Human Neutrophil Peptide Receptors: 
Mobilization Mediated by Phospholipase C

ROBERT D. NELSON, PhD, VANCE D. FIEGEL, BS, and DENNIS E. CHENOWETH, MD, PhD

Incubation of human neutrophils with phospholipase C from Clostridium perfringens caused an increase in the ability of the treated cells to bind the chemotactic peptide, F-Met-Leu-Phe. The increase in binding was related to an increase in specific binding of the ligand. The increase in specific binding was, in turn, related to an increased number of peptide receptors. The dissociation constant (Kd) for the tripeptide was not altered, on the average, by enzyme treatment. The increase in peptide receptor number was related temporally, and possibly mecha-

nistically, to enzyme-stimulated secretory function involving the secondary granules. Phospholipase C treatment did not similarly augment binding of the complement-derived attractant, C5a. Receptor numbers for different chemotactic ligands may therefore be controlled by different mechanisms. Supplementary experiments provided evidence that this phenomenon was attributable to phospholipase C activity and not to contaminating protease(s). (Am J Pathol 1982, 107:202-211)

AN ABILITY to bind synthetic N-formyl-methionyl peptides through specific cell membrane receptors is an established property of human neutrophils and neutrophils from other, but not all, mammalian species. The functional significance of these peptide receptors is thought to relate to their role in chemo-
tactic responses of neutrophils to analogous peptides produced by bacterial pathogens. A single class of such receptors appears to mediate chemotaxis as well as stimulated respiratory and secretory functions induced by these ligands. A majority of studies to date have emphasized aspects of the interaction of neutrophils with one or more members of a series of N-formyl-methionyl peptides and other closely related compounds. Results of these studies have given the impression that the spectrum of ligands with which the peptide receptors interact is rather limited. Several recent reports now demonstrate, however, that these receptors may interact with a number of ligands seemingly unrelated to the N-formyl-methionyl peptide series. Therefore, the limits of structural and compositional diversity for ligands binding to peptide receptors remain currently unresolved.

Another feature of the peptide receptors that remains to be fully understood is the mechanism of expression and functional significance of peptide receptors, which can be exposed by treatment of neutrophils with organic solvents or mobilized by incubation with various secretagogues. The latter phenomenon has been reported by Fletcher and Gallin to occur following incubation of human neutrophils with ionophore A23187, phorbol myristate acetate, or activated serum under conditions that stimulate the release of lysosomal enzyme. We have chosen to explore this phenomenon and its mechanism further, using an al-

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Address reprint requests to Dr. Robert D. Nelson, Box 124 Mayo, University of Minnesota, Minneapolis, MN 55455.
ternative stimulant, phospholipase C (PL-C). The present report describes the results of our experiments employing this agent.

**Materials and Methods**

**Preparation of Cells**

Heparinized blood was obtained from human volunteers with the approval of the University of Minnesota Committee on the Use of Human Subjects in Research. Neutrophils were isolated by the method of Ferrante and Thong. The medium used for washing, treatment, and assessing binding of chemotactic ligand was Hanks' balanced salt solution (HBSS, Gibco Laboratories, Grand Island, NY).

**Treatment With Phospholipase**

Phospholipase A2 (from *Naja naja* venom), C (from *Clostridium perfringens*), and D (from peanut) were obtained from Sigma Chemical Co., St. Louis, Missouri (product numbers P 6139, P 0264, and P 0515, respectively). The treatment protocol involved incubation of 10^7 neutrophils in a 1-ml volume of HBSS containing 0.1-1.0 unit enzyme for 5-60 minutes at 37 C. Treated cells were washed before transfer to the cytotaxin-binding assays. Such treatment did not affect cell viability measured in terms of release of cytoplasmic lactate dehydrogenase.

