Regulation of Receptors and Digestive Activity Toward Synthesized Formyl-Chemotactic Peptide in Human Polymorphonuclear Leukocytes

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A receptor binding and digestive activity of human polymorphonuclear leukocytes (PMNs) toward formyl-methionyl-leucyl-phenylalanine (3H-FMLP) was examined with the following results: (1) Up- and down-regulation and recovery of 3H-FMLP binding activity were demonstrated. (2) Both intact PMN and a lysate prepared from them cleaved the carboxyl terminal amino acid (phenylalanine) of 3H-FMLP. (3) The digestive activity decreased as the receptor binding was inhibited by n-ethylmaleimide and 4-chloromercuribenzoate. (4) Little digestive activity was found in the supernatant from PMN stimulated by FMLP. (5) The released phenylalanine was found in the pellet and supernatant of PMNs. (6) Digestive activity with cathepsin A-like characteristics was found in the lysate of PMN. These observations suggest that FMLP is internalized in lysosomes in a receptor-mediated manner and cleaved by the cathepsin A-like enzyme, the free phenylalanine is released extracellularly, and a part of the dissociated receptors with FMLP may return to the surface or to an intracellular receptor pool. (7) Another finding was that the digestive activity of the lysate of cord blood granulocytes was decreased compared with that of adult blood granulocytes. This decrease may explain in part the impaired chemotaxis of cord blood granulocytes.

POLYMORPHONUCLEAR leukocytes (PMNs) have specific receptor systems for sensing gradients of various chemotactic factors.1 After a receptor binds with a ligand, the receptor–ligand complex mediates signal transductions and is internalized as rapidly as other hormone-receptor systems.2 If a limited number of receptors are internalized with the formation of the receptor–ligand complex, leukocytes cannot sense the directionality. However, PMNs are capable of migrating along a gradient for hours. To explain this discrepancy, the hypothesis is required that either the receptor is synthesized rapidly or reused through a receptor recycling system or has another large reservoir.

Rabbit peritoneal PMNs migrate along the gradient of chemotactic factor even in the presence of protein synthesis inhibitors.3 Zigmond et al4 performed precise kinetic analysis of chemotactic peptide receptor modulation and proposed that receptor recycling may occur in rabbit peritoneal exudate PMNs.

In the present study, we examined their receptor recycling model using human peripheral PMNs. To clarify the interaction among receptor recycling, digestive activity of n-formyl-methionyl-leucyl-phenylalanine (FMLP), and chemotaxis, we examined the ability of both intact PMNs and a PMN lysate to digest FMLP. We demonstrated the decrease of the digestive activity of FMLP in the cord blood granulocyte. This decrease seems to be attributable to a postreceptoral dysfunction, in addition to the decreased number of receptors for FMLP in the cord blood granulocytes.

MATERIALS AND METHODS

Chemicals

The synthesized peptide FMLP was purchased from the Protein Research Foundation, Osaka, Japan. The labeled peptide n-formylmethionyl-leucyl-[3H]phenylalanine (3H-FMLP; specific activity, 46.4 Ci/mmol) was purchased from New England Nuclear Corp, Boston. This labeled peptide was purified on a Dowex 50W × 8 column with 0.01 N HCl.

Disopropyl phosphorofluoridate (DFP), phenylmethylene-sulfonfluoride (PMSF), L-tyrosylamido-2-phenethyl chloromethyl ketone (TPCK), n-ethyl-L-lysine-chloromethyl ketone (TLCK), 4-chloromercuribenzoate (PCMB), n-ethylmaleimide (NEM), pepstatin, ethylenediamine tetra-acetate (EDTA), ethylene glycol tetra-acetate (EGTA), formyl-methionine (f-Met), formyl-methionyl-alanine (f-Met-Ala), formyl-methionyl-valine (f-Met-Val), formyl-methionyl-phenylalanine (f-Met-Phe), and cytochalasin D were purchased from Sigma Chemical Co, St Louis. DFP was dissolved in isopropyl alcohol. PMSF, PCMB, NEM, pepstatin, f-Met, f-Met-Ala, f-Met-Val, f-Met-Phe, and cytochalasin D were dissolved in dimethyl sulfoxide. EDTA and EGTA were dissolved in deionized water.

