0022-1767/82/1282-0956$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 128, No. 2, February 1982
Printed in U.S.A.

ANTI-f Met-Leu-Phe: SIMILARITIES IN FINE SPECIFICITY WITH THE FORMYL PEPTIDE CHEMOTAXIS RECEPTOR OF THE NEUTROPHIL[1]

WAYNE A. MARASCO,[2] HENRY J. SHOWELL, RICHARD J. FREER, AND ELMER L. BECKER

From the Department of Pathology, University of Connecticut Health Center, Farmington, CT 06032, and the Department of Pharmacology, Medical College of Virginia, Richmond, VA 23219

We have prepared antisera in both rabbits and rats against f Met-Leu-Phe conjugated to a variety of carrier proteins. Over 40 peptides with widely varying reactivity for the neutrophil formylpeptide receptor have been tested for their ability to bind to rabbit antibody raised against $fMLP_{10}$-BSA. Structure-activity studies of peptides structurally related to f Met-Leu-Phe demonstrate that the *N*-formyl group is mandatory for maximum antibody binding activity. Methionine in position 1 and phenylalanine in position 3 are found to confer maximum binding activity. Stereoselectivity of the antibody-combining site also has been demonstrated. Comparison of the ability of the peptides to bind to the antibody receptor with their reactivity for the neutrophil has demonstrated a strong correlation in the rank order of reactivity of the numerous synthetic peptides: for the αNH_2-acyl group, $r = 0.94$; for position 1, $r = 0.90$; for position 2, $r = 0.97$; and for position 3, $r = 0.78$. This strong correlation is seen across species lines with both rabbit and rat antibodies. Significant differences, however, in the specificity of the antibody and neutrophil receptors are seen at the carboxyl terminus of phenylalanine, and beyond the phenylalanine ring, $r = 0.29$. In addition, bacterial chemotactic factor-enriched butanol extracts from *Escherichia coli* culture filtrates can also bind to anti-f Met-Leu-Phe, affording additional evidence for the similarity in the structure of the bacterial chemotactic factor to the synthetic chemotactic peptides.

The demonstration by Schiffmann *et al.* (1) that simple formylmethionyl peptides are potent chemoattractants for polymorphonuclear leukocytes and macrophages stimulated a number of investigations aimed at identifying and characterizing a receptor on the plasma membrane of these cells for the oligoformylpeptides. The systematic studies by Showell *et al.* (2) and Freer *et al.* (3) on the structure activity relationships of various synthetic peptides as chemotactic factors and inducers of lysosomal enzyme secretion suggested the presence of a stereoselective receptor on the rabbit neutrophil surface. The synthesis of radiolabeled formyl peptide attractants has since allowed a specific receptor to be demonstrated on rabbit (4), human (5), guinea pig (6), horse (7), and rat (Marasco, W. A., J. C. Fantone, R. J. Freer, and P. A. Ward, manuscript submitted for publication) neutrophils as well as guinea pig macrophages (8). Other work with rabbit (9) and human (10) neutrophils has shown that these receptors are, in fact, on the plasma membrane. Furthermore, a partially purified, low m.w. attractant obtained from *Escherichia coli* culture medium competed for the binding of radiolabeled peptide, suggesting that the synthetic peptides and the naturally occurring bacterial products react with the same receptor (4).

The study of the biochemical characteristics of this receptor as well as production of antibodies to it have been difficult because of the inability to obtain soluble receptor preparations that retain binding affinity for the formyl peptides. In addition, until recently, an abundant and easily obtainable source of receptors has not been available (11–13). However, antibodies against the formyl peptide receptor would be useful, as have other anti-receptor antibodies (14–17), in elucidating the structure and function of the receptor as well as the biochemical events that follow the receptor-peptide interaction (18).

It was the purpose of this investigation to produce antibodies against the formyl peptide chemotaxis receptor of the neutrophil. This has been attempted by first raising antibodies to the potent neutrophil chemoattractant formylmethionyl-leucyl-phenylalanine (f Met-Leu-Phe).[3] The affinity column-purified antibody (idiotype) has then been used as immunogen in an attempt to produce anti-idiotypic antibodies that will cross react with the neutrophil formyl peptide receptor. The success of this endeavor was dependent on at least two possibilities. First, one or more ligand recognition structures or conformations in the anti-f Met-Leu-Phe combining site is sufficiently close in conformation to the binding sites in the neutrophil receptor that anti-idiotype raised against the former would react against the latter. Second, immunization with anti-f Met-Leu-Phe could elicit a subpopulation of anti-idiotypic antibodies that would, by virtue of their "internal image" of f Met-Leu-Phe, bind to both receptor sites. The binding of this population of anti-idiotypic antibodies occurs independently of the idiotypic character of the anti-f Met-Leu-Phe receptor and thus can bind to any receptor capable of binding f Met-Leu-Phe.

In this communication, we report on an extensive immunochemical analysis of the specificity of antibodies raised to the chemotactic peptide, f Met-Leu-Phe, that is designed to test these possibilities. In addition, we will demonstrate by both

Received for publication July 29, 1981.
Accepted for publication November 2, 1981.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported in part by Grant AI-90648 from the United States Public Health Service. Wayne A. Marasco was supported in part by Training Grant T32-A10780 from the United States Public Health Service.
[2] This work was done in partial fulfillment of the requirements for the Ph.D. Present address: Department of Pathology, The University of Michigan, Box M045, Pathology Bldg., 1335 E. Catherine St., Ann Arbor, MI 48109.

[3] Abbreviations used in this paper: $fMLP_x$-carrier protein conjugate, where x refers to the mean number of fMLP molecules bound per molecule of carrier protein (see *Materials and Methods*); f Met-Leu-Phe, fMLP, formylmethionyl-leucyl-phenylalanine; CBZ, carbobenzoxy; Cys(Me), methyl cysteine; Hep, 2-aminoheptanoic acid; Abu, 2-aminobutyric acid; Nva, norvaline; Nle, norleucine; Eth, ethionine; BzA, benzylamide; CMK, chloromethyl ketone; t-Boc, tert-butoxy; Pea, phenethylamine; OBzl, benzyl ester; KLH, keyhole limpet hemocyanin.

radioimmunoassay and bioassay that anti-f Met-Leu-Phe binds and removes the biologically active component of chemotactic factor-enriched extracts from *Escherichia coli* culture filtrates.