**Measurement of Respiratory and Secretory Functions**

Enzyme-mediated stimulation of the respiratory burst was measured as production of ^14^CO_2_ production from 1-14^C-glucose by standard methodology. Siliconized glass scintillation vials were prepared with 4 mCi 1-14^C-glucose (specific activity 5-10 mCi/mmole, New England Nuclear Corp., Boston, Mass) and enzyme or 10^-^ M N-formyl-methionyl-leucyl-phenylalanine (F-Met-Leu-Phe, Calbiochem, LaJolla, Calif) in 0.5 ml total volume of phosphate-buffered saline (PBS) and prewarmed in a water bath to 37 C. Cells were then added, and the vials were capped immediately with stoppers to which were attached 1.5 cm filter papers saturated with 20% NaOH. Incubation at 37 C was continued for 30 minutes, after which time the filter papers were recovered and dried; accumulated ^14^CO_2_ was measured by liquid scintillation spectrometry.

Secretion of lysosomal enzymes, lysozyme and myeloperoxidase, was assessed with the use of cell-free supernatant fluids. Lysozyme was assayed with the use of a reagent kit from Worthington Biochemical Co., Freehold, New Jersey. Myeloperoxidase was quantitated according to the method of West et al. Enzyme release is reported as the percentage of total available enzyme activity, a value obtained by consideration of enzyme activity in a lysate prepared by sonication of an aliquot of cells.

**Cytotaxin-Binding Assays**

F-Met-Leu-3^H^-Phe, specific activity 46.4 or 56.9 Ci/m mole, was obtained from New England Nuclear. The binding assay involved use of a microcentrifuge (Microfuge B, Beckman Instruments, Lincolnwood, Ill), as previously described. Cells and cytotaxin, both precooled to 0 C, were mixed in a conical microfuge tube to provide a final concentration of 10^7 cells and 0.6-60 nM cytotaxin in a 1-ml volume. After incubation at 0 C for 60 minutes, with occasional mixing, the cells were sedimented by centrifugation in the microfuge for 30 seconds, the supernatant fluid was discarded, the cells were suspended in a 1-ml volume of Ready-Solv HP (Beckman), and the radioactivity was quantitated by scintillation spectrometry. Specific binding denotes the amount of bound ligand displaced by a thousandfold excess of unlabeled cytotaxin present for the duration of the assay.

To assess binding of C5a, the anaphylatoxin was purified to homogeneity by the methods of Fernandez and Hugli. The glycopeptide was radiolabeled with 125^I by a solid-phase lactoperoxidase-glucose oxidase method (Enzymobead, Bio-Rad Laboratories, Richmond, Calif) according to the specifications of the manufacturer (Technical Bulletin 1060). The labeled cytotaxin was separated from free 125^I by immunoaffinity chromatography and had a specific activity of 37 ^μ^Ci/μg. The binding assays involved addition of 10 μl 125^I-C5a at concentrations specified in the text, to 90 μl of human neutrophils suspended at a density of 11.1 x 10^6/ml in HBSS containing 0.5% gelatin. After incubation for 20 minutes at room temperature, the cells were sedimented by centrifugation at 10,000g for 30 seconds, and the amount of total bound 125^I-C5a, as femtmoles bound, was calculated by an equation given previously.

**Results**

**Influence of PL-C on Peptide Binding**

Data illustrating the influence of *C perfringens* PL-C on binding of F-Met-Leu-3^H^-Phe to human neutrophils are presented in Figure 1. The labeled tripeptide bound under each of the pretreatment conditions
is expressed as a compound bar. The larger open bars represent total bound ligand, and the smaller stippled and hatched bars represent the nonspecific and specific components of the bound ligand, respectively.

