Superoxide Production Induced in Rabbit Polymorphonuclear Leukocytes by Synthetic Chemotactic Peptides and A23187

The Nature of the Receptor and the Requirement for Ca^{2+}

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Synthetic formyl methionyl chemotactic peptides induce the various manifestations of the respiratory burst: increased O_2 consumption, activation of the hexose monophosphate shunt, and increased production of superoxide (O_2^-) and H_2O_2. They do so alone but to a much greater extent when in the presence of cytochalasin B. Superoxide generation by the chemotactic peptides in the presence of cytochalasin B shows the same relationship of structure to activity as does the stimulation of chemokinesis and chemotaxis, granule enzyme secretion, and neutrophil aggregation by these same agents. Carbobenzoxy-phenylalanyl-methionine, CBZ-Phe-Met, competitively inhibits the induced stimulation of locomotion, granule enzyme secretion, and neutrophil aggregation caused by the synthetic peptides. It also is a competitive inhibitor of O_2^- generation by the same peptides. The structure-activity and the competitive inhibitor studies lead to the conclusion that in polymorphonuclear leukocytes the chemotactic peptides induce superoxide formation and presumably the other manifestations of the respiratory burst by interacting with the same membrane receptor responsible for the stimulation of chemokinesis, chemotaxis, granule enzyme secretion, and neutrophil aggregation. The effectiveness of formyl-methionyl-leucyl-phenylalanine, F-Met-Leu-Phe, in generating O_2^- is greatly reduced but not abolished by removing calcium from the external medium. The calcium ionophore A23187 induces O_2^- generation that requires external calcium and is greatly enhanced by cytochalasin B. From these findings we hypothesize that the proximate cause of the induction of O_2^- formation and other manifestations of the respiratory burst by the chemotactic peptides is the influx into the neutrophil of Ca^{2+} and/or possibly Na^+ previously shown to be induced by the peptides. (Am J Pathol 95:81–98, 1979)

THE N-FORMYL METHIONYL and related synthetic oligopeptides stimulate the locomotion of neutrophils, basophils, and eosinophils as well as macrophages.[1,2] They are both chemotactic and chemokinetic for neutrophils, induce granule enzyme secretion when added with cytochalasin B to neutrophils in suspension,[2] and aggregate these cells.[3] We have presented evidence that all of these functions result from the interaction of the synthetic oligopeptides with a single population of receptors on the

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neutrophil membrane.[2-4] While this paper was being prepared for publication, Hatch et al [5] reported that the N-formyl methionyl peptides also induce chemiluminescence in polymorphonuclear leukocytes and macrophages and postulated that this resulted from the ability of the peptides to stimulate superoxide (O_2^-) formation. They suggested that the stimulation of O_2^- production may have arisen from the interaction of the same peptide receptor mechanism responsible for granule enzyme secretion and stimulated locomotion.

We shall demonstrate that the N-formyl methionyl peptides not only stimulate O_2^- generation in rabbit peritoneal neutrophils but also induce activation of the hexose monophosphate shunt and increased O_2 consumption and H_2O_2 production. We shall present evidence that the O_2^- generation and presumably the other oxidative metabolic changes arise from the interaction of the peptides at the same neutrophil receptor responsible for inducing the other functions of the neutrophil. In addition, we shall describe the role of external Ca^{2+} in the generation of O_2^- by the peptide chemotactic factors.

Materials and Methods

Glycogen, tris (hydroxylmethyl) aminomethane, crystalline bovine serum albumin, dimethyl sulfoxide (DMSO), ferricytochrome C (Type III from horse heart), superoxide dismutase, scopoletin, horseradish peroxidase, Hyamine hydroxide, ethyleneglycol-bis(β-aminoethylether)N,N'-tetraacetic acid (EGTA), and disodium ethylenediamine tetraacetic acid (EDTA) were obtained from Sigma Chemical Co. (St. Louis). [1-C^{14}]-Glucose was obtained from New England Nuclear (Boston). The chemotactic peptides were the same as used previously,[2] as was the carbobenzoxy-phenylanyl-methionine, CBZ-Phe-Met.[3] The peptide agonists were made up as 10 mM stock solutions; a 200 mM stock solution of CBZ-Phe-Met in dimethyl sulfoxide was employed. The solutions were stored in the refrigerator and diluted just before use. The C5a was obtained by trypsin treatment of highly purified C5 as described previously.[6] The N-ethyl maleimide was a product of J.T. Baker Chemical Co. (Phillipsburg, NJ) and 30% hydrogen peroxide was from Fisher Scientific Co. (Fairlawn, NJ). Cytochalasin B, a product of Aldrich Chemical Co. (Milwaukee) was made up as a 5 mg/ml stock solution in dimethyl sulfoxide; its final concentration was 5 μg/ml. The residual dimethyl sulfoxide from the peptides and/or the cytochalasin B was shown not to affect the cells or the measurement of any of the functions tested. The A23187 was a generous gift of Dr. Robert Hamil (Eli Lilly Co., Indianapolis) whom we wish to thank. It was made up to 10 mM in dimethyl sulfoxide.