Cell Preparation

Cell preparation was performed by modification of the method of Büyum5 with slight modification as follows. Cells were isolated from heparinized (10 U/mL) peripheral blood, and polyvinylpyrrolidone K 90 (Wako Pure Chemical Industries, Ltd, Osaka, Japan) in normal saline was used to separate the leukocyte-rich fraction from the erythrocyte sediment.6 Hypotonic shock (0.2% saline for 30 seconds) lysed contaminating red cells. Cells were suspended in 0.9% saline or modified Krebs-Ringer phosphate (Ca 0.5 mmol/L). Blood specimens were obtained from healthy adult volunteers, cord blood of full-term neonates, and patients with chronic granulomatous disease (CGD). The diagnosis of CGD was made by the following criteria: no reduction of nitroblue tetrazazonium, no release of superoxide, and no chemiluminescence. Chemotaxis was investigated in two CGD patients by the agarose plate method and was found to be normal.

3H-FMLP Binding Assay

The binding assay was performed as reported previously with slight modifications.7 One hundred microliters of PMN (105 cells per milliliter) and 80 μL of modified Krebs-Ringer phosphate buffer were preincubated at 4°C for about ten minutes or at 37°C. The PMN suspension and 20 μL of 3H-FMLP (104 mol/L; 200,000 dpm) were incubated for 60 minutes in the 4°C assay, with gentle vortex mixing every 15 minutes, and for 20 minutes in the 37°C assay, with gentle shaking. Incubations were terminated by rapidly diluting the reaction mixture with 3 mL of chilled incubation buffer, followed by rapid filtration of the mixture through a 1.0-μm pore size millipore filter (EAWP, Millipore Corp, Bedford, Mass). They were
washed with 6 mL of chilled buffer, dried, and then placed in 10 mL of scintillant (Scintisole EX-H, Dojindo Laboratories, Kumamoto, Japan).

**Lysate Sample Preparation**

Pellets of PMN were suspended in 0.9% saline to a given density (2 × 10⁷ to 10⁷ cells per milliliter). Each suspension was sonicated for ten seconds with a hand sonicator (hand sonic model UR-20P, Tomy Seiko Corp, Tokyo) on ice, then formate buffer (pH 4.0) was added to a final concentration of 10 mmol/L, and finally the mixture was centrifuged at 10,000 g for 20 minutes. The supernatant was used as the lysate sample. In the preparation of sonicated supernatant, respectively.

± lysate sample at a concentration of the mixture was centrifuged at 10,000 (2 to 10 × 10⁶ cells per milliliter). Each suspension was sonicated for a given time and then centrifuged at 4 °C. The supernatant was given as the lysate sample. In the preparation of lysate sample at a concentration of 5 × 10⁶ adult blood granulocyte (ABG) per milliliter (n = 3), the yields of protein and digestive activity of the lysate sample were 37% ± 3% and 90% ± 5% of the sonicated supernatant, respectively.

**Standard Assay for Peptide Digestion**

In the assay for intact PMNs, 100 μL of PMN (10⁴ cells per milliliter) and 80 μL of buffer were preincubated at 37 °C for a given time. The reaction was initiated with 20 μL of 3H-FMLP (10⁻⁴ mol/L; 200,000 dpm). The mixture was incubated at 37 °C for a given time and then centrifuged at 4 °C. The supernatant was applied on a minicolumn of Dowex 50W X 8 (0.3 mL; H⁺ form). The pellet was sonicated on ice and then centrifuged. This supernatant was applied on a minicolumn as before.

In the assay for lysate PMN, 25 μL of 0.2 mol/L formate buffer (pH 4.0), 145 μL of deionized water, and 10 μL of lysate sample were preincubated at 37 °C for five minutes. The reaction was initiated with 20 μL of 3H-FMLP (5 × 10⁻⁴ mol/L; 50,000 dpm). The mixture was boiled for five minutes to stop the reaction. One milliliter of 0.01 N HCl was added, and then the mixture was cooled on ice and applied on a minicolumn as before. This column was eluted with 16 mL of 0.01 N HCl and subsequently with 2 mL of 0.5 N NaOH, the fractions corresponding to the intact FMLP and its digestion products, respectively. One milliliter of each eluate with HCl and NaOH was placed in a counting vial and mixed well with 10 mL of scintillant (Scintisole EX-H: Dojindo Corp). The activity was calculated and expressed as a percentage of cleavage of FMLP, from which the background activity (usually 2% to 4%) was subtracted.

