Role of Secretory Events in Modulating Human Neutrophil Chemotaxis

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ABSTRACT The relationship between neutrophil polymorphonuclear leukocyte (PMN) locomotion and the exocytosis of neutrophil cytoplasmic granules was studied by assessing these processes in cells migrating through micropore filters and by measuring the effects of degranulating stimuli on PMN chemotaxis, orientation, adhesiveness, and ability to bind the chemoattractant f-Met-Leu-[^{3}H]Phe. Studies of cells migrating through cellulose nitrate filters indicated that concentrations of f-Met-Leu-Phe optimal for exocytosis were greater than those optimal for chemotaxis and actually inhibited cell migration. In other studies incubation of PMNs with concentrations of secretagogues causing exocytosis of 30% or greater PMN lysozyme increased cell adhesiveness and inhibited chemotaxis. PMNs that had secreted more than 30% lysozyme appeared round, did not orient in a gradient of chemoattractant, and were capable of significantly less f-Met-Leu-[^{3}H]Phe binding than were control cells. The decreased binding of f-Met-Leu-Phe was not associated with hydrolysis of chemotactic peptide by washed cells, although peptide hydrolysis was caused by cell products secreted extracellularly after vigorous exocytosis. In contrast, when only 10–15% cellular lysozyme was released f-Met-Leu-Phe binding was enhanced significantly and there was no depression of chemotaxis. The data indicate limited exocytosis of intracellular granule contents is associated with increased availability of PMN chemotactic factor receptors. Vigorous exocytosis is associated with inactivation of chemotactic responsiveness related to increased cell adhesiveness, decreased PMN binding of chemotactic factors, and to hydrolysis of chemoattractants by factors secreted extracellularly.

INTRODUCTION

A relationship between neutrophil locomotion and secretion of granule associated enzymes has been indicated by studies from several laboratories (1–5). Most agents that are chemokinetic are also chemotactic and induce exocytosis of cytoplasmic granule enzymes when the leukocyte membrane is perturbed either by adherence to a surface such as a cellulose nitrate filter or by treatment with cytochalasin B (1–3). Furthermore, there is evidence that chemotactic factors stimulate neutrophil locomotion and secretion by interacting with a common membrane receptor, although concentrations of chemotactic factors initiating chemotaxis are significantly lower than concentrations initiating secretion (4, 6, 7). We have investigated the relationship between neutrophil locomotion and secretion by studying these processes in cells that migrate through cellulose nitrate filters and by studying the effects of degranulating stimuli on cell orientation, chemotaxis, adhesiveness, and the ability of chemotactic factors to bind to the cell. Our findings indicate that granule exocytosis has modulating effects upon neutrophil chemotactic responsiveness, adhesiveness, and the availability of membrane receptors for chemotactic factors.

METHODS

Materials. Ionophore A23187, provided by Robert Hamill (Eli Lilly and Co., Indianapolis, Ind.), was dissolved (10 mg/ml) in dimethyl sulfoxide (Fisher Scientific Co., Pittsburgh, Pa.) and stored at −20°C until diluted in the indicated solutions on the day of the experiment. Phorbol myristate acetate

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(PMA)[1] was obtained from Consolidated Midland Corp. (Brewster, N. Y.) and dissolved in dimethyl sulfoxide. Appropriate dimethyl sulfoxide controls were evaluated for each experimental condition. Concanavalin A (2 times crystallized in saturated NaCl, Miles-Yeda Laboratories, Rehovot, Israel) was dissolved in Hanks' balanced salt solution (National Institutes of Health Media Unit) at 100 times the concentration used in incubations.

Isolation of human neutrophils. Leukocyte suspensions containing over 95% neutrophils were prepared from heparinized blood of healthy volunteers by centrifugation on Hypaque (Winthrop Laboratories, New York)-Ficoll (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) followed by dextran sedimentation and hypotonic lysis of erythrocytes as described previously (8, 9). The resulting neutrophils were washed with Hanks' balanced salt solution before use.

Chemotaxis assay. Initial chemotaxis studies employed the ^{51}Cr radioassay of chemotaxis previously described (10). Neutrophils were incubated for 1 h at 37°C with 1 μCi of ^{51}Cr (New England Nuclear, Boston, Mass.) per 10^6 cells. Two 3.0-μm cellulose nitrate filters (Sartorius, Göttingen, West Germany; distributed by Beckman Instruments Inc., Science Essentials Co., Mountainside, N. J.) were placed in the chemotactic chamber. The ^{51}Cr-labeled neutrophils were washed in Hanks' medium, suspended at a density of 2.3×10^6/ml in Gey's tissue culture medium (pH 7.2) containing 2% bovine serum albumin, 2% penicillin, and streptomycin (Microbiological Associates, Walkersville, Md.), and then placed in the upper compartment of the chemotactic chamber. The chemotactic stimulus was placed in the lower compartment. The chambers were incubated for 3 h at 37°C in 100% humidity and 5% CO_2. Under these conditions neutrophils migrate through the upper and into the lower filter. The lower cellulose nitrate filters were then rinsed and placed in individual gamma counter vials for radioassay. The number of neutrophils migrating into the lower filter are expressed as corrected counts per minute as described previously. All assays were performed in quadruplicate and the data expressed as the mean±SEM of these four values.

In other experiments the morphologic method of Zigmond and Hirsch was used to assess neutrophil chemotaxis (11). The cells were processed as described and then suspended at a density of 2.3×10^6/ml in Gey's tissue culture medium (pH 7.2) containing 2% bovine serum albumin, 2% penicillin, and streptomycin. A single 3.0-μm cellulose nitrate filter (Sartorius) was used and the chemotactic chambers were incubated for 45 min as described above. The filters were then removed from the chambers, rinsed in 0.85% saline, fixed in methanol, and stained with Mayer's hematoxylin and eosin. The filters were dehydrated with increasing concentrations of ethanol, cleared with xylene, and mounted on glass slides with immersion oil. All assays were performed in duplicate. The migration front was determined by measuring the farthest distance traveled by two cells per high-powered field with a standard microscope micrometer. Five such determinations were made for each filter. Measurements from duplicate filters were pooled and the mean and standard error determined. Such pooling of data is justified because the variation of migration fronts within a single filter was greater than variation among duplicate filters (12). The mean values were compared by the Student's *t* test.