Preparation of Cells

Rabbit polymorphonuclear leukocytes were obtained 12 to 16 hours after intraperitoneal injection of 0.1% glycogen as described.[2] They were washed once in Hanks' balanced salt solution, pH 7.2, containing 10 mM tris (hydroxymethyl)aminomethane and 1 mg/ml glucose. Except where noted, the Hanks' buffer was used throughout.

Superoxide (O_2^-) Production

The assay of the peptides' ability to induce O_2^- production was essentially as described by Babior et al.[7] Briefly, 5×10^6/ml peritoneal neutrophils were suspended in Hanks'

buffer containing 1 mg/ml crystalline bovine serum albumin and kept on ice until ready for use. The cells were equilibrated for 10 minutes at 37 C and cytochalasin B was added. Then 0.5 ml cells were dispensed into 12 × 75 mm glass test tubes to which had been added 0.025 ml of either Hanks' buffer or a 1 mg/ml solution of superoxide dismutase, 0.25 ml of a 0.23 mM solution of ferricytochrome C, and 0.05 ml of the appropriate dilution of peptide. When C5a was the stimulus, 0.2 ml of a 0.29 mM cytochrome C solution was employed with 0.1 ml of the appropriate dilution of C5a. In those studies in which the competitive antagonist CBZ-Phe-Met was tested, this was added in the desired concentration to the dilutions of agonist (either F-Met-Leu-Phe or C5a) just before use.

The mixtures were incubated at 37 C for 5 minutes (the reaction was completed within less than 5 minutes), and the reaction was stopped by the addition of 0.5 ml 1 mM N-ethylmaleimide. The tubes were centrifuged at 4 C at 630*g* for 5 minutes and the absorbance of the supernatant was read at 550 mm in a Gilford 300 N spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The amount of O_2^- produced was calculated from the difference in absorbance of the samples with and without superoxide dismutase. This difference was divided by the extinction coefficient for the change between ferricytochrome C and ferrocytochrome C and was multiplied by twice the final volume of the sample (0.825 ml) to give the nanomoles (nmol) of O_2^- produced per 5×10^6 cells.

Measurements of Other Oxidative Metabolic Changes

The assay of the neutrophils' ability to undergo the other metabolic changes (increased O_2^- consumption, hexose monophosphate shunt activation, and H_2O_2 production) was performed in cells prepared essentially as described for the determination of O_2^- production. The only difference was that 3×10^6 PMN were employed when hexose monophosphate shunt activity was measured; 5×10^6 PMN were employed for the determination of the other activities.

The O_2 consumption was determined by means of a continuous assay using an O_2 electrode (Model 53, Yellow Springs Instrument Co., Yellow Springs, Ohio) in control cells and in cells exposed to cytochalasin B and/or F-Met-Leu-Phe. Cells (5×10^6 PMN in 2 ml medium) were incubated in Hanks' medium at 37 C for approximately 15 minutes to obtain a resting level of O_2 consumption. Chemotactic peptide and/or cytochalasin was introduced by injection, and incubation continued for an additional 15 minutes until the stimulated rate of O_2 consumption began to reduce toward baseline. In the presence of F-Met-Leu-Phe and cytochalasin B, increased O_2 consumption was initiated without appreciable delay (not more than 0.25 minutes) and a linear rate of enhanced consumption was measured over approximately 5 minutes. The O_2 electrode was calibrated to read percent saturation of the cell suspension, and the calculated O_2 consumption during the linear phase of response was expressed as nmol/min/5×10^6 cells.

