasin B was used to potentiate the secretory response of the PMNL to fMLP, experiments were done using cells treated with cytochalasin B plus virus buffer. Cytochalasin B did not alter the effect of the virus on the PMNL. In the membrane fluidity assay, where 2 x 10^8 PMNL/ml were used, 2 ml of virus were concentrated 20-fold by ultracentrifugation (100,000 g for 30 minutes).

Chemotaxis and Adherence

A modification of the under-agarose assay of Nelson et al 19 was used to study the effect of influenza virus on the chemotactic response of PMNL. Ten microtiter of virus or buffer-treated PMNL (5 x 10^7/ml) was placed into the center of three equally spaced wells cut into the agar. An equivalent amount of 10^-7 mol/L fMLP (Sigma Chemical Co., St Louis, Mo) or ZAS was placed into the outer well, and the inside well received HBSS. The plates were incubated with 5% CO2 at 37°C for two hours, the cells fixed with 2% glutaraldehyde, the agar removed, and the cells stained with a 1:1 mixture of Wright's stain and distilled water added separately. Cell migration was examined with an inverted microscope on low power, and the chemotactic activity was measured by subtracting the distance cells migrated toward the buffer from the distance migrated toward the chemoattractant. The adherence of PMNL (5 x 10^7/ml) to a 35-mmol/L plastic dish (Corning Glass Works, Corning, NY) was determined using the method of Crowley et al. 21 The number of PMNL sticking to five random fields was calculated using a phase-contrast microscope.

Receptor Binding

The binding of fMLP to receptors on the membrane surface of the PMNL was examined as previously described. 22 Radiolabeled tripeptide as fMLP-([3H])P, specific activity ~50 Ci/mmol, was obtained from New England Nuclear. PMNL were incubated with virus at 30 minutes at 0° or 37°C; control cells were incubated into phosphate-buffered saline under the same conditions. Virus and buffer-treated PMNL, and fMLP-([3H])P, precooled to 0°C, were then mixed in a conical microfuge tube to provide a final concentration of 10^6 cells and 6 mmol/L tripeptide in a 1-mL vol. After incubation at 0°C for 60 minutes (a time providing for maximal binding of the labeled tripeptide to control PMNL), the cells were sedimented by centrifugation in a microfuge (Microfuge B, Beckman Instruments, Lincolnwood, Ill) for 30 seconds, the supernatant fluid was decanted, the cells were suspended in a 1-mL vol of Ready-Solv HP (Beckman), and the radioactivity quantified by scintillation spectrometry. Specific binding denotes the amount of bound ligand displaced by a 1,000-fold excess of unlabeled tripeptide present for the duration of the assay. Binding data are presented as mean cpm ± SD.

Cell Shape Change

The capacity of influenza virus to induce a cell shape change from spherical to bipolar was assayed using the method of Jadwin et al. 13 Briefly, 1 mL of anticoagulated (EDTA, 1.5 mg/mL) whole blood was incubated with 5 μL–100 μL virus, 10^-7 mol/L fMLP, 10 μL ZAS, or buffer for 30 minutes. Monolayer blood films were made with a Larc spinner (Corning Medical, Corning, NY), the slides fixed with methanol, stained with Wright's stain, and examined with a light microscope under high power. A total of 50 PMNL on each slide were counted and classified according to whether they were spherical (round shape with centrally placed nucleus), amorphous (round shape but with irregular cytoplasmic blebs and clumped nucleus), or bipolar (elongated and constricted shape and nucleus toward the leading edge of the cell).

