THE STRUCTURE-ACTIVITY RELATIONS OF SYNTHETIC PEPTIDES AS CHEMOTACTIC FACTORS AND INDUCERS OF LYSOSOMAL ENZYME SECRETION FOR NEUTROPHILS*

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A large number of different kinds of substances are reported to be chemotactic for neutrophils (1). The size, complexity, and unknown structure of most of these have precluded any definitive analysis of the structural or molecular basis of their chemotactic activity. Recently, Schiffmann et al. have reported that simple, synthetic *N*-formyl methionyl peptides are chemotactic for neutrophils and macrophages (2). This has made possible the beginning of a systematic study of the relation of the structure of simple peptides to their chemotactic activity and thus, eventually to directly investigate the primary interaction of chemotactic agents with the neutrophil surface. In addition to their chemotactic activity, substances such as C5a or low molecular weight peptides isolated from *Escherichia coli* culture filtrates induce lysosomal enzyme release from rabbit or human neutrophils in the presence of cytochalasin B (3, 4) or, when the neutrophils are on a suitable surface, in its absence (5, 6).

We have synthesized a series of 24 peptides, all but 2 of them methionyl or *N*-formyl methionyl derivatives. Most of them are di- and tripeptides, with a few tetrapeptides. We shall show that these substances not only increase random movement but are chemotactic as well, that is, they also induce directed movement. In addition, a systematic study of the relation of structure to activity has demonstrated a highly specific dependence of the activity of these peptides upon their structure. The ability of a given peptide to induce migration strictly correlates with its ability to induce lysosomal enzyme secretion from cytochalasin B-treated cells suggesting that the same primary interaction of peptide and cell initiates both activities.

Materials and Methods

Rabbit polymorphononuclear leukocytes (neutrophils) were obtained 12–14 h after the intraperitoneal injection of 0.1% glycogen, as described (7). They were washed in Hanks' balanced salt

* Supported by NIH grants AI 06948 and HL 15697, American Heart Association grant AHA-74-827 (funds contributed in part by the Hartford chapter of the American Heart Association), and NIDR contract N-01-DE-5477.

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solution containing 0.01 M tris(hydroxymethyl) aminomethane, pH 7.2, 1 mg/ml of glucose, and 1 mg/ml of crystalline bovine serum albumin. The tris(hydroxymethyl) aminomethane and bovine serum albumin were obtained from Sigma Chemical Co. St. Louis, Mo. The Hanks' balanced salt solution was the buffer used throughout the work.

Assay of Relative Migratory Activity of Peptides. The migratory assay employed a modified Boyden chamber[1] as described previously (7). Briefly, 1 ml of washed peritoneal neutrophils containing 2.5×10^6 cells was added to the upper compartment of the modified Boyden chamber separated from 1 ml of peptide solution by a 25 mm diameter filter of 0.6 μm average pore size (Schleicher & Schuell Inc., Keene, N. H.). The loaded chambers were incubated for 30 min at 37°C. This time interval was chosen as giving, for the particular batch of filters used, the greatest difference between the number of cells moving into the filter with no peptide in the lower compartment (background) and the maximum number of cells moving into the filter when stimulated by peptide. At the end of the incubation the filters were removed and stained and the number of cells in five high-power (400 magnification) fields (5 HPF) were counted and averaged (7). The results reported are the means of duplicate chambers.

The migration enhancing activities of different peptides were compared essentially as described previously (8, 9). In measuring the relative activity of the peptides, a peptide was diluted so that each concentration was 1/4 that of the next higher concentration. The concentrations of peptides were chosen on the basis of preliminary experiments to cover the range giving minimal to maximal activity. The chemotactic activity plotted against the logarithm of the molar concentration of peptide yielded a sigmoidal-shaped dose-response curve. Different peptides tested at the same time with the same cells gave curves which were parallel to each other in their linear portions and had the same maximum. The activities of the different peptides are reported as the ED_{50}, the concentration of chemotactic factor giving 50% of the maximum activity. The ED_{50} depends on the activity of the peptide and also on the cells used in testing. In order to ensure that the activities determined with different cells were always comparable, a peptide, F-Met-Leu was arbitrarily chosen as the standard peptide and included in each assay. The activity of each peptide was then recorded both as the ED_{50} and the relative activity or potency ratio, that is the ratio of the ED_{50} of F-Met-Leu, run in the same experiment, to the ED_{50} of the given peptide.

Test of Whether the Peptides are Chemotactic. The test of whether or not the peptides are truly chemotactic was carried out as described by Zigmond and Hirsch (10) for two peptides, F-Met-Leu-Phe and F-Met-Met-Met.

In testing F-Met-Leu-Phe, 5×10^6/ml of washed peritoneal neutrophils obtained 12 h after the administration of 0.1% glycogen were suspended in varying concentrations of the peptide and placed in the upper compartment of a modified Boyden chamber. The cells were separated from the bottom compartment containing varying concentrations of the same peptide by a 3.0 μm pore size filter (Millipore Corp., Bedford, Mass.). Duplicate chambers were incubated 60 min at 37°C. The filters were fixed and processed as usual. Using the micrometer of the fine adjustment of the microscope, the distance was measured from the top of the filter to the farthest plane of focus still containing two cells. This distance was determined across five fields and averaged (10). The results reported are the means of duplicate chambers.

In testing F-Met-Met-Met, essentially the same procedure was followed except that 8×10^6/ml of washed peritoneal cells obtained 4 h after administration of 0.1% glycogen were employed. The cells were allowed to migrate into a 3 μm pore size Millipore filter for 80 min at 37°C. For reversibility studies with both peptides, cells were incubated 30 min at 37°C in varying concentrations of a given peptide before washing with buffer or saline and testing in fresh medium.

Induced Lysosomal Enzyme Secretion. Washed neutrophils were resuspended to a final concentration of 1×10^7/ml in Hanks' buffer containing 2 mg/ml of bovine serum albumin and 10 μg/ml cytochalasin B (Aldrich Chemical Co., Milwaukee, Wis.). The cytochalasin B was diluted from

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[1] The Boyden chamber system measures enhanced migration due to either an increase in random locomotion or in directed migration or both. As is shown later, the synthetic peptides we have studied stimulate both forms of movement. Therefore, in an attempt at greater terminological exactitude, in describing our experimental results, we have tried to use the noncommittal terms "enhanced migration," "stimulated movement," etc., except under circumstances where we have experimentally demonstrated the nature of the movement.

a stock solution containing 4 mg/ml in dimethyl sulfoxide. The dimethyl sulfoxide remaining after dilution was shown not to affect the release (5). In duplicate, 0.5 ml of cell suspension was added to 0.5 ml of fourfold serial dilutions of peptide in 12 × 75 mm test tubes at 4°C. The mixtures were then incubated for 5 min at 37°C (the release is completed in 1 min or less under these conditions) and the tubes centrifuged at 2,500 rpm (1,400 *g*) for 5 min at 4°C. The supernates were removed and aliquots taken for measurement of lactic dehydrogenase, lysozyme, and β-glucuronidase as described (5).