In three experiments, total binding of labeled tripeptide at 0°C by neutrophils tested immediately following their isolation averaged 8983 cpm. Of these counts, 2555 cpm (28%) were attributed to nonspecifically bound ligand that could not be displaced by addition of a thousandfold excess of unlabeled tripeptide. By subtraction, the remaining 6248 cpm (72%) were attributed to ligand bound specifically to peptidase receptors. Incubation of neutrophils at 37°C for 30 minutes, in the absence of PL-C, produced an average 36% increase in total binding of F-Met-Leu-^3^H-Phe attributable virtually exclusively to an increase in specifically bound tripeptide. Incubation of the cells at 37°C for 30 minutes, in the present of 1 unit PL-C, produced an average 67% increase in total binding of labeled tripeptide, relative to the 37°C controls. This increment was similarly related to an increase in specifically bound ligand. Thus, PL-C treatment produced an average increase of 84% in specific binding of tripeptide. Lower amounts of PL-C produced lesser effects on the peptide binding phenomenon. Greater amounts of enzyme or longer treatment times were not routinely used, because of occasional aggregation of the treated cells. No increase in binding of labeled tripeptide was observed on exposure of the human neutrophils to PL-C at 0°C for 30 minutes or following pretreatment with phospholipase A2 or phospholipase D under comparable conditions. Further, treatment of bovine neutrophils with 1 unit PL-C at 37°C for 30 minutes did not effect a change in ability to specifically bind the labeled tripeptide.

Our use of a commercial C. perfringens PL-C preparation in the preceding experiments, without further purification of the phospholipase activity, raises questions about the enzyme activity responsible for the phenomenon described. To assess the possibility that a contaminating protease activity might have produced this result, four experimental approaches were taken. Our first approach was to assess protease activity present in this PL-C preparation. A colorimetric method based upon azo-casein as substrate was used for this purpose. The data in Figure 2 derive from one such experiment. Protease from Strep tomyces griseus (pronase P, Sigma, P 5130) and protease from Bacillus subtilis (subtilopeptidase A, Sigma, P 5380) produced the dose-related changes in OD values shown following 3 hours of incubation at 37°C, pH 7.4. Under the same conditions, addition of the C. perfringens PL-C at 125 units/ml produced no increment in OD. For comparison, addition of B. cereus
enzymes over the concentration range of 0.5–2.0 units/ml produced 40% to >80% increases in binding of the tripeptide. Note also that at a concentration of 1 unit/ml, the *C. perfringens* and *B. cereus* PL-Cs produced comparable levels of stimulation of binding of the tripeptide. This similarity in dose effect occurred in spite of a reasonable difference in protease activities contaminating these two PL-C preparations (Figure 2).

A final approach has involved the application of 1,10-phenanthroline (PAL) to inhibit PL-C activity. Inhibition of PL-C activity by PAL occurs through chelation of the zinc ion, a cofactor essential for enzymatic activity. Data in Table 2 summarize the results of one experiment to assess the influence of PAL on the ability of *C. perfringens* PL-C to stimulate specific binding of F-Met-Leu-3H-Phe. Pretreatment of the neutrophils with 1 unit/ml PL-C for 30 minutes caused a 104% increase in subsequent specific binding of the labeled ligand. This treatment was also observed in the release of cytoplasmic lactate dehydrogenase (LDH), indicating some loss of integrity of cytoplasmic membrane. Although the LDH released by PL-C pretreatment was five times that released by the untreated cells, we believe that the enzyme released, in terms of total enzyme (3.5%), is not of sufficient magnitude to invalidate the data obtained in this experiment. Pretreatment with 0.5 mM PAL alone did not alter specific binding of tripeptide relative to the untreated control. In the combined presence of PL-C and PAL, no increment in subsequent specific binding of tripeptide occurred. The 26% decrement observed under the latter conditions was not associated with loss of cell viability assessed as release of LDH. We suggest that these

![Figure 3](image-url)

**Figure 3** – Dose-related influence of *B. cereus* phospholipase C (PL-C) on binding of F-Met-Leu-3H-Phe to human neutrophils. Pretreatment involved incubation of 10⁶ neutrophils in 1 ml fluid volume, with or without PL-C, for 30 minutes at 37°C. The data denote the percentage increase in specific binding of the labeled tripeptide observed in two experiments using *B. cereus* PL-C. The open and solid squares denote the influence of pretreatment with 1 unit/ml *C. perfringens* PL-C on binding of the tripeptide in these experiments.