The activity of the hexose monophosphate shunt pathway (HMS) was measured by the oxidation of [1-C^{14}]-glucose to $^{14}CO_2$ essentially as described by Henson and Oades.[8] Cells in 1.0 ml Hanks' medium without glucose were pipetted into disposable glass tubes which were then sealed by rubber caps. Plastic cups that contained 0.2 ml of Hyamine hydroxide absorbed onto a filter paper were inserted through the caps. Substrates (0.1 ml of 10 mM [1-C^{14}] glucose; 1 μCi/ml) and F-Met-Leu-Phe were introduced into the cell suspensions in rapid succession by injection through the caps. Cells were then incubated for 5 minutes prior to inhibiting the reaction by injection of 1.0 ml of 5N H_2SO_4. The acidified suspensions were incubated for a further 30 minutes at 37 C to collect $^{14}CO_2$ in the Hyamine; the cups were then collected and radioactivity was determined by liquid scintillation counting using Bray's counting solution. Portions of the substrate were also counted and results were expressed as ng $^{14}CO_2$/5 min/sample. Hexose monophosphate shunt activity could only be measured in the presence of F-Met-Leu-Phe. Cytochalasin B inhibited the transport by neutrophils of glucose [9] and so prevented the measurement of $^{14}CO_2$ production from exogeneous [1-^{14}C]-glucose. Since rabbit PMN contain large cyto-

plasmic stores of glycogen, we suppose nevertheless that the HMS pathway is activated in such cytochalasin-treated cells.

Hydrogen peroxide (H_2O_2) was measured by the continuous peroxidase-dependent decrease in fluorescence of scopoletin as described by Root et al.[10] Cells (5×10^{-6} PMN in 2 ml Hanks' medium) were incubated with 0.05 ml of 0.2 mM scopoletin and 0.025 ml of 1 mg/ml horseradish peroxidase. A small baseline production of H_2O_2 could be measured by following the decrease in fluorescence over 15 minutes at 37 C in a Hitachi-Perkin-Elmer MP4 spectrofluorimeter. F-Met-Leu-Phe and/or cytochalasin B (0.1 ml) was then added, and the increased rate of scopoletin consumption was measured over time. With both F-Met-Leu-Phe and cytochalasin B, no lag was detectable between adding the reagents and observing the linear increase in H_2O_2 formation. The increased H_2O_2 production remained linear for 5 to 10 minutes and was routinely followed for 5 minutes. H_2O_2 production was quantified by preparation of a standard curve of decreased scopoletin fluorescence against known concentrations (usually 10 to 200 nmol/2 ml) of H_2O_2 and was expressed as nmol/min/5×10^{-6} cells.

Granule Enzyme Release

The release of the granule enzymes β-glucuronidase and lysozyme as well as the cytoplasmic marker enzyme lactic dehydrogenase (LDH) activity was determined exactly as previously described.[2] Except where noted, no lactic dehydrogenase leakage was induced by either the chemotactic factors or A23187 and is not otherwise reported. The β-glucuronidase release paralleled the lysozyme release and is not reported.

Ca^{2+} Dependence of O_2^- Production and Enzyme Release

For these studies the cells were washed twice in Hanks' buffer without Ca^{2+} or Mg^{2+} and resuspended to 10^7 cells/ml in the same buffer. Just before use, they were diluted to 5×10^6 cells/ml and incubated for 10 minutes at 37 C. To these cells was then added 100 mM EDTA to give a final concentration of 1 mM. The assays for O_2^- formation and enzyme release were performed as described. The presence or absence of Mg^{2+} was shown to have no effect on O_2^- production; therefore, none was added to the Ca^{2+}-free suspensions of cells.

Results

Oxidative Metabolic Changes Induced by F-Met-Leu-Phe

In separate experiments, the ability of 2.5×10^{-8} M F-Met-Leu-Phe to induce O_2 consumption and hexose monophosphate shunt activation, to produce H_2O_2, and to generate O_2^- was determined in the presence (except for hexose monophosphate shunt activation [see above]) and absence of 5 μg/ml of cytochalasin B. In addition, the ability of the peptide to induce granule enzyme release was determined on the same batch of cells within a given experiment. The concentration of 2.5×10^{-8} M F-Met-Leu-Phe was chosen as that which, in the various experiments, gave maximum oxidative metabolic changes in the absence of cytochalasin B as well as in its presence.

The results of a representative group of experiments are seen in Table 1. It is evident that 2.5×10^{-8} M F-Met-Leu-Phe alone was able to induce O_2

Table 1—Oxidative Metabolic Responses and Lysozyme Secretion Induced by F-Met-Leu-Phe in the Presence and Absence of Cytochalasin B

Experiment	Activity	Activity in the presence of				Lysozyme secreted	
		Hanks'	F-Met-Leu-Phe	Cytochalasin B	F-Met-Leu-Phe + cytochalasin B	F-Met-Leu-Phe	F-Met-Leu-Phe + cytochalasin B
1	O_2^- production (nmol/5 × 10^6 PMN/5 min)	0.4	7.9	0.9	33	0	52
2	H_2O_2 production (nmol/5 × 10^6 PMN/5 min)	0	9.0	0.5	12.5	16	79
3	O_2 consumption	0.3	4.6	0.3	9.3	21	98
4	[^{14}C]O_2 production (ng/3 × 10^6 PMN/5 min)	65	174	ND	ND	12	69

The four activities were measured in duplicate or triplicate in separate experiments. In all cases, cytochalasin B, when present, was at a concentration of 5 μg/ml and F-Met-Leu-Phe was at a concentration of 2.5 × 10^{-8} M.