**Digestive Activity of Supernatant of Stimulated PMNs**

PMNs (10⁴ cells per 200 μL) were incubated with 10⁻⁴ mol/L FMLP for 20 minutes at 37 °C after preincubation for 20 minutes at 37 °C. The supernatant samples were divided into two groups: One was treated at 100 °C for five minutes, as the negative control, and the other was not treated so. Other supernatants were prepared without FMLP treatment. One hundred thirty-five microliters of these supernatants were preincubated for 20 minutes at 37 °C and then incubated with 15 μL of 3H-FMLP (10⁻⁴ mol/L; 50,000 dpm) for 20 minutes at 37 °C. After that, the mixtures were applied on a minicolumn as described earlier.

**Identification of the Digestion Product**

After the standard assay for digestive activity of intact PMNs, the supernatant and the sonicated supernatant were spotted on a chromatography paper (Toyo filter paper No. 50, Tokyo) and developed with a solvent system of n-butanol: acetate: H₂O (6:1:1 vol/vol/vol). For identification of the digestion product from a lysate sample, the lysate (20 μg protein), 140 mmol/L formate buffer (pH 4.0), and 5 × 10⁻⁴ mol/L 3H-FMLP (30,000 dpm) were incubated at 37 °C for 30 minutes. To 1 vol of this reaction mixture, 5 vol of 1% picric acid was added to stop the reaction. The mixture was centrifuged and freed of protein. The supernatant was applied on 1.0 mL of Dowex 2 × 8 (Cl⁻ form) and eluted with 5 mL of 0.01 N HCl. All of the eluted sample was applied on 1.0 mL of Dowex SOW x 8 (H⁺ form), washed with 5 mL of 0.01 N HCl, and subsequently eluted with 5 mL of 4 N NH₄OH. The NH₄OH eluate was lyophilized, dissolved in 100 μL of deionized water, and spotted on a thin-layer chromatography plate of silica gel (DC-Ferriplaten Kieselgel 60; Merck, Darmstadt, Germany) and cellulose (micro crystalline cellulose, Tokyo Kasei Industrial Co, Tokyo) and then developed with the solvent system mentioned earlier. The amino acid standards were visualized by spraying with cadmium ninhydrin reagent. The ninhydrin-treated sample band was scraped off or cut into widths of 0.5 cm, placed in vials, mixed with 10 mL of scintillant, and then counted with a liquid scintillation spectrometer (LSC-683, Aloka, Tokyo). Then the RF of the radioactive product was compared with those of the presumed products (3H-FMLP, Leu-Phe, and Phe).

**Subcellular Fractionation Procedure**

Subcellular fractionation was performed by a modification of the method of Klemper et al. and Ulrich et al. PMNs were suspended in disruption buffer (75 mmol/L KC1, 65 mmol/L NaCl, 2.5 mmol/L MgCl₂, 10 mmol/L HEPES buffer [pH 7.4]) and then placed in a cell disruption bomb (PARP 4635 Cell Disruption Bomb, PARP Instrument Corp) at 4 °C and equilibrated at 350 psi for 20 minutes. After release from the disruption bomb, the suspension was collected in an EDTA solution so that the final concentration was 2.5 mmol/L EDTA. To sediment the nuclei and the undisrupted cells, the remaining suspension was centrifuged at 500 g for 30 minutes at 4 °C and designated as pellet 1. The supernatant was centrifuged at 15,000 g for 30 minutes at 4 °C, and the recovered pellet containing lysosomes and mitochondria was designated as pellet 2. The remaining supernatant containing endoplasmic reticulum, plasma membrane vesicles, and the disrupted cell cytosol was designated as the supernatant. Pellets 1 and 2 and the supernatant were sonicated for ten seconds × 3 times on ice before measurement of marker enzymes.

The following marker enzymes were used to follow the subcellular organelles in the fractionation. Lactate dehydrogenase (LDH) activity was assayed as described by Bergmeyer and Bernt, to monitor the fate of the cytosol. The lysosomal enzyme myeloperoxidase (MPO) and DNA were assayed as described by Michell et al. to determine the fate of the lysosomal and nuclear fractions, respectively. Protein was assayed by the method of Lowry et al.