[1] *Abbreviations used in this paper*: Con A, concanavalin A; LDH, lactic dehydrogenase; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocyte; TPCK, tosyl-L-phenylalanyl choromethane.

Chemotactic agents. Partially purified C5a was prepared by methods described previously (13). 4 ml of fresh serum was incubated with 0.3 mg/ml of *Escherichia coli* 0127:B8 endotoxin (lipopolysaccharide B, Difco Laboratories, Detroit, Mich.) for 1 h at 37°C. After addition of EDTA (10 mM, Fisher Scientific Co.) the sample was placed on a Sephadex G-100 column which was eluted with 0.04 M phosphate-buffered 0.85% saline, pH 7.4. Every third fraction was assayed for chemotactic activity. C5a eluted in the 15,000-dalton region. For some experiments sodium caseinate (Difco Laboratories), 5 mg/ml in 0.85% NaCl, or unfractionated *E. coli* endotoxin-activated serum (14) were used as the chemotactic stimulus. The synthetic peptide chemotactic factor f-Met-Leu-Phe (obtained from Dr. R. Freer, Department of Pharmacology, Virginia Commonwealth University, Richmond, Va.) was prepared as previously described (4). f-Met-Leu-[^{3}H]Phe with a sp act of 55 Ci/mM was a gift from Dr. R. Young, New England Nuclear. f-Nle-Leu-[^{3}H]Phe was obtained from New England Nuclear after synthesis from its precursor as already described (15). Random locomotion was assessed with Hanks' medium in the stimulus compartment.

Neutrophil orientation in response to a chemoattractant. Neutrophil orientation in response to a chemoattractant was assessed using the orientation chamber recently described by Zigmond (16). Human neutrophils prepared by the Hypaque-Ficoll technique were subjected to indicated manipulations, washed in Hanks' media, and then permitted to adhere to glass cover slips by incubation for 15 min at 37°C in 100% humidity. Adherent neutrophils were then placed in orientation chambers, with f-Met-Leu-Phe (50 nM) as the chemoattractant. After a 45-min additional incubation, 100 cells were examined by phase microscopy and scored as either oriented toward the stimulus, oriented away from the stimulus, or indeterminant (no cell polarity).

Neutrophil adherence. Polymorphonuclear leukocyte (PMN) adherence to a surface was quantitated by placing 1 cm^3 of ^{51}Cr-labeled PMNs used for the chemotaxis studies into 16-mm diameter wells (Tissue Culture Cluster 24 well, Costar, a Division of Data Packaging Corporation, Cambridge, Mass.) followed by a 30-min incubation at 37°C. The wells were then drained and rinsed three times with Hanks' medium. The adherent cells were lysed by addition of 1 cm^3 distilled water to the wells followed by a 30-min agitation at room temperature. Adherence was quantitated as the amount of ^{51}Cr released by the adherent cells into the distilled water by counting a 0.5-cm^3 aliquot in a gamma counter. All measurements of adherence were performed in triplicate.

Cell viability. Cell viability after exposure to the various compounds was monitored by two techniques: (*a*) the ability to exclude trypan blue dye (Grand Island Biological Co., Grand Island, N. Y.) and (*b*) measurements of the cytoplasmic enzyme lactic dehydrogenase (LDH) released into the extracellular environment. Under the conditions studied in these experiments, ionophore A23187 (10 nM–1 μM), PMA (0.05–50 ng/ml), and concanavalin A (Con A; 5–100 μg/ml) did not affect cell viability.

Assessment of neutrophil receptors for the chemoattractant f-Met-Leu-Phe. Neutrophil binding of f-Met-Leu-[^{3}H]Phe was determined as recently described (6). Human neutrophils (Hypaque-Ficoll) were subjected to various manipulations, washed twice in Hanks' medium, and then suspended at 5×10^6 cells/ml in Hanks' medium. Tosyl-L-phenylalanyl chloromethane (TPCK, Calbiochem, San Diego, Calif.) was added (0.1 mM) to 2 cm^3 (10^7) PMNs and then 50 nM f-Met-Leu-[^{3}H]Phe containing 200,000 cpm of ^{3}H was added to the cells. Control cells contained the above plus 10 μM nonradioactive peptide. The cells were then incubated on ice

for 1 h with intermittent agitation. The interaction of cells with peptide was terminated by flushing 5×10^6 cells on to a GF/B glass fiber filter (Whatman, Inc., Clifton, N. J.) followed by washing twice with 7 cm^3 of ice-cold Hanks' solution. The filters with cells were then counted in a liquid scintillation counter with 10 ml of Aquasol (New England Nuclear) as the counting solution. Data are expressed as net (experimental − control) counts per minute per 5×10^6 neutrophils. Hydrolysis of [^{3}H]Nα-formyl Nle-Leu-Phe-OH (f-Nle-Leu-[^{3}H]Phe) was assessed by adding 1 μCi (50 nM) to cells, without TPCK, and the cells were incubated for 30 min at 37°C. The reaction was terminated by addition of acetic acid (final concentration 50%). Any debris was removed by centrifugation, the supernate was concentrated, and then assayed for hydrolytic products by thin-layer chromatography as recently described (17).

Enzyme measurements. Lysozyme activity was determined by measuring turbidometrically the rate of lysis of *Micrococcus lysodeikticus* (Worthington Biochemical Corp., Freehold, N. J.) at pH 6.2. Enzyme activity was expressed in terms of micrograms per milliliter egg-white lysozyme standard (Worthington Biochemical Corp.) (18, 19). β-Glucuronidase was assayed by measuring the release of phenophthalein from its β-glucuronate (Sigma Chemical Co., St. Louis, Mo.) after 4 h incubation at pH 4.5. Activity was expressed as micrograms phenophthalein released/10^7 neutrophils per 4 h (20). LDH was assayed by measuring the consumption of β-nicotinamide-adenine dinucleotide (NADPH, Sigma Chemical Co.) during the reaction of pyruvate to lactate. LDH activity was expressed in Wroblewski units (21). Enzyme activities were determined in incubation media or in whole cell or granule suspensions after disruption with 0.2% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.). None of the agents studied affected these enzyme assays.

