Characterization of the Chemotactic Defect in Polymorphonuclear Leukocytes Exposed to Influenza Virus In Vitro

By Dorothy L. Moore and Elaine L. Mills

The mechanism by which influenza virus interferes with polymorphonuclear leukocyte (PMN) chemotaxis was investigated. Incubation of human PMN with influenza A virus in vitro for 30 minutes significantly decreased PMN migration under agarose in response to N-formyl-methionyl-leucyl-phenylalanine (FMLP) or zymosan-activated serum. Virus-treated PMN tended to aggregate in the under-agarose assay. Aggregation was avoided by using a more dilute PMN suspension in filter assays. Viral treatment significantly decreased migration through 100-µm thick cellulose nitrate filters but had no effect on migration through 10-µm thick polycarbonate filters or on PMN bipolar shape change. Virus was not chemotactic in the polycarbonate filter assay and did not induce shape change in purified PMN. It was concluded that influenza virus did not interfere with the ability of PMN to recognize a chemoattractant, undergo shape change, and move a short distance but did limit the extent of migration. Inhibition could not be explained by chemotactic deactivation, since the virus was not chemotactic.

Infection with influenza virus has been shown to predispose the host to secondary bacterial infection and to interfere with a number of phagocytic cell functions including chemotaxis. Defective monocyte and polymorphonuclear leukocyte (PMN) chemotaxis have been reported in patients and volunteers infected with influenza A. Defective chemotaxis has also been demonstrated in patients and volunteers infected with influenza B. The mechanism by which this inhibition occurs is not known. Chemotaxis is a complex process involving the recognition of a chemoattractant, the triggering of a series of biochemical events leading to microtubule assembly, a change to a bipolar shape, orientation of this bipolar cell toward the chemical gradient source, increased adhesiveness and deformability, and movement toward the chemoattractant. In addition, degranulation of specific granules onto the plasma membrane surface occurs in association with a decrease in net surface charge. In an attempt to understand at what stage influenza virus interferes with this process, we have examined locomotion by migration under agarose and through filters and have assessed the early phases of chemotaxis by evaluating cell shape change. The possibility that the virus itself might be chemotactic was also investigated.

Materials and Methods

Preparation of PMN. Human PMN were purified (≥98%) from heparinized whole blood of healthy adult volunteers by dextran sedimentation, centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ), and hypotonic lysis of residual erythrocytes as described previously. The PMN pellet was washed twice in approximately 30 vol of Hanks' balanced salt solution (HBSS; Gibco Laboratories, Grand Island, NY) containing 0.1% gelatin. PMN were harvested from allantoic fluid of infected embryonated hens' eggs. For the 100-µm thick filters, PMN were treated with virus or AF for as for the agarose technique, then suspended in MEM at a concentration of 1 × 106/mL. Twenty-five µL vol of FMLP (1 × 10−4 mol/L) or 10-µm thick polycarbonate filters, pore size 5 µm (Neuropore Corp., Pleasanton, CA). For the 100-µm thick filters, PMN were treated with virus or AF and incubated at 37°C for 30 minutes with constant rotation. PMN were collected by centrifugation, counted in trypan blue to assess viability, resuspended in MEM at a concentration of 5 × 105/mL, and plated in triplicate wells in volumes of 10 µL per well. Ten µL volumes of N-formyl-methionyl-leucyl-phenylalanine (FMLP; Sigma Chemical Co) at concentrations of 1 × 10−5 or 5 × 10−7 mol/L, 15 µL vol of zymosan-activated serum (ZAS) prepared from pooled human serum or 10 µL MEMg were placed in adjacent wells. The plates were incubated in 5% CO2 at 37°C for 2½ hours. The cells were then fixed with 2% glutaraldehyde in PBS, the agar was removed, and the plates were stained with Diff-Quick (Harleco, Gibbstown, NJ). Migration distances were measured using an overhead projector and results expressed as the mean migration observed in the triplicate wells.

