consisted predominantly of meteoric water with, at most, only minor amounts of marine water. It is possible, however, that a deeper stratum of groundwater, predominantly marine, is tapped during explosions.
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21. This value has been calculated (*7*). Few determinations are available, and higher chlorine concentrations may exist.
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N-Formylmethionyl Peptide Receptors on Equine Leukocytes Initiate Secretion but Not Chemotaxis

Abstract. *The chemotaxis of leukocytes appears to be initiated by the binding of chemotactic factors to the surface of these cells. N-Formylated peptides induce chemotaxis and lysosomal enzyme secretion of leukocytes; because these peptides are available in a purified radiolabeled form, they have been useful in the characterization of receptors for chemotactic factors. Equine polymorphonuclear leukocytes secrete lysosomal enzymes but do not exhibit chemotaxis in response to the N-formylated peptides, even though they have a high-affinity cell surface receptor for these agents. The specificity of the equine receptor resembles the specificity of the receptor on chemotactically responsive leukocytes from other species. Equine polymorphonuclear leukocytes may thus be an excellent model for the study of the events that lead to a biological response following receptor occupancy.*

Chemotaxis, the ability of certain cells to sense and migrate along chemical gradients, can be demonstrated in many organisms, including bacteria, protozoans, and slime molds, as well as in eukaryotic cells (*1*). In higher organisms, chemotaxis is particularly important for the normal functioning of the immune system because phagocytes such as polymorphonuclear leukocytes (PMN's) and macrophages appear to accumulate at inflammatory sites by responding to chemotactic signals produced there (*2*).

A major advance in the understanding of chemotaxis came about through the discovery that bacteria as well as PMN's and macrophages contain specific high-affinity surface receptors for chemotactic factors (*3*, *3a*). In bacteria, the availability of chemotactically defective mutants has led to the definition of biochemical events that follow the coupling of chemotactic factors to their receptors and result in a unidirectional migratory response (*4*). The availability of a reliable source of eukaryotic cells with specific defects of chemotactic responses may have similar significance for the eventual understanding of chemotaxis in higher life forms. To this end, we surveyed the chemotactic response in vitro of PMN's from several mammalian species. We found that equine PMN's, while able to respond chemotactically to the 5a component of complement (C5a) and zymosan-activated horse plasma (*5*), did not respond to the *N*-formylated chemotactic peptides (*6*), even though there are high-affinity receptors for these peptides on their surface. Since the equine PMN's did secrete lysosomal enzymes in response to the *N*-formylated peptides, leukocytes from this species appear to lack the mechanisms that specifically produce a chemotactic response upon occupancy of the *N*-formylated peptide receptor.

Blood containing 10 units of heparin per milliliter was obtained from healthy horses maintained at the Duke University Medical Center farm. The PMN's were isolated by sedimentation of the blood in dextran T-500 (Pharmacia) followed by Ficoll-Hypaque density centrifugation (*7*). The resultant cells routinely contain > 95 percent PMN's. Chemotaxis was quantified in modified Boyden chambers with 5-μm nitrocellulose filters (*8*), was scored by determining the average number of PMN's that migrated through the filter, and is expressed as the percentage of the maximal response (*8*). Partially purified horse C5a was isolated from zymosan-activated horse plasma by methods used for the isolation of human C5a (*5*, *9*). *N*-Formylated methionyl peptides used were: *N*-formylmethionylleucylphenylalanine (fMet-Leu-Phe), fMet-Met-Met-Met, fNle-Leu-Phe (Nle, norleucine) (Peninsula Laboratories, Palo Alto, California), and fMet-Leu (Andrulis Research Corp., Bethesda, Maryland). Receptor binding studies were performed with fMet-Leu-[^{3}H]Phe (specific activity, 56.9 Ci/mole; New England Nuclear). A glass fiber vacuum filtration method was used with PMN's at a concentration of 5×10^7 per milliliter of phosphate-buffered saline (PBS) (*3a*, *10*).

To test whether equine PMN's contained specific receptors for fMet-Leu-Phe, we incubated isolated PMN's with 1 to 6 n*M* fMet-Leu-[^{3}H]Phe in the presence or absence of 10 μM unlabeled fMet-Leu-Phe. The reaction mixtures were rapidly vacuum filtered onto glass fiber disks and washed, and specific