Cell lysis and granule separation. Neutrophil granules were separated by continuous sucrose gradients with a modification (22) of the method of West et al. (23). Neutrophils suspended in 9 cm^3 0.34 M sucrose (5×10^7 cells/ml) were lysed in the presence of 1 cm^3 sodium heparin (10,000 U/ml, Upjohn Co., Kalamazoo, Mich.) by repeated aspiration through a 20-gauge, 9-cm spinal needle until 85% of cells were lysed as indicated by phase microscopy. Lysates were then passed serially through 5- and 2-μm polycarbonate filters (Nuclepore; Neuro Probe, Inc., Bethesda, Md.) to remove unbroken cells and nuclei. Resulting granule-rich lysates (5 ml) were layered over continuous sucrose gradients with specific gravities ranging from 1.12 to 1.28 in 1 × 3.5-inch cellulose nitrate tubes (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), then centrifuged at 95,000 *g* for 4 h at 4°C. After centrifugation, gradients were pumped out (1.0 ml/min) from the bottom through a 0.8-mm × 14-cm rigid tube inserted through the gradient and held in a fixed position against the bottom of the centrifuge tube and 1.2-ml fractions were collected for enzyme determinations. By this technique, three granule fractions and a particulate fraction that contains enzymes characteristic of plasma membrane can be obtained. The granules have been labeled A, B, and C and it has been shown that granules A and B correspond to primary (azurophil) granules and C to the secondary (specific) granules (23). The various granule fractions were pooled, diluted in 0.34 M sucrose, pelleted by centrifugation at 27,000 *g* for 20 min, washed twice in Hanks' medium, repelleted, and then lysed by three freeze-thaw cycles with dry ice in alcohol and a 37°C water bath.

Protein synthesis. The effect of the protein synthesis inhibitors cycloheximide (Sigma Chemical Co.), puromycin (Sigma Chemical Co.), and Harringtonine (the latter a generous gift from Dr. J. A. R. Mead, National Cancer Institute, National Institutes of Health, Bethesda, Md.), on chemotaxis, secretion of lysozyme, and f-Met-Leu-[^{3}H]Phe binding was studied. The protein synthesis inhibitors were added to the cells at the indicated concentrations and the mixture was incubated for 30 min at 37°C before assessing the indicated function. In related experiments the inhibition of protein synthesis was documented by following the 30-min cell-inhibitor incubation by another 1-h incubation at 37°C in the presence of [^{3}H]leucine (leucine, L-[4,5-^{3}H(N)], New England Nuclear, 1.0 μCi per 10^6 PMNs). Ice-cold trichloroacetic acid (TCA, J. T. Baker Chemical Co., Phillipsburg, N. J.) was then added so that the final concentration was 10%. The resulting protein precipitate was pelleted by centrifugation, washed four times in ice-cold 10% TCA, suspended in 1.0 cm^3 Hanks' buffer, and then a 0.5-cm^3 aliquot was dissolved in Aquasol and counted in a scintillation counter. [^{3}H]Leucine incorporation into the TCA precipitate from cells treated with buffer was compared to cells treated with the protein synthesis inhibitors.

RESULTS

Dose-response of f-Met-Leu-Phe on chemotaxis and lysozyme secretion. We examined the effect of varying concentrations of f-Met-Leu-Phe upon chemotaxis and lysozyme secretion from cells migrating through 3.0-μm cellulose nitrate filters using the radioassay of chemotaxis. The chemotactic response of human neutrophils was similar to that previously described for rabbit neutrophils (4). For chemotaxis a maximal response was noted with 0.01–0.1 μM peptide (Table I). At >0.1 μM f-Met-Leu-Phe the chemotactic response was inhibited. In contrast, lysozyme secretion by neutrophils migrating through cellulose nitrate filters was maximal

TABLE I

*Dose Response of f-Met-Leu-Phe on Chemotaxis and Lysozyme Secretion by Neutrophils in Modified Boyden Chambers**

Concentration of f-Met-Leu-Phe	Chemotaxis (lower filter)	Lysozyme released
	corrected cpm	*% total*
0	250±15	4±1
1 pM	350±25	4±2
10 pM	250±20	—
0.1 nM	300±38	4±1
1 nM	1,250±105	—
10 nM	3,100±320	6±1
0.1 μM	2,750±255	8±2
1 μM	450±8	10±1
10 μM	180±20	11±2

* Chemotactic response with the ^{51}Cr-radioassay of chemotaxis. Lysozyme released into the extracellular medium by PMNs migrating through 3.0-μm cellulose nitrate filters is expressed as percent of total lysozyme contained in all cells placed into the chemotactic chamber. Mean±SEM four determinations for chemotaxis and two experiments for enzyme release.