The total concentration of enzyme was measured by lysing the cells with buffer containing 0.1% Triton X-100 (Beckmann Instruments, Inc., Fullerton, Calif.). The units of enzyme activity were calculated as described (4). We measured the lactic dehydrogenase of each supernate to ensure that the peptides induced no release of this cytoplasmic marker. No release was found in any instance.

The ability of each peptide to induce lysosomal enzyme release was assayed essentially as described for chemotaxis. The lysozyme or β-glucuronidase activity released into the supernate at each concentration of a given peptide plotted against the logarithm of the molar concentration of the peptide gave rise to a sigmoidal dose-response curve. As with chemotaxis, in a given experiment, the curves were parallel in their linear portions and showed the same maximum. The lysosomal enzyme-inducing activity for each peptide was obtained from the dose-response curve as its ED_{50}, the molar concentration of peptide causing 50% of the maximal release of either lysozyme or β-glucuronidase. In order to correct for changes in the responsiveness of different cells, the dose-response curve of the standard peptide, F-Met-Leu was measured whenever any group of peptides was assayed so that the activity relative to the standard could be also calculated.

Synthesis of Peptides. Leu-Trp-Met and Leu-Trp-Met-Arg were purchased from Research Plus, Denville, N. J., and formylated by the method of Sheehan and Yang (11). All other synthetic peptides used in this study were prepared by the Merrifield solid-phase technique (12) essentially as described by Stewart and Young (13). Amino acid resins with the exception of methionine, were prepared by the reaction of the triethylammonium salt of the tert-butyloxycarbonyl (t-Boc)[2] amino acid and the chloromethyl resin in ethanol at 85°C for 24–65 h. The Met resin was prepared by the reaction of the cesium salt of t-Boc methionine in dimethylacetamide for 18 h at room temperature according to the method of Gisin (14). Subsequent couplings were by standard solid-phase methods using dicyclohexylcarbodiimide. Side-chain protecting groups used in this study were glutamic acid (benzyl) and arginine (NO_2). The completed peptides were cleaved from the resin by reaction with liquid hydrogen fluoride (HF) for 30 min at 0°C. 50 equivalents of anisole were added to the HF to provide a free radical scavenger. The HF-treated resin was washed with ethyl ether to remove anisole and its reaction products. The resin was extracted with 1 M acetic acid and lyophilized. Some of the synthetic peptides were only sparingly soluble in 1 M acetic acid and therefore it was necessary to extract them with glacial acetic acid.

The synthetic peptides were purified either by countercurrent distribution (100 transfers in *n*-butanol: acetic acid: water; 4:1:5) or preparative high voltage electrophoresis (pyridine: acetic acid: water; compounds were considered pure if they showed only one spot on high voltage electrophoresis at pH 5.0, and in at least two thin-layer chromatography systems [usually *n*-butanol: acetic acid: water, 4:1:5 or cellulose and chloroform: methanol: acetic acid: water, 65:30:4:1 on silica gel]). In all cases both ninhydrin and nonspecific I_2 stains were used to visualize the peptides. Amino acid content was determined by quantitative analysis on a Beckman 121 C Amino Acid Analyzer (Beckman Instruments, Inc.).

Formylated methionyl peptides were prepared successfully by the method of Sheehan and Yang (10). Unformylated peptides were removed from the reaction mixture by passage of the peptide through Dowex 50 (Dow Chemical Co., Midland, Mich.). Formylated peptides were subject to the same analytical tests and criteria of purity described for the unformylated peptides.

All the peptides studied are L amino acid peptides. They are not so designated in what follows merely because of convenience.

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[2] *Abbreviations used in this paper:* HF, hydrogen fluoride; 5 HPF, five high-power fields; t-Boc, tert-butyloxycarbonyl.

TABLE I

The Nature of the Migration of Neutrophils Induced by F-Met-Leu-Phe and F-Met-Met-Met

Molar concentration above filter	Molar concentration below the filter				
	6.2×10^{-12}	1.85×10^{-11}	5.55×10^{-11}	1.66×10^{-10}	5×10^{-10}
A. F-Met-Leu-Phe					
6.2×10^{-12}	29		46 [35]	64 [47]	75 [61]
1.85×10^{-11}		40		70 [54]	82 [64]
5.55×10^{-11}	31 [55]		60		66 [68]
1.66×10^{-10}	34 [67]	36 [67]		71	
5×10^{-10}	49 [72]	47 [72]	46 [72]		73
	10^{-9}	10^{-8}	10^{-7}	10^{-6}	
B. F-Met-Met-Met					
10^{-9}	27	30 [27]	57 [30]	69 [35]	
10^{-8}	27 [30]	30	47 [30]	62 [35]	
10^{-7}	34 [33]	36 [34]	34	60 [38]	
10^{-6}	38 [52]	34 [52]	48 [52]	56	

The unbracketed figures are the measured mean distance from the top of the filter to the front polymorphonuclear leukocytes (PMNs) in one plane of focus (40 × objective). The figures within the diagonals are the measured mean distance in a uniform concentration of the respective peptides. The latter are used to calculate the distances to the front two PMNs of a hypothetical population undergoing purely random migration as described by Zigmond and Hirsch (8). These are the bracketed figures.