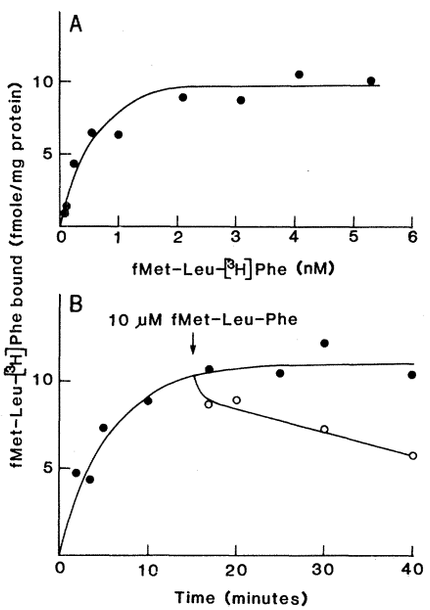

Fig. 1. (A) Binding of fMet-Leu-[^{3}H]Phe to equine PMN's as a function of fMet-Leu-[^{3}H]Phe concentration. Five million equine PMN's were incubated with the indicated concentration of fMet-Leu-[^{3}H]Phe in the presence and absence of 10 μM unlabeled fMet-Leu-Phe for 25 minutes at 25°C. Reactions were terminated by dilution with ice-cold incubation buffer followed by immediate filtration onto glass fiber disks. Specific binding (*11*) was calculated after quantification of radioactivity by liquid scintillation counting. Each value represents the mean of duplicate determinations. (B) Time course of fMet-Leu-[^{3}H]Phe binding to PMN. The fMet-Leu-[^{3}H]Phe (5 n*M*) was incubated with equine PMN's for the indicated time intervals at 25°C, and specific binding (*11*) was assayed (●). To some incubation mixtures (○) a large excess of unlabeled fMet-Leu-Phe (10 μM) was added after 15 minutes of incubation, and fMet-Leu-[^{3}H]Phe binding was assayed at subsequent time intervals as indicated. Each value represents the mean of determinations from two separate incubation mixtures.

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binding was determined (*11*). The PMN's had a high-affinity receptor for fMet-Leu-Phe (Fig. 1A). Scatchard analysis of the data in Fig. 1A and two additional experiments demonstrated that the affinity of fMet-Leu-[^{3}H]Phe for the binding site on equine PMN's was 0.52 ± 0.15 n*M* and that the PMN's contained 630 ± 184 receptors per cell. The kinetics of fMet-Leu-[^{3}H]Phe binding was rapid at 25°C with half-time ($t_{1/2}$) for equilibration of 2.5 to 3.0 minutes (Fig. 1B). The binding of fMet-Leu-[^{3}H]Phe was reversible; a large excess of unlabeled fMet-Leu-Phe (10 μM) added to an equilibrated mixture of fMet-Leu-[^{3}H]Phe and PMN's displaced approximately 50 percent of the bound labeled peptide within 25 minutes (Fig. 1B). The specificity of the binding site for fMet-Leu-[^{3}H]Phe on equine PMN's was examined by determining the ability of a series of *N*-formylated peptides to compete for the site with the labeled ligand. Cells were incubated with 5 n*M* fMet-Leu-[^{3}H]Phe in the presence of buffer alone or buffer containing various concentrations of unlabeled *N*-formylated peptides (Fig. 2C). The peptides inhibited the binding of fMet-Leu-[^{3}H]Phe with order of potency fMet-Leu-Phe > fNle-Leu-Phe > fMet-Met-Met-Met > fMet-Leu, an indication that the equine binding site has the same specificity as the *N*-formylated peptide receptor described for human and rabbit PMN's (*3*, *3a*).