MATERIALS AND METHODS

Oligopeptides. The production and biologic activity of most of these oligopeptides have been reported previously (2, 3, 19). Dr. Elliot Schiffmann (WIDR, Bethesda, MD) generously gave us the f Met-(D)Leu-Phe; f-Nle-Leu-Phe-Nle-Tyr-Lys was a gift from Dr. James Niedel (Duke University). All amino acids are of the (L) configuration unless otherwise mentioned.

Preparation of conjugates. The tripeptide f Met-Leu-Phe (Sigma Chemical Company, St. Louis, MO) was covalently bound to various carrier proteins by using the carbodiimide reaction (20). In brief, f Met-Leu-Phe dissolved in 25 μl of dimethyl sulfoxide (DMSO) was added in a 100-mol excess to a solution of carrier protein in H_2O to which was added 88 to 176 pmol of f Met-Leu-(^{3}H)Phe (56.9 Ci/mmol) New England Nuclear, Boston, MA, as a tracer. The solution of coupling reagent 1-ethyl-3-(3-diethylaminopropyl)-carbodiimide HCl (Sigma Chemical Co.) was added to a total concentration of 200 mg in a total reaction volume of 2 to 5 ml. The pH was found to be critical for covalent coupling and was therefore monitored during the first 2 hr of the coupling procedure. Conjugation performed at neutral pH was more effective than when started at acid pH (4.5). Furthermore, when coupling was started in the acid pH range, f Met-Leu-Phe was often bound, but only a small percentage of these counts were precipitable with 5% trichloroacetic acid (TCA) at 4°C. Thus, f Met-Leu-Phe can be strongly absorbed to a variety of proteins to such an extent that 48-hr dialysis at 4°C with three changes of 2 liters each of borate-saline buffer (160 mM H_3BO_3, 140 mM NaCl, 22 mM NaOH) does not remove unbound fMLP. The percentage of f Met-Leu-Phe bound was therefore determined by TCA precipitability at 4°C. In addition to pH, the molar excess of f Met-Leu-Phe, reaction volume, and protein and carbodiimide concentrations all affect the efficiency of conjugation (data not shown).

Bovine serum albumin, rat albumin, rabbit albumin, and human transferrin were obtained through Sigma Chemical Co. Goat IgG was purified by passage over DEAE followed by gel filtration over Sephacryl S-200. Keyhole limpet hemocyanin (KLH) was obtained from Calbiochem Corp., San Diego, CA. The proteins have all been successfully conjugated to f Met-Leu-Phe, with the moles of fMLP bound being roughly proportional to the m.w. of the carrier protein. The molar ratio of f Met-Leu-Phe bound ranged from 5 for rat albumin to 147 for KLH under the reaction conditions given.

Immunization. Four New Zealand rabbits were each immunized with one of the following immunogens—$fMLP_{10}$-BSA,[3] $fMLP_{12}$-Goat IgG, $fMLP_{147}$-KLH, or $fMLP_8$-rabbit albumin—by approximately 20 intradermal injections of antigen (total protein 500 μg) along the back emulsified in an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, MI). The animals were injected in the same way with antigen in incomplete Freund's adjuvant at two biweekly intervals. Starting 1 wk later, rabbits were bled weekly for 4 wk. Six months after the initial immunization, all rabbits were boosted with the same amount of protein in Freund's incomplete adjuvant and bled 3 wk later. The antiserum we report on ($FMLP_{10}$-BSA) is from this last bleed.

Five DAL (F_1-DA x Lewis) rats were immunized in the same fashion with 500 μg $fMLP_{10}$-BSA in complete Freund's adjuvant by multiple intradermal injections along the back and in the hind footpads. This was repeated after 2 wk with incomplete Freund's adjuvant.

Radioimmunoassay. All experiments were performed in 12 x 75 mm Falcon 2052 tubes, Falcon Plastics Co., Oxnard, CA. Equal volumes of cold ligand or buffer, f Met-Leu(^{3}H)Phe, and antiserum (150 μl final volume) were reacted at room temperature for 30 min before cold (4°C) saturated ammonium sulfate was added to a final concentration of 50% saturation. After 1 hr incubation at 4°C, the reaction tubes were centrifuged at 3000 rpm for 30 min, and 25 μl of supernatant were counted in a Beckman scintillation counter. The concentration of antiserum used in the final radioimmunoassay was chosen by testing the ability of dilutions of antiserum in PBS (2.14 mM NaH_2PO_4 H_2O; 9.15 mM Na_2HPO_4; 268 mM NaCl) to bind 0.88 pmol f Met-Leu-(^{3}H)Phe. The dilution of antiserum was employed that bound 70 to 80% of the ligand. Comparative binding experiments utilized the chosen concentration of antiserum, a constant amount of labeled ligand (0.88 pmol), and variable concentrations of nonradiolabeled f Met-Leu-Phe or various other chemotactic oligopeptides. The ID_{50}, the concentration of nonradiolabeled ligand that gave 50% inhibition of specific f Met-Leu-(^{3}H)Phe binding, was used as the comparative index of binding activity. In this fashion, the various chemotactic oligopeptides could be directly compared with f Met-Leu-Phe in terms of rank order binding reactivity.

Biologic activity of fMLP conjugates. Chemotaxis was performed on rabbit peritoneal exudate cells using the modified Boyden chamber developed by Swanson (21), employing a cell concentration of 2 x 10^6 cells/ml suspended in complete Hanks' medium with 1 mg/ml BSA. Each conjugate was also tested for its ability to induce release of the lysosomal enzymes, B-glucuronidase, and lysozyme from cytochalasin B-treated rabbit neutrophils as described previously (2, 3).

Preparation of protein A-Sepharose CL4B immunoadsorbants. In the experiments testing the ability of anti-f Met-Leu-Phe to bind bacterial chemotactic factor, 150 mg of protein A-Sepharose CL4B (Sigma Chemical Co.) were washed once with 250 ml 1 mM HCl and three times with 200 ml each of borate-saline buffer. One milliliter of normal rabbit serum or rabbit anti-f Met-Leu-Phe serum previously dialyzed against borate-saline buffer was then added and incubated at 37°C for 1 hr. This amount of serum was more than sufficient to saturate the protein A-binding capacity for rabbit IgG. The Sepharose was then repeatedly washed with a total volume of 1 liter borate-saline buffer. After this procedure, no detectable protein could be assayed in the supernatant fluid of the Sepharose, which was resuspended in 1 ml and then allowed to settle. Fifty microliters of the packed Sepharose were then carefully aliquoted, appropriately diluted in suspension, and incubated at room temperature for 30 min with 50 μl buffer and 50 μl of either 0.88 pmol f Met-Leu-(^{3}H)Phe or 1:1800 to 1:3000 final dilutions of bacterial chemotactic factor. (The ED_{50} for lysozyme release of the bacterial factor was 1:3500.) The mixtures were centrifuged at 3000 rpm for 15 min, and aliquots of the supernatant were tested for their ability to induce enzyme release. An aliquot was also taken from the f Met-Leu(^{3}H)Phe-Sepharose mixture and counted to assay loss of radiolabel from the supernatant.

