Phagocytosis-Induced Modulation of Human Neutrophil Chemotaxis Receptors

By T. A. Lane, G. C. Lamkin, and B. E. Windle

We investigated the effect of a major physiologic event, phagocytosis, on the ability of human neutrophils (PMN) to bind the synthetic chemotactic peptide, 3H-formyl-methionyl-leucyl-phenylalanine (3H-FMLP). We found that both latex and opsonized zymosan (OPZ) particles caused dose-dependent suppression of 3H-FMLP binding and abolition of 3H-FMLP binding with concentrations of OPZ greater than 2 mg/ml. This "particle effect" was completely inhibited by incubation of PMN with 10 mM 2-deoxyglucose before, but not after, phagocytosis. The particle effect was partially inhibited (28%) by anaerobic conditions and CGD neutrophils, but not by superoxide dismutase, catalase, or oxygen radical scavengers. In addition, supernatants from PMN that had been incubated with OPZ caused 55% reduction of 3H-FMLP binding by fresh PMN. The effect of PMN-OPZ supernatants was inhibited by 1 mM phenylmethylsulfonylfluoride (PMSF), suggesting an additional role for release of neutrophil proteases in the particle effect. The finding that the particle effect was not inhibited by cytochalasin-B or under conditions in which 3H-FMLP was bound to PMN prior to OPZ, indicated that membrane ingestion was not responsible for phagocytosis-induced suppression of 3H-FMLP binding. Finally, we found that low concentrations of OPZ (0.187 mg/ml) decreased the affinity but not total number of 3H-FMLP receptors, but higher OPZ concentrations (>2 mg/ml) totally abolished 3H-FMLP binding. These data indicate that the act of phagocytosis initiates at least two distinct mechanisms that may result in the modulation of chemotactic peptide receptors and perhaps other membrane proteins on neutrophils.

It has long been known that prior phagocytosis impairs the ability of neutrophils to respond to chemotactic stimuli. This effect is probably multifactorial, involving chemotactic factor inactivation and autotoxicity due to toxic oxygen radicals, granule contents, alterations in energy metabolism, membrane structure, and mechanical ability of the cell to migrate with ingested materials. In the case of bacterial ingestion, however, it is unlikely that postphagocytotic mechanical constraints alone are the cause of decreased chemotaxis. In addition, there is evidence that phagocytosis of particles may result in modulation, sometimes selective, of functional neutrophil membrane proteins, not specifically related to receptors involved in phagocytosis. The studies reported here were undertaken to examine the possibility that phagocytosis might alter chemotaxis by modulating the number or affinity of human neutrophil receptors for a potent synthetic chemotactic factor, formylmethionyl-leucyl-phenylalanine (FMLP), and also to determine the effect, if any, of a physiologic membrane event (phagocytosis) on the homeostasis of the membrane receptor for this chemotactic factor. We report that phagocytosis impairs the binding of tritiated FMLP (3H-FMLP) by autotoxicity due to generation of toxic oxygen radicals during phagocytosis and by a mechanism independent of the generation of toxic oxygen radicals.

MATERIALS AND METHODS

Cell Collection and Preparation

Granulocytes were obtained from heparinized (10 U/ml) fresh whole blood or in the form of buffy coat concentrates from fresh CPD blood as described previously. Granulocytes were purified by sedimentation at room temperature for 30 min with 0.5 volume of 6% hydroxyethylstarch in saline (McGaw Laboratories, Irvine, Calif.). The supernatant leukocyte-rich plasma was placed over Ficoll-Hypaque (Sigma Chemical Co., St. Louis, Mo.) and centrifuged at 400 g for 30 min. After aspiration of the supernatant erythrocytes were lysed by addition of 5 ml of 0.2% NaCl followed after 30 sec by 5 ml of 1.6% NaCl. Cells were resuspended in Basal Eagle's medium with Earle's salts (BME-Microbiological Associates, Bethesda, Md.) for cell function studies and in phosphate-buffered saline, pH 7.3 with 10 mM glucose, 5 mM KCl, 1.5 mM CaCl2, and 0.5 mM MgCl2, at 107 PMN/ml for phagocytosis and subsequent binding assays.

