

## SELECTIVE NEUTROPHIL DESENSITIZATION TO CHEMOTACTIC FACTORS

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### ABSTRACT

In the presence of extracellular calcium and magnesium, a series of chemotactic oligopeptides and C5a caused aggregation of human polymorphonuclear neutrophils (PMNs). This cellular response developed rapidly and began to reverse 2 min after exposure to the chemotactin. In the absence of the bivalent cations, none of the chemotactins stimulated the aggregation response. If cells were first exposed to a chemotactin and then treated with calcium and magnesium, aggregation was detected only after addition of the cations, and the magnitude of the response fell sharply as the interval between the addition of chemotactin and addition of cations was lengthened: when this interval exceeded 2 min, aggregation was barely detectable. This loss of reactivity persisted even when cells were re-exposed to fresh chemotactic factor and washed between the first and second exposures. In all instances, however, loss of cellular reactivity was highly selective: cells preincubated with any chemotactic oligopeptide were hyporesponsive to subsequent stimulation with an oligopeptide but remained fully responsive to C5a; cells preincubated with C5a were hyporesponsive to C5a but retained their responsiveness to the oligopeptides. Because this selectivity parallels the known specificities of these chemotactic factors for their receptors in or on the neutrophil, desensitization may reflect functional loss of receptors after stimulation. Alternatively, this selectivity may indicate that morphologically identical neutrophils contain subpopulations of cells with varying reactivities to receptor-bound chemotactic factors. In either event, desensitization may be useful in functionally defining chemotactic factors and their respective receptors. The rapidity of development of desensitization suggests that it may operate to limit or moderate various *in vitro* and *in vivo* neutrophil responses to chemotactic factors.

**KEY WORDS** neutrophil · chemotactic factors · receptors in or on the cell (5, 7, 16, 28, 33, 36, 40). Synthetic oligopeptide chemotactic factors appear to bind to one receptor; the chemotactic aggregation · desensitization

In stimulating polymorphonuclear neutrophil (PMN) responses such as chemotaxis, exocytosis, and cellular aggregation, certain chemotactic factors first interact with stereospecific, high-affinity biological activities of these chemotactic factors,

and agents which interfere with this binding inhibit cellular responses to the chemotactic factors (1, 2, 28, 33, 36, 39). In the presence of extracellular  $\text{Ca}^{2+}$ , chemotactic factors stimulate PMNs to take up  $\text{Ca}^{2+}$  and increase their exchangeable intracellular  $\text{Ca}^{2+}$  pool (4, 11, 12, 22, 23, 35). It has been postulated that these events trigger or modulate the chemotactic (4, 11, 12, 22), degranulation (14, 15, 23, 35), and aggregation (26, 31) responses of the cell. One important consequence of chemotactic factor-receptor binding, therefore, may be alterations in surface membrane permeability to  $\text{Ca}^{2+}$ .

In this report, we studied the effect on the neutrophil aggregation response of withholding bivalent cations from cells treated with a chemotactic factor. In the presence of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , PMNs rapidly aggregated in response to C5a or the chemotactic oligopeptides; in the absence of these cations, the cells did not aggregate but rather rapidly became desensitized to subsequent treatment with the bivalent cations and fresh chemotactic factor. Desensitization appeared selective for the chemotactic factor receptor stimulated: various chemotactic oligopeptides desensitized the cells to further stimulation with an oligopeptide but not to C5a; C5a desensitized the cells to further stimulation with C5a but not to the chemotactic oligopeptides. Thus, the desensitization procedure functionally defined the two chemotactic factor receptors and may be useful in defining other chemotactic factor receptors. The rapidity of development of desensitization suggests that chemotactic factor influences on the surface membrane permeability to  $\text{Ca}^{2+}$  may be short-lived, and that desensitization may serve to limit in vitro and in vivo PMN responses to chemotactic factors.

## MATERIALS AND METHODS

### *Chemotactic Factors, Buffers, and Reagents*

C5a was generated from zymosan-activated normal human sera and partially purified by gel filtration over Sephadex G100 as previously described (29, 38). The reagent contracted guinea pig ileum, degranulated cytochalasin-B-treated PMNs, and stimulated PMN and monocyte chemotaxis. Virtually all of the chemotactic activity of the reagent could be inactivated by 30-min incubation with anti-human C5 but not by incubation with anti-human C3 or anti-human gammaglobulin (25, 29). The synthetic oligopeptide chemotactic factors *N* $^{\alpha}$ -formyl-methionyl-leucyl-phenylalanine (FMLP), *N* $^{\alpha}$ -formyl-methionyl-methionyl-phenylalanine (FMMP),