Chemotaxis through filters. Migration through filters was assayed with a multiwell chemotaxis chamber (Neuro Probe, Inc, Cabin John, MD) using Toyo 100-µm thick cellulose nitrate filters, pore size 5 µm (Neuro Probe, Inc), and 10-µm thick polyanipyrroolidone-free polycarbonate filters, pore size 5 µm (Nucleopore Corp, Pleasanton, CA). For the 100-µm thick filters, PMN were treated with virus or AF and incubated at 37°C for 30 minutes with constant rotation. PMN were collected by centrifugation, counted in trypan blue to assess viability, resuspended in MEM at a concentration of 5 × 105/mL, and plated in triplicate wells in volumes of 10 µL per well. Ten µL volumes of N-formyl-methionyl-leucyl-phenylalanine (FMLP; Sigma Chemical Co) at concentrations of 1 × 10−5 or 5 × 10−7 mol/L, 15 µL vol of zymosan-activated serum (ZAS) prepared from pooled human serum or 10 µL MEMg were placed in adjacent wells. The plates were incubated in 5% CO2 at 37°C for 2½ hours. The cells were then fixed with 2% glutaraldehyde in PBS, the agar was removed, and the plates were stained with Diff-Quick (Harleco, Gibbstown, NJ). Migration distances were measured using an overhead projector and results expressed as the mean migration observed in the triplicate wells.

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distance of 60 μμ after incubation for one hour and the number of cells migrating through to the undersurface of the filter after 2½ hours. Three to five high-power fields were examined for each well and the results expressed as the mean values from triplicate wells.

For the 10-μμ filters the procedure was similar except that HBSS was used instead of MEMg in the chambers. Incubation was for one hour. The filter was processed and stained by the method of Harvath et al. The number of cells migrating to the undersurface of the filter after 30 minutes was used instead of MEMg in the agarose assay. Random migration was also reduced at this gate when concentrated to 5% serum antibody titrations. Sera from PMN donors were tested for antibody to the virus used in these experiments by hemagglutination inhibition assay. Statistical evaluation. Significance was determined by the Student’s t test for paired data.

RESULTS

Effect on migration under agarose. Treatment with influenza virus reduced PMN chemotactic responses to FMLP and to ZAS in the migration under agarose assay (Table 1). Inhibition occurred at virus concentrations of 100 and 10 HAU:10⁶ PMN, with greater inhibition at the higher concentration. Random migration was also reduced at this virus concentration. No inhibition of migration occurred in a single experiment with virus concentration of 1 HAU:10⁶ PMN (data not shown). Migration inhibition was greater with 1 x 10⁻³ mol/L FMLP, a suboptimal concentration for chemotactic activity, than with 5 x 10⁻³ mol/L FMLP.

Viability of the virus-treated cells was ≥98% by trypan blue exclusion. However, virus-treated cells tended to aggregate when concentrated to 5 x 10⁷ cells/mL for testing in this assay.

Effect on migration through 100 μμ cellulose nitrate filters. To eliminate the problem of PMN aggregation, studies were performed using filter assays that employed

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>Chemoattractant</th>
<th>Virus-Treated PMN</th>
<th>Control PMN</th>
<th>% Inhibition*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h</td>
<td>FMLP 1 x 10⁻⁸ mol/L</td>
<td>105 ± 73</td>
<td>186 ± 118†</td>
<td>46 ± 8</td>
</tr>
<tr>
<td></td>
<td>FMLP 1 x 10⁻⁷ mol/L</td>
<td>88 ± 58</td>
<td>133 ± 73‡</td>
<td>38 ± 9</td>
</tr>
<tr>
<td></td>
<td>FMLP 1 x 10⁻⁶ mol/L</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2½ h</td>
<td>FMLP 1 x 10⁻⁸ mol/L</td>
<td>91 ± 55</td>
<td>165 ± 58‡</td>
<td>48 ± 19</td>
</tr>
<tr>
<td></td>
<td>FMLP 1 x 10⁻⁷ mol/L</td>
<td>37 ± 17</td>
<td>76 ± 38‡</td>
<td>49 ± 12</td>
</tr>
<tr>
<td></td>
<td>FMLP 1 x 10⁻⁶ mol/L</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± SD of values from four separate experiments with PMN from different donors. Migration at one hour was measured at a filter depth of 60 μμ. Migration at 2½ hours was measured at the bottom of the filter. Virus concentration was 100 HAU:10⁶ PMN.
†Difference between virus-treated and control PMN significant at P < 0.05.
‡Difference between virus-treated and control PMN significant at P < 0.02.
CHEMOTACTIC DEFEAT IN PMN