Membrane Fluidity

The membrane fluidity of PMNL (2 x 10^7/mL) was measured at 21°C and 37°C using a Bruker ER-200D electron spin resonance spectrometer (USA Bruker Instruments Inc, Billerica, Mass) and 40 mmol/L (final concentration) of the spin label 5-doxyl stearic acid (5DS; Molecular Probes Inc, Junction City, Ore), as previously described. 23 In additional experiments, virus and buffer-treated cells were stimulated with 10^-7 mol/L fMLP for 10 minutes to determine the effect of influenza virus on membrane fluidity of PMNL in response to a secondary stimulus. The order parameter (S), a measurement of spin label motion, was calculated as previously described. 23

Lactoferrin Release

PMNL (10^4) and virus were suspended in HBSS to a final volume of 1.0 mL, preincubated for 30 minutes at 37°C, and centrifuged at 200 g for 10 min at 22°C. The supernatant was decanted for lactoferrin determination; the PMNL were resuspended in HBSS to a final volume of 1 mL and were incubated with opsonized Staphylococcus aureus for 20 minutes at 37°C or fMLP for 10 minutes at 37°C, following a preincubation of 10 minutes with 5 μmol/L cytochalasin B. Following stimulation with S aureus or fMLP, the PMNL were pelleted and the supernatant assayed for lactoferrin determination. The staphylococcal strain, HS8, was a laboratory strain. 24 Quantitation of lactoferrin was performed by electroimmuno- densitometry as described by Laurell 25 and as modified by Weke. 26 Agarose (type 1 low EEO, Sigma Chemical Co) 1% was made in 0.2 mol/L barbital-tris-glycine buffer, pH 8.6, by heating to 90°C, followed by cooling to 60°C. Rabbit anti-human lactoferrin (Cappel Laboratories, Cochranville, Pa) was added to the agarose solution to make a final concentration of 0.15%–0.20%. Aliquots of 14 cc of antibody-containing gel were poured onto polyester slides (Gel Bond Film, BioProducts, Rockland, Me), 84 x 94 mm. Electrophoresis was performed with an aqueous 0.02 mol/L barbital-tris-glycine buffer, ph 8.6, at 100 V, 10 mA, overnight on an LKB multiphor (LKB Instruments, Rockville, Md) maintained at 4°C ambient temperature. The gel slides were washed three times with PBS (pH 7.0), dried in a 37°C oven, and stained with 0.5% Coomasie Brilliant Blue R (Sigma) made in 4.5:1:4.5 ethyl alcohol 95%-glacial acetic acid:water. The gel slides were destained in 10% glacial acetic acid. Rocket height was measured on millimeter paper in a standard manner. 25 Lactoferrin concentration was calculated by comparison with a standard curve of human lactoferrin (Cappel). The specificity of the antibody for lactoferrin has previously been demonstrated. 27
Electron Microscopy

Virus and buffer-treated PMNL (2.5 × 10⁷/mL) were incubated with 5 μmol/L cytochalasin B (Sigma) for five minutes and then stimulated with 10⁻⁷ mol/L fMLP or buffer for 10 minutes. In other experiments, the virus and buffer-treated cells were incubated with opsonized S. aureus and prepared as previously described at a 20:1 bacteria-to-cell ratio for 30 minutes at 37°C. Cells for immunocytochemistry-studies were fixed at 1–3°C with 0.1 mol/L acetyldehyde-buffered (pH 7.4) paraformaldehyde (2.0%) in the presence of 2% sucrose. Paraformaldehyde as the primary fixative was selected, as greater permeability of immunoreagents into these cells was found when compared to glutaraldehyde, as previously reported for routine cytochemistry. The cells were then washed three times in cold 0.1 mol/L cacodylate buffer with 2% sucrose and incubated for 30 minutes at 37°C with IgG lactoferrin antibody tagged with peroxidase (Cappel, final dilution 1:12,000). The cells were again washed three times, and peroxidase-reaction product was demonstrated by the diaminobenzidine (DAB) reaction of Graham and Karnovsky as applied to human leukocytes.