**RESULTS**

**Study on Intact Polymorphonuclear Leukocytes**

Receptor modulation of PMNs by FMLP. 3H-FMLP binding after repeated incubation with FMLP is shown in Fig 1. The absence of stimuli had no effect on 3H-FMLP binding, and nonspecific bindings were not changed during these procedures. After the first incubation with FMLP for 20 minutes, 3H-FMLP binding increased to about 150% at concentrations of both 10⁻³ and 10⁻⁴ mol/L. Increments ranging from 120% to 140% were observed five or ten minutes after the incubation in other repeated studies. After the subsequent incubation without FMLP for 20 minutes, 3H-FMLP binding of PMNs further increased to more than after the first incubations, in which the samples at the concentration of 10⁻³ mol/L showed much higher elevation compared with those at 10⁻⁴ mol/L. These increases were
already seen 7.5 minutes after the incubation, and maximal binding was seen after incubation for 20 minutes. After the second incubation with FMLP at concentrations of $10^{-7}$ and $10^{-8}$ mol/L, the binding activity decreased to the level prior to the absence of stimuli. Receptor recovery was found after the second incubation without FMLP.

In the next study, after the incubation with 3H-FMLP ($10^{-7}$ mol/L; 400,000 dpm) at 37 °C for 20 minutes, the PMNs were washed and incubated in the buffer for 20 minutes at different temperatures (4 °C and 37 °C), cell-associated radioactivity was measured, and the results were 8,500 ± 500 dpm at 4 °C and 5,500 ± 300 dpm at 37 °C (n = 3). Approximately 3,000 dpm of the residual count in the incubation at 37 °C was found in the supernatant of the mixture, and two thirds of it corresponded to the phenylalanine with the paper chromatographic procedure.

**Time course of digestive activity of intact PMNs.** Percentages of cleavage of 3H-FMLP in the supernatant and in the cell pellet are shown in Fig 2A and B, respectively. 3H-FMLP was cleaved in a time- and dose-dependent manner in the supernatant samples until 30 minutes, while the free phenylalanine in the cell pellet was approximately 40% to 50% at 10 minutes’ incubation and increased slightly after the subsequent incubation. Thus intact 3H-FMLP remained in the pellet samples even after 30 minutes’ incubation.

For confirmation of the products of 3H-FMLP digestion, a paper chromatographic procedure was used. The supernatant and the pellet showed similar profiles with two peaks, of which the leading one was intact FMLP and the other was phenylalanine (Fig 3).

**Digestive activity of the supernatant of stimulated PMNs.** The digestive activity of the supernatant of stimulated and nonstimulated PMNs was 2.1% ± 0.3% and 2.4% ± 0.1%, respectively. The activity of the negative control (heated supernatant) was 0.0% ± 0.2%. Comparison of the digestive activity of the supernatant and PMNs themselves (Fig 2A) showed that the activity of the former was about 10% that of the latter.

**Effects of proteinase inhibitors on the digestive and binding activity for 3H-FMLP of intact PMNs (Fig 4).** DFP, PMSF, and TPCK had no effect, while TLCK and cytochalasin D slightly inhibited both activities at the concentration of 1 mmol/L or 8 μg/200 μL, respectively.
FMLP RECEPTOR REGULATION IN HUMAN PMNs

Fig 4. The effects of proteinase inhibitors on digestive activity and 3H-FMLP binding of intact PMNs. TPCK (○), TLCK (◇), DFP (◇), PMSF (◇), NEM (▲), PCMB (▲), or cytochalasin D (△) was preincubated with PMNs for 20 minutes at 37 °C, and the percentages of cleavage in the supernatant and binding in the pellets were assayed after 20 minutes' incubation with 3H-FMLP (10⁻⁷ mol/L; 200,000 dpm) at 37 °C. In this figure, 100% control cleaving activity and receptor bound indicate 29 ± 4 (percentage of cleavage) and 2.3 ± 0.4 (percentage of bound/total), respectively (▲). To examine the dose effect of these inhibitors, the final concentration was increased from 1 mmol/L to 2.5 mmol/L (◇ — ◇) for DFP, from 2 to 8 μg/200 μL (△ — △) for cytochalasin D and from 0.1 to 1.0 mmol/L for other proteinase inhibitors (○, ●, ▲, △, ▲).

PCMB and NEM at the concentration of 1 mmol/L inhibited both activities similarly.

