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N-Formylmethionyl Peptides as Chemoattractants for Leucocytes

(chemotaxis/bacterial products/neutrophil/macrophage)

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ABSTRACT **Leucocytes such as neutrophils are attracted by *N*-formylmethionine, but not by methionine. Di- and tripeptides containing formylmethionine are strong attractants for both neutrophils and macrophages, whereas the corresponding nonacylated compounds are not chemotactic. The formylated peptides may be related to an incompletely characterized chemotactic material normally produced by bacteria which attract the same animal cells.**

Chemotaxis is the directed migration of cells along a chemical gradient and is demonstrable with a variety of prokaryotic and eukaryotic cells, including bacteria (1), the slime mold (2), and leucocytes (3–6). Bacteria such as *Escherichia coli* are attracted to simple compounds, such as serine, aspartic acid, galactose, and ribose (1). The cells of the slime mold respond to cAMP (2); and leucocytes are attracted to both serum factors such as C5a from complement (4) and compounds elaborated by bacteria (5, 6). Attractants produced by *E. coli* are extremely potent and present in low concentrations in the cell culture medium (7). We have established that some of the active components are small, heterogeneous peptides with blocked amino groups (7), but the low levels of attractants have hampered further characterization. However, since a number of bacteria elaborate such chemotactic substances (8), it seemed likely to us that a class of these compounds characteristic of the cells could be involved. Such prokaryotic cell products could consist of formylmethionyl peptides derived from the NH_2-terminal regions of newly synthesized protein (9, 10), since eukaryotic cells for the most part apparently initiate protein synthesis with nonacylated methionine (11–13). However, mitochondrial and chloroplast preparations are able to utilize formylmethionine in initiating protein synthesis (14). Nevertheless, since leucocytes accumulate at foci of bacterial infections, they may, as eukaryotic cells, be responding in part to just such a typically prokaryotic cell material. In this report we now show that simple formylmethionyl peptides indeed are potent attractants for two types of leucocytes normally involved in the host's resistance to infection, neutrophils and macrophages.

MATERIALS AND METHODS

Production of Bacterial Factor. E. coli was grown to stationary phase in 24 hr in a minimal medium (15). The chemotactically active medium was centrifuged to remove cells and then concentrated by lyophilization. The concentrate was then extracted with butanol/acetic acid (10:1, v/v) and the extract lyophilized. The lyophilized extract containing the active material was dissolved in water and passed through Dowex 50 × 2 to remove impurities and cations. The eluate contained the active material. Salts were removed by passage through Dowex 1 × 2 (16).

Production of C5a. C5a, a chemotactic factor derived from complement, was obtained according to the procedures of Shin *et al.* (17). The only modifications consisted of centrifuging the incubated, complement-inactivated mixture of guinea pig serum and cobra venom factor and then concentrating the supernatant to one-half volume by pressure filtration in a Diaflow apparatus (Amicon Co., Lexington, Mass.), using a filter that retained materials whose molecular weights exceeded 10,000. The concentrated material was then filtered on Sephadex.

Synthetic Chemotactic Substances. The following peptides were obtained from Schwarz/Mann, New York: methionylalanine, methionylalanylserine, methionylglutamic acid, and methionylleucine. *N*-Formylmethionine and other *N*-formylamino acids were purchased from Sigma Chemical Co., St. Louis, Mo., as were a number of formiminoamino acids. Methionyl peptides were formylated by the procedure of Sheehan and Yang (18). Acetylation of amino acids and peptides was carried out according to standard procedures (19).

The following *N*-formylated peptides were obtained from Andrulis Research Co., Bethesda, Md.: *N*-formylmethionylhistidine, *N*-formylmethionyltryptophan, *N*-formylmethionylphenylalanine, and *N*-formylmethionylvaline. The purity of all compounds was checked by thin-layer chromatography on silica gel in *n*-butanol/acetic acid/water, (4:1:1, v/v/v) using the iodoplatinate visualization reagent (20).