To assure ultrastructural specificity of antilactoferrin immunoprecipitate, PMNL subsequent to challenge were incubated with nonimmune rabbit serum followed by the second stage conjugate and peroxidase cytochemistry. Because the immunocytochemistry involved the same enzyme reaction as myeloperoxidase, it was important to discriminate between these two. This was accomplished by treating the fixed PMNs with 10% acetic acid overnight at 4°C to inhibit the myeloperoxidase. Subsequent to myeloperoxidase inhibition, the cells were washed in the cacodylate buffer and carried through immunocytochemistry as described above. Results are reported as the mean ± 1 standard deviation. Statistical evaluation was done using the paired Student’s t test.

RESULTS

Chemotaxis and Adherence

The chemotactic activity of PMNL incubated with PR8 and Texas 77 virus for 30 minutes was markedly decreased when compared to control cells (Table 1). This depressed chemotactic activity was due to a decreased directed migration, as random migration was equivalent in cells treated with virus or buffer (data not shown). The number of virus-incubated PMNL that adhered to a plastic surface was significantly decreased compared with control cells (Table 1).

Receptor Binding

To test whether the decreased chemotactic activity of virus-treated PMNL to fMLP was due to a decrease in the amount of ligand attached to peptide receptors, the effect of Texas 77 on fMLP binding was measured. In three replicate experiments, preincubation of PMNL with virus at either 0°C or 37°C had no influence on nonspecific binding of fMLP-(³H)P. Similarly, preincubation of PMNL with virus at 0°C did not alter specific binding of the labeled tripeptide: 5,282 ± 380 cpm v 5,258 ± 1,538 cpm for buffer and virus-treated cells, respectively; P > .05. In contrast, preincubation of PMNL with virus at 37°C augmented subsequent specific binding of the labeled tripeptide by approximately 20%: 5,282 ± 380 cpm v 6,325 ± 450 cpm for buffer and virus-treated cells, respectively; P < .05.

Cell Shape Change

PMNL that undergo chemotaxis change from a spherical configuration to bipolar in shape. Approximately 40% of PMNL became bipolar in shape in response to stimulation with PR8, ZAS, and fMLP for 30 minutes, whereas less than 5% of the cells became bipolar when maintained in the buffer alone (Table 2, experiment 1). When cells were incubated with PR8 for 30 minutes and then stimulated with ZAS or fMLP, there was no significant change in the percentage of cells that became bipolar as compared to cells incubated with buffer prior to stimulation with ZAS or fMLP (Table 2, experiments 2 and 3). Similar results were obtained when PMNL were treated with Texas 77. Cells treated with ZAS or fMLP for 30 minutes and then exposed to ZAS, fMLP, PR8, or Texas 77 for 30 minutes did not have an increased percentage of bipolar cells (data not shown).

Membrane Fluidity

The effect of influenza virus on PMNL membrane fluidity was studied, as abnormalities of PMNL membrane fluidity, as measured by electron spin resonance (ESR), have been reported in Chediak-Higashi patients with abnormal chemotaxis and degranulation. The membrane fluidity of PMNL incubated with PR8 virus for 30 minutes was not significantly altered when compared to buffer-treated cells as measured by ESR at 21°C (S = 0.658 ± 0.021 and 0.658 ± 0.010, respectively; P > .05). Subsequent stimulation of virus and buffer-treated PMNL with fMLP did not significantly alter the order parameter (S = 0.659 ±
The presence of lactoferrin within the cell and on the plasma membrane was detected by incubating PMNL with a lactoferrin antibody tagged with peroxidase and then localizing the enzyme with the DAB reaction. Using this technique, buffer-treated cells had electron-dense material in granules (reflecting myeloperoxidase and lactoferrin) and in the cytoplasm surrounding the granules. In addition, a fine deposit on the plasma membrane was noted on all nonactivated cells (Fig 1A). The small amount of peroxidase-positive material on the cell surface is felt to represent nonspecific attachment of the lactoferrin antibody to Fc receptors, as it appeared on all of the cells treated with the specific antibody as well as nonimmune rabbit serum. The fine deposit was not due to cellular myeloperoxidase, because a previous study done to examine the release of myeloperoxidase in virus-treated cells exposed to S aureus used the DAB technique without the lactoferrin antibody and did not have this reaction product on the cell surface. Furthermore, the delicate surface reaction was observed on cells incubated with acetid to inhibit the myeloperoxidase (Fig 2, A, B, and C). Electron-dense precipitate in these cells was observed as a delicate surface deposition and as an immunoprecipitate in a small number of PMNL granules. Occasionally, enzyme-positive mitochondria could be observed. In these cases, the reaction product was a reflection of mitochondrial oxidases.