The lysozyme is reported as percent of total activity. The blank (release in Hanks' buffer) was subtracted in each instance. The total enzyme activity in units of lysozyme in Experiments 1, 2, 3, and 4 was, respectively, 850, 1175, 1200, and 1725 units/5 × 10^6 cells after 5 minutes of incubation.

ND = not done.

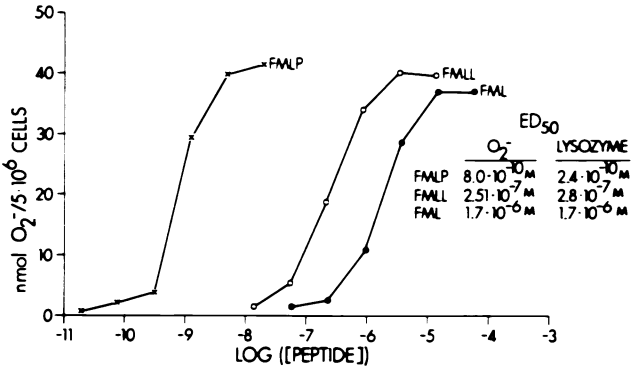
consumption, O_2^- production, and H_2O_2 production that was approximately one fourth to two thirds that produced in the presence of cytochalasin B. In some experiments the peptide was able to induce lysozyme release in the absence of cytochalasin B, although this was always considerably less than in its presence. Moreover, in Experiment 1 no enzyme release was detectable although considerable O_2^- formation was evident. The ability found here for a chemotactic factor to sometimes induce granule enzyme release in the absence of cytochalasin B when neutrophils were in suspension is in contradiction to our previous experience and is in part due to the high concentrations of chemotactic factor tested.

In the work to be described, only the ability of the peptides to induce O_2^- generation was tested and only in the presence of cytochalasin B.

Comparison of the Superoxide-Inducing Activities of Various Peptides

Previous work showed that plotting the extent of stimulated locomotion or of lysosomal secretion against the logarithm of the concentration of the peptide gave a sigmoidal dose-response curve.[2] Different peptides tested for a given activity at the same time with the same cells gave curves that were parallel in their linear portion and had the same maximum. This allowed either activity of a given peptide to be expressed as an ED50, ie, the concentration of peptide giving 50% of the maximum activity.[2] The same procedure was followed with respect to the production of superoxide by the peptides. The log-dose responses curves for three representative peptides, ie, formyl methionyl-leucyl-phenylalanine (F-Met-Leu-Phe), formyl methionyl-leucyl-leucine (F-Met-Leu-Leu), and formyl methionyl leucine (F-Met-Leu), are shown in Text-figure 1. The same general dose-response relationship holds for stimulated O_2^- production as previously found for stimulated secretion, locomotion, and aggregation. This allows the activity of the peptides stimulating O_2^- production also to be expressed as an ED50.

The ED50 for O_2^- formation was determined for seven peptides. These peptides were chosen to cover as wide a range of activities as possible based on our prior knowledge of their abilities to induce enzyme release and locomotion. In every case, the ED50 for O_2^- formation was determined in four to five experiments, similar to the one described in Text-figure 1. The results of these experiments are summarized in Table 2, which shows the mean ED50 ± SE (standard error) for each peptide. The wide spread in activity is apparent; the ED50 for the most active peptide studied, F-Met-Leu-Phe, is 8.0×10^{-10} M and 1.3×10^{-5} M for the least active, Met-Met-Met-Met. Table 2 also shows the ED50s for the stimu-



TEXT-FIGURE 1 — The assay of the ability of three peptides, F-Met-Leu-Phe (FMLP) (X—X), F-Met-Leu-Leu (FMLL) (o—o), and F-Met-Leu (FML) (•—•), to induce O_2^- production. (Cytochalasin B present; blanks subtracted).