Results

Demonstration that the Synthetic Peptides are Chemotactic. Increased penetration of cells into or through the filter of a Boyden chamber system under the stimulus of a chemical agent is generally taken as indicating the chemotactic activity of the agent. Zigmond and Hirsch have pointed out that the increased migration could result from a response directed by the chemical gradient, that is from chemotaxis, or from increased random locomotion or both (10). These authors have described a technique for determining if the movement of the cell into the filter of a Boyden chamber system under the influence of different gradients is greater than could be expected on the basis of increased rates of locomotion alone and to that extent is due to a true chemotactic response. Table I shows the results of tests of two representative synthetic peptides, F-Met-Leu-Phe and F-Met-Met-Met. In Table I, the unbracketed values are the experimentally determined distances into the filter that the front two cells of the population moved when various concentrations of either F-Met-Leu-Phe (A) or F-Met-Met-Met (B) were placed above and below the filter. The bracketed values are the predicted distances the front two cells would move in a random walk process in which the only variable was the rate of random locomotion. These values were determined, as described (10), from the distance the cells moved in the absence of a gradient (values within the diagonal lines). The bracketed values thus represent the migration one would expect from cells that were not exhibiting chemotaxis, that is were moving by random locomotion alone.

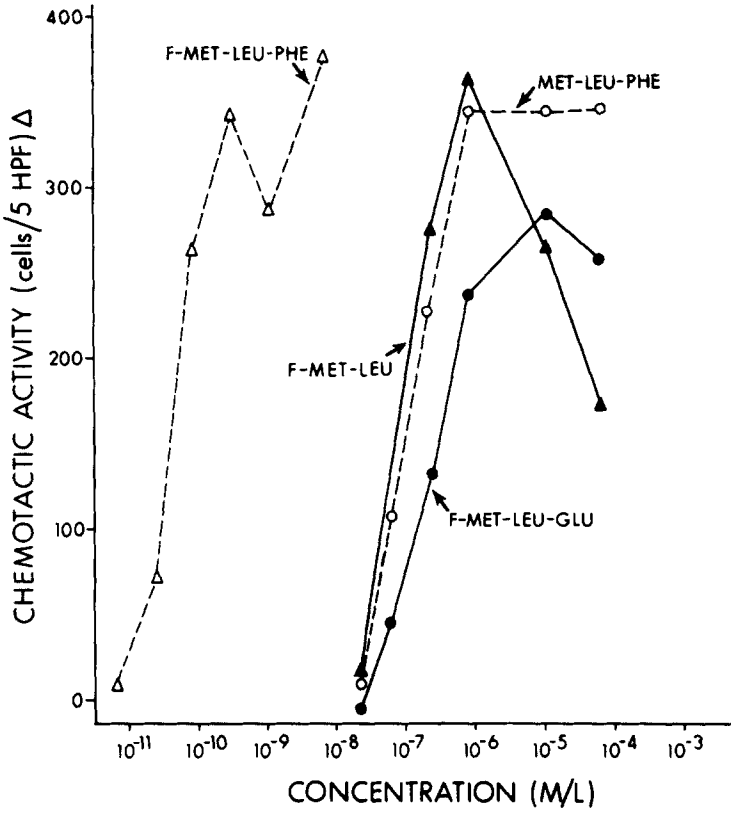

FIG. 1. The assay of the migration inducing (chemotactic) activity of four peptides: F-Met-Leu-Phe (△---△), F-Met-Leu (▲—▲), Met-Leu-Phe (○---○), and F-Met-Leu-Glu (●—●).

The overall results with both peptides were the same. Over the concentration ranges given in Table I, the effects of the two peptides on locomotion were rapid and largely reversible (results not shown). Both peptides stimulate random movement of the neutrophil as shown by the penetration obtained when no gradient was present. Comparison of experimental values with predicted values shows that both peptides are also chemotactic. The cells moved significantly farther than predicted (greater than 10 μm) when the concentration of peptide below was greater than above the filter (positive gradient) and not as far as predicted when the reverse was the case (negative gradient). With each peptide, two additional experiments gave essentially the same result.

Comparison of the Chemotactic Activities of the Various Peptides. Fig. 1 shows the results of a representative experiment in which the migration enhancing activity of four peptides were compared. In the experiment of Fig. 1, the $ED_{50's}$ of F-Met-Leu-Phe, F-Met-Leu, F-Met-Leu-Glu, and Met-Leu-Phe were found to be, respectively, 4.1×10^{-11} M, 3.0×10^{-7} M, 4.2×10^{-7} M, and 1.3×10^{-6} M. Thus, for this experiment, F-Met-Leu-Phe was 7,300 times; F-Met-Leu-Glu, 0.71 times; and the Met-Leu-Phe, 0.43 times as active as F-Met-Leu.

The chemotactic activity of the 24 peptides was determined as just described. In every case, the activity of each peptide was determined in at least three

TABLE II
Chemotactic Activity of Synthetic Peptides

	ED_{50} ± SE	Relative Activity* ± SE
1. F-Met	$2.1 \pm 0.62 \times 10^{-3}$	0.0003 ± 0.00017
2. F-Met-Leu	$4.0 \pm 0.45 \times 10^{-7}$	1
3. F-Met-Leu-Phe	$7.0 \pm 1.7 \times 10^{-11}$	5,050 ± 820
4. F-Met-Leu-Glu	$1.3 \pm 0.38 \times 10^{-6}$	0.37 ± 0.17
5. F-Met-Leu-Arg	$3.6 \pm 1 \times 10^{-7}$	0.98 ± 0.11
6. F-Met-Leu-Leu	$4.8 \pm 1.3 \times 10^{-8}$	9.2 ± 2.7
7. F-Met-Phe	$4.1 \pm 0.95 \times 10^{-7}$	1.3 ± 0.33
8. F-Met-Phe-Leu	$5.4 \pm 1.9 \times 10^{-8}$	10 ± 2.4
9. F-Met-Phe-Met	$1.5 \pm 0.33 \times 10^{-9}$	169 ± 50
10. F-Met-Met	$8.8 \pm 2.3 \times 10^{-7}$	0.57 ± 0.21
11. F-Met-Met-Met	$5.1 \pm 0.62 \times 10^{-9}$	70 ± 32
12. F-Met-Met-Met-Met	$3.0 \pm 0.13 \times 10^{-10}$	760 ± 82
13. F-Met-Met-Ala	$5.4 \pm 1.8 \times 10^{-7}$	1 ± 0.3
14. F-Met-Met-Phe	$2.1 \pm 0.49 \times 10^{-10}$	1,900 ± 260
15. F-Leu-Trp-Met	$2.5 \pm 1.5 \times 10^{-8}$	17 ± 5.6
16. F-Leu-Trp-Met-Arg	$1.1 \pm 0.4 \times 10^{-6}$	0.25 ± 0.06
17. Met-Leu-Phe	$6.7 \pm 1.9 \times 10^{-7}$	0.48 ± 0.08
18. Met-Leu-Glu	$2.7 \pm 1.0 \times 10^{-3}$	0.00017 ± 0.00005
19. Met-Leu-Leu	$2.1 \pm 0.24 \times 10^{-4}$	0.0015 ± 0.0003
20. Met-Phe-Leu	$2.4 \pm 1.1 \times 10^{-4}$	0.003 ± 0.0015
21. Met-Met-Met	$1.0 \pm 0.39 \times 10^{-4}$	0.003 ± 0.0008
22. Met-Met-Met-Met	$1.3 \pm 0.29 \times 10^{-6}$	0.19 ± 0.06
23. Met-Met-Ala	$1.9 \pm 0.39 \times 10^{-4}$	0.003 ± 0.00008
24. Met-Met-Phe	$9.0 \pm 3.9 \times 10^{-7}$	0.7 ± 0.29