Virus-treated cells had the enzyme reaction product in vacuoles containing the virus in addition to the other sites previously noted for the control cells (Fig 1B). Incubation of cells with 5 μmol/L cytochalasin B prior to addition of virus had no effect on uptake of the virus

### Table 2. The Effect of Influenza Virus on PMNL Shape Changes*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Incubation</th>
<th>Stimulant</th>
<th>Percent Spherical</th>
<th>Percent Amorphous</th>
<th>Percent Bipolar</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Buffer</td>
<td>ZAS</td>
<td>28.6 ± 8.0</td>
<td>32.0 ± 5.2</td>
<td>39.4 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>Buffer</td>
<td>fMLP</td>
<td>29.4 ± 4.2</td>
<td>34.0 ± 7.2</td>
<td>36.6 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>Buffer</td>
<td>PR8</td>
<td>31.4 ± 6.4</td>
<td>24.0 ± 6.0</td>
<td>44.6 ± 9.4</td>
</tr>
<tr>
<td>2</td>
<td>Buffer</td>
<td>ZAS</td>
<td>25.4 ± 6.2</td>
<td>26.6 ± 9.2</td>
<td>48.0 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>PR8</td>
<td>ZAS</td>
<td>25.4 ± 7.6</td>
<td>28.6 ± 3.0</td>
<td>46.0 ± 6.0</td>
</tr>
<tr>
<td>3</td>
<td>Buffer</td>
<td>fMLP</td>
<td>33.4 ± 9.0</td>
<td>18.0 ± 5.2</td>
<td>48.6 ± 13.4</td>
</tr>
<tr>
<td></td>
<td>PR8</td>
<td>fMLP</td>
<td>41.4 ± 12.2</td>
<td>18.6 ± 6.2</td>
<td>47.4 ± 3.0</td>
</tr>
</tbody>
</table>

*PMNLs were incubated with virus or buffer for 30 minutes and then stimulated with ZAS, fMLP, virus, or buffer for an additional 30 minutes. These data were representative of results obtained in three or more separate experiments.

†P < .01 when PMNLs that were stimulated with ZAS, fMLP, or PR8 are compared to cells stimulated with buffer.

Electron Microscopy

The presence of lactoferrin within the cell and on the plasma membrane was detected by incubating PMNL with a lactoferrin antibody tagged with peroxidase and then localizing the enzyme with the DAB reaction. Using this technique, buffer-treated cells had electron-dense material in granules (reflecting myeloperoxidase and lactoferrin) and in the cytoplasm surrounding the granules. In addition, a fine deposit on the plasma membrane was noted on all nonactivated cells (Fig 1A). The small amount of peroxidase-positive material on the cell surface is felt to represent nonspecific attachment of the lactoferrin antibody to Fc receptors, as it appeared on all of the cells treated with the specific antibody as well as nonimmune rabbit serum. The fine deposit was not due to cellular myeloperoxidase, because a previous study done to examine the release of myeloperoxidase in virus-treated cells exposed to S aureus used the DAB technique without the lactoferrin antibody and did not have this reaction product on the cell surface. Furthermore, the delicate surface reaction was observed on cells incubated with acetid to inhibit the myeloperoxidase (Fig 2, A, B, and C). Electron-dense precipitate in these cells was observed as a delicate surface deposition and as an immunoprecipitate in a small number of PMNL granules. Occasionally, enzyme-positive mitochondria could be observed. In these cases, the reaction product was a reflection of mitochondrial oxidases.