Table 3. Effect of Influenza Virus on PMN Migration Through 100 μm Cellulose Nitrate Filters: Leading Front

<table>
<thead>
<tr>
<th>Chemoattractant</th>
<th>Virus-Treated PMN</th>
<th>Control PMN</th>
<th>% Inhibition*</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMLP 1 x 10^-8 mol/L</td>
<td>88.8 ± 7.8</td>
<td>90.4 ± 5.2</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>FMLP 1 x 10^-7 mol/L</td>
<td>82.4 ± 9.2</td>
<td>78.2 ± 10.6</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>FMLP 1 x 10^-6 mol/L</td>
<td>37.0 ± 8.5</td>
<td>36.2 ± 2.3</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>None</td>
<td>40.4 ± 7.2</td>
<td>42.4 ± 5.8</td>
<td>4 ± 1</td>
</tr>
</tbody>
</table>

*Mean ± SD of values from four separate experiments with PMN from different donors. Incubation time was one hour. Virus concentration was 100 HAU:10^6 PMN.
†Difference between virus-treated and control PMN significant at P < 0.05.

more dilute PMN suspension (1 x 10^6/mL). There was no significant aggregation at this cell concentration, with >90% of the PMN remaining free in suspension.

The virus concentration used was 100 HAU:10^6 PMN, and migration was measured by two methods: (1) the number of PMN migrating a given distance and (2) the position of the leading front. There was significant reduction in the number of PMN migrating 60 μm at one hour or completely through the filter at 2½ hours (Table 2). At one hour the position of the leading front was slightly reduced with FMLP 1 x 10^-7 mol/L only (Table 3). By 2½ hours the leading front had reached the undersurface of the filter with both virus-treated and control PMN.

It was noted that with these filters maximum migration occurred at FMLP 1 x 10^-4 mol/L, and no chemotaxis occurred at FMLP 1 x 10^-6 mol/L.

Effect on migration through 10 μm polycarbonate filters. For the 10-μm polycarbonate filters, MEMg was replaced by HBSS in the chambers, since migration was found to be more consistent in the absence of protein. Preliminary experiments with virus concentrations of 100 HAU:10^6 PMN did not show any inhibition; therefore higher virus concentrations were tested. In three separate experiments using PMN from different donors, no inhibition of migration could be detected, even at virus concentrations three to 20 times greater than had been used in the previous assays (Table 4).

With these filters, maximum migration occurred at FMLP 1 x 10^-4 mol/L, in contrast to the results obtained with the 100-μm cellulose nitrate filters.

Effect on bipolar shape change. PMN treated with virus at concentrations of 320 and 2,048 HAU:10^6 PMN showed normal percentage bipolar shape change in response to FMLP (40.7 ± 11.1 v control 41.3 ± 9.9) and to ZAS (27.0 ± 10.6 v control 31.2 ± 10.3). Similar results were obtained at room temperature and at 37°C, with the exception of increased spontaneous shape change in unstimulated PMN at 37°C.

Influenza virus as a chemotactic agent. Influenza virus did not act as a chemotactic agent when tested in the 10-μm polycarbonate filter assay. PMN migration was 282 ± 176 cells/mm² with influenza virus and 241 ± 195 cells/mm² with control AF.

Influenza virus induced PMN shape change when added to whole blood but not when added to purified PMN, PMN in autologous plasma, or whole blood leukocyte preparations (Table 5). In addition, plasma from virus-treated whole blood did not induce shape change in purified autologous PMN.