RESULTS

Biologic activity of fMLP conjugates. Figure 1 shows that both fMLP-BSA and fMLP-human transferrin in the presence of cytochalasin B caused lysozyme release; the carrier proteins, however, in the same concentration range had no effect (data not shown). As is evident, these conjugates are less active on a molar basis by approximately fivefold than the f Met-Leu-Phe itself. In addition, although not shown here, all of the conjugates demonstrated chemotactic activity, in agreement with previous reports (20, 22).

Characterization of anti-fMLP antiserum. Each rabbit immunized with a different FMLP conjugate produced anti-f Met-Leu-Phe antibody. The titer of antiserum varied significantly with each rabbit and was in the rank order anti-$fMLP_{10}$-BSA > anti-$fMLP_{147}$-KLH > anti-$fMLP_{12}$ goat IgG > anti-$fMLP_5$-rabbit albumin. Because $fMLP_{10}$-BSA consistently generated the highest titer anti-fMLP binding activity, this antiserum was used most extensively for the analysis of antibody-combining site

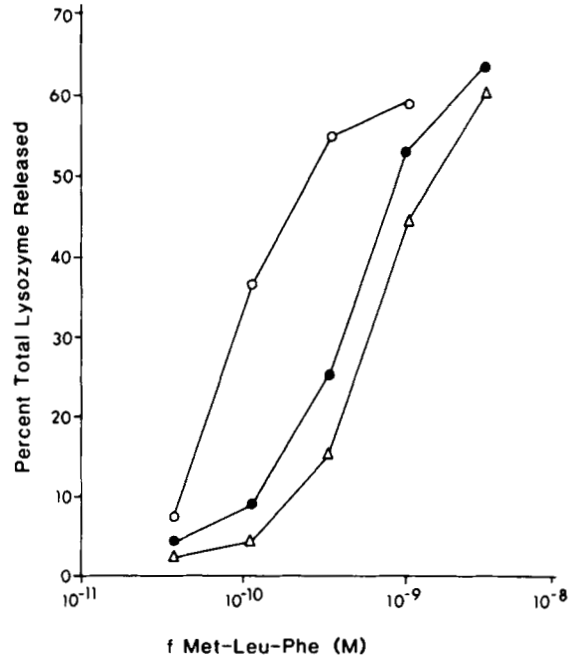

Figure 1. Comparison of the ability of BSA-fMLP (●) or transferrin-fMLP (△) conjugates and free fMLP (○) to induce lysozyme release from rabbit peritoneal exudate cells. The conjugates BSA-fMLP and transferrin-fMLP covalently bound 9.8 and 2.4 mols fMLP per mol carrier protein, respectively, as determined by TCA precipitability. The results given are the mean of triplicate determinations.

specificity. Scatchard and Sips analysis of a 1:15 dilution of the fMLP-BSA antiserum yielded an equilibrium constant $K_0 = 7.9 \times 10^7\ M^{-1}$ with an index of heterogeneity a = 0.98 (23, 24). Specific antibody concentration of different bleeds varied from 100 to 500 μg/ml (24). Similar results were obtained with rat anti-fMLP (data not shown).

Comparison of the effects of modification in the structure of f Met-Leu-Phe on binding to Anti-f Met-Leu-Phe. As pointed out, structure-activity studies on the neutrophil formyl peptide receptor indicate that a nearly perfect correlation exists between binding activity and biologic activity such that biologic activity of peptides is directly proportional to their binding (2–5, 18). Because the ED_{50} values for lysozyme release are uniformly available, we have chosen to use them as the measure of peptide binding to the neutrophil receptor in comparing the rank order of peptide binding to the neutrophil formyl peptide receptor and to the antibody-combining site.

Modification of α-NH_2 group. Previous work has demonstrated the unique role of N-formylation in enhancing biologic and binding activity with the neutrophil. As can be seen in Table I, removal of the formyl moiety, as in Met-Leu-Phe, or of the α amino group, as in desamino-Met-Leu-Phe, decreased antibody binding 7000-fold. Acetylation of the tripeptide (acetyl-Met-Leu-Phe), did not substitute for formylation but resulted in a 700-fold decrease in binding. The lessened decrease in binding of the last compound may be due to a partial recognition of the carbonyl moiety by the antibody. The highest concentration of the 2-ethyl-hexanoyl derivative tested (10^{-4} M) gave no detectable binding, indicating that the steric bulk of the formyl group cannot explain its critical importance, because replacement of the CHO-NH by CH_3-CH_2 caused a complete loss of binding activity at the concentrations tested. The correlation coefficient, r, comparing the antibody-binding capacity of five peptides modified in the α-NH_2 group with their ability to induce lysozyme release is 0.94. Thus these data suggest that there is some unique property of the formyl group responsible for its dramatic effect on both receptors.

Modifications of the side chain in position 1. Varying the length of both aliphatic and sulfur containing straight side chains in the first position from the α-amino terminal end results in a 700 fold range of binding activities (Table II). The aliphatic derivatives constantly increase in binding activity from f Gly-Leu-Phe (zero carbons in side chain) to f Hep-Leu-Phe (five carbons). The peptides with sulfur-containing side chains show a slightly different pattern. For example, the distance of the bulky sulfur group from the peptide backbone is important because appreciable loss of activity for the antibody is seen in going from methionine (-CH_2CH_2-S-CH_3) to methyl cysteine (-CH_2-S-CH_3). However, the antibody-combining site does not distinguish between methionine and ethionine in position 1. If one compares the aliphatic to the corresponding isosteric sulfur-containing analogues, one sees that f Cys(Me)-Leu-Phe shows the same binding characteristics as the norvaline (Nva) derivative in which the carbon is substituted for the sulfur. However, f Met-Leu-Phe and f Eth-Leu-Phe bind antibody three to four times better than their aliphatic counterparts.

Both derivatives with branched four-carbon side chains, f Leu-Leu-Phe and f Ile-Leu-Phe, show approximately equal reactivity, but they are less active than the corresponding straight chain, aliphatic or sulfur-containing derivatives. The number of carbons in the branched group seems more important than either β or γ branching, because the β branched four-carbon side chain Ile and γ branched Leu derivatives are more active than the three-carbon β branched Val in f Val-Leu-Phe. However, despite some subtle differences, the high correlation coefficient, r = 0.90, given by the 13 peptides tested indicates there are overall similarities in structure in this part of the antibody and neutrophil receptor site.