Assay for Chemotaxis. Neutrophils. Cells were taken from rabbit peritoneal exudates (8). The proportion of neutrophils obtained in this manner averaged 80%. The assay was based upon the modified Boyden chamber procedure (8). Cells were added to the upper well at a concentration of 2.2×10^6/ml of Gey's balanced salt solution containing 2% bovine serum albumin. Materials to be tested as attractants were added in Gey's solution to the lower well. A Millipore filter (Millipore Corp., Bedford, Mass.), with an average pore diameter of 5 μm, separated the two compartments. In order to allow cell migration to proceed, the Boyden chambers were then incubated for 2 hr at 37° in a humid atmosphere of 95% O_2 and 5% CO_2. The filters were then removed, stained with hematoxylin, and examined microscopically for cells that had migrated to the underside. These were counted, and the results expressed as the average number of neutrophils within

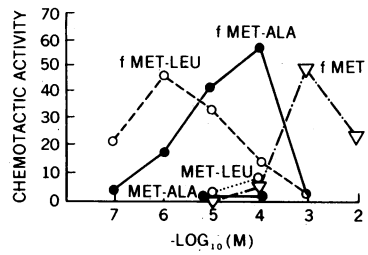

FIG. 1. Chemotactic activity of neutrophils in response to formylated and unformylated peptides. Activity is expressed as the mean of triplicate samples (*Materials and Methods*). Negative control activity was 3.2. Positive control (C5a) activity was 46.4. The SEM did not exceed 10% for values above 10.

10 fields at a magnification of ×800 for triplicate samples. The SEM did not exceed 10% for values above 10.

Macrophages. Chemotactic substances were tested against macrophages in modified Boyden chambers as reported (21). Cells were obtained from peritoneal exudates of guinea pigs that had been injected with 1% shellfish glycogen 4 days before. The cells, containing at least 80% macrophages, were placed on the top surface of a 5 μm polycarbonate filter (Nuclepore Inc., Wallabs, San Rafael, Calif.), and the substance to be tested was placed on the opposite side of the filter in the lower chamber. With these cells 90 min was allowed for migration, after which the filters were processed and cell migration was measured by microscopic examination. Macrophage chemotactic activity is defined as the mean number of macrophages that have migrated through the filter in 20 oil immersion fields in each of triplicate filters ±SEM. The SEM did not exceed 10% for values above 10.

RESULTS

Neutrophil Chemotaxis. The results in Table 1 show that the simplest member of the class, *N*-formylmethionine, but not methionine, was chemotactic for neutrophils over a range from 0.1–10 mM. A likely explanation for the decreased cell migration at the highest concentration is that the chemical gradient was diminished from the increased rate of diffusion of the attractant, resulting in a greater degree of saturation of the cells in the upper well of the Boyden apparatus. Furthermore, when formylmethionine was added together with cells in the upper well of the Boyden chamber, it blocked neutrophil migration in response to either bacterial chemotactic factor or C5a. Methionine, on the other hand, was without effect. Since formylmethionine was found to be chemotactic itself and did not affect cell viability, as determined from the trypan blue staining reaction, it seems likely that this simple acylamino acid competes for the same leucocyte receptor to which the other chemoattractants may bind.

Since formylmethionine required relatively high levels for activity, we tested peptides containing the formylmethionyl (fMet) residue as attractants for neutrophils. These particular peptides were selected chiefly because the residues following methionine have been found in the NH_2-terminal positions of *E. coli* proteins (22). These compounds were assayed for activity over a range from 10^{-7} M to 10^{-2} M (Fig. 1). It is evident that the formylmethionyl peptides were more active than formylmethionine alone by several orders of magnitude. The derivative containing leucine was particularly effective, having shown activity at 10^{-7} M. In contrast, the unformylated peptides were inactive and, when added to cells, did not inhibit chemotaxis to the formylated compounds (not shown). Again, the reduced response of the cells to a higher level of attractant may be explained by the decrease of the chemical gradient caused by its greater rate of diffusion across the filter from the lower well of the Boyden chamber.

TABLE 1. *N-Formylmethionine as attractant and competitor in neutrophil chemotaxis*

Compound tested	Additions to cells‡	Chemotactic activity§
None	None	0.9 ± 0.4
Formylmethionine, 0.1 mM	None	7.0 ± 0.5
Formylmethionine, 1 mM	None	50.5 ± 3.7
Formylmethionine, 10 mM	None	25.7 ± 3.7
Methionine, 1 mM	None	3.0 ± 0.6
Methionine, 10 mM	None	1.8 ± 0.5
Bacterial factor*	None	62.5 ± 5.0
Bacterial factor	10 mM fMet	1.2 ± 0.5
Bacterial factor	10 mM Met	51.5 ± 2.7
C5a†	None	34.1 ± 3.9
C5a	10 mM fMet	6.3 ± 0.4
C5a	10 mM Met	33.2 ± 1.7

* Twenty microliters of purified *E. coli* factor were added to the lower unit of a Boyden chamber.