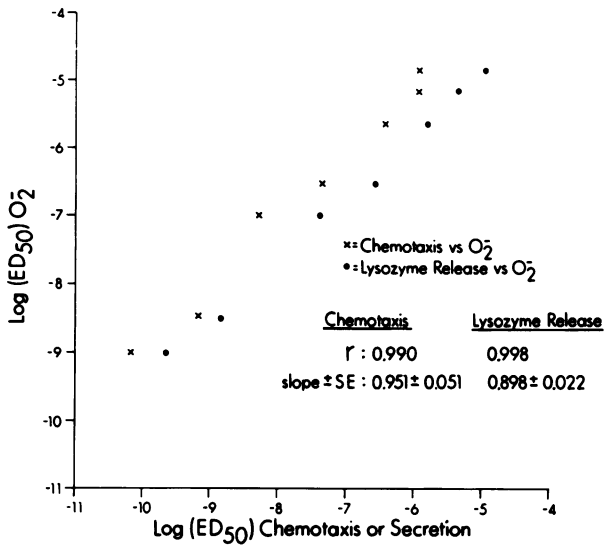
lation of lysozyme and β-glucuronidase secretion. These latter values agree with those previously determined.[2]

The ED50 values for O_2^- formation of Table 2 plotted against the ED50s for stimulation of locomotion and for lysozyme secretion give the results in Text-figure 2. The ED50 values for lysozyme secretion were those of Table 2, but those for stimulation of locomotion were those previously determined.[2] The extremely close correlation between O_2^- forming and releasing ability or migration stimulating activity is evident. The correlation coefficients for O_2^- formation and lysozyme secretion and O_2^- stimulation and locomotion are indistinguishable from 1, being 0.99_0 and 0.89_8, respectively. As expected from the close correlation between lysozyme and β-glucuronidase secretion previously demonstrated,[2] an equally close correlation was found between the ED50s for O_2^- generation and for β-glucuronidase secretion (not shown).

It is also shown in Table 2 that, on the average, somewhat higher concentrations of peptide are required to induce O_2^- generation than to give a corresponding degree of lysozyme secretion. The mean ratio of the

Table 2—Comparison of the Ability of Peptides to Stimulate O_2^- Formation and Enzyme Secretions in Rabbit Neutrophils

Peptide	O_2^- $ED_{50} \pm SE$	Lysozyme $ED_{50} \pm SE$	β-Glucuronidase $ED_{50} \pm SE$
F-Met-Leu-Phe	$8.0 \pm 0.86 \times 10^{-10}$	$2.4 \pm 0.31 \times 10^{-10}$	$2.6 \pm 0.32 \times 10^{-10}$
F-Nleu-Leu-Phe	$3.3 \pm 0.66 \times 10^{-9}$	$1.5 \pm 0.2 \times 10^{-9}$	$1.9 \pm 0.2 \times 10^{-9}$
F-Met-Met-Met	$1.1 \pm 0.26 \times 10^{-7}$	$4.3 \pm 1.4 \times 10^{-8}$	$3.1 \pm 0.63 \times 10^{-8}$
F-Met-Leu-Leu	$2.9 \pm 0.33 \times 10^{-7}$	$2.8 \pm 0.41 \times 10^{-7}$	$4.5 \pm 2.1 \times 10^{-7}$
F-Met-Leu	$2.2 \pm 0.18 \times 10^{-6}$	$1.7 \pm 0.17 \times 10^{-6}$	$1.9 \pm 0.21 \times 10^{-6}$
F-Met-Leu-Glu	$6.9 \pm 0.21 \times 10^{-6}$	$5 \pm 1.3 \times 10^{-6}$	$7.2 \pm 0.29 \times 10^{-6}$
Met-Met-Met-Met	$1.3 \pm 0.22 \times 10^{-5}$	$1.2 \pm 0.19 \times 10^{-5}$	$1.7 \pm 0.47 \times 10^{-5}$

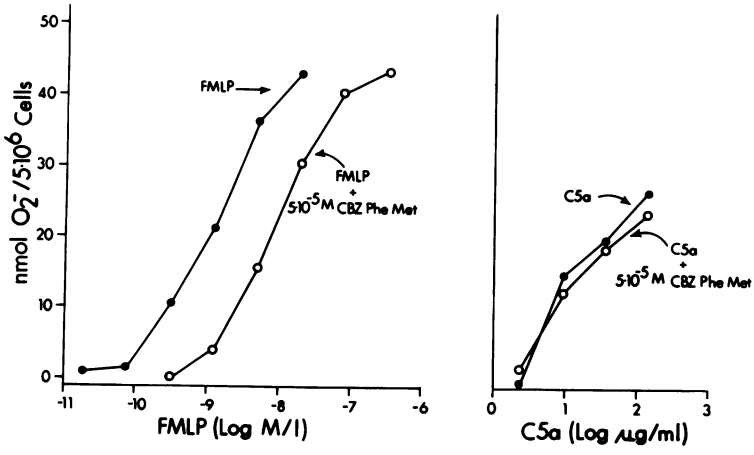


TEXT-FIGURE 2 — Correlation of the ability of seven peptides in the presence of cytochalasin B to induce O_2^- formation with their ability to stimulate locomotion (X) and to induce granule enzyme secretion (•).