Virus-treated cells had the enzyme reaction product in vacuoles containing the virus in addition to the other sites previously noted for the control cells (Fig 1B). Incubation of cells with 5 μmol/L cytochalasin B prior to addition of virus had no effect on uptake of the virus

### Table 3. The Effect of Influenza Virus on the Release of Extracellular Lactoferrin From PMNL*

<table>
<thead>
<tr>
<th>Cell Stimulants</th>
<th>Lactoferrin Released (μg/mL/20 min)</th>
<th>Percent Deposition†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>0.1 ± 0.0</td>
<td>—</td>
</tr>
<tr>
<td>Virus</td>
<td>1.8 ± 1.1</td>
<td>—</td>
</tr>
<tr>
<td>Buffer + fMLP</td>
<td>16.8 ± 1.8</td>
<td>65</td>
</tr>
<tr>
<td>Virus + fMLP</td>
<td>5.9 ± 1.5</td>
<td>65</td>
</tr>
<tr>
<td>Buffer + S aureus</td>
<td>7.3 ± 1.3</td>
<td>45</td>
</tr>
<tr>
<td>Virus + S aureus</td>
<td>4.0 ± 0.7</td>
<td>45</td>
</tr>
</tbody>
</table>

*PMNLs were incubated with virus or buffer for 20 minutes, centrifuged, and the supernatants were assayed for lactoferrin release. In other experiments, PMNLs were incubated with virus or buffer and then stimulated with opsonised S aureus for 30 minutes, or cytochalasin B followed by fMLP for 10 minutes, and the supernatants assayed for lactoferrin release. The data shown are the mean ± 1 SD of 3 experiments. Closely agreeing duplicate determinations were obtained for each experiment.

†The percent depression was determined by comparing the amount of lactoferrin released in cells treated with buffer followed by fMLP or S aureus to cells treated with virus followed by fMLP or S aureus.

‡P < .02 by Student's t test when buffer-treated cells are compared to virus-treated cells.
Fig 1. (A) Control, nonactivated, neutrophil reacted for peroxidase with the diaminobenzidine reagent following treatment with the antilactoferrin antiserum tagged with peroxidase. Electron-dense product (reflecting myeloperoxidase and lactoferrin) is in granules in the cytoplasm as discrete patches near peroxidase-negative granules (see arrow within the cell) and as a fine deposit on the plasma membrane (arrow) (8.025 x). (B) Neutrophils exposed to virus only prior to undergoing immunoelectron microscopy. The DAB reaction product, in addition to the sites described for the control cells, is localized in the large virus-containing vacuole (arrow). Virus particles are also seen on the surface of the PMNL (11,250x). (C) Buffer-treated neutrophils exposed to *S aureus* prior to undergoing immunoelectron microscopy. In addition to the sites noted for the control cells, the DAB immunoreaction product is found as a heavy flocculent deposit in the phagocytized bacteria (arrow, s) and as a delicate flocculent precipitate in large intracellular vacuoles (arrow) (7,800x). (D) Buffer-incubated neutrophils treated with fMLP prior to undergoing immunoelectron microscopy. In addition to the sites noted for the control cells, the immunoreaction product is a heavy flocculent precipitate at localized patches on the surface of the stimulated cells (arrow) (12,150x). (E) Neutrophils treated with virus prior to being exposed to the bacteria. Reaction product exists as a delicate precipitate in vacuoles containing virus (thin arrows), in the cytoplasm as patches surrounding reaction-negative granules (o), and as a delicate precipitate at the cell surface (arrow). The overall distribution of reaction product is a combination of that noted with control and the virus-treated cells. Very little reaction product was associated with the phagocytized staph (compare with C above). Occasionally, a delicate product was observed around a given bacteria (double arrow) (7,500x). (F) Neutrophils exposed to virus prior to fMLP treatment. DAB reaction product is shown in the virus-containing vacuoles (arrow), but very little fMLP-induced surface membrane reaction product was observed. Contrast this to D above (8,625x).
or release of lysozomal enzymes around the virus. A previous article showed that unopsonized influenza virus can enter cells by an endocytic process that is not altered by as much as 30 μg/mL cytochalasin B. Control PMNL exposed to S aureus had the reaction product in vacuoles containing the bacteria as well as the other areas noted in the control cells (Fig 1C). When control PMNL were stimulated with fMLP, the reaction product was found as large discrete clumps on the cell surface as well as at the sites noted for the control cells (Fig 1D). Virus-treated PMNL subsequently exposed to S aureus or fMLP had significant amounts of reaction product in virus-containing vacuoles, but had very little reaction product in the phagosomes containing the bacteria (Fig 1E) or on the surface of the cell (Fig 1F). This indicates that in the virus-infected cells the release of primary or specific granules into phagosomes containing bacteria and onto the cell surface was inhibited. Greater than 90% of the cells in which virus particles were observed had a marked decrease in release of lactoferrin into phagosomes and onto the surface membrane of the cell.