*N* $^{\alpha}$ -formyl-methionyl-methionyl-methionyl-methionine (FMMMM), and *N* $^{\alpha}$ -formyl-norleucyl-leucyl-phenylalanine (FNLLP) were obtained and used as previously described (8, 34). The four oligopeptides had chemotactic activities of 9.4, 3.1, 2.2, and 1, respectively (8, 34). The tritium-labeled oligopeptide, *N* $^{\alpha}$ -formyl-norleucyl-leucyl [*p*-tritio]-phenylalanine ( $[^3\text{H}]$ FNLLP), was synthesized to high specific activity (14 Ci/mmol) as previously described (8). Each of the oligopeptides at 10 mM was dissolved in dimethylsulfoxide. In the final concentrations used here (0.3% or less), the solvent did not influence PMN function. The buffer used throughout these studies was a modified Hanks' balanced salt solution containing (mM): NaCl, 130; KCl, 5.5;  $\text{Na}_2\text{HPO}_4$ , 0.6;  $\text{NaH}_2\text{PO}_4$ , 0.6; glucose, 10; and Tris, 25. Where indicated,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  were added as chloride salts in a final concentration of 1.4 and 0.7 mM, respectively. Chemicals were reagent grade.

### *Chemotactic Assays*

Assays of chemotactic substances were performed using modified Boyden chambers, Millipore filters (Millipore Corp., Bedford, Mass.) of 0.65- $\mu\text{m}$  porosity, and dextran-sedimented human leukocytes (27). Chambers were incubated for 45 min at 37°C, and chemotaxis was scored by averaging the number of cells migrating 10  $\mu\text{m}$  or farther into the filter in five high-power fields. The dose-response curves obtained over a  $10^3$ -fold dilution of chemotactic substance were plotted and from these curves the concentration of chemotactin which stimulated 50% of the maximal chemotactic response ( $\text{ED}_{50}$ ) was extrapolated (24). Data are reported as the  $\text{ED}_{50}$ .

### *Aggregation Assay*

Normal human whole blood was centrifuged over Ficoll-Hypaque discontinuous gradients to obtain leukocyte populations consisting of 98.4% PMNs (27). The neutrophil preparation was freed of contaminating erythrocytes by hypotonic lysis, washed in isotonic saline, and suspended in the buffer at a cell concentration of 4,600/ $\mu\text{l}$   $\pm$  1,000/ $\mu\text{l}$  (range). All reagents were made 37°C and pH 7.4 before use, and all experiments were performed in a 37°C room. 1 ml of the PMN suspension was placed in a plastic vial and stirred continuously with a magnetic bar. After 1–4 min (time specified), the suspension was treated with 1.4 mM  $\text{Ca}^{2+}$  and 0.7 mM  $\text{Mg}^{2+}$  (final concentrations) and, after 1 min more, with a small volume (50  $\mu\text{l}$  or less) of chemotactic substance. In some experiments, the order of adding the bivalent cations and chemotactic substance was reversed, and the interval between the two additions was varied. In other experiments, cell suspensions were exposed to fresh chemotactic factors 1 min after adding the bivalent cations to cells preincubated for 4 min with a chemotactic factor. Finally, in some experiments, cells were washed after 4 min of preincubation with a chemotactin but before addition of the bivalent cations.

Immediately before and at  $1/4$ ,  $1/2$ , 1, 2, 4, 8, and 15 min after adding a chemotactin or the bivalent cations (depending on the particular experimental design), 20- $\mu$ l samples were taken from the PMN suspension, immediately diluted in 10 ml of Isoton (Coulter Electronics Inc., Hialeah, Fla.), and analyzed in a model ZBI Coulter Counter (Coulter Electronics Inc.). The counter aspirates 500  $\mu$ l from the Isoton-diluted cell suspension and electronically enumerates and sizes (by volume) each particle (i.e., single cell or cell clump) in the aspirate. The counter can be set to selectively enumerate only particles with a volume size within a specified range. To detect aggregation of cells, we performed two counts on each Isoton-diluted suspension. The first count, called T, was performed with the following settings for amplification, aperture, upper threshold, and lower threshold:  $1/4$ , 4, infinity, and 10, respectively; the second count, called A, was performed with the following values:  $1/4$ , 4, infinity, and 80, respectively. With these settings, the T count enumerates all particles larger than  $\sim 20\%$  of the size of the average PMN, and the A count enumerates all particles larger than  $\sim 180\%$  of the average PMN size (27). Thus, T is the total particle concentration (i.e., aggregated plus unaggregated cells); A is the aggregated cellular concentration; and  $100 \times A/T$  is the large particle percentage (LPP). We have found that various chemotactic factors stimulate PMNs in suspension to (a) aggregate into doublets and triplets of cells, and (b) swell individually (25–27, 31). The former effect creates particles with volume sizes twice and three times the size of the average PMN; the latter effect enlarges the particle sizes by no more than 20% (25, 27). Hence, the A and LPP values reflect aggregation but not cellular swelling (27).

