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CHEMOATTRACTANT EFFICACY: OXIDATION OF STIMULUS BY RESPONDING CELLS

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SUMMARY. Although all human neutrophils have receptors for formyl-methionyl-leucyl-phenylalanine, only 20-30% migrate in vitro at optimal attractant concentrations. Since this attractant can be oxidized by myeloperoxidase from stimulated neutrophils, we determined if its efficacy was increased by protection from oxidation. Efficacy was increased by reducing agents and by molecules with oxidizable sulfur, 10^{-5}M methionine or 1.5×10^{-6}M albumin. The antioxidant effect was on the attractant, not the cells: albumin in the cell compartment did not increase responses. Furthermore, antioxidants had no effect on efficacy of fNle-Leu-Phe-Nle-Tyr-Lys, an attractant that also stimulated superoxide release but had no oxidizable sulfur.

The accumulation of neutrophils at sites of inflammation is thought to be mediated in part by migration toward chemoattractants generated in the inflammatory locus. However, as reported from our laboratory (1), only 20 to 30% of human blood PMNs migrate toward the chemoattractant, fMet-Leu-Phe, in chemotaxis chambers, despite the fact that all neutrophils have receptors for fMet-Leu-Phe (1,2). Among possible explanations for this finding, one is that in the course of the response to the chemotactic gradient some of the cells are desensitized by the attractant before they complete their migration to the attractant side of the membrane. Another possibility is that neutrophils comprise at least two populations, one of which does not respond to attractant binding with directed movement. In addition to these explanations related to neutrophil responsiveness, experiments on chemoattractant efficacy led us to the conclusion that the chemotactic response could be limited by neutrophil-mediated oxidation of the stimulus.

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ABBREVIATIONS. fMet-Leu-Phe: formylMethionyl-Leucyl-Phenylalanine; fNle-Leu-Phe-Nle-Tyr-Lys: formylNorleucyl-Leucyl-Phenylalanyl-Norleucyl-Tyrosyl-Lysine; HBSS: Hanks balanced salt solution; HSA: human serum albumin.

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MATERIALS AND METHODS

Reagents. We purchased fMet-Leu-Phe and fNle-Leu-Phe-Nle-Tyr-Lys from Peninsula Laboratories, Belmont, CA. Peptide stock solutions in ethanol were made at concentrations of $3x10^{-3}$M and 10^{-3}M, respectively. For dilutions in HBSS, 250 ul volumes were evaporated to dryness under a nitrogen stream, redissolved in 15 ul 1,2-propanediol, diluted in 5ml HBSS, filtered through 0.45 um Millipore filters (Millipore Corp., Bedford, MA) and frozen in aliquots at -80°C. In contrast to ethanol, the amount of 1,2-propanediol in the highest concentration of peptide used did not increase random migration.

L-methionine, L-ascorbic acid, superoxide dismutase and catalase were purchased from Sigma Chemical Co., St. Louis, MO; 2-mercaptoethanol was from LKB Instruments, Rockville, MD; and bovine serum albumin (fatty acid free, Fraction V) was from Miles Laboratories, Naperville, IL. Human serum albumin was obtained as the third major A_{280} peak that eluted during chromatography of human serum on Sephadex G-200.

Leukocyte preparation. Human neutrophils were obtained from heparinized blood of healthy donors (10 units of heparin per ml of blood). One volume of 5% dextran solution was added to 3 volumes of blood in 50 ml polypropylene tubes. After 40 min at room temperature, the leukocyte-rich plasma layer was removed and centrifuged for 10 min at 500 x g in 15 ml polypropylene tubes. The cell pellet was resuspended and washed twice with 15 ml volumes of 0°C HBSS without calcium or magnesium. The suspension contained 73 to 97% neutrophils which were more than 97% viable (assessed by trypan blue dye exclusion).