Side chain modification in position 2 of benzyl esters (OBzl) and free acids. By using a limited number of derivatives, variations in the amino acid in position 2 again show an effect of the length of the straight alkyl chain on antibody binding (Table III). In the benzyl ester derivatives of f Met-Leu-Phe, the highest concentration of f Met-Gly-Phe-OBzl that was soluble (10^{-4} M), gave no detectable binding. Lengthening the aliphatic side chain on the second position amino acid from one to three carbons constantly increased activity, the same effect seen with position 1 of the tripeptide. The sulfur-containing methyl cysteine (Cys(Me)) in f Met-Cys(Me)-Phe-OBzl showed binding activity nearly identical to its aliphatic analog f Met-Nva-Phe-OBzl.

Of the available branched chain derivatives (see *Materials and Methods*), f Met-Leu-Phe-OBzl is the most active, with f

TABLE I

Effect of modification in the α NH_2-group of the tripeptide, Met-Leu-Phe, on binding to anti-f Met-Leu-Phe

Peptide	Binding Inhibition ID_{50}[a] ± SEM[b]
f Met-Leu-Phe	$7.4 \pm 0.4 \times 10^{-8}$
Acetyl-Met-Leu-Phe	$5.0 \pm 0.43 \times 10^{-5}$
Met-Leu-Phe	$3.0 \pm 0.64 \times 10^{-4}$
Desamine-Met-Leu-Phe	$\gg 1.0 \times 10^{-4}$
CBZ-Met-Leu-Phe	$3.0 \pm 0.46 \times 10^{-4}$

[a] ID_{50} = concentration required to displace 50% of specifically bound f Met-Leu-(^{3}H)Phe.
[b] Values are the mean of triplicate determinations ± SEM.

TABLE II

Effects of modifications in position 1 of the tripeptide, f Met-Leu-Phe, on binding to anti-f Met-Leu-Phe

Peptide	Binding Inhibition ID_{50}[a] ± SEM[b] (M)
f Gly-Leu-Phe	$4.2 \pm 0.25 \times 10^{-5}$
f Ala-Leu-Phe	$7.3 \pm 1.14 \times 10^{-6}$
f Abu-Leu-Phe	$2.4 \pm 0.39 \times 10^{-6}$
f Nva-Leu-Phe	$5.8 \pm 0.41 \times 10^{-7}$
f Nle-Leu-Phe	$3.0 \pm 0.29 \times 10^{-7}$
f Hep-Leu-Phe	$2.0 \pm 0.17 \times 10^{-7}$
f Cys(Me)-Leu-Phe	$5.8 \pm 0.98 \times 10^{-7}$
f Met-Leu-Phe	$7.2 \pm 0.39 \times 10^{-8}$
f Eth-Leu-Phe	$6.5 \pm 0.49 \times 10^{-8}$
f Ile-Leu-Phe	$7.5 \pm 0.45 \times 10^{-7}$
f Leu-Leu-Phe	$9.0 \pm 0.6 \times 10^{-7}$
f Val-Leu-Phe	$1.9 \pm 0.2 \times 10^{-6}$
f Phe-Leu-Phe	$5.18 \pm 0.24 \times 10^{-6}$

[a] ID_{50} = concentration required to displace 50% of specifically bound f Met-Leu-(^{3}H)Phe-OH.
[b] Values are the mean of triplicate determinations ± SEM.

TABLE III

Effects of modifications in position 2 of benzyl esters and the free acids of the tripeptide, f Met-Leu-Phe, on binding to anti-f Met-Leu-Phe

Peptide	Binding Inhibition ID_{50}[a] ± SEM[b] (M)
f Met-Gly-Phe-OBzl	$\gg 10^{-4}$
f Met-Ala-Phe-OBzl	$1.5 \pm 0.43 \times 10^{-5}$
f Met-Abu-Phe-Obzl	$2.0 \pm 1.0 \times 10^{-6}$
f Met-Nva-Phe-OBzl	$1.3 \pm 0.4 \times 10^{-7}$
f Met-Val-Phe-OBzl	$\gg 10^{-4}$
f Met-Ile-Phe-OBzl	$1.3 \pm 0.2 \times 10^{-5}$
f Met-Pro-Phe-OBzl	$\gg 10^{-4}$
f Met-Cys(Me)-Phe-OBzl	$2.6 \pm 1.0 \times 10^{-7}$
f Met-Abu-Phe OH	$7.0 \pm 1.6 \times 10^{-6}$
f Met(D)Leu-Phe-OH	$4.4 \pm 0.02 \times 10^{-5}$
f Met-Met-Phe-OH	$3.4 \pm 0.43 \times 10^{-6}$
f Met-Leu-Phe-OH	$7.2 \pm 0.39 \times 10^{-8}$

[a] ID_{50} = concentration required to displace 50% of specifically bound f Met-Leu-(^{3}H)Phe-OH.
[b] Values are the mean of triplicate determinations ± SEM.

Met-Ile-Phe-OBzl being much less active. The different effect of these two amino acids in position 2 is in contrast to the similarity of their effect when in position 1 and suggests that β-branching is important in position 2 but not in position 1. In line with this latter suggestion, the β-branched derivative f Met-Val-Phe-OBzl showed undetectable binding at the concentrations tested.

The stereoselectivity of the antibody-combining site was demonstrated by comparing the activity of f (L)Met-(D)Leu-(L)Phe with f (L)Met-(L)Leu-(L)Phe. The (D)Leu derivative was found to be 600-fold less active than the immunogen f (L)Met-(L)Leu-(L)Phe.

Substituting proline, a nonpolar amino acid known to disrupt α-helix formation, in position 2 resulted in undetectable binding, suggesting that position 2 may be of major importance in the aligning of the formyl methionyl group in the receptor site. Finally, f Met-Abu-Phe-OBzl was found to be threefold more active than the corresponding free acid, thus directly demonstrating an effect of the benzyl ester moiety on binding activity.

The correlation analysis of antibody binding and lysozyme release for peptides varying in position 2 shows an excellent correlation in rank order reactivity, r = 0.97 for the four free-acid derivatives. In contrast, the corresponding benzyl ester compounds show a poor correlation between antibody and neutrophil reactivity (r = 0.36).