To facilitate comparisons between different experiments, the maximum change in the large particle percentage (MLPP) was calculated. The MLPP is:

$$\text{MLPP} = \text{LPP}_m - \text{LPP}_0$$

where  $\text{LPP}_m$  is the largest LPP found at either  $1/4$ ,  $1/2$ , 1, 2, 4, 8, or 15 min after adding a chemotactin to the PMN suspension, and  $\text{LPP}_0$  is the LPP found just before the addition.

### Washing Procedure

In some studies, 1.5 ml of a bivalent cation-free PMN suspension was incubated with a chemotactic factor for 4 min and then immediately transferred with a glass pipette to a plastic conical tube and centrifuged for 10 s in an Eppendorf Microcentrifuge (Brinkmann Instruments, Inc., Westbury, N. Y.) at 8,000  $g$ . More than 95% of the supernatant fluid was removed, the cells were resuspended in 1.5 ml of the buffer and recentrifuged, and the supernatant fluid was again removed. The cell button was resuspended in 1.0 ml of buffer. The suspension was transferred to plastic vials and treated as in the above experiments. This washing procedure took  $< 5$  min and

appeared not to alter PMN function. It was important to determine the efficacy of washing in freeing cells of a chemotactic substance. When PMN suspensions were incubated with  $5 \times 10^{-6}$  M FNLLP, 1% of which was tritium-labeled, the washing procedure freed the cells of 99.93% of the original label.

### Thin-Layer Chromatography

In some experiments, the PMN suspensions were preincubated with  $1 \times 10^{-7}$  M tritium-labeled [ $^3\text{H}$ ]FNLLP for 4 and 30 min under conditions similar to those in the aggregation assay. The cell suspension was centrifuged with the Brinkmann Microcentrifuge, and the supernatant fluid was run on glass-backed silica gel (Sigma Chemical Co., St. Louis, Mo.) plates to study degradation of the tripeptide. The supernatant fluid was applied to the plates and allowed to run for 3 h in an *n*-butanol/water/acetic acid (6:1:1) solvent system as described in reference 8. Nonformylated peptides and amino acids were located by ninhydrin staining of the plates. The stained areas of the plates were scraped off, placed in 5 ml of Aquasol (New England Nuclear, Boston, Mass.), and measured for fluorescence in a Searle Mark III Scintillation Counter (Searle Analytic, Inc., Des Plaines, Ill.) recording at an efficiency of  $> 60\%$ .

## RESULTS

### Neutrophil Aggregation

Neutrophils preincubated for 2 min in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -containing buffer aggregated after exposure to  $5 \times 10^{-6}$  M FMLP or 50  $\mu\text{l/ml}$  C5a. This effect was shown by the rapid rise in the LPP of PMN suspensions found after adding either agent (Fig. 1, solid lines). Changes in the LPP peaked within 2 min of the addition and abated thereafter. Dose-response curves for FMLP indicated that this response was not detected below  $1.7 \times 10^{-8}$  M, increased progressively with incremental increases in FMLP concentrations between  $1.7 \times 10^{-8}$  and  $5 \times 10^{-7}$  M, and plateaued above  $5 \times 10^{-7}$  M; similar curves for C5a showed that the response was not detected below 5  $\mu\text{l/ml}$  and increased progressively with increases in C5a concentrations between 5 and 50  $\mu\text{l/ml}$ , the highest concentration used (not shown). In appropriate concentrations, FMMP, FMMMM, and FNLLP induced similar changes in the LPP. These responses were not found when cells were treated with various nonchemotactic proteins, oligopeptides, and other reagents (24–28, 30, 31).

### Cation Requirements and Loss of Cell Reactivity

In the absence of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ,  $5 \times 10^{-6}$  M

FMLP (Fig. 1, lowest line of upper panel) and 50  $\mu\text{l/ml}$  C5a (Fig. 1, lowest line of lower panel) did not aggregate the cells. A similar lack of reactivity was found when cells were treated with various amounts of C5a, FMLP, or other chemotactic oligopeptides in the absence of bivalent cations (not shown). Apparently, bivalent cations as well as proper concentrations of a chemotactic factor are required for the aggregation response. When the normal sequence of adding the bivalent cations and chemotactic factor was reversed, aggregation was detected only after addition of the cations. The interrupted lines of Fig. 1 show that changes in the LPP were successively blunted when the bivalent cations were added simultaneously with, or at 1/2, 1, or 2 min after, addition of FMLP (upper panel) or C5a (lower panel). Adding the bivalent cations at 4 or 8 min after either chemotactic factor also resulted in no response or a minimal response (not shown). Therefore, delay in adding bivalent cations for 2 min or longer results in an almost total loss of cellular reactivity.