† C5a (0.3 ml) in 20 mM phosphate-buffered saline (pH 7.2) was added to the lower unit in Boyden chamber.

‡ Components were added to the upper unit of Boyden chamber.

§ Chemotactic activity is expressed as the average neutrophil migration in triplicate samples (*Materials and Methods*).

Also tested for activity were a number of other formylmethionyl peptides, which included some with polar and nonpolar substituents. The results (Table 2) are expressed as the activity at the lowest effective concentration of each compound. Only those activities that are at least four times as active as the negative control have been listed. In general, each of the peptides tested exhibited an activity against concentration profile similar to those in Fig. 1. Of those compounds containing hydrophobic groups, fMet-Phe was active at levels as low as 10^{-9} M and fMet-Leu-Gly was effective at 10^{-7} M, as was fMet-Leu (Fig. 1). fMet-Glu and fMet-Ala-Ser, bearing a negative charge of two and one, respectively, at neutral pH, were active at 10^{-5} M. However, fMet-His, with a basic substituent, was much less active (10^{-3} M). The tryptophan derivative, containing a basic group, is also hydrophobic, and this may account for its high efficacy (10^{-7} M).

In order to determine whether other acyl groups could be substituted for formyl in active compounds, *N*-acetylmethionylleucine (AcMet-Leu) was tested and shown to be effective at 10^{-6} M, but it had about one-tenth the activity of the corresponding formylated compound (Fig. 1).

Additional related compounds assayed for their chemotactic potential and found not to be active over a concentration range of 10^{-6} M to 10^{-3} M included: methionine, *N*-acetylmethionine, methionine sulfoxide, methionine sulfone, the *N*-formylated derivatives of the latter two compounds, the sulfone of fMet-Leu, formiminoglycine, formiminoaspartic acid, formiminoglutamic acid, *N*-formylaspartic acid, and *N*-formylglycine; *N*-formylleucine showed slight activity at 10^{-3} M (about three times that of background). Also, *N*-acetyl-

TABLE 2. *Chemotactic activity of N-acylmethionyl peptides for neutrophils*

N-Acylmethionyl peptide	Lowest effective concentration (M)	Chemotactic activity*
fMet-Trp	10^{-7}	25.1 ± 0.8
fMet-Leu-Gly	10^{-7}	18.4 ± 1.2
fMet-Phe	10^{-9}	29.9 ± 2.4
fMet-Glu	10^{-5}	25.1 ± 0.8
fMet-Ala-Ser	10^{-5}	22.7 ± 1.3
fMet-His	10^{-3}	49.0 ± 3.3
fMet-Val	10^{-5}	24.4 ± 2.2
AcMet-Leu	10^{-6}	39.4 ± 2.0

* Chemotactic activity at the lowest effective concentration is expressed as the average neutrophil migration in triplicate samples (*Materials and Methods*). The negative control activity was 4.0. The positive control for C5a was 55.0. All values expressed are significant as compared to the control.

glycylglycine, *N*-acetylglycylglycylglycine, and pyrollidonoylglycine were all inactive.

Macrophage Chemotaxis. Like the neutrophil, the macrophage plays a major role in the host's resistance to infection. Consequently, it was of interest to know whether that cell responded chemotactically to formylmethionyl peptides. The results of such an experiment (Fig. 2) show that the formylated peptides were indeed attractants for the macrophage, while the unformylated peptides had little or no activity. As in the neutrophil system, the leucine derivative was also very potent for the macrophage. Neither *N*-formylmethionine nor its sulfoxide nor its sulfone was active (not shown). We have also found that fMet-Leu, fMet-Trp, and fMet-Phe all strongly attracted human macrophages at concentrations as low as 10^{-6} M, similar to the levels at which these compounds produced chemotaxis in neutrophils.