Modifications in position 3. Table IV demonstrates the importance of the phenylalanine residue in the third position for binding of the tripeptide to the antibody. As can be seen, regardless of whether leucine or methionine is in position 2, replacement of phenylalanine in position 3 by leucine or alanine in the f Met-Leu series, or by methionine or glutamic acid in the f Met-Met series, causes a severe loss of binding activity for these peptides. Furthermore, replacement of phenylalanine with tyrosine causes a 10-fold loss of binding activity.

Two tetrapeptides containing alanine "spacer" groups between methionine and phenylalanine were synthesized, and their activity was tested. As seen in Table IV, f Met-Leu-Ala-Phe and f Met-Ala-Leu-Phe are 125- and 250-fold less active, respectively, than f Met-Leu-Phe. These results demonstrate that not only the presence of methionine in position 1 and phenylalanine in position 3, but also the relative position with respect to one another, are important for binding to the antibody. The correlation between antibody and neutrophil reactivity for eight peptides varying in position 3 is r = 0.78.

Modifications in the terminal carboxylic acid of phenylalanine and in amino acids beyond position 3. A free α-carboxyl group on the phenylalanine in position 3 does not appear to be required for binding to the antibody because the phenylethylamine (Pea), benzylamide (B_zA), and benzyl ester (OBzl) derivatives are as active as the free acid (Table V). The chloromethyl ketone of f Nle-Leu-Phe was also not significantly different in binding activity from the corresponding free acid. In contrast, appreciable variation is seen for these peptides with the neutrophil (3, 19).

TABLE IV

Effects of modification in position 3 of the tripeptide, f Met-Leu-Phe, on binding to anti-f Met-Leu-Phe

Peptide	Binding Inhibition ID_{50}[a] ± SEM[b] (M)
f Met-Leu-Phe	$7.2 \pm 0.39 \times 10^{-8}$
f Met-Leu-Leu	$2.5 \pm 0.25 \times 10^{-5}$
f Met-Leu-Ala	$\gg 10^{-4}$
f Met-Met-Phe	$1.94 \pm 0.64 \times 10^{-6}$
f Met-Met-Met	$5.1 \pm 2.4 \times 10^{-5}$
f Met-Met-Glu	$\gg 10^{-4}$
f Nle-Leu-Tyr	$1.95 \pm 0.14 \times 10^{-6}$
f Nle-Leu-pCl-Phe	$2.87 \pm 0.12 \times 10^{-7}$
f Met-Leu-Ala-Phe	$9.6 \pm 0.12 \times 10^{-6}$
f Met-Ala-Leu-Phe	$1.8 \pm 0.34 \times 10^{-5}$

[a] ID_{50} = concentration required to displace 50% of specifically bound f Met-Leu-(^{3}H)Phe-OH.

[b] Values are the mean of triplicate determination ± SEM.

TABLE V

Effects of modifications in the terminal carboxylic acid of phenylalanine and in amino acids beyond position 3 of the tripeptide, f Met-Leu-Phe, on binding to anti-f Met-Leu-Phe

Peptide[a]	Binding Inhibition ID_{50} ± SEM[b] (M)
f Met-Leu-Phe	$7.2 \pm 0.4 \times 10^{-8}$
f Met-Leu-Pea	$1.2 \pm 0.2 \times 10^{-7}$
f Met-Leu-Phe-BzA	$1.1 \pm 0.01 \times 10^{-7}$
f Nle-Leu-Phe-CMK	$4.76 \pm 0.84 \times 10^{-7}$
f Nle-Leu-Phe	$3.0 \pm 0.2 \times 10^{-7}$
f Nle-Leu-Phe-Nle-Tyr-Lys	$3.2 \pm 0.2 \times 10^{-7}$
f Met-Met-Met	$8.0 \pm 0.45 \times 10^{-5}$
f Met-Met-Met-Met	$4.0 \pm 0.5 \times 10^{-5}$
f Met-Leu-Phe α-N-Lys	$1.4 \pm 0.3 \times 10^{-7}$
f Met-Leu-Phe-ε-N-Lys	$1.2 \pm 0.2 \times 10^{-7}$

[a] ID_{50} = concentration required to displace 50% of specifically bound f Met-Leu-(^{3}H)Phe-OH.

[b] Values are the mean of triplicate determinations ± SEM.

The extent of recognition beyond the third position also has been investigated. The tripeptide f Nle-Leu-Phe and the hexapeptide f Nle-Leu-Phe-Nle-Tyr-Lys gave nearly identical ID_{50} values. Furthermore, the tetrapeptide f Met_4 was found to be only twofold more active than the tripeptide f Met_3. Lysine in position 4 also did not appreciably alter binding activity compared with the parent tripeptide f Met-Leu-Phe when linked to the tripeptide, by either its α or ε nitrogen. These results suggest that there is minimal immunologic recognition of the tripeptide beyond position 3. In contrast, marked variation in activity of the neutrophil is seen with peptide modified beyond position 3.

These results show a very distinct difference in antibody and neutrophil receptor specificity for peptides modified in the carboxyl group of phenylalanine or beyond the phenylalanine residue (r = 0.29).

Immunologic recognition of naturally occurring bacterial chemotactic factors by anti-fMLP. To test whether naturally occurring bacterial chemotactic factors could compete for binding to anti-f Met-Leu-Phe, chemotactic factor-enriched butanol extracts from *E. coli* culture filtrates were investigated. As shown in Figure 2*A*, pronase-sensitive, bacterial chemotactic factor can compete for binding to anti-f Met-Leu-Phe. This result parallels our findings with the neutrophil receptor (4).

The butanol extracts used were quite impure (25); therefore, we determined whether the material in the butanol extract binding to anti-f Met-Leu-Phe was the biologically active factor. As shown in Figure 2*B*, incubating varying concentrations of anti-f Met-Leu-Phe bound to protein A-Sepharose with f Met-Leu-(^{3}H)Phe or bacterial chemotactic factor resulted in a dose-dependent decrease of lysozyme releasing activity with both factors and a concomitant loss of radiolabeled f f Met-Leu-(^{3}H)Phe. A strong correlation between the loss of radiolabeled f Met-Leu-(^{3}H)Phe and decrease of biologic activity is seen, r = 0.98.

In addition to bacterial chemotactic factor, we investigated the chemotactic factors C5a, pepstatin (26), and substance P (27) for their ability to bind anti-f Met-Leu-Phe. C5a, at a concentration 100-fold in excess of that needed for maximum biologic activity, 10^{-4} M pepstatin, and 10^{-3} M substance P could not compete for the binding of 0.088 pmol f Met-Leu-(^{3}H)Phe to antibody (data not shown).