ED50s for O_2^- generation to the ED50 for lysozyme release for the seven peptides is $1.7_5 \pm 0.32$.

Ability of CBZ-Phe-Met to Specifically Block O_2^- Production

CBZ-Phe-Met can reversibly bind to the peptide receptor on the neutrophil but cannot induce any of the neutrophil functions tested, ie, stimulation of migration or granule secretion or neutrophil aggregation.[3] It acts as a competitive antagonist for, and is specific to, the peptide



TEXT-FIGURE 3—Effect of the competitive inhibitor CBZ-Phe-Met (5×10^{-5} M) on O_2^- generation by varying concentrations of F-Met-Leu-Phe (*left*) and by varying concentrations of C5a (*right*).

receptor on the neutrophil.[3] It thus can serve as another test of whether a given function is being induced by interaction at the same peptide receptor. We have demonstrated (results not shown) that, by itself, CBZ-Phe-Met is not able to induce the formation of O_2^- when tested at concentrations as high as 5×10^{-4} M. However, as seen in Text-figure 3, it inhibits O_2^- induced by F-Met-Leu-Phe, causing a parallel shift to the right of the dose-response curve as expected for a competitive antagonist.[11] From the results in Text-figure 3, it is possible to calculate K_d, the dissociation constant of the competitive antagonist, using the following equation [11]:

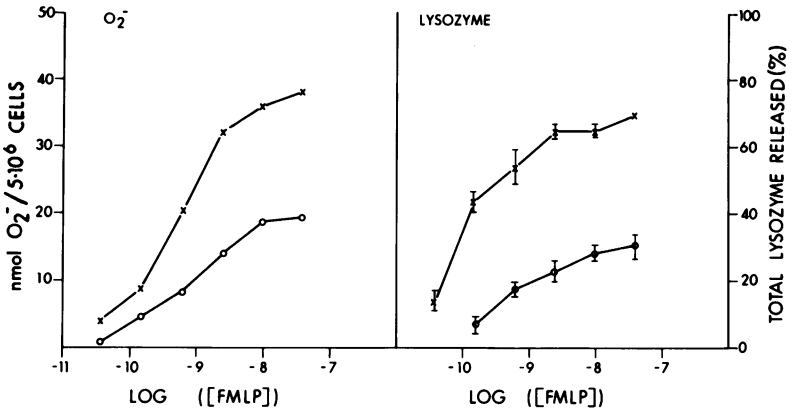
$$\frac{A_i}{A_o} - 1 = \frac{B}{K_d}$$

where A_o is the concentration of agonist, ie, F-Met-Leu-Phe, giving a certain degree of activity in the absence of the inhibitor; A_i is the concentration of agonist yielding the same degree of activity in the presence of a given concentration, B, of the inhibitor; and K_d is the apparent dissociation constant for the antagonist. From the ED50s obtained in the presence and absence of 5×10^{-5} M CBZ-Phe-Met in the experiment in Text-figure 3 and in a second, similar experiment, we calculated a value of 0.8×10^{-5} M for K_d. This does not differ significantly from the value of 1.0×10^{-5} M K_d for the binding constant of the same agent.[3] It is also the same as the K_d from similar experiments in which the ability of CBZ-Phe-Met to inhibit the stimulation of locomotion, granule enzyme secretion, and neutrophil aggregation induced by the chemotactic peptides was measured.[3]

It is also shown in Text-figure 3 that the same concentration of CBZ-Phe-Met that inhibited the O_2^- production by F-Met-Leu-Phe had no effect on O_2^- production by C5a. The same lack of effect of CBZ-Phe-Met was seen on C5a stimulation of locomotion, enzyme secretion, and aggregation [3] and confirms the conclusion from binding studies that C5a and the chemotactic peptides interact at different receptors.[12,13]