* Relative to F-Met-Leu = 1.0.

separate experiments similar to the one pictured in Fig. 1. The results of these experiments are compiled in Table II which shows the mean ED_{50} ± SE (standard error) and the mean activity ± SE of each peptide relative to the standard, F-Met-Leu.

The high activity attainable and the wide spread in activities of the various peptides are clearly evident; the mean ED_{50} of F-Met-Leu-Phe, the most active peptide tested being $7.0 \pm 1.7 \times 10^{-11}$ M and the least active, Met-Leu-Glu is $2.7 \pm 1.0 \times 10^{-3}$ M, a 26 million-fold difference in activity. Another way of stating the same thing is that the F-Met-Leu-Phe is 5,000-fold more active than the reference compound, F-Met-Leu, whereas Met-Leu-Glu is only 1.7×10^{-4} as active. The other peptides fall more or less regularly between these extremes.

Inspection of Table II reveals the very great specificity in the relationship of structure to activity, small structural changes being capable of creating large differences in activity. For example, F-Met-Leu-Phe and F-Met-Phe-Leu differ only in the inversion of the terminal two amino acids yet F-Met-Leu-Phe is 500 times more active than F-Met-Phe-Leu. Similarly, F-Met-Met-Phe is approximately 10 times as active as F-Met-Phe-Met.

Moreover, there are striking regularities in the relationship of structure to activity. In confirmation of the report of Schiffmann et al. (2), the presence of a formyl group on the *N*-terminal methionyl residue enhances activity: F-Met-Leu-Phe is approximately 10,000-fold more active than the corresponding Met-

Leu-Phe; F-Met-Phe-Leu is around 3,000-fold more active than Met-Phe-Leu; and F-Met-Met-Met is approximately 20,000 times more active than Met-Met-Met. Among the dipeptides studied, addition to F-Met of a neutral amino acid, either methionine, leucine, or phenylalanine increased activity to the same extent, approximately 3,000-fold. Among the tripeptides tested, a terminal phenylalanine had the most striking effect. F-Met-Leu-Phe is 5,000 times more active than F-Met-Leu, whereas F-Met-Leu-Leu is only 9 times as active. F-Met-Leu-Glu and F-Met-Leu-Arg are equal to or one-third as active as F-Met-Leu, respectively. This same large effect of terminal phenylalanine is seen in other tripeptides. For example, F-Met-Met-Phe is approximately 3,500–4,000 times more active than the parent dipeptide, F-Met-Met; addition of another methionyl residue to F-Met-Met to give F-Met-Met-Met increases activity only 120- to 150-fold. Addition of an alanine residue is without effect, F-Met-Met-Ala having the same activity as F-Met-Met itself.

As is also seen in Table II, increasing the number of methionyl residues in the peptide chain progressively increases activity at least up to F-Met-Met-Met-Met. The increment in activity tends to be less with each methionine residue added suggesting that if the series were to be continued, maximal activity might be attainable with a peptide containing only five or six methionyl residues. It is not clear at this time whether the effectiveness of the methionine in the series is due to its containing a sulfur atom or a hydrophobic side chain or both.

Schiffmann et al. in their initial work suggested that *N*-acylated methionine is required for chemotactic activity (2). Table II shows that F-Leu-Trp-Met and F-Leu-Trp-Met-Arg are both active, the first with an ED_{50} of 2.5×10^{-8} M and the second with an ED_{50} of 1×10^{-6} M. These are not among the most active peptides obtained but indicate that there is no absolute requirement for an F-Met residue for high activity. Moreover, the 40-fold drop in activity on the addition of basic arginine residue to F-Leu-Trp-Met indicates that the great dependence of activity on structure extends to these peptides as well.

Synthetic Peptides as Inducers of Lysosomal Enzyme Secretion. The peptides listed in Table II were also tested for their ability in the presence of cytochalasin B to induce the secretion of the lysosomal enzymes, β-glucuronidase and lysozyme. Fig. 2, illustrates the results obtained in one experiment in which five peptides were tested for their ability to induce secretion of lysozyme and β-glucuronidase. As already described, the dose-response curves were parallel in their linear portion and showed the same maximum. From such curves, the $ED_{50's}$ and relative activities were calculated. Each peptide was similarly assayed on at least three separate occasions.

Table III gives for the entire group of 24 peptides the mean lysosomal enzyme-releasing activities ± SE relative to the activity of F-Met-Leu, as well as the mean $ED_{50's}$ ± SE. Five peptides, F-Met, Met-Leu-Glu, Met-Phe-Leu, Met-Met-Ala, and Met-Leu-Leu did not induce release of either lysosomal enzyme at concentrations of 1×10^{-3} M or higher.

The structure activity relationships for the release of lysozyme or β-glucuronidase are the same and are also the same as that for chemotaxis. The last is shown in Figs. 3 and 4, where the logarithms of the $ED_{50's}$ for chemotaxis of 19 peptides are plotted against the logarithm of the $ED_{50's}$ for β-glucuronidase (Fig.