Study on the Lysate of Polymorphonuclear Leukocytes

pH profile of the digestive activity of the lysate sample. As shown in Fig 5, the activity was found in the pH range of 3.0 to 7.0 but not from 7.5 to 10.0. Among the available buffers, formate and acetate buffers were more suitable than the others. The maximum activity was obtained at pH 4.0, and 80% of the activity was observed in a pH range of 3.0 to 5.0, for which formate or acetate buffer (pH 4.0) was used.

pH dependency and heat lability of digestive activity of the lysate sample. The activities were measured in 10 mmol/L formate (pH 4.0) and 10 mmol/L potassium phosphate buffer (pH 7.5) at various temperatures for two incubation times, 15 and 30 minutes. In the formate buffer, the activity seemed to be stable up to 30 minutes at 50 °C. In the phosphate buffer, the activity was unstable even at 30 °C for 15 minutes (Fig 6). Based on these results, the samples were prepared with 10 mmol/L formate buffer (pH 4.0) in the present study. The activity was found to be stable for more than three months at −20 °C when kept in this buffer.

Effect of ionic strength of the formate buffer. The activity of the enzyme decreased in parallel with an increase

Fig 5. pH profile of the cleaving activity. Various buffers (140 mmol/L) at the given pHs (○—○, acetate; ●—●, formate; ▲—▲, citrate; ■—■, succinate; ●—●, HEPES; ▲—▲, phosphate; △—△, Tris; ▲—▲, citrate-phosphate; ★—★, glycine) and the sample (20 μg protein) in saline solution were preincubated for five minutes at 37 °C. The reaction was initiated with 5 × 10⁻⁷ mol/L 3H-FMLP, and then the mixture incubated for ten minutes at 37 °C.

Fig 6. The pH dependency and heat lability of the digestive activity. Potassium phosphate buffer, pH 7.5 (●), or formate buffer, pH 4.0 (▲), at a final concentration of 10 mmol/L was added to the samples after sonication and centrifugation. These samples were incubated for 15 minutes (open symbols) and 30 minutes (closed symbols) at a given temperature. The activity with 4 μg protein of the sample was assayed by the method described in Materials and Methods.

Fig 7. The kinetics of the reaction. The lysate (6 μg protein), 25 mmol/L (●) and 75 mmol/L (○) formate buffer (pH 4.0), and a given concentration of 3H-FMLP were incubated for five minutes at 37 °C. The reaction mixture contained 1% of DMSO at the highest concentration of FMLP since DMSO was necessary to dissolve FMLP at this concentration and did not inhibit the reaction. 1/S equals (10⁻⁸ mol of FMLP per liter)⁻¹. 1/V equals (picomoles of FMLP per microgram protein per minute)⁻¹.
in the ionic strength of the formate buffer (25 to 150 mmol/L). Twenty-five millimoles per liter of formate buffer (pH 4.0) was used for the standard assay procedures.

Time course of the reaction. The rate of reaction was linear until almost six minutes of incubation, and therefore, an incubation time of five minutes was used in the present study.

Dose dependency of the reaction. The rate of reaction was linear from 2 to 8 μg protein/200 μL of assay mixture.

Kinetics of the reaction. Figure 7 shows a Lineweaver-Burk plot. At each concentration, 25 and 75 mmol/L, of formate buffer, the Vmax was about 75 mmol/min/μg protein. By calculation according to Segel, the true Km of FMLP was determined to be 20 to 25 μmol/L. These kinetic studies revealed that the employed assay method with 5 × 10⁻⁷ mol/L FMLP involved a first-order reaction and competitive inhibition by formate.

Thin-layer chromatographic pattern of the digestion products of FMLP. Table 1 shows the RF values of FMLP and its digestion products on thin-layer chromatography on silica gel and cellulose. The results suggested that the substance with this digestive activity belonged to the carboxypeptidase category.

Gel filtration pattern and isoelectric focusing pattern. The gel filtration patterns in Fig 8 show two peaks of activity. The high-molecular and low-molecular ones account for 5% and 70% of the total activity, respectively. The molecular weights of the higher and lower ones were approximately 1 million and 200,000 daltons, respectively. The higher one seemed to form aggregates consisting of enzyme molecules and other protein. From the isoelectric focusing pattern (Fig 9), the isoelectric point of the substance with this activity was found to be about 5.0.