#### Desensitization and its Selectivity

This loss in PMN reactivity was also found when cells were re-exposed to fresh chemotactic factor. Cells were incubated in bivalent cation-free buffer

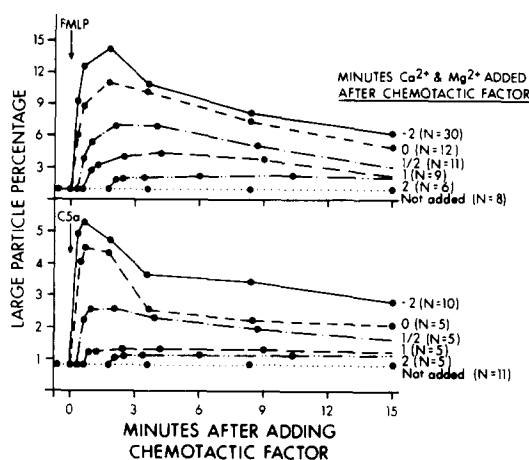


FIGURE 1 Effect of the time and sequence of addition of chemotactic factor and bivalent cations on the large particle percentage of PMN suspensions. Cells were exposed to  $5 \times 10^{-6}$  M FMLP (upper panel) or 50  $\mu\text{l/ml}$  C5a (lower panel) and, at the indicated times before and afterwards, treated with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . The bottom curve of each panel indicates experiments in which cells were not exposed to the bivalent cations. The numbers in parentheses are the number of separate experiments performed.

for 4 min with 50  $\mu\text{l/ml}$  C5a (Fig. 2, upper panel) or  $5 \times 10^{-6}$  M FMLP (Fig. 2, lower panel) and then treated with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . 1 min thereafter, cells were again exposed to 50  $\mu\text{l/ml}$  C5a,  $5 \times 10^{-6}$  M FMLP, or no chemotactic factor. Cells preincubated with C5a aggregated minimally upon re-exposure to C5a (Fig. 2, lowest curves of upper panel) and cells preincubated with FMLP were similarly unreactive to re-exposure to FMLP (Fig. 2, lowest curves of lower panel). In striking contrast to these results were the results found when PMNs were preincubated with C5a or FMLP and then exposed to the opposite chemotactic factor. Cells preincubated with C5a were normally responsive to FMLP, and cells preincubated with FMLP showed a prominent, sustained response to C5a (Fig. 2, two upper curves of each panel). Thus, loss in PMN reactivity was highly selective. This selective loss of PMN reactivity also occurred when cells first incubated with comparatively low concentrations of FMLP or C5a before exposure to  $5 \times 10^{-6}$  M FMLP or 50  $\mu\text{l/ml}$  C5a. Cells preincubated with  $1.7 \times 10^{-7}$  to  $5 \times 10^{-6}$  M FMLP were hyporesponsive to further stimulation with the FMLP but normally responsive to further stimulation with the C5a (Fig. 3, upper panel), and PMNs preincubated with 5 to 50  $\mu\text{l/ml}$  C5a

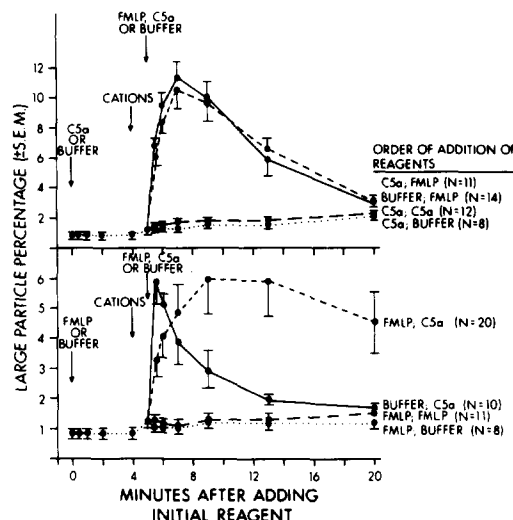


FIGURE 2 Specificity of neutrophil desensitization. Cells were preincubated with C5a, FMLP, or buffer for 4 min and then treated with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . 1 min thereafter, cells were exposed to fresh C5a, FMLP, or buffer. In these experiments, the concentrations of C5a and FMLP were 50  $\mu\text{l/ml}$  and  $5 \times 10^{-6}$  M. The numbers in parentheses are the number of separate experiments performed.