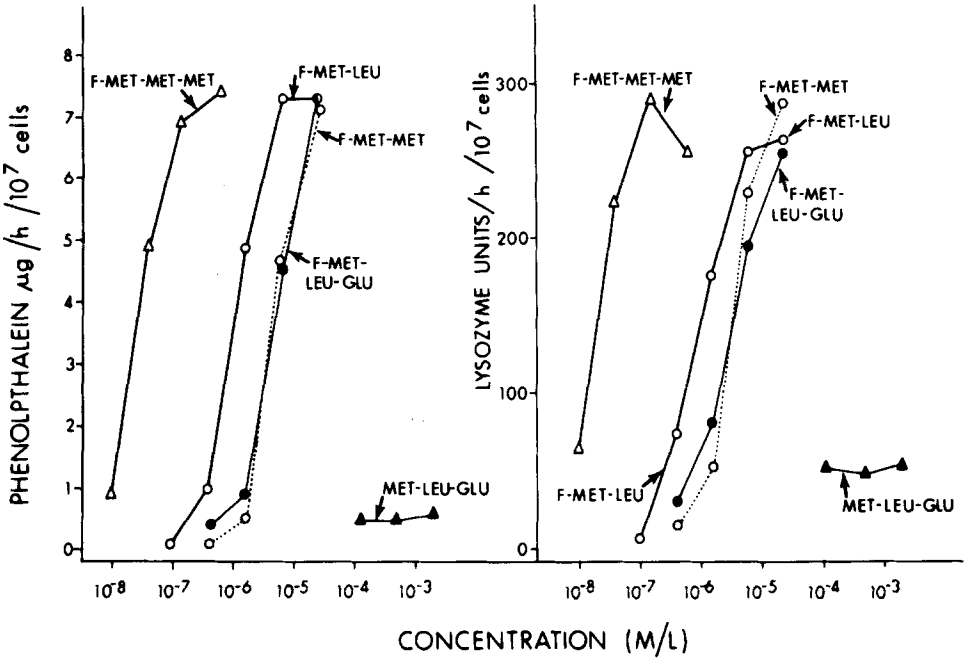

FIG. 2. The assay of the ability of five peptides: F-Met-Met-Met (△—△), F-Met-Leu (○—○), F-Met-Met (○---○), F-Met-Leu-Glu (●—●), and Met-Leu-Glu (▲—▲) to release β-glucuronidase (left-hand frame) and lysozyme (right-hand frame).

3) or lysozyme (Fig. 4) release. The extremely close correlation between chemotactic activity and releasing ability for both enzymes is apparent. The correlation coefficients for chemotaxis and lysozyme secretion and for chemotaxis and β-glucuronidase are both 0.98. These very high correlations extend over a greater than one million-fold range in activity. The linear regression equation relating the logarithm of the ED_{50} for lysozyme release (Y_L) to the logarithm of the ED_{50} for chemotaxis (X) is:

$$Y_L = (0.95 \pm 0.27)\,X + 0.45.$$

The corresponding equation relating the logarithm of the ED_{50} for β-glucuronidase release (Y_G) to that for chemotaxis (X) is:

$$Y_G = (0.97 \pm .30)\,X + 0.67.$$

The slopes of these equations are indistinguishable from 1, indicating that the enzyme-releasing and chemotactic activity of the peptides are linearly proportional to each other.

Table III also shows that higher concentrations of peptide are required to give lysosomal enzyme release than a corresponding chemotactic response. The mean ratio of the ED_{50}'s for enzyme release to the ED_{50} for chemotaxis for all 19 peptides is 7.53 ± 1.17 for lysozyme and 9.29 ± 1.58 for β-glucuronidase. This says that on the average, rabbit neutrophils require approximately 7.5 times

TABLE III
Lysozomal Enzyme-Releasing Activity of Synthetic Peptides

	Lysozyme		β Glucuronidase	
	ED_{50} ± SE	Relative activity* ± SE	ED_{50} ± SE	Relative Activity* ± SE
1. F-Met	$>1 \times 10^{-2}$	>0.00001	$>1 \times 10^{-2}$	>0.00001
2. F-Met-Leu	$1.7 \pm 0.17 \times 10^{-6}$	1	$1.9 \pm 0.21 \times 10^{-6}$	1
3. F-Met-Leu-Phe	$2.4 \pm 0.31 \times 10^{-10}$	5,120 ± 480	$2.6 \pm 0.32 \times 10^{-10}$	4,470 ± 610
4. F-Met-Leu-Glu	$5 \pm 1.3 \times 10^{-6}$	0.24 ± 0.02	$7.2 \pm 0.29 \times 10^{-6}$	0.23 ± 0.02
5. F-Met-Leu-Arg	$2.2 \pm 0.45 \times 10^{-6}$	0.76 ± 0.05	$2.6 \pm 0.7 \times 10^{-6}$	0.72 ± 0.04
6. F-Met-Leu-Leu	$2.8 \pm 0.41 \times 10^{-7}$	6.9 ± 0.88	$4.5 \pm 2.1 \times 10^{-7}$	4.3 ± 0.7
7. F-Met-Phe	$1.5 \pm 0.39 \times 10^{-6}$	0.99 ± 0.08	$2 \pm 0.42 \times 10^{-6}$	0.82 ± 0.06
8. F-Met-Phe-Leu	$3.3 \pm 0.7 \times 10^{-7}$	5.9 ± 1.1	$3.5 \pm 1.6 \times 10^{-7}$	4.6 ± 1
9. F-Met-Phe-Met	$2.6 \pm 0.5 \times 10^{-8}$	92 ± 14.7	$3.5 \pm 0.6 \times 10^{-8}$	77 ± 11
10. F-Met-Met	$2.9 \pm 0.47 \times 10^{-6}$	0.39 ± 0.06	$7.5 \pm 3.5 \times 10^{-6}$	0.28 ± 0.05
11. F-Met-Met-Met	$4.3 \pm 1.4 \times 10^{-8}$	41 ± 2.8	$3.1 \pm 0.63 \times 10^{-8}$	41.9 ± 10.6
12. F-Met-Met-Met-Met	$2.4 \pm 0.34 \times 10^{-9}$	1,080 ± 340	$3 \pm 0.73 \times 10^{-9}$	980 ± 290
13. F-Met-Met-Ala	$1.5 \pm 0.5 \times 10^{-6}$	2.3 ± 0.88	$1.5 \pm 0.45 \times 10^{-6}$	1.4 ± 0.15
14. F-Met-Met-Phe	$1.9 \pm 0.4 \times 10^{-9}$	1,630 ± 260	$1.8 \pm 0.24 \times 10^{-9}$	1,380 ± 230
15. F-Leu-Trp-Met	$5.7 \pm 0.5 \times 10^{-7}$	4.2 ± 1.4	$7.3 \pm 0.75 \times 10^{-7}$	3.6 ± 0.4
16. F-Leu-Trp-Met-Arg	$1.1 \pm 0.17 \times 10^{-5}$	0.16 ± 0.02	$1.9 \pm 0.55 \times 10^{-5}$	0.15 ± 0.03
17. Met-Leu-Phe	$8.9 \pm 1.4 \times 10^{-7}$	1.5 ± 0.11	$9.7 \pm 0.12 \times 10^{-7}$	1.3 ± 0.17
18. Met-Leu-Glu	$>5 \times 10^{-3}$	>0.0005	$>5 \times 10^{-3}$	>0.0005
19. Met-Leu-Leu	$>1 \times 10^{-3}$	>0.0001	$>1 \times 10^{-3}$	>0.0001
20. Met-Phe-Leu	$>1 \times 10^{-3}$	>0.0001	$>1 \times 10^{-3}$	>0.0001
21. Met-Met-Met	$8.8 \pm 4.4 \times 10^{-4}$	0.004 ± 0.001	$7.2 \pm 1.3 \times 10^{-4}$	0.0035 ± 0.001
22. Met-Met-Met-Met	$1.2 \pm 0.19 \times 10^{-5}$	0.2 ± 0.03	$1.7 \pm 0.47 \times 10^{-5}$	0.17 ± 0.03
23. Met-Met-Ala	$>1 \times 10^{-3}$	>0.0001	$>1 \times 10^{-3}$	>0.0001
24. Met-Met-Phe	$8.9 \pm 3.3 \times 10^{-6}$	0.41 ± 0.09	$8 \pm 1.2 \times 10^{-6}$	0.29 ± 0.08