Effects of proteinase inhibitors, formyl-peptides, amino acids, ions, and solvent media on digestive activity of 3H-FMLP (Table 2). DFP and PCMB inhibited the reaction at 0.1 mmol/L. The other available proteinase inhibitors (PCMB, NEM, pepstatin, EDTA, and EGTA) did not inhibit digestion. The inhibition with 1 mmol/L PCMB was reversed by 1% of 2-mercaptoethanol to some extent. These inhibition patterns were reported by Doi, who explain that chloromercuribenzoate may cause steric hindrance at the active site of lysosomal carboxypeptidase A by reacting with a neighboring cysteinyl residue. The enzyme activity was not inhibited by dimethyl sulfoxide (DMSO) or isopropyl alcohol at less than 1% vol/vol.

Of the available formyl-peptides, f-Met-Phe (0.1 mmol/L) and f-Met-Ala (1 mmol/L) inhibited the activity to 40% and 20%, respectively, but f-Met and f-Met-Val failed to do so. None of the amino acids (lysine, leucine, glutamine, or phenylalanine) inhibited the peptidase activity. Thus the enzyme activity may be inhibited by the product of formyl-peptides, and not the amino acids.

As shown in Table 2, SCN⁻ (100 mmol/mL) and SO₄²⁻ (50 mmol/mL) inhibited 80% and 70% of the activity, respectively. At 1,000 mmol/L NaCl, it was inhibited about 70%. These results suggested that the rate of inhibition of the peptidase activity depended on the type of anion and became more prominent as the ionic strength increased.

Subcellular localization. With the present procedure (350 psi, 20 minutes, 4 °C), the range of disruption of the neutrophils was from 65% to 70% (n = 3) as judged by microscopic examination. The disruption of 52 × 10⁶ cells yielded 37.6 mg of total protein, distributed as follows: pellet 1, 13.8 mg; pellet 2, 3.3 mg; and the supernatant, 12.4 mg. The total recovery of protein was 82%. In Table 3, protein and other marker enzymes are expressed as percentages of total protein or total activity.

The digestive activity (percentage of cleavage per microgram of protein · five minutes) was 0.53, 0.24, 1.86, and 0.1 in each fraction of the homogenate, pellets 1 and 2, and the supernatant, respectively. These results suggested that the digestive activity for FMLP was located in pellet 2, the lysosomal fraction.

Comparison of digestive activity of adult blood granulocytes (ABGs), cord blood granulocytes (CBGs), and granulocytes of CGD patients (CGDGs). Digestive activity of ABGs, CGDGs, and CBGs (percentage of cleavage per microgram protein · five minutes; mean ± SD) was 7.8% ± 2.2% (n = 11), 7.4% ± 1.4% (n = 10), and 5.8% ± 1.5% (n = 9), respectively. The activity of each granulocyte type is shown in Fig 10.

The activity of ABGs was close to that of CGDGs, but that of CBGs was significantly lower (P < .01) than that of ABGs.

DISCUSSION

We found that FMLP binding was increased by the first incubation with FMLP and further elevated by subsequent incubation without FMLP. The elevated binding then
decreased to a level corresponding to the unstimulated one after the second incubation with FMLP, and the binding increased again after the following incubation without FMLP. The first phenomenon (up regulation) may be due to the interaction between induction of spare receptors and internalization of the receptor–ligand complex. Fletcher and Gallin18 found that incubation of human PMNs with degranulating stimuli increased the availability of receptors for FMLP but decreased the apparent affinity for FMLP significantly, and the fractions containing the specific granules were the intracellular pool of FMLP receptors, the source of new surface membrane receptors.17 However, many unknown biological mechanisms may be involved in this induction process for the spare receptors. The second phenomenon suggested that down regulation and recovery of receptors (receptor recycling) were generated in human PMNs. This concept was supported by Zigmond et al,4 who conducted precise kinetic analysis of chemotactic peptide receptors in rabbit peritoneal exudate PMNs. However, receptor recycling has not been clearly shown in human PMNs, and more precise kinetic analysis will be necessary to clarify these phenomena. Lane et al18 reported that a decrease in FMLP receptors was not detected after preincubation with FMLP. On the other hand, Niedel et al19 found that the cell surface binding was decreased after stimulation with formyl-chemotactic peptide and remained at the low level during the subsequent incubation for two hours at 37°C without chemotactic peptide. The observation by Lane et al seemed to correspond to our finding of a slight increase in FMLP binding in the first incubation. However, because the data were not shown in their report, further confirmation is impossible. The discrepancy between Niedel’s and our observation may be in part attributable to the difference in digestive velocity between n-formyl-norleucyl-leucyl-phenylalanyl-leucyl-125I-tyrosyl-lysine and FMLP used in the studies. In our study, approximately one third of the FMLP was released from PMNs and about 40% of it was cleaved as monoiodotyrosine after the same incubation.