Inability of specific competitive antagonists of the neutrophil formyl peptide chemotaxis receptor to compete for binding to

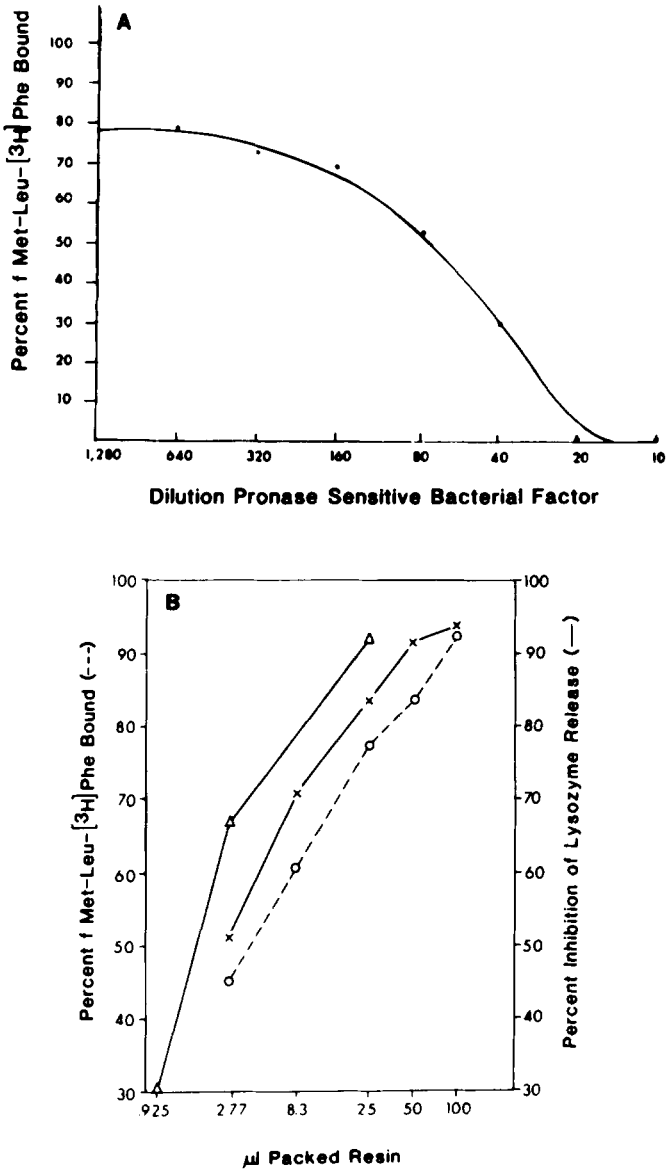

Figure 2. A, the ability of varying concentrations of butanol extracts of *E. coli* culture filtrates enriched with bacterial chemotactic factor to compete with 0.088 pmols f Met-Leu-(^{3}H)Phe for binding to anti-f Met-Leu-Phe. *B*, loss of radiolabeled f Met-Leu-Phe (○——○) and of the biologic activity of f Met-Leu-Phe (x——x) and of bacterial chemotactic factor (△——△) by reaction with anti-f Met-Leu-Phe bound to protein A.

anti-f Met-Leu-Phe. A number of competitive antagonists of the formyl peptide receptor are available that are able to inhibit both the biologic response and binding of f Met-Leu-(^{3}H)Phe to the neutrophil receptor, e.g., carbobenzoxy (CBZ) Phe-Nle, CBZ Phe-Met, and t butoxy-Phe-Leu-Phe (3, 18). None of these specific antagonists at the highest concentration tested (10^{-4} M) demonstrated detectable binding to anti-f Met-Leu-Phe. This does not appear to be due to a lack of sensitivity of the radioimmunoassay, because the antibody binding of agonists of lower affinity for the neutrophil receptor than these antagonists can be detected. The Ki's for these antagonists, as measured by inhibition of lysozyme release, are 1.8×10^{-6} M, 6×10^{-6} M, and 6×10^{-7} M, respectively (3, 18). These Ki's are higher than some of the ID_{50} values for agonists tested successfully for competition of antibody binding. For example, the dipeptide f Met-Leu, with an ED_{50} for lysozyme release of $(1.7 \pm 0.17) \times 10^{-6}$ M (2), gives detectable antibody binding at 10^{-4} M (data not shown). Furthermore, slight yet detectable antibody binding of 1 mM f Met is seen (data not shown) in spite of the fact that it's ED_{50} for chemotaxis is only 2.1×10^{-3} M (2).

The minimum size of the peptide needed for good binding activity for both receptors appears to be the tripeptide as evidenced by a 7000-fold loss of binding of biologic activity in the neutrophil (2) and greater than 10,000-fold loss of binding activity to the antibody with f Met-Leu (data not shown).

DISCUSSION

One approach in understanding the specificity of small peptides and their cell surface receptors is through the production of antibodies to carrier-bound haptens. This approach assumes that the free hapten maintains the conformation originally present in the carrier-bound form used for immunization. Therefore, antibodies raised to a peptide can resemble in specificity that of the cellular receptor, i.e., should recognize the preferred conformation of the bound hapten. Indeed, numerous data exist in the literature that demonstrate that antibodies can have similar fine specificity to the corresponding cell surface receptor (28–31).

Our results demonstrate that the synthetic chemotactic tripeptide can be conjugated to a variety of carrier proteins. Immunization of rabbits and rats with the f Met-Leu-Phe conjugates resulted in the production of high-titer, moderate-affinity IgG antibodies ($K_0 = 7.9 \times 10^7$ M^{-1}). In addition, these antibodies appear to be of restricted heterogeneity as determined by Scatchard and Sips plot analysis; Sips heterogeneity index, a = 0.98 (23, 24). We have previously reported that affinity column purified anti-f Met-Leu-Phe is of restricted electrophoretic heterogeneity (22).

The ability of anti-f Met-Leu-Phe to bind bacterial chemotactic factor is of importance for two reasons. First, anti-f Met-Leu-Phe immunoadsorbents can now be used as a purification step for this small peptide (22). Second, part of the anti-f Met-Leu-Phe combining site is specific for the prokaryotic initiating signal peptide (formylmethionine) that is believed to direct the transport of the polypeptide across the appropriate membrane during its synthesis (32). Thus, *in vivo* contact of f Met-Leu-Phe-sensitized lymphocytes with prokaryotic signal peptides from endogenous bacteria such as *E. coli* is one possible explanation for our unpublished observation that high-titer anti-f Met-Leu-Phe antibody persisted for many weeks without subsequent antigen boosts. Moreover, these same f Met-Leu-Phe-sensitized T and B cells may interact with PMN and macrophages to regulate the phlogistic response through a receptor of common formylmethionine peptide specificity (2, 3, 8).