Cleavage of 3H-FMLP

Cleavage of 3H-FMLP was examined by electrophoresis in a formic acid/acetic acid (1/1) buffer, pH 2.0, on Sephaphore-III paper (Gelman Instrument Corp., Ann Arbor, Mich.) at 300 V for 25 min. Cleavage products were identified using labeled and unlabeled FMLP, leucyl-phenylalanine, and phenylalanine standards (Sigma and New England Nuclear, Boston, Mass.). Percent cleavage was defined as cpm phenylalanine peak ÷ (cpm 3H-FMLP peak + cpm phenylalanine peak). Using this system there was less than 8% cleavage of fresh 3H-FMLP. Similar results were obtained using thin-layer chromatographic methods as previously described; however, with this technique, cleavage of fresh 3H-FMLP was occasionally as high as 15%.

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3H-FMLP Binding Assay

Except for initial experiments (see Results) and unless otherwise indicated, the conditions used for the measurement of 3H-FMLP receptors in these studies were as follows: 10^8 neutrophils in a total volume of 1.2 ml buffer, were incubated with 20 nM 3H-FMLP (New England Nuclear) with (tube 1) or without (tube 2) 10^-4 M FMLP in 2.5 ml polycarbonate test tubes (Falcon Plastics, Cockeysville, Md.). In order to prevent the possibility that 3H-Phe, cleaved from 3H-FMLP, might bind to PMN (data available on request) we included 10^-4 M phenylalanine during binding analysis. Cells were added last, the tubes were capped, mixed, and placed at a 45° angle for 60 oscillations per minute for 12 min. After incubation, tubes were placed in an ice bath, duplicate 500 μl aliquots were removed and placed over GF/C filters (Whatman Liquid Scintillation counter with an efficiency of 39%. Quench corrections were made using an external standard. The number of containing 10 ml of Aquasol (New England Nuclear) on a Beckman aliquots were removed and placed over GF/C filters (Whatman added last, the tubes were capped, mixed, and placed at a 45° angle ville, Md.). In order to prevent the possibility that 3H-Phe, cleaved to 30 mm at 37°C with 50 nM FMLP followed by 3 washes in buffer fluid was aspirated by suction and the filters were washed with 10 of 1.2 ml buffer. Filters were air dried and counted in vials ml of iced buffer. Filters were air dried and counted in vials containing 10 ml of Aquasol (New England Nuclear) on a Beckman. Liquid Scintillation counter with an efficiency of 39%. Quench corrections were made using an external standard. The number of 3H-FMLP counts specifically bound was defined as the number of counts bound by cells from tube 2 minus the counts bound by cells (in the presence of 1000-fold excess unlabelled FMLP) in tube 1. Under the conditions of this assay, incubation of neutrophils for up to 30 min at 37°C with 50 nM FMLP followed by 3 washes in buffer and immediate 3H-FMLP binding analysis resulted in a 13% + 8% increase (p < 0.05) in 3H-FMLP specifically bound to neutrophils (4 experiments). Parallel experiments in which washing and binding was performed at 4°C yielded similar results.

Phagocytosis and 3H-FMLP Binding

Zymosan particles (Sigma) were opsonized as described previously and resuspended in saline at 40 mg/ml. Latex particles (0.794 μm, Difco) were diluted in saline to a concentration of 6.6 x 10⁶ particles/ml unless otherwise stated. For these experiments, 2.5 ml of neutrophils (10⁸ PMN/ml) were incubated at 37°C for 15 min with 0.25 ml of dilutions of opsonized zymosan (OPZ), latex particles, or buffer, following which 1.0 ml aliquots were removed for standard binding studies. Neither latex nor opsonized zymosan particles bound 3H-FMLP in the absence of cells. Anaerobic experiments were performed under nitrogen (PN₂ < 10⁻⁵). All solutions were brought to equilibrium with nitrogen overnight before each experiment. Under anaerobic conditions, cytochrome-c (Sigma) reduction by phagocytosing neutrophils, as previously described, was inhibited by more than 90%. The phagocytic index, as described previously, was unaltered by anaerobiosis.

Supernatants from phagocytosing neutrophils were prepared by incubation of 2 x 10⁸ PMN in a final volume of 2.5 ml buffer with 1 mg/ml (final concentration) opsonized zymosan or an equal volume of 0.9% saline. Tubes were shaken at 37°C for 15 min, followed by rapid cooling on ice, and centrifugation for 10 min at 450 g. To insure removal of all particles, the supernates were passed through a 0.45 μm filter (Millipore Corp., Bedford, Mass.) and used immediately. We incubated 1 ml of supernatant or boiled supernatant with 2 ml of buffer containing 2 x 10⁸ PMN for 15 min at 37°C, followed by standard 3H-FMLP binding analysis.