The mean lysozyme release as percent of total lysozyme ± standard deviation was 88 ± 19%; the mean β glucuronidase release as percent of total β-glucuronidase was 53 ± 7.0%. The mean total lysozyme activity ± standard deviation from 10^7 neutrophils was 440 ± 180; the mean total β-glucuronidase activity was 15 ± 3.3 μg phenolphthalein released/h from 1×10^7 neutrophils.

* Relative to F-Met-Leu = 1.0.

more peptide for lysozyme release than for migration enhancement and 9.3 times more for β-glucuronidase release.

As one would expect from the curves of Fig. 3 and inspection of the data of Table III, there is also a close correlation between the ED_{50} for β-glucuronidase release and that for lysozyme. For all peptides, the mean ratio of the ED_{50} for β-glucuronidase to that for lysozyme is 1.26 ± 0.095. In other words, an approximately 25% greater concentration of a given peptide is required to give 50% of maximal β-glucuronidase secretion than is required for 50% of maximal lysozyme release. This difference is statistically significant.

Discussion

Agents may induce cells to move into or through a micropore filter by stimulating random or directed locomotion (chemotaxis) or both. Substances that increase random locomotion but are not chemotactic have been reported by several workers (15–17). Thus, in describing any substance as "chemotactic," on the basis of its ability to stimulate movement in a Boyden chamber system, it becomes essential to provide evidence that, in fact, the agent is capable of inducing directed movement. The data of Table I demonstrate that two of the synthetic peptides studied here, F-Met-Leu-Phe and F-Met-Met-Met, not only increase random movement but also induce a true chemotactic response. More-

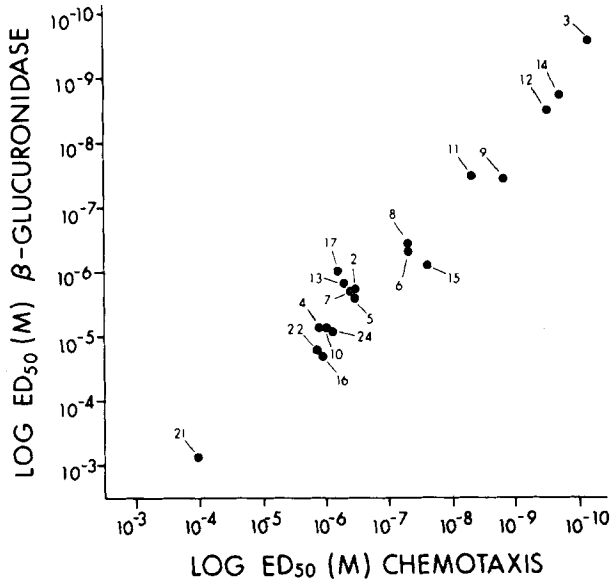

FIG. 3. The relation between the migration inducing (chemotactic) activity of 19 peptides and their ability to induce β-glucuronidase release. F-Met-Leu (2), F-Met-Leu-Phe (3), F-Met-Leu-Glu (4), F-Met-Leu-Arg (5), F-Met-Leu-Leu (6), F-Met-Phe (7), F-Met-Phe-Leu (8), F-Met-Phe-Met (9), F-Met-Met (10), F-Met-Met-Met (11), F-Met-Met-Met-Met (12), F-Met-Met-Ala (13), F-Met-Met-Phe (14), F-Leu-Trp-Met (15), F-Leu-Trp-Met-Arg (16), Met-Leu-Phe (17), Met-Met-Met (21), Met-Met-Met-Met (22), and Met-Met-Phe (24).

over, in unpublished work, Zygmond has confirmed by direct morphologic observation that F-Met-Met-Met is chemotactic. It is reasonable to believe that these two peptides are representative of the other peptides and thus, that at least part of the movement induced by the class of peptides studied here is directed locomotion, i.e., that these peptides are truly chemotactic.

Certain of the peptides investigated are active at very low concentrations. F-Met-Leu-Phe, the most active compound obtained so far, has an ED_{50} for chemotaxis of 7×10^{-11} M, and with suitable cells its activity is detectable at less than 1×10^{-11} M. This can be contrasted with the activity of C5a, one of the more potent natural chemotactic factors which in our hands is active with rabbit neutrophils in the range of 10^{-7}–10^{-8} M. Our chemotactic assay uses 2.5×10^{6} neutrophils in 1 ml. A value of 1×10^{-11} M chemotactic peptide corresponds to approximately 2,400 molecules of peptide per neutrophil. What the concentration would be at the leading edge of the gradient under these circumstances is not known but should be distinctly lower. These calculations not only demonstrate the very great activity of F-Met-Leu-Phe but suggest that for the most active obtainable peptide one molecule per cell might be sufficient. A program of synthesis to obtain such maximally active peptides is currently under way based on the structure-activity relationships revealed in this work.