Five critical areas have been defined for the interaction of the formyl peptides with the neutrophil receptor (19). These areas are in the vicinity of the formyl group, the methionine side chain in position 1, the leucine side chain in position 2, the phenylalanine in position 3, and the phenylalanine carbonyl group. In the first four of the five areas, the peptides react very similarly with the antibody and the neutrophil receptor, implying a corresponding similarity of conformation of the antibody and neutrophil receptors in these four areas.

Within these four critical areas, the same model we have proposed for the neutrophil receptor (19) fits the antibody with some only minor differences. Like the neutrophil, the antibody has a specific requirement for the formyl group, possibly due to the ability of the latter to provide a hydrogen (H) bond to react with a putative acceptor in the receptor. Also like the neutrophil, the evidence is compatible with the notion that the methionine in position 1 fills a hydrophobic pocket in the

antibody of a depth sufficient to occupy a side chain of four carbon atoms. In this pocket, there also may be a discrete area of positive charge corresponding to the electron-rich sulfur of the methionine. This explains the decidedly greater activity for both receptors of the derivative with methionine compared with that with norleucine. In the latter, a carbon is substituted for the sulfur. With the leukocyte receptor, we have postulated that the pocket may be of limited capacity in the area of the γ carbon, because branching at that point is little tolerated, but for the opposite reason there is no restriction in the area of the β carbon. The antibody data suggest that the pocket in the combining site is of restricted capacity in the area of both the β and the γ carbon.

With regard to the leucine in position 2, we propose a hydrophobic interaction for the antibody receptor as we have done for the neutrophil. As the results with the benzyl esters show (Table III), β branching induces very large decreases in activity, suggesting a restriction in the corresponding area of the receptor. In this, the antibody and neutrophil receptor are unlike.

The phenylalanine side chain in position 3 appears to reside in a hydrophobic pocket in the antibody as well as in the neutrophil receptor. Charged residues in this position greatly reduce the activity to the antibody (Table IV) as well as the neutrophil, whereas neutral residues are acceptable. Because of the distinct increase in activity caused by phenylalanine, it is also possible that properties other than hydrophobicity, such as aromaticity, are important in this area in both the antibody and neutrophil receptor. Substituting tyrosine for phenylalanine brings a distinct fall in antibody-binding activity as well as in reactivity with neutrophils (10, 19). This suggests that the highly hydrophobic pocket can not tolerate the polar OH group of tyrosine. This suggestion is compatible with the finding that the pCl phenylalanine derivative shows no loss of activity with antibody and only a relatively small loss of neutrophil reactivity.

Despite the major similarities and minor differences between the two receptors in the first four critical areas of interaction, large differences appear once we go beyond them. The coefficient of correlation, r, between the ID_{50} as a measure of antibody binding and the ED_{50} for enzyme release from neutrophils (19), for the benzyl esters of four peptides differing in position 2 is only 0.36 compared with that for the corresponding free acids of 0.97. The phenylalanine carbonyl is important for neutrophil, but not for antibody reactivity, as shown by the marked decrease in reactivity with the neutrophil of f Met-Leu-Pea (19) compared with the very little change in reactivity with the antibody (Table V). The binding activities for antibody of the tetrapeptides f Met-Met-Met-Met and f Met-Leu-Phe-Lys, and of the hexapeptide f Nle-Leu-Phe-Nle-Tyr-Lys, do not differ from their respective parent tripeptides, but their neutrophil reactivity does (3). However, these differences between antibody and neutrophil reactivity, although large, are all explicable on the basis that the antibody receptor fits the tripeptide f Met-Leu-Phe and possibly a small portion of the carrier protein used in immunization, whereas the neutrophil receptor can accommodate distinctly more than the tripeptide. The broader specificity of the neutrophil receptor compared with the antibody receptor is also suggested by the finding that pepstatin, substance P, and the specific antagonists react with the neutrophil but not the antibody.

The heterogeneity index in the Sips plot of a = 0.98 indicates that the anti-f Met-Leu-Phe antibody is homogeneous in binding affinity. However, the limitations of the $(NH_4)_2SO_4$ assay used to determine this value have been pointed out (23, 24), and it is quite possible that the antibody is more heterogeneous than the Sips plot indicates. This in turn raises the possibility that the particular pattern of reactivity found is a function of the particular distribution of antibody affinities present in the antiserum employed. This is rendered unlikely by the finding that a pool of rat antiserum raised against $fMLP_{10}$-BSA when tested with 10 peptides gave a pattern of reactivity very similar (r = 0.80) to that given by the rabbit antibody shown here (33). In addition, other rabbit antibody gave similar results (33).

As pointed out, the conformation of part of the anti-f Met-Leu-Phe receptor and that of the neutrophil formyl peptide receptor appear to be similar. As described in the introduction, it then follows that antibody raised against the anti-f Met-Leu-Phe combining site could be expected also to have anti-neutrophil formylpeptide receptor reactivity. In accord with this expectation, the following paper demonstrates that anti-idiotypic antibody raised against purified anti-f Met-Leu-Phe antibody also specifically reacts with the formyl peptide receptor of the neutrophil (34).