Although the mechanism of receptor recycling is not clearly understood, the following process might be involved: binding between receptor and ligand, internalization of the receptor–ligand complex, digestion of the ligand in the cells, release of its product, and return of the receptor to the cell surface. In these processes, digestion of FMLP by PMNs may play an important role.

Based on this assumption, the digestive activity for FMLP of intact PMNs and a lysate was examined. The intact cells showed the ability to digest the carboxyl terminal amino acid of FMLP. The finding of little digestive activity in the supernatant from stimulated PMNs suggested that the digestion of 3H-FMLP mostly occurred within the cells. The same observation had been reported by Bronzna et al.20

Digestive activity with the following characteristics was found in the lysate of PMNs: (1) It cleaved the carboxyl terminal amino acid; (2) showed optimum activity at pH 4.0 (corresponding to intralysosomal pH); (3) was inhibited by DFP and PCMB; (4) was heat stable at acid pH but not at neutral pH; and (5) was found in the lysosomal fraction. These characteristics led us to believe that this substance was a cathepsin A-like enzyme.21 It also had the following additional characteristics: (6) It was inhibited by formyl-methionyl peptide; (7) inhibited by some anions (COO-, Cl-, SCN-, and SO42-); (8) the molecular weight of the substance showing this activity was about 200,000 daltons; (9) its isoelectric point was 5.0; and (10) the Km value for FMLP was about 20 to 25 μmol/L.

Inhibition by some anions was reported for other enzymes.22,23 Jenkins and D’Are24 extensively studied the inhibition of glutamic aspartic transaminase with the displacement of various anions. The results suggested that the buffer ions not only prevented substrate access, but were also apparently not freely dissociable. Although the biological significance of these effects is obscure, this inhibition by anions was thought to be an important characteristic of this cathepsin A-like enzyme.
The results are expressed as percentage of relative cleavage rates in the presence of inhibitors compared with the absence of inhibitors. The intra-assay coefficient of variation was less than 5%. In all experiments, the final concentrations of DMSO and isopropyl alcohol were less than 1%. Under these conditions, the activity was not inhibited.

Table 3. Distribution of Digestive Activity, Protein, and Marker Enzymes Among Subcellular Fractions From Nitrogen-Disrupted Human PMNs

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Digestive Activity (%)</th>
<th>Protein (%)</th>
<th>DNA (%)</th>
<th>MPO (%)</th>
<th>LDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pellet 1</td>
<td>41 (0.24)</td>
<td>47</td>
<td>100</td>
<td>63</td>
<td>24</td>
</tr>
<tr>
<td>Pellet 2</td>
<td>50 (1.86)</td>
<td>11</td>
<td>0</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td>Supernatant</td>
<td>9 (0.10)</td>
<td>42</td>
<td>0</td>
<td>9</td>
<td>76</td>
</tr>
</tbody>
</table>

Protein and other marker enzymes are expressed as the percentage of total protein or total activity. The digestive activity of FMLP is expressed as percentage of cleavage per microgram of protein - five minutes. The method of subcellular fractionation was described in Materials and Methods. Pellet 1 corresponds to the nuclei and undisturbed cell fraction; pellet 2, the lysosomal and mitochondrial fraction, supernatant; the endoplasmic reticulum, plasma membrane vesicle and disrupted cytosol fraction.

Kinetic studies on the peptidase showed a Km value for FMLP (2.0 to $2.5 \times 10^{-5}$ mol/L) much less than those for other substrates, such as n-benzyl-oxyacarbonyl-glutamyl-phenylalanine (10 mmol/L), that are usually used for assaying cathepsin A enzymes. This indicates that FMLP might be the natural substrate for this enzyme. Although the Km value is higher than the FMLP-mediated function, such as chemotaxis ($10^{-8}$ to $10^{-7}$ mol/L) and superoxide production ($10^{-4}$ to $10^{-6}$ mol/L), the Km value obtained would not be disrete from those functions if FMLP were internalized and condensed in the lysosome.