Not only are the synthetic peptides capable of high activity but the relation of structure to activity is an exceedingly specific one, very small changes in structure making large changes in activity. Moreover, this specificity exhibits a

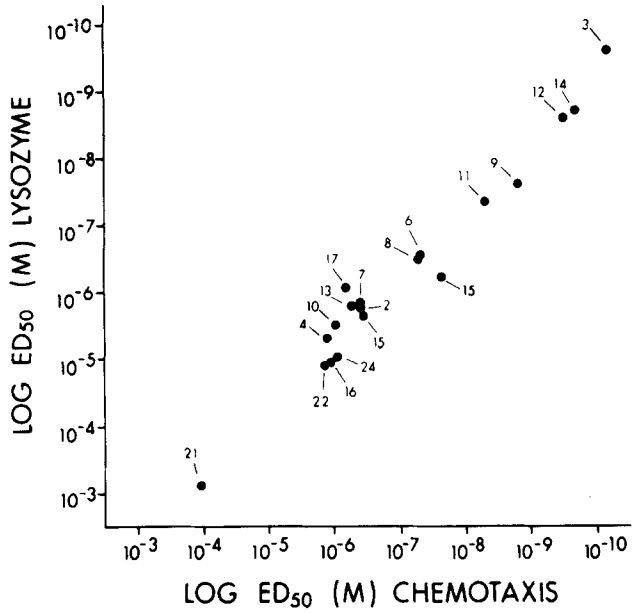

FIG. 4. The relation between the migration-inducing (chemotactic) activity of 19 peptides and their ability to induce lysozyme release. The numbers indicate the same peptides as in Fig. 3.

definite regularity and pattern; the activity of a given peptide depends not only on its constituent amino acids but on the place of these amino acids in the peptide chain. This can be seen by considering the requirements for maximum chemotactic activity of a tripeptide of the general structure, $A_1A_2A_3$ as revealed by this study.

From the work of Schiffmann et al. (2) and the work reported here, it is clear that for maximum activity the positively charged terminal amino group must be neutralized. Moreover, the previous finding that *N*-formyl methionyl peptides are distinctly more active than *N*-acetyl methionyl peptides (2) suggests that decreasing the bulk of the group acylating the *N*-terminal amino group increases activity. This in turn suggests that deletion of the *N*-terminal amino group might increase activity still more. This suggestion is in process of being tested.

As mentioned, methionine in the NH_2-terminal position has been considered to be required for activity (2). As shown here, F-Leu-Trp-Met has reasonably high activity (ED_{50}, 2.5×10^{-8} M) indicating that there is no absolute requirement for methionine in this position. Moreover, F-Leu-Trp-Met and F-Met-Phe-Leu have essentially the same activity, the ED_{50}'s being 2.5×10^{-8} M and 5.4×10^{-8} M, respectively. If one considers that phenylalanine and tryptophan in the second position are equivalent by virtue of their hydrophobic character (see below), then the findings just alluded to suggest that it is the nonpolar character of methionine which is of importance in the NH_2-terminal position. This is given some credence by the prior finding that F-leucine is chemotactic although not as active as F-methionine (2). If this suggestion is correct, then F-norleucine might

serve as well or almost as well as F-methionine. The test of this idea is now under way.

The dipeptides F-Met-Leu, F-Met-Phe, and F-Met-Met all have essentially the same activity (Table II). The positively charged F-Met-His or the negatively charged F-Met-Glu were found to be distinctly less active than F-Met-Leu (2). These findings suggest that in the second position of the peptide chain, a neutral amino acid with a sufficiently nonpolar side chain is required for maximal activity.

Although any neutral, sufficiently nonpolar amino acid seems to confer maximum activity when in the second position, this does not seem to be the case for amino acids in the third position. In F-methionyl tripeptides, a terminal phenylalanine, seems to be particularly and peculiarly effective and this capacity does not seem to reside wholly in its hydrophobic character. F-Met-Leu-Phe is 5,000 times more active than F-Met-Leu, whereas F-Met-Leu-Leu with a terminal nonpolar amino acid residue is only 9 times more active. Similarly, F-Met-Met-Phe is 3,400–4,400 times more active than F-Met-Met, whereas adding another methionyl residue to form F-Met-Met Met increases activity only 125 times and the activity of F-Met-Met-Ala is essentially the same as F-Met-Met. (Table I). In these tripeptides, if only the hydrophobic character of the terminal phenylalanine was the cause of their greatly increased activity one would expect F-Met-Leu-Leu and F-Met-Met-Met with their terminal hydrophobic side chains to approach the activity of F-Met-Leu-Phe and F-Met-Met-Phe; instead, they are 30–500 times less active.

Moreover, the striking effect of phenylalanine is seen only when it is in the third position in the peptide chain; in the second position, it contributes nothing more to the activity than do the other neutral nonpolar amino acids studied. This is evident when phenylalanine is the terminal amino acid of a dipeptide, F-Met-Phe having the same activity as F-Met-Leu and F-Met-Met. It is also evident when phenylalanine is present in the second position of a tripeptide; F-Met-Phe-Leu and F-Met-Phe-Met are 500 and 10 times less active, respectively, than F-Met-Leu-Phe and F-Met-Met-Phe, the corresponding tripeptides with phenylalanine in the third position.

It is not known whether this specific effect of phenylalanine in the terminal position of the tripeptide is due to its aromaticity or to its stereospecificity. Also it is not known whether the high activity of phenylalanine in the tripeptides is due to its being in the third position from the NH_2-terminal end or because it is terminal.

The findings just discussed are most simply explained and strongly support the hypothesis that the primary interaction of synthetic peptide and neutrophil leading to either chemotaxis or lysosomal enzyme release is the binding of the peptide to a stereospecific receptor on the neutrophil surface. This hypothesis is in accord with the great specificity of the structural requirements for activity and, even more, the manner in which the activity depends not only on the amino acids present but also on their position in the peptide chain. The very low concentrations, 10^{-10}–10^{-11} M at which the most highly active peptides work is compatible with an extremely firm binding of these agents to the putative neutrophil receptor, i.e., to the receptor-peptide association constant being quite

high. The evidence for a stereospecific receptor is indirect, but the work presented here forms the basis for obtaining the requisite direct evidence.