DISCUSSION

A number of small *N*-formylmethionyl peptides have been found to be potent chemoattractants for both neutrophils and macrophages. *N*-Formylmethionine itself has considerably less activity for neutrophils and none for macrophages. Our studies permit some inferences on the requirements for activity in these compounds:

(*i*) A requirement for *N-acylation* is indicated by the observation that neither the nonacylated peptides nor free methionine show activity. A specificity for *formylation* is suggested by the findings that in the neutrophil system fMet-Leu is more active than AcMet-Leu, and that formylmethionine is active while acetylmethionine is not.

(*ii*) A requirement for *methionine* is suggested by the observations that of all the formylamino acids tested, only formylmethionine was active, and that of the acetylated peptides assayed, only AcMet-Leu was active while AcGly-Gly, for example, was not. It also appears that the sulfur of methionine must not be oxidized, since neither the sulfone nor sulfoxide of formylmethionine nor the sulfone of fMet-Leu attracted neutrophils.

(*iii*) A requirement for *a minimum size* is suggested by the observation that the most active fMet peptides contain at least two residues.

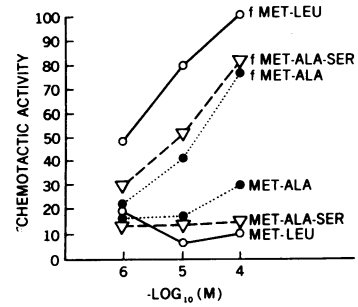

FIG. 2. Chemotactic activity of macrophages in response to formylated and unformylated methionyl peptides. Activity is expressed as the mean of triplicate samples (*Materials and Methods*). Negative control activity was 8.0. The SEM did not exceed 10% for values above 10.

(*iv*) A degree of specificity for *hydrophobic residues* is suggested from the finding that such nonpolar residues as Leu, Phe, and Trp in fMet peptides increased their activities by some two orders of magnitude over those of peptides with polar residues (Table 2). Wilkinson (23) has emphasized the importance of hydrophobic groups in enhancing the chemotactic potential of certain proteins, and it may be that nonpolar residues facilitate entry of the chemoattractant into the membrane bilayer. It does not seem likely that the differences in activities among the peptides result from their differing rates of diffusion in the Boyden chamber, since the rate-limiting process is probably cell migration.

The competition experiments between formylmethionine and other chemotactic factors (Table 1) suggest that the same site on the cell interacts with a variety of attractants of widely differing sizes, since formylmethionine has a molecular weight of 177, the bacterial factor has a molecular weight range from 500 to 1500 (7), and C5a has a weight of about 15,000 (4). These considerations, as well as our demonstration that small acylated peptides are chemotactic, would not accord with Wilkinson's inference that macromolecular size is required for chemoattractants of leucocytes (23).

It is appropriate to consider whether these synthetic acylated peptides are related to naturally produced bacterial chemotactic factors. While it is attractive to propose that the eukaryotic cell recognizes a prokaryotic cell product derived from NH_2-terminal regions of nascent proteins, there is no direct evidence for this to date. The bacterial factor has not been fully characterized. The evidence is indirect that these attractants are formylmethionyl derivatives. The chemical properties of the bacterial factor and the synthetic peptides are similar; both the bacterial factor (E. Schiffmann, H. Showell, B. Corcoran, P. A. Ward, E. Smith, and E. L. Becker, in preparation) and some of the active peptides contain amino acids that have been found to succeed methionine at the NH_2-terminal position of bacterial proteins (22) and, in some cases, of fragments of proteins from mutants of *E. coli* (24). In addition, while the formylmethionine at the NH_2-terminal position of nascent bacterial protein is in general subsequently deformylated (25), it is possible that enzymatic cleavage of small fMet peptides from protein may occur (9, 10).

While the assay conditions used to study migration vary with different types of cells, both neutrophils and macrophages were found to respond in similar fashion to different

compounds. This suggests that similar receptors for these compounds exist in these cells. The competition observed between the complement-derived attractant and *N*-formylmethionine in the neutrophil may have a similar basis. This result may be compared with the behavior of bacteria such as *E. coli*, which in the presence of "receptor saturating" concentrations of one kind of attractant still respond to a gradient of another chemotactic compound (1). Both C5a and the bacterial factor induce the secretion of lysosomal enzymes from migrating cells (26), and Dr. Elmer L. Becker of the University of Connecticut Health Center has informed us that fMet peptides have a similar action in the presence of cytochalasin B. Since such a release of these cell products is an important factor in producing inflammation, it may be possible to alter the leucocytes' participation in this process by local or systemic administration of fMet peptides.

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