TABLE I  
*Presence and Specificity of Neutrophil Desensitization Induced by C5a and Various Oligopeptide Chemotactic Factors\**

Aggregating agent	Desensitizing agent (used in pretreating cells)		
	None	C5a (50 $\mu$ l/ml)	FMLP ( $1.7 \times 10^{-7}$ M)
C5a (50 $\mu$ l/ml)	5.1 $\pm$ 1.0 (10)‡	1.1 $\pm$ 0.2 (11)§	4.9 $\pm$ 1.2 (20) <sup>NS</sup>
FMLP ( $1.7 \times 10^{-7}$ M)	4.8 $\pm$ 1.0 (9)	6.1 $\pm$ 0.7 (5) <sup>NS</sup>	1.8 $\pm$ 0.3 (6)§
FMLP ( $5 \times 10^{-6}$ M)	10.6 $\pm$ 1.0 (14)	9.6 $\pm$ 1.1 (11) <sup>NS</sup>	4.1 $\pm$ 1.3 (6)§
FMMMM ( $5 \times 10^{-6}$ M)	10.1 $\pm$ 2.2 (6)	7.5 $\pm$ 2.0 (5) <sup>NS</sup>	ND

\* The cells were incubated with the indicated concentration of desensitizing agent for 4 min and then exposed to 1.4 mM  $Ca^{2+}$  and 0.7 mM  $Mg^{2+}$ . 1 min thereafter the cells were treated with the indicated concentration of aggregating agent and the maximal change in the large particle percentage was recorded.

‡ Maximal change in the large particle percentage.  $\pm$  SEM. The number of separate experiments performed is in parentheses.

§  $P < 0.05$  compared to cells preincubated with no desensitizing agent but exposed to the same concentration of aggregating agent.

|| NS, no significant difference compared to cells preincubated with no desensitizing agent but exposed to the same concentration of aggregating agent.

¶ ND, not done.

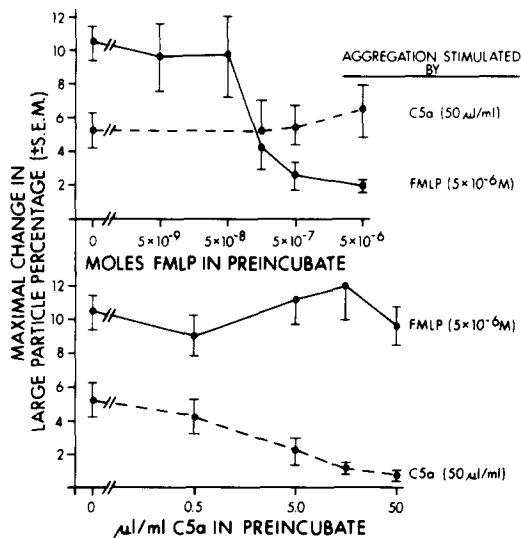


FIGURE 3 Effect of the concentration of chemotactic factor in the preincubate on the desensitization of neutrophils. Cells were preincubated for 4 min with the indicated concentration of FMLP (upper panel) or C5a (lower panel), treated with  $Ca^{2+}$  and  $Mg^{2+}$  for 1 min, and then exposed to  $5 \times 10^{-6}$  M FMLP (solid lines) or 50  $\mu$ l/ml C5a (interrupted lines). Each point is the mean of the maximal change in the large particle percentage found for at least six experiments.

were hyporesponsive to further stimulation with the C5a but normally responsive to further stimulation with the FMLP (Fig. 3, lower panel).

Apparently, PMNs incubated with a chemotactic factor in the absence of bivalent cations rapidly become desensitized to that chemotactic factor. In experiments performed in a manner similar to

those of Fig. 2, cells were preincubated with diverse oligopeptide chemotactic factors for 4 min, treated with bivalent cations, and 1 min thereafter were re-exposed to fresh chemotactic factor. Table I shows that cells preincubated with FMLP, FMMMM, FMMP, or FNLLP were refractory to subsequent stimulation with FMLP or FMMMM but retained their responsiveness to C5a; cells preincubated with C5a became refractory to subsequent stimulation with C5a but retained their responsiveness to FMLP and FMMMM. These data indicate that the specificity of loss in PMN reactivity parallels the specificity of PMN receptors for the chemotactic factors.