DISCUSSION

Although previous studies have shown that in vitro incubation of PMNL with influenza virus causes depressed chemotactic activity, the mechanism by which this occurs is not understood. The capacity of PMNL to move through a microfilter is dependent on both the cells’ ability to respond to a chemotactic gradient and its deformability. The finding in this study that virus-treated PMNL have decreased chemotactic activity, as measured under-agarose, indicates that the decreased chemotactic activity of the cell is not due to decreased deformability; in contrast to the microfilter technique, the under-agarose assay does not require that cells move through small spaces in response to a chemoattractant gradient.

A role for specific granules in modulating membrane responses of PMNL has been postulated. Boxer et al have described a patient with recurrent infections who had a marked decrease in the quantity of specific granules in his PMNL. Abnormalities of various membrane responses included a decreased change in the cell surface charge of fMLP-stimulated cells (ie, his cells stayed more negative than control), decreased adherence to nylon fibers and endothelial cells, and decreased directed chemotaxis (random migration was normal). Lactoferrin has been shown to bind to the PMNL membrane and to reduce the net negative charge.

Fig 2. (A) Immunoperoxidase reaction for antilactoferrin in control PMNL following myeloperoxidase inhibition with acetic acid. Reaction product exists as a diffuse membrane density (arrow at tangential plane), within select granules (arrow, a) and occasionally in mitochondria. This electron micrograph is magnified 30,000× in order to make the reaction product on the membrane easily discernible. (B) Immunoperoxidase reaction for antilactoferrin in control PMNL following acetic acid treatment. Note reaction product at the periphery of cytoplasmic granules (17.250×). (C) Immunoperoxidase reaction for antilactoferrin in staph-challenged PMNL. The preparation had been treated with acetic acid to inhibit myeloperoxidase prior to immunocytochemistry. Reaction product exists as a flocculent surface precipitation (arrow) and surrounding staph (s) in a phagosome (14.250×).
surface charge, thereby enhancing adherence of cells to various surfaces.\textsuperscript{15,27} Other proteins found in specific granules have also been reported to have an effect on adherence and chemotaxis of PMNL.\textsuperscript{10,33-35}

In cells exposed to virus, followed by subsequent stimulation with soluble or particulate stimuli, there is a marked inhibition of degranulation. As examined by electron microscopy, the amount of lactoferrin still contained within the granules is substantial, and therefore, the inhibition of release of specific granules does not appear to be due to exhaustion of cellular stores. Additional support for this statement comes from data obtained in the immunoelectrophoresis assay, which showed that the virus by itself caused release of only a small amount of lactoferrin from the cell (Table 3). The mechanism by which the virus inhibits degranulation remains to be determined. The decreased release of specific granules onto the PMNL membrane after stimulation of the cell with fMLP could cause the surface charge to be relatively more negative, and thereby decrease adherence and chemotaxis to fMLP and other chemoattractants. The finding that PMNL treated with virus alone release small quantities of lactoferrin yet have decreased adherence suggests that an additional mechanism may be involved in altering the cells' adherence to surfaces.