PL-C at 25 units/ml produced an increment in OD of 0.020. A second approach involved testing the effect of preexposure of neutrophils to a known protease (pronase P) on subsequent binding of the labeled tripeptide. In two experiments, a 30-minute pretreatment of neutrophils with *C. perfringens* PL-C increased subsequent specific binding of F-Met-Leu-3H-Phe by 59% and 80% (Table 1). Protease at 1 unit/ml inhibited subsequent specific binding of the ligand by 33% and 4%, and lesser concentrations of the protease had only a marginal positive influence (≤4%) on specific binding of the tripeptide.

A third approach to this question involved a comparison of the abilities of PL-C enzymes from two sources, *C. perfringens* and *B. cereus*, to stimulate specific binding of F-Met-Leu-3H-Phe. Our rationale for this comparison is based upon the assumption that PL-C enzymes from two unrelated sources would contain either different containing proteases or different levels of a single protease activity. If contaminating protease(s) were responsible for stimulation of peptide binding, the abilities of these enzyme preparations to stimulate specific binding should then be related to protease activities and independent of their PL-C activities. Data from two experiments, summarized in Figure 3, illustrate the dose effect of *B. cereus* PL-C on subsequent binding of the labeled tripeptide. Pre-exposure of neutrophils to these en-

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Pretreatment: enzyme, dose (units/ml)</th>
<th>Specific binding F-Met-Leu-3H-Phe</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No enzyme</td>
<td>4115</td>
</tr>
<tr>
<td></td>
<td>PL-C, 1</td>
<td>6535</td>
</tr>
<tr>
<td></td>
<td>Protease, 0.1</td>
<td>2751</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>4267</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>4153</td>
</tr>
<tr>
<td>2</td>
<td>No enzyme</td>
<td>4873</td>
</tr>
<tr>
<td></td>
<td>PL-C, 1</td>
<td>8789</td>
</tr>
<tr>
<td></td>
<td>Protease, 0.1</td>
<td>4698</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>4967</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>4950</td>
</tr>
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</table>
Table 2—Influence of Phenanthroline (PAL) on Specific Binding of F-Met-Leu-3H-Phe on Human Neutrophils Stimulated by PL-C Pretreatment

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Specific binding F-Met-Leu-3-Phe CPM</th>
<th>% control</th>
<th>LDH release % total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL-C</td>
<td>PAL</td>
<td>2300</td>
<td>0.7</td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>4697</td>
<td>+104 3.5</td>
</tr>
<tr>
<td>-</td>
<td></td>
<td>2185</td>
<td>- 5 1.6</td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>1713</td>
<td>- 28 1.5</td>
</tr>
</tbody>
</table>

results collectively, provide reliable indirect support for an ability of clostridial PL-C to stimulate specific binding of F-Met-Leu-Phe to human neutrophils and provide a basis for further consideration of the mechanism by which this phenomenon occurs.

Effect of PL-C on Peptide Receptor Number

A mechanism to provide for the PL-C-mediated increase in specific binding of F-Met-Leu-3H-Phe could involve an increase in the number of specific receptors available for binding the ligand. Alternatively, under conditions for the binding assay which do not provide for full saturation of all existing receptors, an increase in the affinity of the receptor for the labeled tripeptide would produce the same result. To assess these alternatives, experiments were performed to provide for Scatchard analysis of this phenomenon. These analyses were performed with the use of 5 concentrations of the labeled tripeptide. The r values for the lines drawn were consistently > 0.95. Data obtained are presented and summarized in Table 3. The peptide receptor number for untreated neutrophils from 5 different individuals averaged 34,903 (range 25,585–40,575). Pretreatment of neutrophils from these individuals with 1 unit PL-C for 30 minutes increased the average receptor number to 71,112 (range 45,559–97,073) (P < 0.02). The affinity of peptide receptors of untreated cells for the tripeptide, expressed here as the dissociation constant (KD), averaged 2.7 × 10^-8 M. The Kp value for receptors present on treated cells averaged 2.5 × 10^-8 M. Although a comparison of these average values would suggest that PL-C did not influence KD, consideration of the Kp values obtained in the individual experiments illustrates that enzyme treatment has a variable influence on this receptor quality. In experiments 1, 3, and 4, enzyme treatment caused only a marginal increase or decrease in the KD value. In experiments 2 and 5, the Kp value was more significantly decreased or increased, respectively.