Chemotaxis experiments employed a migration under agarose assay, as described previously. In these experiments, 5 ml of neutrophils (10⁸ PMN/ml) were incubated with opsonized zymosan at a final concentration of 0.0 mg/ml, 0.167 mg/ml, or 1.6 mg/ml opsonized zymosan at 37°C for 15 min, followed by 3H-FMLP binding analysis or resuspension in buffer at 2.5 x 10⁹ PMN/ml for chemotaxis.

In some experiments, inhibitors and scavengers of toxic oxygen radicals were employed. Superoxide dismutase, catalase, ethanol, mannitol, cytochalasin-B, and colchicine were of the highest purity available and were purchased from Sigma.

RESULTS

Initial 3H-FMLP Binding Studies

In initial studies we suspended granulocytes in buffer at 10⁸ PMN/ml. A volume of 0.5 ml of cells was added to 12 x 75 mm polypropylene tubes (Falcon) and to this was added 20 nM 3H-FMLP (New England Nuclear). Unlabeled FMLP (final concentration 10⁻⁴ M) was added to a second tube with an equal concentration of cells and 3H-FMLP. Tubes were incubated at 37°C in a shaking water bath for 12 min. Specific 3H-FMLP binding to neutrophils was determined as in Materials and Methods. Under these conditions, 60% cleavage of 3H-FMLP was observed in the supernatant buffer after a 12-min incubation of neutrophils with 3H-FMLP. The cleavage product was identified as 3H-phenylalanine.

Effect of Enzyme Inhibitors on 3H-FMLP Cleavage

In view of the success, in rabbit neutrophils, of inhibition of chemotactic peptide cleavage by certain inhibitors, we examined the effect of these and other agents on 3H-FMLP cleavage by human neutrophils. The following agents were employed: 0.2, 1.0, and 2.0 mM 1-(1-tosylamido-2phenyl) ethylchloromethyl ketone (TPCK), and 0.1, 1.0, and 5.0 mM N-alpha-(p-chloromercuriphenyl sulfonyl acid (PCPSA), or phenylmethyl sulfonylfluoride (PMFS). Assays were performed at 10⁸ PMN/ml, and cells were preincubated with agents for 20 min at 37°C, following which 20 mM 3H-FMLP was added and all tubes were incubated again at 37°C, for 12 min. In addition, the effect of reducing incubation temperature to 4°C and cell number to 10⁷ PMN/ml was examined. At the end of incubation, tubes were centrifuged at 7000 g for 1 min, and 2 μl of supernate was examined for 3H-FMLP cleavage. Only 5 mM PCPSA, TPCK, and PMFS reduced 3H-FMLP cleavage (by 49%, 39%, and 45%, respectively), and in each of these cases, partial inhibition of cleavage was accompanied by decreased (76%, 38%, and 32% of control, respectively, 3H-FMLP binding; hence, these agents were of no overall value. NEM (0.1 mM) also reduced 3H-FMLP binding by 66%, without influencing 3H-FMLP cleavage. Incubation at 4°C decreased cleavage at 12 min by 45% compared to the control,
but at this temperature, 3H-FMLP binding was reduced and equilibration of 3H-FMLP binding to neutrophils required 30-min incubation, hence there was no overall advantage to the use of 4°C incubation for these studies. In contrast, reduction of neutrophil concentration in the assay from $10^8$ PMN/ml to $10^6$ PMN/ml, while maintaining the total PMN number at $10^7$ PMN per assay, resulted in a 74% reduction in relative 3H-FMLP cleavage (absolute cleavage was 14% of total 3H-FMLP added) and specific binding per PMN increased by 181%.

Since initial studies revealed decreased 3H-FMLP cleavage when neutrophils were incubated at 4°C, binding equilibrium studies were performed at this temperature and also at 24°C and 37°C. These studies indicated that, whereas binding equilibrium was reached by 12–15 min at 24°C and 37°C, at 4°C binding equilibrium was not achieved until 30–40 min.

Using final assay system (see Materials and Methods) human neutrophils bound 3H-FMLP in a saturable fashion. In 12 experiments, the mean dissociation constant ($K_d$) of 3H-FMLP receptors was $1.58 \pm 0.98$ (SD) $\times 10^{-8}$ M and number of receptors per neutrophil was $33,600 \pm 10,000$ (SD).