Chemotactic assay. Neutrophil chemotaxis was assayed with a multiwell chemotaxis chamber (Neuro Probe, Inc., Bethesda, MD) and 10 um thick, 3 um pore polyvinylpyrolidone-free polycarbonate filters (Nuclepore Corp., Pleasanton, Calif.)(3). The number of neutrophils per well was $5x10^4$. Chemoattractants and protecting reagents were warmed to 37°C before addition to chemotaxis chambers. After incubation for 40 min at 37°C in humidified air, filters were removed, dried, and stained with Diff-Quick (American Scientific Products, McGaw Park, IL). Migrated neutrophils were counted with an image analyzer (Optomax, Inc., Hollis, NH)(4).

RESULTS AND DISCUSSION

The lower curve of Figure 1A shows the neutrophil chemotactic response to fMet-Leu-Phe; at the optimal concentration of 10^{-6}M, 30% of the input neutrophils migrated. As shown by the upper curve, when 10^{-4}M 2-mercaptoethanol was present in the fMet-Leu-Phe attractant wells, chemotactic responses were increased at peptide concentrations between 10^{-8}M and 10^{-6}M. The curve was not shifted on the concentration axis; the peak migration of 65% occurred at 10^{-6}M fMet-Leu-Phe. L-ascorbic acid (10^{-4}M) and catalase (25U/ml), but not superoxide dismutase, increased the chemotactic response to fMet-Leu-Phe as effectively as 2-mercaptoethanol (Table I). Similar enhancement was obtained by addition of agents with oxidizable sulfur, 10^{-5}M methionine or $1.5x10^{-6}$M serum albumin. At the concentrations used, none of these substances alone in the attractant well caused neutrophil migration. The results with the reducing agents

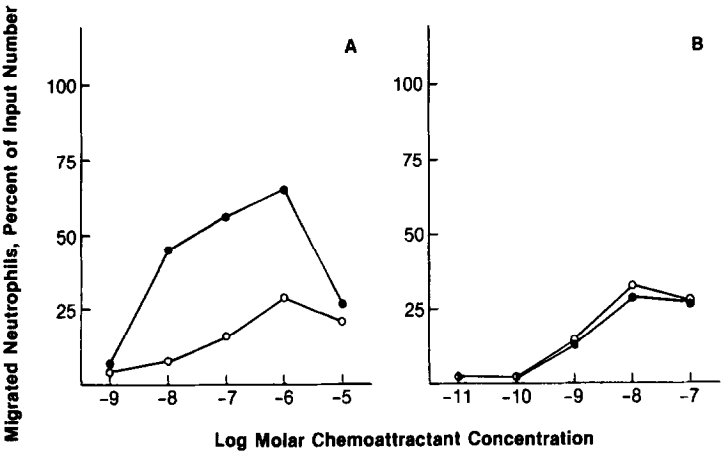

Figure 1. Effect of 10^{-4}M 2-mercaptoethanol in the attractant wells on chemotactic efficacy of (A) fMet-Leu-Phe, and (B) fNle-Leu-Phe-Nle-Tyr-Lys. Closed circles: with 2-mercaptoethanol. Open circles: without. All data were from duplicate wells; no standard error of the mean exceeded 5%.

suggested that the increased migration to fMet-Leu-Phe was due to protection of attractant or cells from oxidation by stimulated neutrophils. The increased response with catalase (which destroys H_2O_2) but not superoxide dismutase (which dismutates superoxide to H_2O_2), suggested that the oxidizing system was myeloperoxidase and H_2O_2. Two experiments indicated that the target of the oxidizing system was not the neutrophil, but the readily oxidizable sulfur in the methionine residue of fMet-Leu-Phe. In contrast to the enhancing effect of 2-mercaptoethanol on the chemotactic response of neutrophils to

Table I

Effect of anti-oxidants on neutrophil response to 10^{-6}M fMet-Leu-Phe

Reagent Tested	Concentration	Percent of Neutrophils Migrated
2-mercaptoethanol	10^{-4}M	65 (2)
l-methionine	10^{-5}M	63 (3)
l-ascorbic acid	10^{-4}M	64 (7)
catalase	25U/ml	63 (2)
superoxide dismutase	5ug/ml	25 (2)
human serum albumin	$1.5x10^{-6}$M	60 (1)
bovine serum albumin	$1.5x10^{-6}$M	61 (2)
HBSS		29 (1)

100 ul of $2x10^{-6}$M fMet-Leu-Phe was mixed with 100 ul of reagent to make the concentrations indicated in the Table. Chemotactic responses were then measured. Numbers in parentheses are standard errors of the mean for duplicate chemotaxis chamber wells.