Influence of PL-C on Neutrophil Respiratory Function

To study, in turn, the mechanism by which PL-C produces an increase in peptide receptor number, we determined the relationship of this phenomenon to effects of the enzyme on neutrophil respiratory and secretory functions. Data in Figure 4 illustrate the effect of PL-C on hexose monophosphate shunt activity measured as generation of 3CO2 from 1-14C-glucose. PL-C at a concentration of 1 unit/ml elicited a respiratory burst in these terms of 7500 cpm. This level of stimulation was 68% of that obtained using 10^-5 M F-Met-Leu-Phe as the stimulant. Phospholipase D at a concentration of 1 unit/ml, which did not effect an increment in binding of labeled tripeptide, elicited a respiratory burst of 5400 cpm, or 72% that produced by the PL-C.

To examine the possible mechanistic relationship between the ability of PL-C to stimulate the respiratory burst and increase peptide receptor number, we tested the influence of PL-C on respiratory activity and peptide binding function of neutrophils from an individual with chronic granulomatous disease (CGD). Phospholipase C did not stimulate CGD neutrophil respiratory activity (data not shown). The data in Figure 5 illustrate that total binding of F-Met-Leu-3H-Phe by neutrophils from both the healthy control and the CGD donor was significantly increased by pretreatment with 1 unit PL-C at 37 C for 30 minutes (P < 0.001) and that these increments were attributable to increases in specific binding of the labeled ligand.

Table 3—Influence of Phospholipase C on Peptide Receptor Number and KD for F-Met-Leu-Phe

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cells</th>
<th>Receptor number</th>
<th>Dissociation constant (KD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>25,585</td>
<td>1.6 × 10^-5 M</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>62,187</td>
<td>1.7 × 10^-5 M</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>32,207</td>
<td>3.3 × 10^-5 M</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>45,559</td>
<td>1.6 × 10^-5 M</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>38,943</td>
<td>1.8 × 10^-5 M</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>57,732</td>
<td>1.3 × 10^-5 M</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>37,204</td>
<td>2.8 × 10^-5 M</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>97,073</td>
<td>2.5 × 10^-5 M</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>40,575</td>
<td>3.9 × 10^-5 M</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>93,009</td>
<td>5.5 × 10^-5 M</td>
</tr>
<tr>
<td>Summary data*</td>
<td>Control</td>
<td>34,903</td>
<td>2.7 × 10^-5 M</td>
</tr>
<tr>
<td></td>
<td>± 2,719</td>
<td>± 0.4 × 10^-5 M</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>71,112</td>
<td>2.5 × 10^-5 M</td>
</tr>
<tr>
<td></td>
<td>± 10,162</td>
<td>± 0.8 × 10^-5 M</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± SEM.
Influence of PL-C on Neutrophil Secretory Function

The influence of PL-C on lysosomal enzyme secretion, assessed as release of myeloperoxidase and lysozyme in the absence of cytochalasin B, is illustrated by the data in Figure 6. In three experiments, 1 unit/ml PL-C stimulated secretion of myeloperoxidase to an average level approximately twice that released spontaneously. This level of release did not change over the time of the assay. In contrast, release of lysozyme increased from approximately 5% to 12.5% of total enzyme over the 60-minute time period. Under comparable conditions, PL-D did not stimulate the secretion of either enzyme. The data in this figure also illustrate the temporal relationship of the influence of PL-C on specific binding of F-Met-Leu-3H-Phe and release of lysozyme. The correlation coefficient relating these effects was 0.97.