#### *Retention of Biological Activity of Chemotactic Factors during Incubation with Cells*

It is possible that during the 4-min incubation period PMNs inactivate the chemotactic factors and/or generate inhibitors to them. However, we found that the supernatant fluid of PMN suspensions exposed to FMLP or C5a for 4 min retained its chemotactic and aggregating activities when assayed against fresh cells (Table II). Moreover, the supernatant fluid was able to desensitize selectively fresh PMNs (Fig. 4). Finally, thin-layer chromatography of the supernatant fluid of [ $^3H$ ]FNLLP-treated PMN suspensions could detect no or minimal degradation of the labeled oligopeptide during the 4 min it was incubated with the cells, although definite degradation was detected after 30 min of incubation (see Table III), as has been reported previously (2). It seems,

TABLE I, continued

Desensitizing agent (used in pretreating cells)			
FMLP ( $5 \times 10^{-6}$ M)	FMMMM ( $5 \times 10^{-6}$ M)	FMMMP ( $5 \times 10^{-6}$ M)	FNLLP ( $3 \times 10^{-5}$ M)
$7.1 \pm 1.5$ (20) <sup>NS</sup>	$6.0 \pm 1.0$ (3) <sup>NS</sup>	$6.0 \pm 2.1$ (4) <sup>NS</sup>	$9.7 \pm 4.6$ (4) <sup>NS</sup>
ND <sup>¶</sup>	$0.6 \pm 0.7$ (3) <sup>§</sup>	$0.2 \pm 0.2$ (3) <sup>§</sup>	$0.8 \pm 0.3$ (3) <sup>§</sup>
$1.7 \pm 0.4$ (8) <sup>§</sup>	ND	ND	ND
$1.0 \pm 0.5$ (3) <sup>§</sup>	$3.2 \pm 1.5$ (5) <sup>§</sup>	$1.5 \pm 0.5$ (3) <sup>§</sup>	$2.1 \pm 1.3$ (3) <sup>§</sup>

TABLE II

Comparison of the Chemotactic and Aggregating Activities of FMLP, C5a, and the Supernatant Fluid of FMLP- or C5a-Treated Neutrophils

Chemotactic substance	Activity measured	
	Chemotaxis* ED <sub>50</sub>	Aggregation† MLPP
FMLP	$5.9 \times 10^{-9} \pm 2.1 \times 10^{-9}$ M (3) <sup>§</sup>	$4.8 \pm 1.0$ (9)
Supernate of FMLP-treated cells	$4.4 \times 10^{-9} \pm 1.2 \times 10^{-9}$ M (4)	$5.9 \pm 1.4$ (6)
C5a	$3.1 \pm 2.0$ $\mu$ l/ml (3)	ND <sup>  </sup>
Supernate of C5a-treated cells	$2.7 \pm 1.4$ $\mu$ l/ml (3)	ND

\* Chemotaxis of dextran sedimented human leukocytes was performed as described in Materials and Methods. 1 ml of  $5 \times 10^{-6}$  M FMLP, 50  $\mu$ l/ml C5a, or of supernatant fluid taken from cell suspensions incubated with  $5 \times 10^{-6}$  M FMLP or 50  $\mu$ l/ml C5a for 4 min was serially diluted over a  $10^5$ -fold range and assayed for chemotactic activity. The ED<sub>50</sub> was extrapolated from dose-response curves.

† Neutrophils were preincubated with 1 ml of buffer for 4 min, treated with Ca<sup>2+</sup> and Mg<sup>2+</sup>, and 1 min thereafter exposed to  $1.7 \times 10^{-7}$  M FMLP or 35  $\mu$ l of supernatant fluid taken from cells preincubated with  $5 \times 10^{-6}$  M FMLP for 4 min. Since the 35  $\mu$ l of supernatant fluid was added to 1 ml of PMN suspension, the calculated final concentration of FMLP in these experiments was  $1.7 \times 10^{-7}$  M.

§ Mean  $\pm$  SEM for the number of separate experiments given in parentheses.

|| ND, not done.

therefore, that during the 4-min incubation period the fluid phase of the PMN suspensions contains full chemotactic, aggregating, and desensitizing activity and that prominent degradation of the chemotactin or generation of inhibitors does not occur under the experimental conditions used here.

#### Desensitization after Cell Washing

The data suggest that loss in PMN reactivity to chemotactic factors is a cellular phenomenon. This suggestion is supported by experiments in which cells were washed after exposure to a chemotactin. Cells were preincubated with  $5 \times 10^{-6}$  M FMLP or 50  $\mu$ l/ml C5a in buffer free of bivalent cations

for 4 min and then rapidly washed. (The washing procedure removed >99.9% of labeled FNLLP; see Materials and Methods.) Cells were resuspended in fresh buffer for 1 min, treated with bivalent cations, and 1 min thereafter were exposed to fresh FMLP ( $5 \times 10^{-6}$  M), C5a (50  $\mu$ l/ml), or neither chemotactin. The PMNs preincubated with either chemotactic factor before washing showed no tendency to aggregate when resuspended in fresh buffer and treated with bivalent cations (not shown). Cells preincubated with FMLP before washing were hyporesponsive to FMLP but not to C5a (Fig. 5, upper panel), and cells preincubated with C5a before washing were hyporesponsive to C5a but not to FMLP (Fig. 5,