REFERENCES

1. Schiffmann, E., B. A. Corcoran, and S. M. Wahl. 1975. N-formylmethionyl peptides as chemoattractants for leukocytes. Proc. Natl. Acad. Sci. 72: 1059.
2. Showell, H. J., R. J. Freer, S. H. Zigmond, E. Schiffmann, S. Aswanikumar, B. A. Corcoran, and E. L. Becker. 1976. The structure-activity relations of synthetic peptides as chemotactic factors and inducers of liposomal enzyme secretion for neutrophils. J. Exp. Med. 143:1154.
3. Freer, R. J., A. R. Day, E. Schiffmann, S. Aswanikumar, H. J. Showell, and E. L. Becker. 1980. Further studies of the structural requirements for synthetic peptide chemoattractants. Biochemistry 19:2404.
4. Aswanikumar, A., B. A. Corcoran, E. Schiffmann, A. R. Day, R. J. Freer, H. J. Showell, and E. L. Becker. 1977. Demonstration of a receptor on rabbit neutrophils for chemotactic peptides. Biochem. Biophys. Res. Comm. 74: 810.
5. Williams, L. T., R. Snyderman, M. C. Pike, and R. J. Lefkowitz. 1977. Specific receptor sites for chemotactic peptides on human polymorphonuclear leukocytes. Proc. Nat'l. Acad. Sci. 74:1204.
6. Chenoweth, D. E., J. G. Rowe, and T. E. Hugli. 1979. Complement mediated chemotaxis of human, rabbit, guinea pig and procine neutrophils. Fed. Proc. 38:1454. (Abst.)
7. Snyderman, R., and M. C. Pike. 1980. N-formylmethionyl peptide receptors on equine leukocytes initiate secretion but not chemotaxis. Science 209: 493.
8. Snyderman, R., and E. J. Fudman. 1980. Demonstration of a chemotactic factor receptor on macrophages. J. Immunol. 124:2754.
9. Sha'afi, R. I., K. Williams, M. C. Wacholtz, and E. L. Becker. 1978. Binding of the chemotactic synthetic peptide (^{3}H)-Formyl-Norleu-Leu-Phe to plasma membrane of rabbit neutrophils. F.E.B.S. Lett. 91:305.
10. Niedel, J. S., S. Wilkinson, and P. Cuatrecasas. 1979. Receptor-mediated uptake and degradation of I^{125}-chemotactic peptide by human neutrophils. J. Biol. Chem. 254:10700.
11. Niedel, J., I. Kahane, L. Lachman, and P. Cuatrecasas. 1980. A subpopulation of cultured human promyelocyte leukemia cells (HL-60) displays the formyl peptide chemotactic receptor. Proc. Natl. Acad. Sci. 77:1000.
12. Fontona, J. A., D. G. Wright, E. Schiffman, B. A. Corcoran, and A. B. Deisseroth. 1980. Development of chemotactic responsiveness in myeloid precursor cells: studies with a human leukemia cell line. Proc. Natl. Acad. Sci. 77:3664.
13. Pike, M. C., D. G. Fischer, H. S. Koren, and R. Snyderman. 1980. Development of specific receptors for N-formylated chemotactic peptides in a human monocyte cell line stimulated with lymphokines. J. Exp. Med. 152: 31.
14. Shui, R. P. C., and H. G. Friesen. 1976. Interaction of cell-membrane prolactin receptor with its antibody. Biochem. J. 157:619.
15. Jacobs, S., K. J. Chang, and P. Cuatrecasas. 1978. Antibodies to purified insulin receptor have insulin-like activity. Science 200:1238.
16. Myers, P. R., M. Donlon, K. McCarthy, P. Livengood, and W. Shain. 1976. Evidence for a dopamine receptor antibody. Biochem. Biophys. Res. Commun. 72:1311.
17. Pillion, D. J., and M. P. Czech. 1978. Antibodies against intrinsic adipocyte plasma membrane proteins activate D-glucose transport independent of interaction with insulin binding site. J. Biol. Chem. 253:3761.
18. Becker, E. L. 1979. A multifunctional receptor on the neutrophil for synthetic chemotactic oligopeptides. J. Reticuloendothel. Soc. (Suppl. 26):701.
19. Freer, R. J., A. R. Day, N. Muthukumaraswamy, D. Pinon, A. Wu, H. J. Showell, and E. L. Becker. 1981. Formyl peptide chemoattractants: a model of the receptor in rabbit neutrophils. Biochemistry. In press.

20. Isturiz, M. A., A. L. Sandbery, E. Schiffmann, S. M. Wahl, and A. L. Notkins. 1978. Chemotactic antibody. Science 200:554.
21. Swanson, M. J. 1977. A simple multiple chamber apparatus for measuring chemotaxis of polymorphonuclear leukocytes utilizing centrifugation of the chambers before incubation. J. Immunol. Methods 116:385.
22. Marasco, W. A., H. J. Showell, and E. L. Becker. 1980. Biological and immunologic properties of covalently bound fMLP on various carrier proteins. Fed. Proc. 39:879. (Abstr.)
23. Stupp, Y., T. Yoshida, and W. E. Paul. 1969. Determination of antibody-hapten equilibrium constants by an ammonium sulfate precipitation technique. J. Immunol. 103:625.
24. Yoshida, T., W. E. Paul, and B. Benacerraf. 1970. Genetic control of the specificity of anti-DNP antibodies. I. Differences in the specificity of anti-DNP antibody produced by mammalian species. J. Immunol., 105:306.
25. Schiffmann, E., H. J. Showell, B. A. Corcoran, P. A. Ward, E. Smith, and E. L. Becker. 1975. The isolation and partial characterization of neutrophil chemotactic factors from *Escherichia coli*. J. Immunol. 114:1831.
26. Nelson, R. D., S. K. Ackerman, V. D. Fiegel, M. P. Bauma, and S. D. Douglas. 1979. Cytotaxin receptors of neutrophils: evidence that F-methionyl peptides and pepstatin share a common receptor. Infect. Immun. 26:996.
27. Marasco, W. A., H. J. Showell, and E. L. Becker. 1981. Substance P binds to the formyl peptide chemotaxis receptor on the rabbit neutrophil. Biochem. Biophys. Res. Commun. 99:1065.
28. Tanenbaum, S. W., M. Mage, and S. M. Beiser. 1959. Unity in the specificity of enzyme and antibody induction by the same determinant groups. Proc. Natl. Acad. Sci. 45:922.
29. Van Vunakis, H., J. T. Farrow, H. B. Gjika, and L. Levine. 1971. Specificity of the antibody receptor site to D-lysergamide. Model of a physiological receptor for lysergic acid diethylamide. Proc. Natl. Acad. Sci. 68:1483.
30. Faraj, B. A., Z. H. Israili, and N. E. Knight. 1976. Specificity of an antibody directed against d-methamphetamine. Studies with rigid and non-rigid analogs. J. Med. Chem. 19:20.
31. Sullivan, S., H. Akil, S. J. Watson, and J. D. Barchas. 1977. Antibodies to enkepahlins: coupling of antigens and a specific methionine-enkephalin radioimmunoassay. Commun. Psychopharmacol. 1:605.
32. Bennett, J. P., K. P. Hirth, E. Fuchs, M. Sarvas, and G. B. Warren. 1980. The bacterial factors which stimulate neutrophils may be derived from procaryote signal peptides. F.E.B.S. Lett. 116:57.
33. Marasco, W. A. 1981. Structure activity studies in regard to the use of anti-idiotypic antibodies as possible anti-cell surface receptor reagents. Ph.D. Thesis. University of Connecticut Health Center, Farmington, CT.
34. Marasco, W. A., and E. L. Becker. 1982. Anti-idiotype as antibody against the formyl peptide chemotaxis receptor of the neutrophil. J. Immunol. 128: 963.