Influence of PL-C on Binding of C5a

We have also considered the effect of PL-C on binding of 125I-C5a to neutrophils to determine whether PL-C stimulated mobilization of peptide receptors reflects a specific or a generalized influence on neutrophil cytotoxin binding function. In three experiments, pretreatment of neutrophils with 1 unit PL-C at 37°C for 30 minutes reduced subsequent binding of 125I-C5a by an average 29 ± 4% (mean ± SEM). This loss of ability to bind C5a appeared to be related to a decrease in receptor number, as shown by the data in Figure 7. In this experiment, increasing the amount of 125I-C5a added to the binding assay from 25 to 100 ng/ml did not further change the percentage of the decrease in the level of ligand bound.

Discussion

The results described in this report demonstrate 1) that pretreatment of human neutrophils with clostridial PL-C causes an increase in total binding of F-Met-Leu-Phe; 2) that this influence of clostidial PL-C is not due to protease contamination of the commercial enzyme preparation; 3) that the increase in total bound tripeptide is due to an increase in specific binding of ligand; 4) that the increment in specific binding of tripeptide is associated with an increase in peptide receptor number; 5) that the mechanism of expression of new receptors is independent of stimulated respiratory function but may be related to stimulation of secretion involving the secondary granules; and 6) that binding of an alternative cytotoxin, C5a, is not similarly augmented by pretreatment with PL-C. On the basis of these results, we suggest that this influence of PL-C on human neutrophil peptide receptor number and the mechanism of this phenomenon are analogous to secretagogue-mediated mobilization of peptide receptors, described recently by Fletcher and Gallin.15

Experimental results described in Figure 1 illustrate the phenomenon of PL-C–stimulated increase in total binding of F-Met-Leu-3H-Phe to human neutrophils. They also illustrate that this increase is due to specific binding of the ligand, that is, ligand that can be displaced by addition of an excess of unlabeled tripeptide. The upper limit of this phenomenon was not determined, because of difficulties associated with cell aggregation, which frequently accompanied the use of greater enzyme concentrations and/or longer treatment periods. These results suggest that PL-C in some way causes the expression of peptide receptors previously inactive or unavailable for interaction with ligand or modifies the strength of binding of F-Met-Leu-Phe with existing receptors.

The data in Table 3 demonstrate the appearance of additional peptide receptors following PL-C treatment. In these experiments, an average doubling of
peptide receptor was observed. In individual experiments the level of increase varied from approximately 50% (2 experiments) to more than 100% (3 experiments). This result mimics that reported by Fletcher and Gallin,15 who used $5 \times 10^{-8}$ M ionophore A23187 or 1.25% activated serum for a 30-minute treatment period. The number of peptide receptors per neutrophil before treatment in our experiments falls approximately midway between those reported by Niedel et al27 and by Fletcher and Gallin.15 These numbers, in turn, all exceed the 2000 receptors reported by Williams et al.1 The basis of these discrepancies is not known but could reflect the consequence of some unidentified stimulating condition(s) in some cell isolation protocols or condition(s) inducing loss of receptors in others. The observation by us (Figure 1) and by Fletcher and Gallin15 that incubation of isolated neutrophils at 37°C alone can also produce a limited increase in specific binding of tripeptide supports the possibility that isolation conditions may affect peptide receptor number. It is possible, therefore, that none of the determinations of peptide receptor number for untreated or treated cells accurately reflect the base number of peptide receptors of human neutrophils in vivo or the full potential of neutrophils for mobilization of peptide receptors.