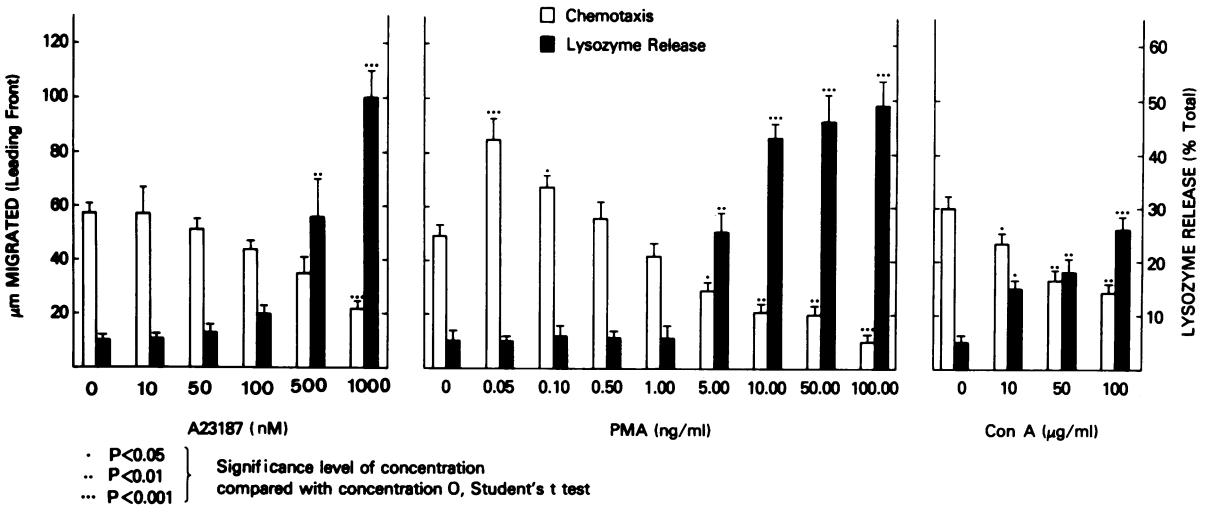

FIGURE 1 Effect of incubating neutrophils with the degranulating stimuli A23187, PMA, or Con A for 30 min on lysozyme release into the extracellular milieu and chemotactic responsiveness. Post-secretory cells were washed twice and chemotaxis was assayed by the leading front morphologic technique, with partially purified C5a as the chemoattractant. Mean±SEM, four experiments.

at concentrations of f-Met-Leu-Phe which inhibited chemotaxis.

The inverse relationship between exocytosis and neutrophil chemotaxis. Fig. 1 shows that concentrations of ionophore A23187, PMA, and concanavalin A, which induced secretion of >30% of the total neutrophil lysozyme, significantly inhibited neutrophil chemotaxis. Less than 10% of the azurophil enzyme β-glucuronidase was released under these conditions. It is to be noted that with low concentrations of PMA (0.05 and 0.1 ng/ml), conditions not associated with detectable secretion, chemotaxis was enhanced, confirming an earlier report (24).

In other studies, in which secretion was induced by exposing cells in suspension to the chemotactic peptide f-Met-Leu-Phe and cytochalasin B, and C5a was used as the chemoattractant, inhibition of locomotion was noted under conditions of lysozyme release (Fig. 2).

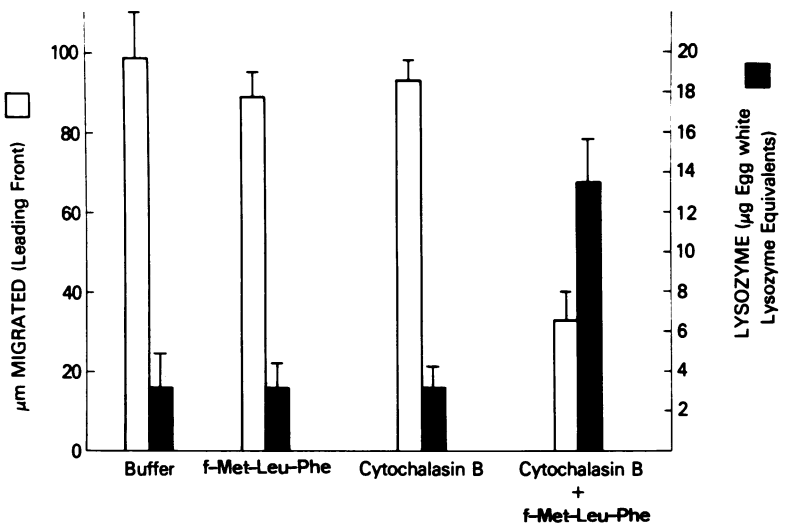

FIGURE 2 Effect of exposing neutrophils to cytochalasin B (5 μg/ml) and f-Met-Leu-Phe (1 μM) on lysozyme release and chemotactic responsiveness. Lysozyme release is expressed as micrograms per egg white lysozyme equivalents (see Methods). Post-secretory cells were washed twice and chemotaxis was determined by the leading front morphologic technique, with partially purified C5a as the chemoattractant. Mean±SEM, four determinations.

Less than 15% release of β-glucuronidase occurred under these conditions and there was no detectable release of the cytoplasmic enzyme lactate dehydrogenase. To test the possibility that the inhibition of locomotion after exocytosis was caused by the feedback action of a secreted granule product, neutrophil granule lysates were incubated with fresh cells which were then assessed for chemotactic responsiveness to f-Met-Leu-Phe (10 nM). As shown in Table II, incubation of neutrophils with specific granule lysates (fraction C), which contained the granule enzymes most readily released during the conditions of exocytosis, did not inhibit chemotaxis. Slight but significant ($P < 0.05$) inhibition of migration by an azurophil granule (B-granule in Table II) was noted.

The ability of cells to orient in a gradient of f-Met-Leu-Phe (50 nM) after incubation with A23187, PMA, Con A, or C5a plus cytochalasin B, was assessed (Table III). After such incubation cells appeared round and did not assume their characteristic polarity in the chemoattractant gradient. Incubation with C5a or cytochalasin B alone, conditions not associated with exocytosis of lysosomal granules, did not affect the ability of PMNs to orient in a gradient of f-Met-Leu-Phe.

Effect of exocytosis on neutrophil adherence. Incubation of PMNs with A23187, PMA, or f-Met-Leu-Phe plus cytochalasin B increased cell adhesiveness to plastic (Table IV). Whereas 12% of control cells adhered to the plastic wells, the adherence of cells exposed to A23187 (1 μM), PMA, Con A, or f-Met-Leu-Phe (10 μM) plus cytochalasin B was 34, 42, 49, and 38%, respectively. Incubation of PMNs with cytochalasin B alone reduced the percent adherent cells to 3% ($P < 0.001$). PMNs incubated with f-Met-Leu-Phe alone and then washed twice and suspended in fresh media adhered more than control cells ($P < 0.01$) but less well than cells exposed to f-Met-Leu-Phe plus cytochalasin B ($P < 0.01$).