**Effect of Phagocytosis on 3H-FMLP Binding**

Exposure of neutrophils to either OPZ or latex particles for 15 min at 37°C prior to measuring 3H-FMLP receptors, resulted in dose-dependent (Fig. 1) and time-dependent (Fig. 2) suppression of 3H-FMLP binding. This effect, herein after referred to as the particle effect, did not occur with unopsonized zymosan particles and was not accompanied by increased 3H-FMLP cleavage during the subsequent binding analysis (neutrophils alone—15% 3H-FMLP cleaved in 30 min, neutrophils and 1 mg/ml OPZ—12% 3H-FMLP cleaved). In 3 experiments, receptors were not regenerated over a 6-hr interval. The particle effect was unaltered by performing binding analysis at 4°C (3 experiments), but was completely abolished by incubation of neutrophils with 10 mM 2-deoxyglucose (2DG) for 15 min at 37°C prior to addition of opsonized zymosan (Fig. 3). Under these conditions,
particle attachment was normal by light microscopy, but phagocytosis was inhibited by 88% and cytochrome-c reduction was abolished. Addition of 10 mM 2DG after phagocytosis also inhibited cytochrome-c reduction but did not inhibit the particle effect.

We reasoned that the particle effect might be due to autooxidation of neutrophil membrane receptors by toxic oxygen radicals generated during phagocytosis. This was examined in several ways. First, neutrophils were preincubated in the presence of superoxide dismutase (SOD), catalase, ethanol, or mannitol (4 experiments each). Preliminary experiments showed that the concentration of SOD used in these studies completely inhibited cytochrome-c reduction by phagocytosing neutrophils and that the concentration of catalase used in these experiments inhibited hydrogen peroxide generation (measured by Scopoletin oxidation, as described by Root and coworkers\(^a\)) by stimulated neutrophils, by 85%. As shown on Table 1, the particle effect was not inhibited by any of these agents. Additional dose–response experiments employing 5, 10, 25, or 50 mM ethanol or mannitol or 0.1, 1, 10, or 100 U of catalase failed to reveal any evidence of a trend toward inhibition of the particle effect. Similar results were obtained if these agents (or 2-deoxyglucose, see above) were added after phagocytosis.

Next, we examined the influence of anaerobic conditions on the particle effect. Anaerobiosis was confirmed by demonstration of greater than 90% inhibition of cytochrome-c reduction by phagocytosing neutrophils (see Materials and Methods). In four experiments, anaerobiosis inhibited the particle effect by 28% ± 12% (Fig. 4). Likewise, in one experiment, chronic granulomatous disease (CGD) neutrophils exhibited normal 3H-FMLP binding and inhibited the particle effect by 27%. The authors' experience with this assay indicates that the 27% inhibition of the particle effect using CGD neutrophils is far greater than the expected variation in the system on any given day. The CGD patient's neutrophils were characterized as typical for CGD by virtue of having less than 10% of normal capacity to stimulate cytochrome-c reduction during phagocytosis, and were unable to reduce nitroblue tetrazolium or consume oxygen during phagocytosis. In addition, phagocytosis of OPZ and chemotaxis toward FMLP by these cells was normal. The patient was not infected at the time of the study. Taken together these data indicated that the particle effect was partially but not completely dependent on generation of toxic oxygen radicals.

A previous report\(^11\) demonstrating selective receptor aggregation in response to particle attachment suggested the possibility that a similar mechanism might have caused the decreased 3H-FMLP binding observed in these experiments. This was examined by incubating cells with or without 10 \(^-6\)M colchicine prior to phagocytosis. In two experiments (Table 2), colchicine itself had no effect on 3H-FMLP binding, and incubation of PMN with colchicine prior to phagocytosis did not alter the particle effect.

We next examined whether complete particle ingestion was necessary for the particle effect. As shown

### Table 1. Influence of Oxygen Radical Inhibitors on the Particle Effect

<table>
<thead>
<tr>
<th>Condition</th>
<th>Buffer Control</th>
<th>Buffer OPZ</th>
<th>SOD (30 (\mu)g/ml) Control</th>
<th>SOD (30 (\mu)g/ml) OPZ</th>
<th>Catalase (10 (\mu)g/ml) Control</th>
<th>Catalase (10 (\mu)g/ml) OPZ</th>
<th>Ethanol (50 mM) Control</th>
<th>Ethanol (50 mM) OPZ</th>
<th>Mannitol (20 mM) Control</th>
<th>Mannitol (20 mM) OPZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent Inhibition</td>
<td>.010 ± .006</td>
<td>.035 ± .012</td>
<td>.035 ± .008</td>
<td>.004 ± .008</td>
<td>.022 ± .008</td>
<td>.012 ± .002</td>
<td>.026 ± .006</td>
<td>.015 ± .006</td>
<td>.014 ± .002</td>
<td>.008 ± .002</td>
</tr>
</tbody>
</table>

Neutrophils were preincubated at 37°C for 15 min with indicated agent, followed by addition of buffer or OPZ (final concentration 1.6 mg/ml) and incubation for 15 min at 37°C, followed by 3H-FMLP binding analysis (see Methods). Percent inhibition refers to (pmole 3H-FMLP bound/10^7PMN in buffer – pmole 3H-FMLP bound/10^7PMN with OPZ)/pmole 3H-FMLP bound in buffer. Four experiments each.