The data in Table 3 also identify changes in the strength of interaction between F-Met-Leu-Phe and peptide receptors induced by enzyme treatment. We conclude from this limited number of experiments that the influence of PL-C on $K_D$ for the tripeptide ligand is highly variable. Finding no consistency in terms of the direction of change, we choose to withhold final judgment on the influence of PL-C on this receptor quality at this time. Although Fletcher and Gallin15 reported that the binding affinity of stimulated neutrophils for tripeptide is reduced relative to the control cells, these investigators also observed a similarly variable pattern of change. The average level of change they reported was not great, and the statistical significance of this difference was marginal. Clarification of this phenomenon therefore demands further experimentation. If the receptors mobilized in response to effects of various stimulants are meant to augment available receptors or replace receptors lost following interaction with peptide ligand, and if the mobilized receptors are to participate in mediating the same responses of the cell to the same ligand, then there may be no reason to expect that the receptor affinity for the ligand should be grossly altered. Or, if a significant change in this function can occur, then one must also consider the possibility that this change in affinity for ligand might alternatively reflect a different physicochemical environment of the newly mobilized receptors on the cell membrane rather than an altered state of these receptors.

The mechanism by which PL-C produces an increase in peptide receptor number could be direct, involving conversion of inactive "pro-receptors" to a functional form or catabolism of cell membrane components to expose previously masked receptors. Recent reports by Goetzl28 and Liao and Freer14 support these concepts. Such direct mechanisms would require that the pro-receptor or masking material have a phosphoglyceride component, since PL-C hydrolyzes the bond between phosphoric acid and glycerol of such substrates; ie, PL-C cleaves choline phosphate from phosphatidyl choline. In this regard,
Figure 6—Influence of C. perfringens phospholipase C (PL-C) on human neutrophil secretory function and specific binding of F-Met-Leu-{sup}[3]H-Phe. Secretion of myeloperoxidase (O) and lysozyme (■) over the 60-minute incubation period was assessed as the percentage of total enzyme available in an equal aliquot of untreated cells. Values denoted as □ and ■ reflect the levels of myeloperoxidase and lysozyme present in supernatants derived from cells incubated in the absence of PL-C for 60 minutes. The symbol ○ denotes the percentage increase in specific binding of labeled tripeptide. The data presented reflect mean ± SEM values calculated from the results of three independent experiments.

It is of interest to note that neither phospholipase A₂ nor phospholipase D produced an increase in binding of labeled tripeptide. Phospholipases of the A class specifically hydrolyze the fatty acid from the 2 position, and phospholipase D removes the X-group, i.e., choline of phosphatidyl choline, to leave a phosphatidic acid. Neither of these direct mechanisms could be discounted on the basis of the experiments described using human neutrophils. Our failure to cause bovine neutrophils to specifically bind F-Met-Leu-{sup}[3]H-Phe by pretreatment with PL-C might suggest that direct enzymatic activity also does not produce an increase in human neutrophil peptide receptor number. Yet, since untreated bovine neutrophils fail to specifically bind tripeptide, these cells may also lack either PL-C sensitive pro-receptors or masked receptors for the tripeptide. Additional experiments to date to block stimulation of human neutrophil respiratory and secretory functions, while preserving enzyme function, have been unsuccessful.

Alternatively, PL-C may effect an increase in peptide receptor number indirectly by stimulating some neutrophil function that, in turn, results in the expression of preexisting receptors. Our experiments appear to discount a mechanistic association between increased receptor number and PL-C-mediated stimulation of the respiratory burst. Phospholipase D was observed to stimulate a respiratory burst (Figure 4) but did not produce an increase in the binding of labeled tripeptide. Further, CGD neutrophils exposed to PL-C did not increase their respiratory activity but did exhibit a normally increased ability to bind labeled tripeptide (Figure 5). Exposure of the CGD neutrophils to PL-C did, however, stimulate release of lysozyme. Fletcher and Gallin more recently reported similar findings from experiments involving pretreatment of CGD neutrophils with other stimulating agents.