Effect of secretion on binding of f-Met-Leu-[³H]Phe. As shown in Fig. 3 incubation of neutrophils with concentrations of A23187 or PMA sufficient to cause secretion of lysosomal granule contents and inhibition of chemotaxis significantly inhibited binding of f-Met-Leu-[³H]Phe despite the presence of TPCK during the binding assay, an agent expected to protect the ³H-ligand from proteolytic degradation. Low concentrations of the two secretagogues, which induced release of less than 20% total lysozyme, had no inhibitory effect and in some experiments enhanced f-Met-Leu-[³H]Phe binding. This enhanced binding was often observed even without detectable lysozyme release. The time course of inhibition of f-Met-Leu-[³H]Phe binding was also studied (Fig. 4). After brief exposure to the secretogogue, often before lysozyme release was

TABLE II
Effect of Neutrophil Granule Lysates on Neutrophil Locomotion

Granule fraction*	Lysozyme	Chemotaxis (lower filter)
	μg/nl	*corrected cpm*
None (buffer)	0	785±85
Azurophil granules		
A	10	842±38
B	5	690±30
Specific granules		
C	11.5	891±75

* Sucrose gradient fractions identified as described in (25). Granules were obtained as described, washed in Hanks' medium, lysed by sonication and suspended with PMNs at the indicated concentration of lysozyme. After incubation for 30 min at 37°C the lysate-PMN preparations were added to chemotactic chambers with 10 nM f-Met-Leu-Phe as the chemotactic stimulus.

TABLE III
Effect of Secretion on Neutrophil Orientation in a Gradient of f-Met-Leu-Phe

Secretagogue*	Cell orientation‡		
	Oriented toward stimulus	Indeterminant	Oriented away from stimulus
None (buffer)	63	30	7
	61	30	9
A23187 (1 μM)	0	95	5
	3	93	4
PMA (50 ng/ml)	1	99	0
	8	90	2
Con A			
(50 μg/ml)	27	56	17
(100 μg/ml)	6	94	0
C5a (5 μg/ml)	64	30	6
Cytochalasin B (5 μg/ml)	70	20	10
C5a (5 μg/ml)			
+ Cytochalasin B	3	96	1
(5 μg/ml)	1	99	0

* Neutrophils were exposed to the indicated substance for 30 min at 37°C, the cells were then washed twice in Hanks' medium, suspended in fresh Gey's medium at a density of 2.5×10^6 cells/ml.

‡ Numbers represent percent of cells oriented toward the chemoattractant f-Met-Leu-Phe (50 nM) when incubated in the presence of a chemoattractant gradient for 30 min at 37°C (see Methods). Horizontal rows represent single experiments; 100 cells counted for each experiment.

TABLE IV
*Effect of Secretion on Cell Adherence**

Secretagogue	Cell adherence		
	cpm/0.5 cm³	% adherent	*P* value‡
None	457±37	12	—
A23187			
(1 μM)	1,292±30	34	<0.001
(0.1 μM)	840±75	22	<0.02
PMA (20 ng/ml)	1,617±89	42	<0.001
Con A (50 μg/ml)	1,867±28	49	<0.001
f-Met-Leu-Phe (10 μM) + cytochalasin B (5 μg/ml)	1,272±78	33	<0.001
(1 μM) + cytochalasin B (5 μg/ml)	1,013±63	26	<0.001

* ^{51}Cr PMN adherence to plastic after treatment with secretagogues for 30 min at 37°C (see Methods). Mean±SEM, three determinations. The cells (3.0×10^6) added to the adherence wells contained 3,844 cpm/0.5 cm³.
‡ Significance level between experimental and control mean values, Student's *t* test.

detected, f-Met-Leu-Phe binding was enhanced. With more prolonged incubation and secretion of more than 30% total cellular lysozyme inhibition of f-Met-Leu-Phe binding occurred.

Although the inhibition of f-Met-Leu-[^{3}H]Phe binding could have been related in part to cell aggregation and decreased available membrane for peptide binding, the magnitude of the inhibition of binding was too great to invoke this as the sole mechanism. For example, concentrations of PMA (5–10 ng/ml) and A23187 (0.1 μM) at which binding was inhibited, are less than those required to cause significant cell aggregation as viewed by microscopy or assessed in an aggregometer. Furthermore, in studies done with cells exposed to higher concentrations of degranulating stimuli large cell aggregates did occur but these were removed by filtration of cells through four-ply surgical gauze. After this filtration, aggregates of two to five cells were seen but binding was inhibited over 90% when only 25% of cells existed as aggregates.

The effect of various concentrations of a number of other secretagogues including f-Met-Leu-Phe plus cytochalasin B, C5a plus cytochalasin B, and Con A on f-Met-Leu-[^{3}H]Phe bindings was studied. Enhancement of f-Met-Leu-Phe binding was seen with each secretagogue when 10–15% lysozyme secretion occurred. When the data from all the experiments were pooled and plotted as a function of the amount of lysozyme released, the enhancement of f-Met-Leu-[^{3}H]Phe binding with limited exocytosis was significant (Fig. 5). The inhibition of binding noted with greater than 30% lysozyme secretion was usually associated with β-glucuronidase release but this was not always the case; concentrations of PMA causing 50% lysozyme release did not cause significant β-glucuronidase release. In addition, we were unable to show decreased f-Met-Leu-[^{3}H]Phe binding by pretreatment of cells with nonradioactive f-Met-Leu-Phe (1–10 μM) plus cytochalasin B (5 μg/ml) when compared to PMN pretreatment with f-Met-Leu-Phe (1–10 μM) without cytochalasin B. This was presumably because at such relatively high concentrations of f-Met-Leu-Phe too much of the nonradioactive peptide remained bound to the cell.