*pmole specific 3H-FMLP bound/10^7PMN ± SE.

![Fig. 4. Effect of respiratory burst inhibition on phagocytosis-induced suppression of 3H-FMLP binding to neutrophils. (A) Neutrophils (10^7/ml) were incubated under aerobic or anaerobic conditions in the presence of OPZ, 1.6 mg/ml or buffer for 15 min at 37°C, followed by measurement of specific 3H-FMLP binding. Values represent mean ± SE of three experiments. (B) Normal or CGD neutrophils (10^7/ml) were incubated with OPZ, 1.6 mg/ml or buffer for 15 min at 37°C, followed by measurement of specific 3H-FMLP binding. Values represent mean of duplicate determinations.](attachment://image.png)
above (Fig. 3), incubation of neutrophils with 10 mM 2-DG, which inhibited phagocytosis and the respiratory burst, but not particle attachment, abolished the particle effect. On the other hand, as shown on Table 2, preincubation of neutrophils for 30 min at 37°C with 5 μg/ml cytochalasin-B (which inhibited phagocytosis in these experiments by 80% but did not inhibit particle attachment or ATP levels) did not inhibit the particle effect. These results indicated that complete particle engulfment was not necessary for the particle effect and suggested that the particle effect was not simply due to ingestion of substantial portions of membrane containing 3H-FMLP receptors, resulting in fewer receptors available for 3H-FMLP binding. To confirm this we examined the effect of OPZ on cells prelabeled with 3H-FMLP. These experiments were performed in an anaerobic chamber in order to obviate the possibility of 3H-FMLP (or membrane receptor) oxidation by toxic oxygen radicals during phagocytosis. Neutrophils were preincubated by incubating with 3H-FMLP for 15 min at 37°C, at which time total counts bound to the cells were measured. Opsonized zymosan was added to one tube of neutrophils and an equal volume of buffer to the control. After 15 min at 37°C, binding was examined in cells from both tubes. In four experiments (Fig. 5) 3H-FMLP binding to control cells decreased by 9% during incubation without OPZ. In contrast, cells exposed to opsonized zymosan decreased binding by 36%. As expected, concurrent experiments under aerobic conditions gave even greater inhibition (60% ± 5%) of 3H-FMLP binding. Taken together, these studies strongly suggested that extensive membrane ingestion prior to neutrophil labeling was not necessary for the particle effect.

**Effect of Neutrophil–OPZ Supernate on 3H-FMLP Binding**

Since rapid degranulation and release of granule contents accompanies phagocytosis, we examined the effects of supernates from neutrophils incubated with OPZ on 3H-FMLP binding by fresh neutrophils. Supernates prepared from neutrophil–OPZ mixtures as described in Materials and Methods contained no OPZ particles and did not stimulate cytochrome-c reduction by neutrophils. As shown on Table 3, in 11 experiments, we found that incubation of neutrophils for 15 min at 37°C with neutrophil–OPZ supernates,

### Table 2. Influence of Cytochalasin-B and Colchicine on the Particle Effect

<table>
<thead>
<tr>
<th></th>
<th>Buffer</th>
<th>OPZ</th>
<th>Percent Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>.168*</td>
<td>.063</td>
<td>63</td>
</tr>
<tr>
<td>Cytochalasin-B (15 μg/ml)</td>
<td>.138</td>
<td>.045</td>
<td>67</td>
</tr>
<tr>
<td>Control</td>
<td>.184</td>
<td>.065</td>
<td>66</td>
</tr>
<tr>
<td>Colchicine</td>
<td>.185</td>
<td>.081</td>
<td>56</td>
</tr>
</tbody>
</table>

*pmole specific 3H-FMLP bound/10⁷PMN. Numbers represent mean of 2 experiments. Neutrophils were preincubated at 37°C for 30 min with indicated agent, followed by addition of buffer or OPZ (final concentration 0.67 mg/ml) and incubation for 15 min at 37°C, followed by 3H-FMLP binding analysis (see Methods). Percent inhibition refers to (pmole 3H-FMLP bound/10⁷PMN in buffer – pmole 3H-FMLP bound/10⁷PMN with OPZ)/pmole 3H-FMLP bound/10⁷PMN in buffer.