The data in Figure 6 illustrate the ability of PL-C to stimulate release of lysozyme and the temporal relationship of this secretory response and the increase in binding of labeled tripeptide (r = 0.97). The secretory response must involve the secondary granules selectively, since PL-C treatment did not produce a corresponding increase in release of myeloperoxidase from primary granules. The interior
membrane of secondary granules may therefore possess receptors that bind N-formyl peptide, and these may become available for binding peptide as the granules fuse with the cell membrane and open to the extracellular space. These results suggest, but do not prove, a mechanistic relationship between these events. Proof of this mechanistic relationship will depend upon experiments involving neutrophils selectively deficient in secondary granules or secretory function, and studies to directly verify that the interior membrane of secondary granules can indeed specifically bind peptide ligand.

The data in Figure 7 illustrate that neutrophil receptors for C5a are not similarly increased by PL-C treatment. Rather, PL-C mediated a decrease in binding of [125I]-C5a that could not be overcome by the addition of more ligand. This observation suggests that mechanisms for modulation of receptor numbers for N-formyl peptides and C5a must differ.

Receptors mobilized by exposure of human neutrophils to agents stimulating secretory function may provide a source of receptors available to replace cell membrane receptors lost following binding of ligand and internalization of the receptor–ligand complex. Whether such receptors are or can become functional in terms of neutrophil migratory, respiratory, and/or secretory responses of the neutrophil remains unknown at the moment. Comparing the influence of PL-C on peptide receptors observed in this study with the observation by Wilkinson that PL-C treatment selectively depresses the response of human neutrophils to F-Met-Leu-Phe suggests that the enzyme-mobilized peptide receptors may not function in chemotaxis.

Two additional considerations merit attention in this discussion. Schiffman et al. recently reported that pretreatment with PL-A, but not PL-C, stimulated subsequent specific binding of tritiated tripeptide to rabbit peritoneal exudate neutrophils. These results are in direct conflict with our findings. This discrepancy could be due to the use of neutrophils from two different mammalian species or to a property unique to the rabbit exudative cells. It could alternatively be due to differences in conditions used for pretreatment. The report cited does not provide information on the sources and activities of the enzymes used; hence a proper comparison of treatments cannot be made. Information provided on enzyme dosage is useful, however. In our experiments, 1 unit/ml of the clostridial PL-C produced the greatest stimulation of tripeptide binding without excessive cellular aggregation or loss of viability under the conditions described. This dose is equivalent to 4 µg/ml of protein. Use of PL-C at 250 µg/ml by Schiffman et al. therefore represents a tremendous excess of enzyme relative to our experience and may, in some way, account for these differences.

Finally, on the basis of our results, it is possible to speculate about the substrate(s) that provide for this influence of clostridial PL-C on human neutrophils. The clostridial enzyme is known to preferentially attack choline-containing phospholipids, such as phosphatidylcholine (lecithin), sphingomyelin, choline plasmalogens, and, to a much lesser degree, ethanolamine phospholipids. The B. cereus PL-C has a broad specificity toward phosphatides, being equally active toward phosphatidylcholine and phosphatidylethanolamine and less active toward phosphatidylserine. Since both enzymes share a strong preference for phosphatidylcholine, and since they produced a comparable dose-related influence on stimulation of specific binding of tripeptide (Figure 3), it is tempting for us to think that choline-containing phospholipids may be the target substrate involved. They may not be an exclusive substrate through which peptide receptor function of human neutrophils can be stimulated, however. In preliminary testing of the influence of phosphatidylino-sitol-specific PL-C (prepared from Staphylococcus aureus by Dr. Martin G. Low, Medical College of Virginia) we have determined that pre-exposure of human neutrophils to this enzyme, too, can produce an increment in specific binding of the tripeptide ligand in association with stimulation of release of lysozyme. Much additional work is required for full detailing of the mechanism of this phenomenon.
References


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