Additional experiments were performed to determine

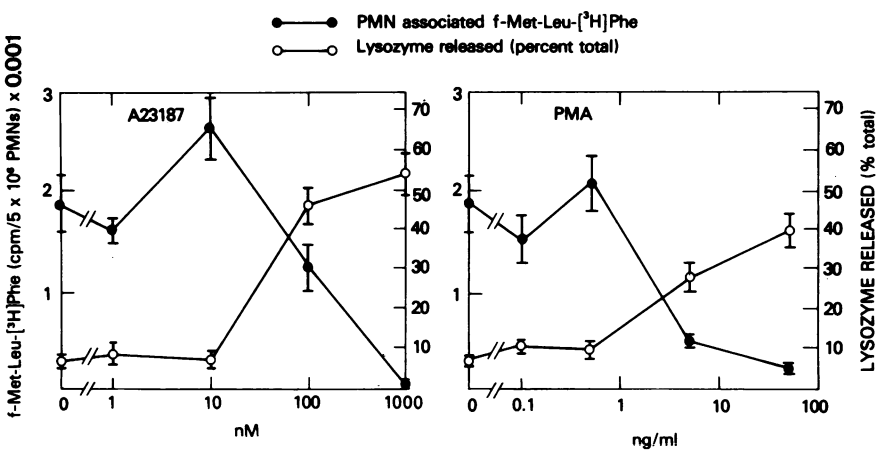

FIGURE 3 Effect of A23187 and PMA on neutrophil lysozyme release and f-Met-Leu-[^{3}H]Phe binding to post-secretory PMNs. Cells were incubated with the indicated concentrations of secretagogue for 30 min, washed twice, filtered through four-ply gauze, and then the binding studies were carried out as described in Methods. Mean±SEM, two determinations.

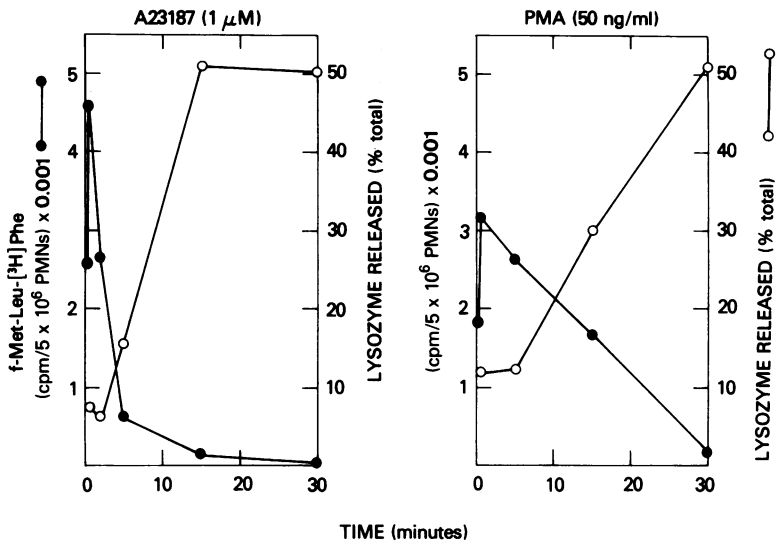

FIGURE 4 Effect of incubating PMNs with A23187 or PMA for varying amounts of time on f-Met-Leu-[^{3}H]Phe binding. Data are the mean of duplicate determinations, one experiment.

whether the secretogogues inhibited binding by competing directly with the labeled peptide. Cells were incubated with A23187 (1 μM) or PMA (50 ng/ml) for 30 min at 4°C, instead of at 37°C, or in an environment free of divalent cations, conditions that inhibited secretion. The cells were then washed in the routine manner and when assessed for f-Met-Leu-[^{3}H]Phe binding, no inhibition was observed. In other studies A23187 (1 μM) or PMA (50 ng/ml) was added during binding. In contrast to nonradioactive f-Met-Leu-Phe (10 μM), which competitively inhibited binding, neither A23187 nor PMA competed for f-Met-Leu-[^{3}H]Phe binding sites.

Hydrolysis of a chemotactic peptide f-Nle-Leu-[^{3}H]Phe by post-secretory cells. In the experiments shown in Fig. 5 decreased f-Met-Leu-[^{3}H]Phe binding accompanied vigorous exocytosis, despite measures to limit ligand hydrolysis by inclusion of TPCK. To further evaluate the possibility that peptide hydrolysis contributed to the decreased binding we assessed the ability of post-secretory cells and their products to hydrolyze the related chemoattractant f-Nle-Leu-[^{3}H]Phe as described in Methods. Neutrophils exposed to A23187, PMA, or f-Met-Leu-Phe plus cytochalasin B and then washed twice in Hanks' medium did not hydrolyze radiolabeled peptide differently from control cells. In contrast, cell-free supernates from post-secretory cells caused peptide hydrolysis with degradation of f-Nle-Leu-Phe to Phe and Leu-Phe residues when significant secretion of β-glucuronidase, an azurophil granule enzyme, occurred (Fig. 6). This suggested a hydrolase that could destroy the chemotactic peptide was stored in the azurophil granules. The various granule contents obtained by sucrose gradient centrifugation of cell homogenates, were therefore tested for their ability to cause peptide hydrolysis. Azurophil "B" granule fractions caused significant hydrolysis of f-Nle-Leu-[^{3}H]Phe. No hydrolysis was noted by other cellular fractions. It is noted that in other studies the hydrolytic products of f-Met-Leu-Phe, Leu-Phe, and Phe did not inhibit f-Met-Leu-[^{3}H]Phe binding.

Effect of inhibitors of protein synthesis on neutrophil chemotaxis, exocytosis, and f-Met-Leu-Phe binding. The enhanced f-Met-Leu-[^{3}H]Phe binding seen with limited exocytosis suggested that during specific granule secretion fusion of granule membrane with cytoplasmic membrane provides new membrane and possibly receptors for chemotactic factors. Such a mechanism for membrane turnover, which has been proposed to be essential for leukocyte chemotaxis (25), might occur independently of protein synthesis. Experiments were therefore designed to assess the role of protein synthesis on chemotaxis, secretion and f-Met-Leu-Phe binding. As shown in Fig. 7, concentrations of three different inhibitors of protein synthesis that blocked [^{3}H]leucine incorporation into TCA-precipitable protein had no significant effect on chemotactic responses to *E. coli* endotoxin-activated serum. Similarly the same concentration of protein synthesis inhibitors did not inhibit exocytosis in response to the secretagogues A23187 (0.1 μM) or f-Met-Leu-Phe (1 μM) plus cytochalasin B (5 μg/ml) and had no effect on f-Met-Leu-[^{3}H]Phe binding.