Effect of Ca^{2+} on O_2^- Production

Chemotactic-factor–induced granule secretion from neutrophils is greatly enhanced by external Ca^{2+}.[14,15] Cohen et al demonstrated that the activation of the granulocyte O_2^- generating system by digitonin requires external Ca^{2+}.[16] We therefore compared the ability of F-Met-Leu-Phe and cytochalasin B to induce O_2^- formation and granule release in the presence and absence of external Ca^{2+}. As is evident in Text-figure 4, the absence of added Ca^{2+} and Mg^{2+} and the presence of 1 mM EDTA greatly depresses, but does not abolish, the ability of chemotactic factors to induce O_2^- generation just as it does granule secretion. In experiments not shown

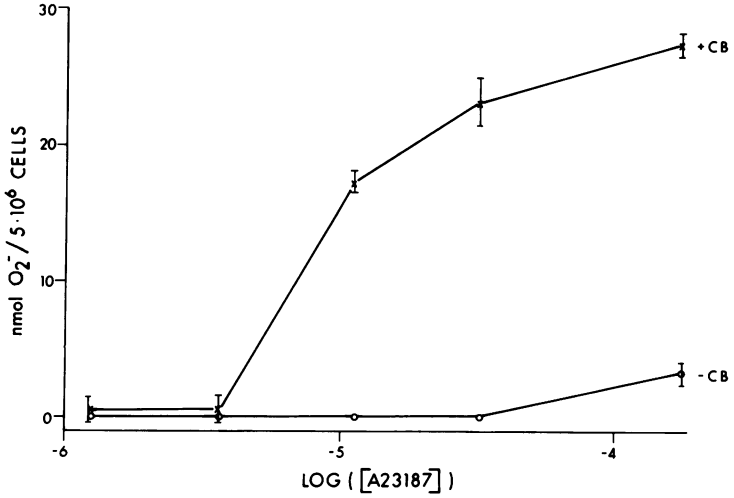


TEXT-FIGURE 4—Comparison of the effect on O_2^- generation of the presence of Ca^{2+} and Mg^{2+} (X—X) or 1 mM EDTA (o—o), induced by F-Met-Leu-Phe in the presence of cytochalasin B (*left*). Same comparison of the effect on lysozyme secretion (*right*).

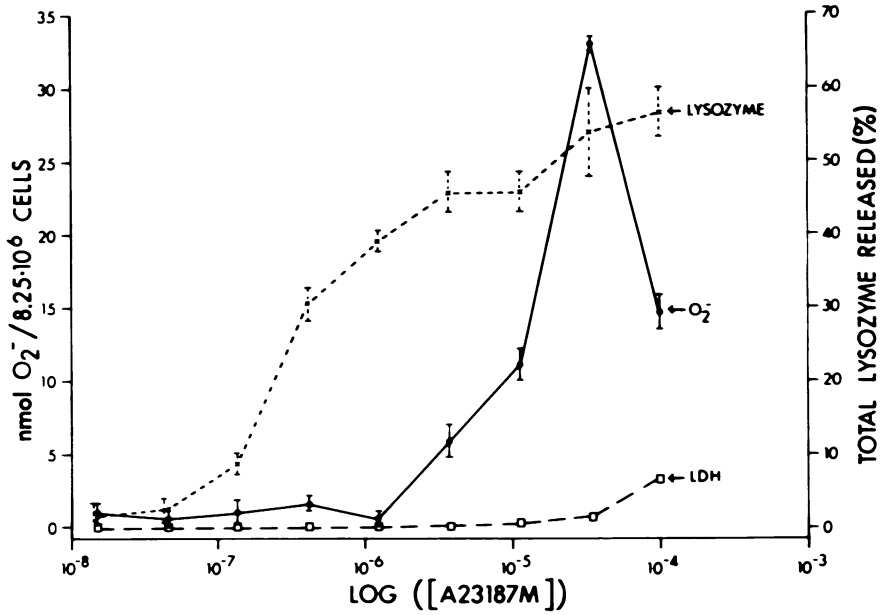
here, 1 mM EDTA in the presence of 1 mM Mg^{2+} gave the same depression of O_2^- generation by F-Met-Leu-Phe and cytochalasin B as did EDTA in the absence of both cations. These experiments indicate that external Ca^{2+} is required for the optimal stimulation of O_2^- production and that Mg^{2+} cannot substitute.

Effect of Cytochalasin B on O_2^- Production by A23187

The Ca^{2+} ionophore A23187 induces an increase in oxygen consumption from guinea pig peritoneal polymorphonuclear leukocytes and hu-



TEXT-FIGURE 5—The effect of the presence (X—X) and absence (o—o) of 5 μg/ml cytochalasin B on the generation of O_2 by A23187.