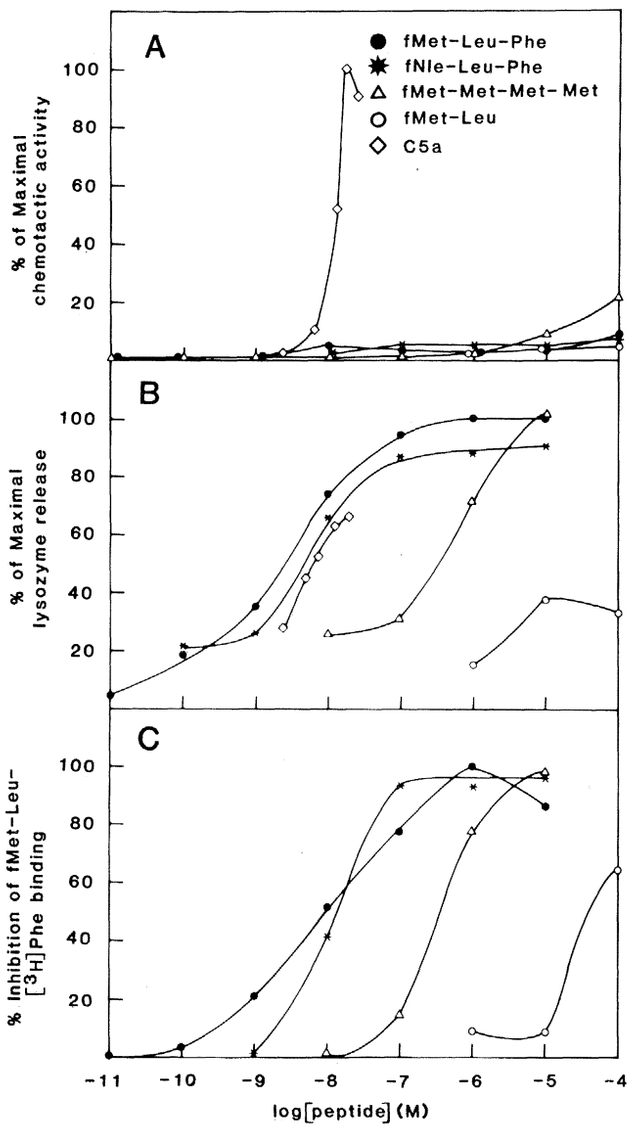

Fig. 2. Comparison of the biological activities of a series of *N*-formylated peptides with their ability to compete for fMet-Leu-[^{3}H]Phe binding. (A) Chemotactic response of equine PMN's to C5a and the *N*-formylated peptides as measured in modified Boyden chambers. Percent of maximal chemotactic activity was calculated as $S/M \times 100$, where M is the maximal chemotactic response in the presence of $1.25 \times 10^{-8} M$ C5a (247 PMN's per high-power field, × 780) and S is the response in the presence of submaximal concentrations of C5a or the various *N*-formylated peptides. Each point represents the mean of triplicate determinations. (B) Induction of lysosomal enzyme release in equine PMN's by *N*-formylated peptides and C5a. PMN's treated with cytochalasin B (10 μM) were incubated with various concentrations of the indicated peptides or C5a for 15 minutes at 37°C, after which lysozyme activity was measured in the cell supernatant. Percent of maximal lysozyme release was computed from the amount of lysozyme activity released in the presence of $10^{-6} M$, fMet-Leu-Phe as maximum (8 μg/ml equivalent lysozyme units). Each value represents the mean of duplicate incubation conditions. (C) Ability of *N*-formylated peptides to compete for fMet-Leu-[^{3}H]Phe binding on equine PMN's. Cells were incubated with 5 n*M* fMet-Leu-[^{3}H]Phe in the presence or absence of various concentrations of the indicated peptides for 25 minutes at 25°C, after which specific binding was determined (*11*). Results are expressed as percent inhibition of fMet-Leu-[^{3}H]Phe binding. Each point reflects the mean of duplicate determinations.

To test whether receptor-ligand binding induced a chemotactic response, we exposed equine PMN's to a variety of *N*-formylated peptides known to be chemotactic for other types of leukocytes (*6*, *10*). Equine PMN's did not respond chemotactically to any *N*-formylated peptide tested over a wide dose range (Fig. 2A), even though the same peptides elicited chemotactic responses in human PMN's and monocytes. That equine cells were indeed able to migrate chemotactically was demonstrated by their response to horse C5a (Fig. 2A). Horse plasma treated with the complement activator zymosan was also a potent chemoattractant for equine PMN's (data not shown). Cellular orientation toward the chemotactic factors was measured (*12*); equine PMN's oriented toward C5a and activated horse plasma, but not toward fMet-Leu-Phe.

We next determined that the *N*-formylated peptides could produce the secretion of lysosomal enzymes from equine PMN's (*13*). Cells were incubated with the peptides plus cytochalasin B, and the amount of lysozyme released was measured (*14*) (Fig. 2B). The *N*-formylated peptides induced a dose-dependent secretion of lysozyme from equine PMN's with the same order of potency observed for inhibition of fMet-Leu-[^{3}H]Phe binding. A correlation coefficient of .999 was obtained when the effective concentrations of each peptide for producing half-maximal lysozyme release and half-maximal inhibition of binding were compared. Release of β-glucuronidase (*13*) was also observed in the presence of the peptides. No release of the cytoplasmic enzyme marker lactate dehydrogenase was observed in the presence of peptides and cytochalasin B. The doses of these peptides and the amount of C5a necessary to release lysosomal enzymes from equine PMN's were similar to those reported for release of lysosomal enzymes from human and rabbit PMN's (*6*, *12*).