The latter characterization made it more probable that FMLP might be digested by a cathepsin A-like enzyme. The lysosomal digestion of FMLP had been reported by other investigators. We examined the binding and digestion of FMLP in intact PMNs using several inhibitors to determine whether or not FMLP was digested in lysosomes in intact PMNs. Among the inhibitors used in the present study (NEM, PCMB, DFP, TPCK, TLCK, PMSF, and cytochalasin D), we could not find a specific one for which the digestive activity, but not the binding activity, was inhibited (Fig 5). The suppression of receptor binding by NEM, but not by PCMB, had been reported by Lane et al, but both of them had inhibitory activities toward FMLP binding. Although, as is shown in our study, DFP inhibited the digestive activity of the lysate sample and was thought to penetrate into the intact PMNs, it did not inhibit either binding or digestive activity in intact cells. Aswanikumar et al demonstrated that hydrolysis of formyl-methionyl peptides was sensitive to TPCK in rabbit peritoneal exudate PMNs. We could not confirm this in human PMNs, as was reported by Lane et al. It was reported that the cytochalasin-inhibited group usually inhibited internalization of receptor-ligand complexes but not the binding between them. In our study, cytochalasin D did not affect the receptor and ligand relationship as expected.

Dissociation between the extent of digestion (29% cleavage) and that of binding (2.3% bound/total) of FMLP by intact cells might be explained in two ways: (1) there are two separate modes of digestion of peptide, one by way of a surface enzyme that can be inhibited by NEM and the other by way of the lysosomal enzyme that was characterized in our study; (2) a cycle of uptake of FMLP and release of phenylalanine after digestion by intact cells is more rapidly generated than we expected. If the first possibility is true, the activity of the lysosomal enzyme will play a more important role than that of the surface enzyme, when PMNs migrate along the chemotactic gradient with recycling of receptors.
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(internalization of receptors and induction of spare receptors).

With regard to the relationship between chemotaxis and proteinase activity, Becker and Ward,29 Goetzl,30 Ward and Becker,31 and Aswanikumar et al32 reported that a membrane-bound serine esterase was essential in a chemotactic response of PMNs to various chemotactic factors.

Furthermore, Niedel et al33 demonstrated that chymotrypsin inhibitors and analogous substrates were one- to twofold logs more potent as inhibitors of chemotaxis than as inhibitors of formyl-peptide binding. They suggested that the mechanism of inhibition of chemotaxis was independent of inhibition of the binding. In our study, TPCK, TLCK, and PMSF inhibited neither the digestive activity nor the binding of FMLP in intact PMNs. Based on these observations and other reports, it is suggested that serine esterase is involved in chemotaxis but only a little in binding and digestion of FMLP.

Another regulatory mechanism of chemotaxis was reported by Tsan and Deison.3 They suggested that during phagocytosis human PMNs oxidized FMLP to its sulfoxide derivative, through a myeloperoxidase-mediated antimicrobial system, and after the sulfonation, the synthetic peptide lost its chemotactic activity. In our present study, digestive activity was assayed in the lysates of both CGDGs and CBGs. It was known that CGDGs show normal chemotactic movement but are defective in $O_2^\cdot$-releasing activity during phagocytosis,37 and CBGs show impaired chemotaxis and deficient oxidative metabolism.3,14-38 As shown in Fig 10, the digestive activity was normal in CGDGs and significantly reduced in CBGs. These data suggest that the digestive activity might be more involved in chemotactic movement than the oxidative mechanisms.

We speculated that polymorphonuclear leukocytes may be capable of migrating along a chemotactic gradient for hours with receptor recycling mechanisms and with the induction of spare receptors. In this process, the digestive activity of the cathepsin A-like enzyme may be important. However, the relationship between any two of these three factors—receptor recycling, digestive activity, and chemotaxis—is still not clear at present. We speculate that in the future a deficiency in this cathepsin A-like enzyme will be found in patients with impaired chemotaxis and we hope that the interaction of these processes will be clarified further.

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Regulation of receptors and digestive activity toward synthesized formyl-chemotactic peptide in human polymorphonuclear leukocytes

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