**Fig. 5. Effect of phagocytosis on receptor-bound 3H-FMLP.** Neutrophils (10⁷/ml) were preincubated with 20 nM 3H-FMLP under anaerobic conditions for 12 min at 37°C, followed by addition of OPZ, 1.6 mg/ml or buffer and continued incubation at 37°C for 15 min. Specific binding was measured after addition of 1 mM unlabeled FMLP to tubes. Values represent mean ± SE of four experiments.

### Table 3. Effect of Neutrophil–OPZ Supernatant on 3H-FMLP Binding by Neutrophils

<table>
<thead>
<tr>
<th></th>
<th>pmole 3H-FMLP Bound/10⁷PMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMN alone (11)</td>
<td>.122 ± .016*</td>
</tr>
<tr>
<td>PMN + OPZ (11)</td>
<td>.005 ± .002</td>
</tr>
<tr>
<td>PMN + supernatant (11)</td>
<td>.055 ± .10</td>
</tr>
<tr>
<td>PMN + boiled supernatant (11)</td>
<td>.158 ± .019</td>
</tr>
<tr>
<td>PMN + PMSF (3)</td>
<td>.111 ± .022</td>
</tr>
<tr>
<td>PMN + PMSF + supernatant (3)</td>
<td>.108 ± .021</td>
</tr>
</tbody>
</table>

Neutrophils were incubated at 37°C for 15 min with OPZ (1 mg/ml), 1 ml of neutrophil–OPZ supernatant, 1 ml of boiled supernatant, 1 mM PMSF, or supernatant preincubated with 1 mM PMSF. Numbers in parenthesis are number of experiments.

*pmole 3H-FMLP bound/10⁷PMN ± SE.
†pmole 3H-FMLP bound/10⁷PMN as percent of PMN alone.
decreased binding of 3H-FMLP to fresh neutrophils by a mean of 55%, and that this effect was abolished by 1 mM PMSF. PMSF inhibited neither phagocytosis nor cytochrome-c reduction at this concentration. On the other hand, incubation of neutrophils with PMSF before phagocytosis did not inhibit the particle effect on these cells, either under aerobic or anaerobic conditions (data not shown).

Since a previous report indicated that lysozyme decreased chemotaxis, we examined the effect of human lysozyme (kindly supplied by Dr. Elliot Osserman) on 3H-FMLP binding. Under conditions previously shown to inhibit chemotaxis (500 µg/ml), incubation of neutrophils for 15 min at 37°C with lysozyme had no effect on 3H-FMLP binding.

Effect of Phagocytosis on 3H-FMLP Receptor Concentration and Affinity

Next, we examined whether the postphagocytic suppression of chemotactic receptor binding was associated with decreased total 3H-FMLP receptors per neutrophil or altered binding affinity of membrane receptors, by measuring the binding and saturation of 3H-FMLP to postphagocytic neutrophils. In a typical experiment (Fig. 6), at a dose of 0.167 mg OPZ/ml, there was a 4.6-fold increase in the concentration of 3H-FMLP required to half saturate the receptors of postphagocytic neutrophils. In 7 such studies at 0.167 mg OPZ/ml, the mean dissociation constant increased from 1.7 ± 0.3 nM in control neutrophils to 4.7 ± 1.2 nM in postphagocytic neutrophils, a 2.7-fold increase. There was less than 10% variation in the total number of receptors (28,200 receptors/cell in the control and 25,900 receptors/cell in postphagocytic neutrophils). On the other hand, higher concentrations of OPZ (Fig. 6) substantially diminished or completely abolished 3H-FMLP binding. Although there was variation in the potent OPZ batches, incubation of neutrophils with a concentration of OPZ in excess of 2 mg/ml consistently abolished 3H-FMLP binding.

Finally, in order to determine if the effect of phagocytosis on 3H-FMLP binding was accompanied by similar changes in chemotaxis, we incubated neutrophils with either 0, 0.167, or 1.6 mg/ml OPZ, exactly as described for binding studies (see Materials and Methods). Following incubation, we examined neutrophil chemotaxis toward FMLP in concentrations ranging from 10⁻⁸ M to 10⁻⁶ M. In two experiments with 0.167 mg/ml OPZ, we observed a 25% reduction in maximal chemotaxis and a threefold increase in the concentration of FMLP required to stimulate half maximal migration. In parallel experiments, 3H-FMLP binding (20 nM 3H-FMLP) of the same samples of neutrophils was reduced by 65% in the presence of 0.167 mg/ml OPZ. In two experiments with 1.6 mg/ml OPZ, neutrophil migration was abolished (3H-FMLP binding was not measured).