These data demonstrate that equine PMN's have a high-affinity receptor for *N*-formylated oligopeptides and that the specificity of this receptor for the peptides is similar to that reported for human and rabbit PMN's (*3*, *3a*). In marked contrast to the human and rabbit cells, however, equine PMN's do not orient or migrate to the *N*-formylated peptides, although the cells are capable of responding to other chemotactic agents. Despite the inability of the *N*-formylated peptides to induce chemotaxis in equine PMN's, they do initiate another receptor-coupled event, the secretion of lysosomal enzymes. Equine PMN's will thus

provide an exceptionally important model for the study of the biology and biochemistry of chemotactic peptide receptor-mediated functions in mammalian cells. Although the occupancy of *N*-formylated peptide receptors induces one receptor-coupled process, it fails to induce another that is thought to be initiated by the same mechanism. The reason for this discrepancy is unknown. It is possible that the coupling processes that link this receptor with the effector mechanisms for the two different biological functions are discrete, with the transducer for secretion intact while the one for chemotaxis is nonfunctional. Alternatively, the number of receptors available may be insufficient for chemotaxis, but sufficient for secretion. In studies of chemotactic factor receptors on chemotactically responsive cells, the estimates have ranged from 2000 to 250,000 receptors per cell. Equine PMN's contain < 1000 receptors per cell. Although these cells clearly have enough *N*-formylated peptide receptors to initiate secretion, they may not have a sufficient number to sense a gradient across their surfaces. Chemotaxis, unlike secretion, may require an asymmetric distribution of chemoattractant binding sites on the cell surface for directional movement. It is also possible that secretion and chemotaxis induced by the *N*-formylated peptides are initiated by similar but independent receptors.

In any case, the transduction signals resulting from the binding of the *N*-formylated oligopeptides to their receptors differ for chemotaxis and secretion in equine PMN's—the former signal being ineffective while the latter signal is operative. It should now be possible, by comparing equine and human PMN's, to separate the biochemical events following *N*-formylated oligopeptide receptor occupancy that result in secretion alone and those that result in both secretion and chemotaxis.

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9. Horse plasma was incubated with zymosan (5 mg/ml) for 60 minutes at 37°C, then for 30 minutes at 56°C. After the zymosan was removed by centrifugation, 4 ml of activated plasma was applied to a Sephadex G-100 column, and the fractions were eluted with 0.01*M* sodium phosphate-buffered isotonic saline, *p*H 7.2. Active fractions were pooled and stored at −20°C (*5*).
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14. Horse PMN's were suspended in phosphate-buffered isotonic saline, *p*H 7.4, at a concentration of 2.5×10^6 to 3.0×10^6 cells per milliliter in the presence or absence of 10^{-5}*M* cytochalasin B. After incubation for 15 minutes at 37°C, buffer or chemotactic factors were added, and incubation was continued for an additional 15 minutes at 37°C. Enzyme release was terminated by immersing the tubes in an ice bath, followed by centrifugation. Lysozyme was assayed in the supernatant by measuring turbidometrically the lysis of *Micrococcus lysodeikticus* at *p*H 7.0 after 15 minutes at 37°C [D. Shugar, *Biochim. Biophys. Acta* **8**, 302 (1952)]. β-Glucuronidase was assayed by measuring the release of phenolphthalein from its β-glucuronate after an 18-hour incubation at *p*H 4.5 [W. H. Fishman, B. Springer, R. Brunetti, *J. Biol. Chem.* **173**, 449 (1948)].
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Ion Pairing Techniques: Compatibility with On-Line Liquid Chromatography–Mass Spectrometry

Abstract. *The mass spectrometric properties of a series of model ion pairs were examined. In the cases studied it was possible to vaporize the ion pair consituents and to produce spectra corresponding to those of the unpaired materials. These findings offer a convenient means for derivatizing certain ionic compounds and demonstrate the feasibility of analyzing ionic species by on-line liquid chromatography–mass spectrometry.*

The direct analysis of ionic species by conventional mass spectrometric (MS) techniques [that is, electron impact ionization (EI) or chemical ionization (CI)] is difficult if not impossible, and chemical derivatization is usually required for these types of compounds. This requirement complicates the analysis of large and important classes of compounds, particularly those of biological and pharmaceutical origin that are ionic in character.

With high-performance liquid chromatography (HPLC), ionic compounds are being successfully analyzed by ion pairing techniques (*1*), but there is limited information on the MS characteristics of the resultant neutral derivatives. The direct combination of reversed phase HPLC and MS has recently been accomplished (*2*), and we report here our findings regarding the MS properties of model ion pairs that demonstrate both the compatibility of on-line LC-MS with ion pairing techniques and the potential for using ion pair derivatives for the direct MS analysis of ionic compounds.

As a model system for this study, we chose ion pairs formed from *n*-alkyl sulfates and sulfonates (counterions) and primary amines (solutes). This system is relatively general and is routinely applied to the HPLC analysis of biological and pharmaceutical materials. To be ideally compatible with MS analysis, both constituents of the ion pair should be volatilized under normal MS operating conditions and should provide spectra indicative of or consistent with the structure of the unpaired materials.

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