The suggestion has been made that nonpolar side chains are of key importance in chemotactic recognition, there being no particular requirement for stereospecific binding (1). The importance of nonpolar side chains is seen in the requirement for acylation of the NH_2-terminal group and for amino acids with a hydrophobic side chain in the first and in the second position of the peptide chain. However, other results with the chemotactic peptides are not in agreement with the hypothesis that only hydrophobicity is of importance. In the tripeptides studied here a terminal phenylalanine contributes more to the activity than can be described to its nonpolar character; in addition, the quantitative nature of the contribution of phenylalanine depends on its position in the peptide chain demonstrating that more is required for activity in these peptides than hydrophobicity. Moreover, if it were merely a matter of hydrophobicity there should be little or no difference in activity between *N*-formyl and *N*-acetyl methionyl peptides. On this same basis, one would expect F-Met-Phe-Leu to be as active as F-Met-Leu-Phe and F-Met-Phe-Met to be as active as F-Met-Met-Phe instead of them being, respectively, 500 and 10 times more active.

A wide variety of agents are reported to be chemotactic, proteolytic enzymes, proteins, denatured proteins, polypeptides derived from complement (1), cyclic AMP, (19–21), prostaglandins (22), and oxidized fatty acids (23). The chemotactic activity of some of these agents has been disputed (24) and for some, e.g. oxidized fatty acids, there is no present evidence of the type adduced in this paper, that they are truly chemotactic. Thus, although our work clearly suggests the existence of a stereospecific receptor for the peptides studied here it does not preclude the possibilities that there are other receptors for other agents or, as suggested by several authors (1, 18), that some of these agents may not require a stereospecific binding to the neutrophil surface. The results of this study provide the means to decide among these and other possibilities.

Wissler et al. working with a chemotactic binary peptide system have suggested that cellular recognition of chemotactic factors depends on the energy change as the factor goes from one conformation to another (24). The active peptides studied here are probably too small to have any large preferred conformation in solution suggesting either that the concept of Wissler et al. cannot be generally applicable or that the putative conformational changes must occur after the peptide has bound to the surface.

As pointed out in the introduction, previous work has shown that the same substance can induce both chemotaxis and lysosomal enzyme secretion from neutrophils (3–6). The present work demonstrates an essentially exact correlation between the chemotactic activity of these synthetic peptides and their ability to induce lysosomal enzyme release over a more than one million-fold range in activities (Figs. 3 and 4). This implies that these two neutrophil functions are triggered by the same primary interaction; possibly, the binding of the peptides to the same putative neutrophil receptor. Both lysosomal enzyme release and chemotaxis are the result of long sequences of biochemical reactions, some parts of which must be different. This work although suggesting that the initial interaction is the same for chemotaxis and lysosomal enzyme secretion

throws no light on how far beyond this primary reaction, if at all, the two functions share a common sequence.

There is also a close correlation between the release of lysozyme and the release of β-glucuronidase, implying that the same initial interaction of cell and peptide suffices to induce release of both enzymes. Approximately one-third of the lysozyme of rabbit neutrophils comes from primary granules, and the remainder from secondary granules (25). In many experiments, the peptides and cytochalasin B induced the release of almost 100% of the lysozyme, indicating that these peptides are capable of inducing release of this enzyme as well or almost as well, from primary as from secondary granules. Approximately 25% more peptide is required to release 50% of the total β-glucuronidase than the same proportion of lysozyme, indicating that the release from the two types of granules is not rigidly coupled. This is seen more clearly in other experimental circumstances; the ratio of the maximum percentage of these same two enzymes released by bacterial chemotactic factor from rabbit neutrophils on filters in the absence of cytochalasin B can vary over a 20-fold range (reference 5; and H. J. Showell and E. L. Becker, unpublished observations) and Goldstein et al. have reported that Ca^{2+} can release a high proportion of lysozyme from human neutrophils with little or no release of β-glucuronidase (26).

In addition to chemotaxis and lysosomal enzyme release, a third function of the neutrophil is phagocytosis. Chemotactic agents, including F-Met-Leu, are able to inhibit the initial rates of erythrophagocytosis by neutrophils in what appears to be a competitive fashion (27). There is also the unpublished evidence of Henson and Oades that C5a when on a particle enhances phagocytosis (28). One hypothesis is that the same initial interaction of chemotactic agent with cell is not only able to induce chemotaxis and lysosomal enzyme secretion but trigger phagocytosis as well. The availability of the series of synthetic peptides allows us to test this hypothesis. This test is now under way.

Summary

24 di-, tri-, and tetrapeptides have been synthesized as a start of a systematic study of the structural requirements for chemotactic activity and lysosomal enzyme-releasing ability in rabbit neutrophils. All but two of them are *N*-formyl methionyl peptides.

Using the method of Zigmond and Hirsch (10), two representative peptides, F-Met-Leu-Phe and F-Met-Met-Met, were shown to stimulate directed, as well as, random locomotion; thus, they were truly chemotactic. The various peptides showed a wide spread in activity. F-Met-Leu-Phe, the most active peptide studied, had an ED_{50} for induced migration of 7×10^{-11} M and for lysozyme and β-glucuronidase release of 2.4×10^{-10} M and 2.6×10^{-10} M, respectively; the least active, Met-Leu-Glu was 26 million times less active in these respects. The relation of activity to structure is exceedingly specific, very small changes in structure making large changes in activity. Moreover, this specificity exhibits a definite regularity and pattern; the activity of a given peptide depends not only on its constituent amino acids but on the position of the amino acid in the peptide chain. Most striking in this last regard is the high activity conferred by phenylalanine when it is in the carboxyl terminal position of a tripeptide,

whereas, as the second amino acid from the NH_2 terminal end whether in a tripeptide or a dipeptide, it contributes no more to the activity than other amino acids with hydrophobic side chains such as leucine or methionine.

The high activity and the specificity and nature of the structural requirements strongly suggest that the primary interaction of peptide and neutrophil leading to either chemotaxis or lysosomal enzyme release is a binding of the peptide with a stereospecific receptor on the neutrophil surface. Whether all chemotactic factors act through the same receptor is not known.

An essentially exact correlation exists between the concentrations of the various synthetic peptides required to induce migration and their ability to induce release of lysozyme or β-glucuronidase. This implies that these two neutrophil functions are triggered by the same primary interaction; possibly, the binding of the peptides to the same putative receptor. A higher concentration of a given peptide is required to stimulate lysosomal enzyme release than a corresponding migratory response. A slightly but significantly higher concentration of peptide is required to induce β-glucuronidase secretion than lysozyme release.

We wish to thank Mr. Mark Sigman and Mr. Jeff Radding for their expert technical assistance and Mr. Robert Musson for devising a program for calculating predicted distances of stimulated random locomotion in the method of Zigmond and Hirsch.

Received for publication 20 January 1976.

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