DISCUSSION

Our findings indicate that the chemotactic responsiveness of human neutrophils is influenced significantly

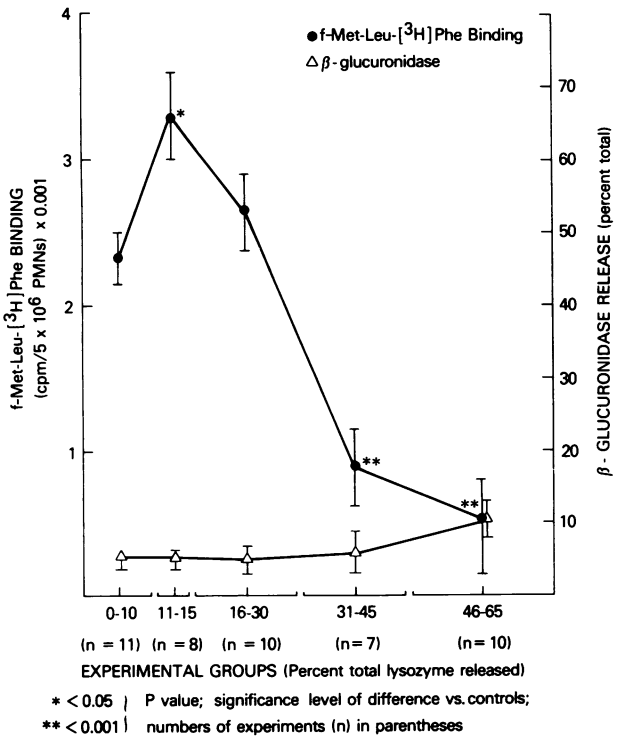

FIGURE 5 Composite data relating degranulation by all the secretagogues studied (see text) to lysosomal enzyme release and f-Met-Leu-[^{3}H]Phe binding. The data were arbitrarily grouped by the percent total lysozyme release as indicated and the data represent mean±SEM of the number of experiments shown in parentheses. *P* values are the significance level of difference from controls, Student's *t* test.

by the exocytosis of cytoplasmic granules. In studies with varying concentrations of the chemoattractant f-Met-Leu-Phe, it was found that concentrations of chemoattractant optimal for inducing granule exocytosis are greater than concentrations optimal for chemotaxis and that the higher concentrations actually inhibit chemotaxis, confirming previous findings of others (4). These initial studies suggested that inhibition of chemotaxis was related to secretion of granule associated enzymes.

In later experiments it was found that incubation of neutrophils with degranulating stimuli, under conditions sufficient to cause secretion of more than 30% cellular lysozyme, inhibited chemotaxis and impaired the ability of post-secretory cells to orient in a gradient of chemoattractant and to bind radiolabeled chemotactic peptide. These findings, together with our recent observation that the membrane hyperpolarization induced by chemotactic factor is inhibited by degranulating stimuli (26), indicate that the depressed chemotaxis in post-secretory cells involves the first step of the chemotactic response; i.e., the interaction of the chemotactic factor with the cell. Whether the decreased binding of chemoattractant by post-secretory cells is the re-

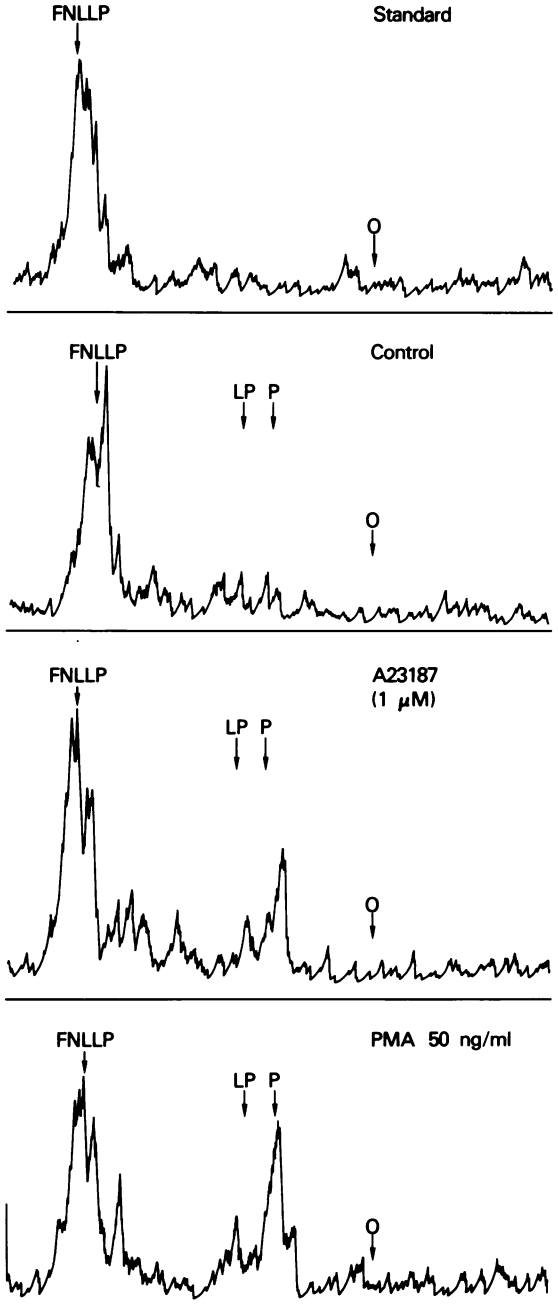

FIGURE 6 Hydrolysis of f-Nle-Leu-[^{3}H]Phe (FNLLP) by PMN post-secretory products. Panels show ^{3}H distribution on thin-layer chromatography plates of control peptide standard FNLLP incubated in experimental medium (upper panel) or peptide exposed to medium from cells incubated 30 min in control medium, A23187 or PMA (lower three panels). O indicates the origin, and P and LP represent the migration of Phe and Leu-Phe standards, respectively. It is noted that in this experiment cells incubated with A23187 released 65% total lysozyme, 20% β-glucuronidase, and 10% LDH; cells exposed to PMA release 70% lysozyme, 38% β-glucuronidase, and 12% LDH whereas control cells released 6% lysozyme, 5% β-glucuronidase, and 3% LDH.