Antibody titers of PMN donors. Hemagglutination inhibition titers of sera from PMN donors ranged from 1:40 to 1:320. There was no correlation between antibody titer and effect of virus on PMN function.

DISCUSSION

Previous studies demonstrating in vitro inhibition of chemotaxis by influenza virus have used cellulose nitrate filter® or migration under agarose® assays. The results of our studies indicate that exposure of PMN to influenza virus in vitro did not interfere with the early stages of chemotaxis, such as the ability to recognize a chemoattractant and undergo bipolar shape change and to deform and migrate the short distance through the 10-μm polycarbonate filter. On the other hand, there was reduction of migration when movement occurred over a greater distance, such as through

Table 4. Effect of Influenza Virus on PMN Migration Through 10-μm Polycarbonate Filter

<table>
<thead>
<tr>
<th>Virus per 10^6 PMN</th>
<th>Chemoattractant</th>
<th>Migration (PMN/mm²)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>320 HAU</td>
<td>FMLP 1 x 10^-4 mol/L</td>
<td>540 ± 120</td>
</tr>
<tr>
<td></td>
<td>FMLP 1 x 10^-7 mol/L</td>
<td>1236 ± 220</td>
</tr>
<tr>
<td></td>
<td>FMLP 1 x 10^-6 mol/L</td>
<td>1784 ± 320</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>216 ± 76</td>
</tr>
<tr>
<td>2048 HAU</td>
<td>FMLP 1 x 10^-4 mol/L</td>
<td>608 ± 436</td>
</tr>
<tr>
<td></td>
<td>FMLP 1 x 10^-7 mol/L</td>
<td>1012 ± 368</td>
</tr>
<tr>
<td></td>
<td>FMLP 1 x 10^-6 mol/L</td>
<td>1652 ± 244</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>400 ± 260</td>
</tr>
</tbody>
</table>

*Mean ± SD of values from three separate experiments with PMN from different donors. No significant differences between virus-treated and control PMN.
Virus also inhibits degranulation of PMN. Adherence may limit the speed or distance of migration. The second group, which used more dilute cell suspensions, showed the inhibition was less when a more potent concentration of FMLP was used.

Interpretation of results obtained in the agarose assay was confounded by virus-induced PMN aggregation. Aggregation of PMN by influenza virus was first reported in 1950 with guinea pig cells, and aggregation of human PMN by influenza A or B virus has been noted by others. The role this plays in limiting PMN migration in the under-agarose assay is not known. If migration inhibition were due only to aggregation, one would expect virus concentration to be the limiting factor determining the degree of inhibition, independent of the chemotactic stimulus. In fact, the degree of inhibition was less when a more potent concentration of FMLP was used.

To avoid aggregation chemotaxis was studied in a filter system, which used more dilute cell suspensions. Inhibition was observed with the 100-μm cellulose nitrate filter but not with the 10-μm polycarbonate filter. While both filters measure the early stages of chemotaxis, cells move over a much greater distance in the thick filter and may therefore be subject to modulating factors. Harvath and Leonard have discussed the differences in characteristics of PMN migration through these two filters. A fixed proportion of approximately 20% to 40% of the total PMN population migrates through the 10-μm filter, with optimal response occurring at 1 × 10^{-6} mol/L FMLP. With the 100-μm filters only about 15% of PMN respond, with optimal migration occurring at 5 × 10^{-8} mol/L FMLP and inhibition at 1 × 10^{-6} mol/L. These authors postulate two populations of PMN. One group responds to 1 × 10^{-6} mol/L FMLP and migrates the short distance through the 10-μm filter but no further, perhaps because of chemotactic deactivation. The other group responds to 5 × 10^{-8} mol/L FMLP and migrates to the leading front in the thick filters. If such subpopulations do exist, our results with the 10- and 100-μm filters may be explained by a preferential effect of influenza virus on the second group.