Table II
Test of the protective effect of HSA in cell or attractant wells

Top Well	Bottom Well	Percent of Neutrophils Migrated
HBSS	HBSS	2 (1)
"	fMet-Leu-Phe	17 (2)
"	fMet-Leu-Phe + HSA	65 (6)
"	HSA	2 (1)
HSA 10^{-6}M	HBSS	1 (0)
"	fMet-Leu-Phe	13 (0)
"	fMet-Leu-Phe + HSA	57 (3)
"	HSA	2 (0)

Final concentrations of fMet-Leu-Phe and of HSA were 10^{-7}M and 1.5×10^{-6}M respectively. Neutrophils were mixed with HSA before addition to chemotaxis wells. Numbers in parentheses are standard errors of the mean for duplicate chemotaxis chamber wells.

fMet-Leu-Phe (Fig. 1A), there was no effect on the chemotactic response to fNle-Leu-Phe-Nle-Tyr-Lys (Fig. 1B), an attractant that also stimulated superoxide release but had no methionine residue. If the effect of 2-mercaptoethanol were to prevent oxidative damage to neutrophils, results with the 2 different peptides should have been comparable. Furthermore, as shown in Table II, serum albumin enhanced the chemotactic response to fMet-Leu-Phe when it was added to the attractant well, but not when it was added to the cells. These experiments are consistent with the reports of Clark et al. that a mixture of H_2O_2, a halide, and partially purified neutrophil myeloperoxidase could inactivate methionine containing attractants by oxidation to the sulfone or sulfoxide (5), that the addition of fMet-Leu-Phe to neutrophils was a sufficient stimulus to cause oxidative inactivation of the attractant during a 60 min incubation, and that this could be prevented by anti-oxidants, catalase or methionine (6).

The hexapeptide, fNle-Leu-Phe-Nle-Tyr-Lys, was first reported by Niedel et al. (7) to be a potent attractant for human neutrophils. Since both the hexapeptide and fMet-Leu-Phe inhibit binding of radioiodinated fNle-Leu-Phe-Nle-Tyr-Lys, it appears that the peptides interact with the same receptor. However, as shown in Fig. 1, the chemotactic efficacy of the hexapeptide, with or without 2-mercaptoethanol is lower than that of 2-mercaptoethanol-protected

fMet-Leu-Phe. This is despite the fact that the affinity of radioiodinated hexapeptide for neutrophils is approximately 10-fold higher than that of fMet-Leu-Phe. Thus, chemotactic efficacy cannot be predicted from binding constants determined on whole cell populations, either because of high and low affinity sites with different functional responses or because of factors operating subsequent to ligand-receptor binding.

The increased efficacy of fMet-Leu-Phe when serum albumin was added is consistent with protection from oxidation. It is of interest that chemoattractants may bind to proteins and retain biological activity. This was reported by Wilkinson for low molecular weight attractants bound to serum albumin (8), by Honda et al. for a macrophage attractant that was bound to a serum globulin (9), and by Goetzl et al. (personal communication) for LTB_4 bound to a high molecular weight protein.

Our data lead to the following conclusions: (1) The chemotactic efficacy of fMet-Leu-Phe is limited by neutrophil-mediated oxidation of the methionine residue, which causes a decrease in the peptide concentration gradient in the region of the membrane that separates cell from attractant compartments.

(2) Comparison of responses to a chemoattractant with and without added 2-mercaptoethanol will determine if the attractant contains an oxidizable residue that is required for chemotactic activity. These considerations are also pertinent in the comparison of efficacy of different attractants.

(3) Although the limited chemotactic efficacy of fMet-Leu-Phe for human neutrophils is due in part to oxidation, additional factors are involved, since protection from oxidation does not lead to migration of 100% of the cells.
(4) It has been suggested that oxidative inactivation of chemoattractants could modulate the inflammatory response (6). However, from our data it is apparent that the concentration of albumin in mammalian tissue spaces would be sufficient to protect attractants from oxidative inactivation *in vivo*.

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