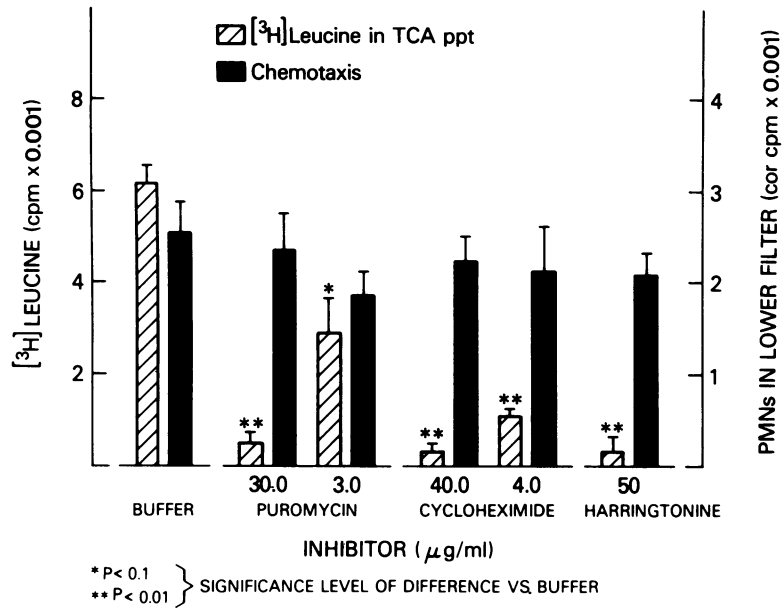

FIGURE 7 Effect of puromycin, cycloheximide, and Harringtonine on [^{3}H]leucine incorporation into TCA-precipitable protein (TCA ppt) and on chemotaxis as measured with the ^{51}Cr-radioassay. Mean±SEM, three determinations. Cor cpm, corrected counts per minute.

sult of destruction, shedding, internalization, or conformational change of existing receptors is not clear from these studies.

In addition to the decrease in the capacity of postsecretory neutrophils to respond to a chemotactic stimulus, there is evidence that the chemoattractants themselves may be inactivated by the secreted contents of the granules. We have shown that products of neutrophil secretion include at least two C5a inactivators that are stored in the azurophil granules of neutrophils and are released under conditions of vigorous exocytosis (27). It is of interest in this regard that after vigorous exocytosis, with release of the azurophil granule enzyme β-glucuronidase, secretory products are found that also hydrolyze the synthetic peptide f-Nle-Leu-Phe and appear to be derived from the azurophil granules.

Release from PMNs of a neutrophil-immobilizing factor has been reported to cause irreversible inhibition of neutrophil migration but no inhibition of cell adherence or phagocytosis (28, 29). We did not, however, detect a neutrophil-immobilizing factor in isolated specific granules; the granule population whose contents were preferentially released during our incubation conditions. Depressed PMN chemotactic responsiveness has also recently been reported to result from exposure of neutrophils to purified human lysozyme (30). However, the concentration of lysozyme required to inhibit locomotion appears to be too high (30–200 μg/ml) to explain the inhibition of chemotaxis seen under our experimental conditions. Increased cell adhesiveness, which we have shown to accompany exocytosis of neutrophil granules, confirming previous reports (31, 32), has also been associated with depression of neutrophil locomotion (32). Inhibition of chemotaxis accompanying vigorous exocytosis can thus be related to multiple phenomena: alterations of the cytoplasmic membrane with increased cell adhesiveness and decreased binding of chemoattractant as well as inactivation of chemotactic factors by lysosomal products secreted extracellularly.

During our experiments we were impressed by the enhanced binding of the chemotactic peptide f-Met-Leu-[^{3}H]Phe to the cell when only 10–15% of the total cellular lysozyme was released. Exocytosis of specific granules is associated with fusion of the granule membrane with the cytoplasmic membrane and this may provide a mechanism for membrane turnover during chemotaxis. A possible role of cytoplasmic membrane turnover during leukocyte chemotaxis has been discussed recently by Stossel who suggested that synthesis of new membrane or fusion of granule membranes with the plasma membrane could be mechanisms for new membrane turnover and replenishment of cytoplasmic membrane receptors (25). Our data on enhanced f-Met-Leu-Phe binding with secretion of 10–15% lysozyme content are consistent with the latter suggestion, although it is to be noted that in some experiments f-Met-Leu-[^{3}H]Phe binding was enhanced before detectable enzyme release. Therefore, the enhanced binding could also be related to membrane events that are independent of secretion. Moreover, we were unable to show that inhibitors of protein syn-

thesis decreased chemotaxis, secretion of lysosomal granules, or f-Met-Leu-Phe binding. Protein synthesis, therefore, does not appear to be necessary for these events. These considerations suggest that inhibition of chemotaxis in post-secretory cells may result, in part, from a depletion of the source of available new membrane. Alternatively, the enhanced chemotaxis observed in the presence of low concentrations of cytochalasin B (13, 33) may be related to the mobilization of chemoattractant receptors which is postulated to occur with minimal secretion.

It is evident that neutrophil chemotactic responses and the mobilization and exocytosis of neutrophil granules are closely interrelated. The coordination of these cellular processes would appear to be critical components of host defenses. The possibility that some of the reported neutrophil chemotactic defects or the mechanism of action of some antiinflammatory agents is related to these processes is intriguing and worthy of further study.

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