Influenza has been shown in previous studies to inhibit PMN adherence to plastic tissue culture plates. Since migration is dependent on optimal adherence, defective adherence may limit the speed or distance of migration. The virus also inhibits degranulation of PMN specific and non-specific granules by preventing phagosome-lysosome fusion. Exocytosis of specific granules has been shown to play a role in modulating locomotion. These granules are normally released in small amounts during a chemotactic response. Virus interference with this response may limit the extent of migration. Exposure of normal neutrophils to limited concentrations of degranulating stimuli results in increased receptor binding of chemoattractants, probably as a result of translocation of specific granule membrane to the plasma membrane. The data presented here could be explained by virus interfering with the expression of new receptors on the PMN surface. We did not examine receptor binding. However, it has previously been shown that binding of chemoattractant is increased in PMN treated with influenza virus.

All PMN donors in this study had serum antibody to influenza A virus, but free antibody would have been removed in the PMN purification process. We cannot eliminate a role for PMN-bound antibody in virus-induced PMN dysfunction.

Viruses are not thought to be chemotactic, although virus-infected cells may be. It has previously been shown that influenza virus induces PMN bipolar shape change in whole blood, suggesting that this virus may be chemotactic and therefore may interfere with migration by the process of chemotactic deactivation. In our studies the virus was not chemotactic and did not cause shape change in purified PMN, although it did cause PMN shape change in whole blood. It appeared that virus interaction with some component of whole blood induced PMN shape change. This component was not present in plasma or whole blood leukocyte preparations or in plasma from virus-treated whole blood. It may be that PMN shape change was the result of virus interaction with erythrocytes. This hypothesis could not be tested with the assay being used because distortion and aggregation of the washed erythrocytes made visualization of PMN shape change impossible. It has previously been noted that damaged erythrocytes are chemotactic. Guinea pig erythrocytes pretreated with influenza virus have been shown to activate the alternative pathway of human complement, but complement is unlikely to have been involved in the present studies because of the presence of EDTA.

The relevance of these in vitro findings to the inhibition of chemotaxis observed in vivo is not known, nor is it known whether or not significant PMN-virus interaction occurs in the blood stream. Maximum depression of chemotaxis has been reported at days 6 to 11 of illness in human volunteers and at days 4 to 8 in chinchillas. At this time virus concentrations in the nasopharynx have reached their peak, and antibody is beginning to appear. Direct interaction between PMN and virus would most likely occur in the respiratory tract, where high concentrations of virus are found. In vivo inhibition may be the indirect result of cellular or humoral changes induced by the disease process, as occurs in a number of other bacterial and viral infections. It remains to be determined whether the inhibition occurring in vivo is similar to that observed here or if defects in the early stages of chemotaxis, demonstrable by shape change or migration through thin filters, also occur.

REFERENCES


Table 5. Stimulation of PMN Bipolar Shape Change by Influenza Virus

<table>
<thead>
<tr>
<th>Cells Tested</th>
<th>% Bipolar PMN *</th>
<th>Virus Mean ± SD</th>
<th>Control AF Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood</td>
<td>24.0 ± 4.6</td>
<td>4.7 ± 3.8†</td>
<td></td>
</tr>
<tr>
<td>Purified PMN</td>
<td>9.3 ± 5.4</td>
<td>8.4 ± 4.0</td>
<td></td>
</tr>
<tr>
<td>Purified PMN in autologous plasma</td>
<td>12.3 ± 4.2</td>
<td>11.6 ± 5.9</td>
<td></td>
</tr>
<tr>
<td>Whole-blood leukocyte preparation</td>
<td>5.5 ± 5.1</td>
<td>5.0 ± 5.9</td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± SD of values from three to five experiments performed with blood from different donors.
†Difference between virus and control AF significant at P < 0.01 by paired t test.
27. Bessis M, Burte B: Positive and negative chemotaxis as observed after the destruction of a cell by U.V. or laser microbeams. Tex Rep Biol Med 23:204, 1965
Characterization of the chemotactic defect in polymorphonuclear leukocytes exposed to influenza virus in vitro

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