DISCUSSION

The discovery that N-formyl-methionyl oligopeptides are potent chemotactic agents for neutrophils has facilitated investigation of the activation and modulation of neutrophil chemotaxis. The measurement of receptors for tritiated chemotactic peptides on human and rabbit neutrophils, however, is complicated by neutrophil-associated proteolytic activity that cleaves chemotactic peptides. In rabbit neutrophils, cleavage of chemotactic peptides was inhibited by certain protease inhibitors, however, using human neutrophils (at 37°C), we and others found that protease inhibitors did not completely inhibit proteolytic activity and failed to enhance 3H-FMLP binding. At 4°C, however, TPCK did enhance 3H-FMLP binding by human neutrophils. Interestingly, both nonpenetrating and penetrating sulfhydryl reagents, PCMPSA and NEM, caused reduction of 3H-FMLP binding, indicating a possible role for free sulfhydryls in association with the 3H-FMLP receptor. This requires further investigation.

Neutrophil chemotactic peptide receptor binding is also complicated by observations that at 37°C, receptor-bound chemotactic peptides are internalized. Under the conditions of our assay, however, we detected no decrease in 3H-FMLP receptors after preincubation of neutrophils with FMLP. The reasons
for the difference between our findings and those previously reported is unclear but may be due to the use of different peptides (N-formyl-methionyl-leucyl-phenylalanine versus N-formyl-norleucyl-leucyl-phenylalanine-norleucyl-125I-tyrosyl-lysine). In addition, we found maximal binding to be similar when measured either at 4°C or 37°C. Finally, the values we obtained for the 3H-FMLP dissociation constant and total receptor number per neutrophil are similar to values reported by others using 4°C incubation during binding analysis. Measurement of chemotactic peptide receptors at 37°C may not be optimal for most purposes; however, we chose to use 37°C in these studies, since this allowed us to directly examine the influence of phagocytosable particles under physiologic conditions on cells with prebound 3H-FMLP.

The well documented postphagocytic chemotactic defect is probably multifactorial. Among the possible causes are loss of chemotactic factor receptors by membrane ingestion or damage to these receptors by products of neutrophil activation. In these studies we found that phagocytosis caused dose- and time-dependent loss of 3H-FMLP receptor activity on human neutrophils and that receptor activity did not return during up to 6 hr of subsequent incubation or upon addition of a metabolic inhibitor (2-deoxyglucose) after phagocytosis. The suppression of 3H-FMLP receptor activity during phagocytosis is a nonspecific event to the extent that other membrane proteins have been previously reported to be suppressed during phagocytosis. Likewise, although we also demonstrated decreased chemotaxis in postphagocytic neutrophils, the relationship between this and altered 3H-FMLP receptor binding must be interpreted with some caution, in view of the protean effects of phagocytosis on neutrophil function (see above).

It has been reported that autooxidation by activated neutrophils can suppress several cell functions, including chemotaxis, and that the postphagocytic chemotaxis defect is not found in CGD neutrophils, indicating a potential role for the participation of toxic oxygen radicals generated during phagocytosis in autooxidation of the neutrophil membrane. The data reported here indicate a definite, but not exclusive, role for toxic oxygen radicals in the suppression of 3H-FMLP receptors during phagocytosis, since the effect was partially, but not completely, inhibited by anaerobic conditions or by CGD cells. These data and the finding that the particle effect was not inhibited by addition of 2DG after phagocytosis indicated that the particle effect was not simply due to oxidation of 3H-FMLP to the sulfoxide derivative by oxygen radicals generated during phagocytosis, but was due to a direct effect on the neutrophil itself.

In view of the inhibition of the particle effect under anaerobic conditions, it is not clear why superoxide dismutase, catalase, and hydroxyl radical scavengers failed to equally inhibit the particle effect. The concentration of superoxide dismutase that we used was sufficient to inhibit phagocytosis-stimulated cytochrome-c reduction by more than 90%. This is similar to the degree of cytochrome-c reduction inhibition during phagocytosis, which we obtained using anaerobic conditions. Likewise, we used a concentration of catalase sufficient in preliminary studies (data not shown) to inhibit an amount of hydrogen peroxide in excess of 200-fold greater than that reported to be generated (per minute) by stimulated neutrophils and sufficient mannitol and ethanol to substantially inhibit hydroxyl radical generated by stimulated neutrophils. Perhaps local concentrations of the inhibitory agents in membrane areas adjacent to particles was insufficient to inhibit high local concentrations of toxic oxygen radicals. In any event, our finding that the use of strict anaerobic conditions or CGD cells partially inhibited the particle effect suggests that the failure of SOD, catalase, and scavengers to inhibit processes potentially related to oxygen radical formation must be interpreted with some caution.