The increased binding of fML-(\textsuperscript{3}H)P observed for cells preexposed to virus at 37°\textdegree C could be due to an effect of virus on either peptide receptor numbers or affinity of existing receptors for the ligand. Although our experiments do not discriminate between these alternatives, they do indicate that the decreased chemotactic activity in virus-treated PMNLs is not associated with decreased receptor–ligand interaction. Based on other information available, it is possible that the phenomenon described is attributable to mobilization of peptide receptors as a consequence of stimulation of PMNL secretory function by endocytosis of the virus particles. Note that exposure of PMNLs to virus alone causes a stimulation of lactoferrin release from secondary granules (Table 3). A mechanistic relationship of mobilization of peptide receptors and stimulation of secretory function involving secondary granules has been proposed by Fletcher and Gallin\textsuperscript{11} and Nelson et al.\textsuperscript{36} Studies are ongoing to determine how influenza virus stimulates peptide binding.

An unexpected finding was that influenza virus induced approximately 40% of the cells to become bipolar in shape. Orientation of the cell is necessary, but not sufficient, for chemotaxis to occur.\textsuperscript{37,38} Studies have indicated that when PMNLs are exposed to a chemoattractant in vitro, less than 50% of the cells will change shape and migrate, indicating that there is a migrating and nonmigrating subpopulation of PMNL.\textsuperscript{39} The number of PMNL that became bipolar in shape was similar for cells stimulated with influenza virus as compared to cells exposed to the chemoattractants fMLP and ZAS. It is possible that the cells that changed shape in response to the virus were the migrating subpopulation of PMNL and that these cells were no longer capable of responding to another stimulus. When cells are exposed to high concentrations of a chemoattractant, there is depressed chemotactic responsiveness to multiple chemoattractors (ie, nonpreferential deactivation), whereas when cells are exposed to low concentrations of a chemoattractant, there is depressed chemotactic responsiveness to only that specific factor (ie, preferential deactivation\textsuperscript{40-42}). Whether influenza virus can act as a chemoattractant for PMNL and thereby nonpreferentially deactivate the cells remains to be determined.

Polymorphonuclear leukocytes from humans and mice with the Chediak-Higashi syndrome are similar to PMNL treated with influenza virus, in that there are abnormalities of chemotaxis, degranulation, and bacterial killing.\textsuperscript{43,44} Increases in membrane fluidity, as measured using ESR with 5-doxyl stearate and 12-doxyl stearate, have been reported in cells from hosts with Chediak-Higashi syndrome.\textsuperscript{21} We were unable to demonstrate significant changes in membrane fluidity using ESR with 5-doxyl stearate, in virus-treated cells alone, or in virus-treated cells subsequently stimulated with fMLP. Therefore, the PMNL dysfunction induced by the virus does not appear to be due to alterations in membrane fluidity.

In summary, the findings indicate that influenza virus does not depress chemotactic activities of PMNL by decreasing the binding of the chemoattractant to the cell or by altering membrane fluidity. The virus does induce PMNL to become bipolar in shape, and cells incubated with virus for 30 minutes have a decreased degranulation response to soluble and particulate stimuli. Thus, the virus could depress migration of PMNL by interfering with lysosomal degranulation and/or by deactivating the chemotactically active subpopulation of PMNL.

ACKNOWLEDGMENT

We thank Giannina Wiegand for technical assistance and Louise Nixon for secretarial assistance.

REFERENCES


Characterization of the effect of influenza virus on polymorphonuclear leukocyte membrane responses

JS Abramson, JW Parce, JC Lewis, DS Lyles, EL Mills, RD Nelson and DA Bass