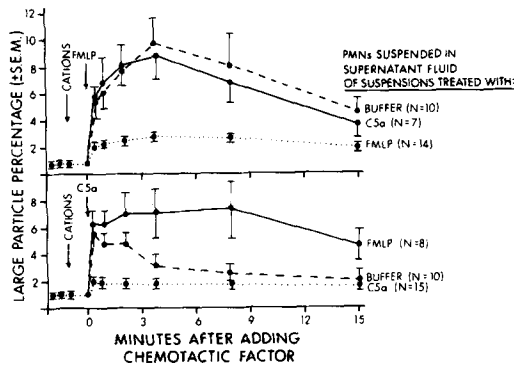


FIGURE 4 Desensitizing activity of supernatant fluids obtained from PMN suspensions treated with FMLP or C5a. Fresh PMNs were placed in the supernatant fluid obtained from cell suspensions preincubated for 4 min with  $5 \times 10^{-6}$  M FMLP, 50  $\mu$ l/ml C5a, or no chemotactic factor. After 1 min the cells were treated with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  and 1 min later with  $5 \times 10^{-6}$  M FMLP (upper panel) or 50  $\mu$ l/ml C5a (lower panel). The number in parentheses is the number of separate experiments performed.

TABLE III  
Degradation of Tritium-labeled FNLLP Into Phenylalanine and Unidentified Products by Neutrophils\*

min of Incubation	Percent of total label co-migrating with		
	FNLLP	Phe	Unidentified†
0	>80 (3)§	$4.9 \pm 0.5$ (3)‡	$6.0 \pm 0.6$ (3)
4	>80 (2)	5.0 (2)	7.5 (2)
30	62	11.6 (1)	13.6 (1)

\* 4,600 Cells/ $\mu$ l were incubated with  $1.0 \times 10^{-7}$  M FNLLP (tritiated in the para position of phenylalanine [phe]) for the indicated time. After incubation, the supernatant fluid of the neutrophil suspensions was assayed for degradation of FNLLP by measuring the percentage of label which co-migrated with FNLLP and phenylalanine.

† Percentage of label migrating to spots not identified as FNLLP or phenylalanine.

§ Number of separate experiments in parentheses.

‡  $\pm$  SEM.

lower panel). Fig. 5 does show that washed cells appeared to regain some of their responsiveness to the desensitizing chemotactic agent compared to unwashed cells (see Fig. 2). Nevertheless, the data suggest that a major portion of the loss in PMN reactivity results from cellular mechanisms.

## DISCUSSION

Eosinophils (13), basophils (3, 19, 32), mast cells

(9, 10), and neutrophils (17, 37) lose their ability to directionally migrate and/or degranulate in response to immunologic stimuli when they are preincubated with these stimuli under conditions which do not support these responses. In these instances, loss of reactivity is specific for the stimuli with which the cells were preincubated and persists after cell washing: the cells themselves have become desensitized or deactivated towards certain stimuli. We preincubated PMNs with chemotactic factors under conditions not supporting cellular aggregation (i.e., lack of extracellular  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) and studied the effect of this treatment on the normally occurring aggregation response to chemotactic factors when conditions supporting aggregation were restored (i.e., addition of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ). We found that PMNs preincubated with chemotactic factors rapidly lost their ability to aggregate in response to bivalent cations (Fig. 1). This loss persisted when cells were retreated with fresh chemotactic factor after exposure to bivalent cations (Fig. 2) and occurred

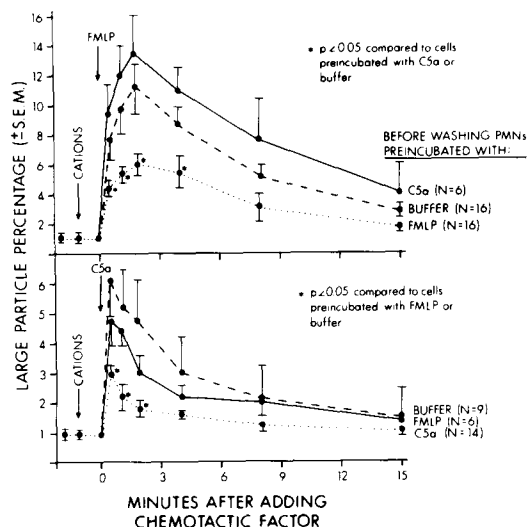


FIGURE 5 Persistence of neutrophil desensitization after washing. Cells were preincubated with FMLP ( $5 \times 10^{-6}$  M, C5a (50  $\mu$ l/ml), or no chemotactic factor for 4 min in bivalent cation-free buffer and then rapidly washed and resuspended in fresh buffer. After 1 min cells were treated with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  and 1 min later exposed to  $5 \times 10^{-6}$  M FMLP (upper panel) or 50  $\mu$ l/ml C5a (lower panel). The number in parentheses is the number of separate experiments performed. Cells preincubated with either or no chemotactic factor did not aggregate when exposed to buffer after washing and treating with bivalent cations (not shown).