Additional experiments suggested that the residual particle effect observed under anaerobic conditions was not simply due to membrane ingestion and loss of receptors into the phagocytic vacuole. Thus, 2-deoxyglucose, which inhibited glycolysis and phagocytosis, abolished the particle effect, while cytochalasin-B, which inhibited only phagocytosis, did not inhibit the particle effect at all. Similar findings have been reported in the case of amino acid transport proteins in neutrophils exposed to phagocytic stimuli. Finally, membrane ingestion could not account for suppression of 3H-FMLP binding by neutrophils prelabeled with the peptide under anaerobic conditions, before addition of particles. Taken together, these experiments suggested an additional mechanism for phagocytosis-induced suppression of 3H-FMLP binding that was independent of oxidative cytotoxic mechanisms.

We found that whole supernatants from neutrophils incubated with OPZ reduced 3H-FMLP binding, and this was inhibited by a protease inhibitor. This suggested that exocytosis of proteases during phagocytosis may have initiated autoproteolysis of phagocytosing neutrophils, thereby altering their membrane properties. It seems equally possible that release proteolytic enzymes might alter or damage adjacent neutrophils or nonphagocytic cells. It is not clear why PMSF failed to inhibit the particle effect on neutrophils to which OPZ (and not PMN–OPZ supernate) was added. In this regard it is noteworthy that Amrein and Stossel found that autoproteolysis was not
completely inhibited even in resting PMN by PMSF, however, diisopropylfluorophosphate, which may penetrate neutrophils more effectively, did inhibit proteolysis. This requires further investigation. Our studies also indicated that the previously reported inhibitory effect of lysozyme on chemotaxis is not mediated by altered binding of chemotactic peptide.

Finally, we found that at low concentrations of OPZ, the decreased 3H-FMLP binding was due to decreased 3H-FMLP receptor binding affinity with no change in total receptor numbers at saturation. This is somewhat similar to the decreased affinity of 3H-FMLP binding reported in the presence of degranulating stimuli or after neutrophil storage; however, we did not observe an increase in total receptor numbers after phagocytosis. We found that total 3H-FMLP receptors were either unchanged or somewhat decreased after phagocytosis in the presence of low concentrations of OPZ. On the other hand, in the presence of high concentrations of OPZ (greater than 1.5 mg/ml of OPZ), binding of 3H-FMLP was abolished in nearly all experiments. The reason for this difference is not clear; however, there are several possibilities. Neutrophil membrane immunoglobulin receptors may be lost (by shedding or internalization) and subsequently replaced by additional receptors. Similarly, recent reports indicate that chemotactic peptide receptors are internalized during binding and that these receptors reappear after the binding stimulus is removed. It is possible that low concentrations of phagocytose particles result in FMLP receptor internalization and appearance of new receptors, perhaps from the membranes of neutrophil granules. Alternatively, changes in membrane ion content, cyclic nucleotides, cytoskeleton, lipid composition, or other phagocytosis-induced events might alter the binding affinity of 3H-FMLP receptors. Some of these possibilities are currently under investigation. At higher concentrations of OPZ, receptors may also be destroyed by generation of toxic oxygen radicals or by proteases released from granule contents.

The precise relationship between OPZ concentration during phagocytosis and altered binding or destruction of the 3H-FMLP receptor and the dependence of these events on toxic oxygen radicals is not addressed by the investigations reported here; however, these studies do indicate that increasing phagocytic activity of neutrophils resulted in decreased ability to bind certain chemotactic peptides and decreased chemotaxis. In addition, these studies suggest that phagocytic stimuli may modulate neutrophil chemotaxis at sites of inflammation by a mechanism independent of the generation of toxic oxygen radicals. Studies in this laboratory are currently in progress to further characterize the precise role of phagocytic and other cell-activating stimuli in modulating the affinity and number of FMLP receptors on human neutrophils and the relationship this bears to modulation of chemotaxis.

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REFERENCES


30. Lane TA, Windle BE: Decreased affinity for a chemotactic factor in stored granulocyte concentrates. Transfusion (in press)


Phagocytosis-induced modulation of human neutrophil chemotaxis receptors

TA Lane, GC Lamkin and BE Windle