when cells were preincubated with relatively low concentrations of the chemotactin before adding the bivalent cations and fresh chemotactic factor (Fig. 3). Desensitization to the chemotactic factors was strikingly specific: cells preincubated with one of the chemotactic oligopeptides were hyporesponsive to the oligopeptides but not to C5a; cells preincubated with C5a were hyporesponsive to C5a but not to the oligopeptides (Table I). During the 4-min preincubation period, the fluid phase of the PMN suspensions retained its chemotactic (Table II), aggregating (Table II), and desensitizing activities (Fig. 4) and we could find no evidence for the generation of chemotactic factor inhibitors nor for the degradation of tritium-labeled FNLLP (Table III). Rather, the PMNs themselves were hyporesponsive to the chemotactic factors and this effect persisted after the cells were washed (Fig. 5). PMNs handled in this way did not release lysosomal enzyme contents (27, 31). We conclude that the aggregation response of PMNs can be desensitized to specific chemotactic factors in a manner similar to the desensitization of the chemotactic and degranulation responses of eosinophils, basophils, mast cells and PMNs. The desensitization studied here appears to reflect the specificity of the chemotactic factors for their respective receptors in or on the neutrophil. A stimulus-induced, functional loss of chemotactic factor receptors may underlie the desensitization phenomenon.

If neutrophils are functionally homogeneous in their reactivity to different chemotactic factors, then, apparently, stimulation by receptor-bound chemotactins renders the cell hyporesponsive to further stimulation by agents interacting with the same receptor but not by agents interacting with a different receptor: the ability of the PMN to detect one type of stimulation has been abrogated by a functional loss of one type of receptor. After binding a chemotactic factor, a receptor may be degraded, shed, or internalized, or it may be continuously occupied without further stimulation. However, radiolabeled chemotactic factors remain associated with the PMN surface membrane (1) and in continuous equilibrium with the extracellular milieu (1, 39). Certain effects of receptor binding, therefore, may be short-lived (as is suggested by the transiency of the aggregation response) and insusceptible to repetitive stimulation by the same receptor. Changes in the surface membrane permeability to  $\text{Ca}^{2+}$  may be such an effect. Chemotactic factors stimulate an

influx and intracellular accumulation of  $\text{Ca}^{2+}$  (4, 11, 12, 22, 23, 35). These events may trigger the aggregation response (26, 31) and, obviously, would not occur in the absence of extracellular  $\text{Ca}^{2+}$ . Rapid but short-lived effects on the membrane permeability to  $\text{Ca}^{2+}$  may explain the rapid and short-lived aggregation response and the equally rapidly developing desensitization phenomenon. Selective desensitization may indicate an inability of the receptor to repeatedly alter membrane permeability under conditions in which a previously unstimulated receptor could.

In the aggregation assay, as in some chemotactic and degranulation assays, only a small proportion of the cells can be definitely identified as responding to a chemotactic factor (18, 33, 40). Morphologically homogeneous PMNs may consist of subpopulations of cells with varying reactivity to different chemotactins. Thus, the aggregation response to a particular type of chemotactin may involve a particular subset of cells; desensitization may indicate that cells once stimulated are refractory to all chemotactic factors, and the specificity of desensitization may result from the response of cells not sensitive to the particular chemotactic factor in the preincubate. Changes in membrane permeability to  $\text{Ca}^{2+}$ , therefore, may occur only once in a cell and the cell may then become insusceptible to further changes regardless of the type of subsequent chemotactic stimuli.

Whether the specificity of desensitization results from receptor specificity of functionally homogeneous cells or from subpopulation specificity of functionally heterogeneous cells, our data indicate that specific desensitization does occur and is importantly related to the role of bivalent cations in cellular responsiveness. Desensitization may be useful in defining receptor specificities of chemotactic factors. Desensitization may also serve to limit *in vitro* and *in vivo* responses to chemotactic factors. The rapid reversal of the aggregation response and its close timing with the development of desensitization suggest that cells are becoming desensitized to the chemotactic stimulus as they disaggregate. Animals infused with chemotactic factors (29) or complement activators such as cobra venom factor (20, 29), zymosan (20), or antigen-antibody complexes (21) rapidly develop transient neutropenia. Similar neutropenia occurs in patients undergoing extracorporeal hemodialysis (6). In each of these conditions, chemotactic factors may circulate freely and be responsible for the neutropenia. The transient nature of this



neutropenia may indicate that cellular desensitization is occurring.

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