TEXT-FIGURE 6—Comparison of effect of the concentration of A23187 in the presence of cytochalasin B on the generation of O_2^- (●—●), the secretion of lysozyme (X---X), and the leakage of lactic dehydrogenase (LDH) (□—□).

man peripheral blood neutrophils that requires the presence of external Ca^{2+}.[17] As just demonstrated, external Ca^{2+} has a role in sustaining the production of O_2^- induced by chemotactic peptides and can induce an increase in O_2 consumption. We therefore tested the ability of A23187 to induce O_2^- production from rabbit polymorphonuclear leukocytes.

As seen in Text-figure 5, A23187 alone does not induce O_2^- formation from rabbit peritoneal PMN except at concentrations at which it starts to damage the cells as indicated by the leakage of LDH (not shown, but see Text-figure 6). As also seen in Text-figure 5, cytochalasin B greatly enhances the ability of the ionophore to cause O_2^- formation, allowing the generation of O_2^- to proceed at concentrations of the ionophore at which there is no evidence of cell damage.

A23187 also induces granule enzyme secretion from polymorphonuclear leukocytes and this too is enhanced by cytochalasin B.[15] Comparison of the ability of A23187 and cytochalasin B to induce O_2^- generation and granule enzyme secretion is seen in Text-figure 6. A23187 induces granule enzyme secretion much more readily than it induces O_2^- production; 10^{-6} M A23187, a concentration that induces almost maximal enzyme secretion, causes no detectable O_2^- generation. The two responses also differ greatly in the overall nature of their dependence on the concentration of the ionophore (Text-figure 6). The dose-response curve for lysosomal

enzyme secretion reaches a maximum or nearly a maximum at approximately 4×10^{-6} M A23187; increasing concentrations up to 10^{-4} M have little further effect. This confirms previous findings.[15] A sharp maximum in O_2^- production is reached at approximately 3×10^{-5} M; increasing the concentration to 1×10^{-4} M causes an abrupt decline. The latter is associated with the cytotoxicity of this high concentration of ionophore, as evidenced by leakage of the cytoplasmic marker enzyme lactic dehydrogenase, suggesting that O_2^- production, at least induced by A23187, may be more dependent on the integrity of the cell than is lysosomal enzyme secretion.

Discussion

The synthetic chemotactic oligopeptides not only stimulate neutrophils to form O_2^- but also cause in these cells other manifestations of the "respiratory burst": activation of the hexose monophosphate shunt pathway, increased O_2 consumption, and increased H_2O_2 production (Table 1). In this, the peptides are similar to other chemotactic factors.[18-20]

We have presented two kinds of evidence that the ability of the synthetic oligopeptides to induce neutrophils to generate O_2^- arises from their interaction with the same neutrophil receptor that causes stimulation of locomotion, granule enzyme secretion, and cell aggregation. The first is the extension to O_2^- production (Text-figure 2) of the finding that there is essentially exact correlation between the ability of chemotactic peptides to stimulate locomotion, secrete granule enzymes, and cause cell aggregation.[2,3] This is true with peptides that differ over an almost 100,000- (Text-figure 2) to 26,000,000-fold [2] range of activities. The second kind of evidence is the ability of the competitive antagonist CBZ-Phe-Met to inhibit O_2^- formation in the same competitive fashion (Text-figure 4) that it inhibits stimulated locomotion, lysosomal enzyme secretion, and neutrophil aggregation. CBZ-Phe-Met does so with a calculated dissociation constant, K_d, of 0.8×10^{-5} M for O_2^- production that is essentially indistinguishable from the 1×10^{-5} M K_d calculated from its inhibition of the other neutrophil functions or its inhibition of the binding of the radiolabeled chemotactic peptide [^{3}H]-formyl-norleucyl-leucyl-phenylalanine.[3]

One possible, essentially trivial, explanation for the ability of the chemotactic peptides to induce O_2^- generation is that the latter results from the degranulation induced by the peptides. Credence is given this explanation by the finding (Table 1) of considerably more O_2^- formation when cytochalasin B was present in addition to the chemotactic peptide, the same circumstance required for the chemotactic factor to induce appreciable enzyme secretion. This explanation is also supported by the

enhancement of O_2^- production by A23187 (Text-figure 5) and by the effect of external Ca^{2+} on both enzyme secretion and O_2^- production (Text-figure 4). This explanation would also be in accord with the concept of some that O_2^- formation results from the activation of a NADPH oxidase in one or another leukocyte granule.[21,22]

However, other evidence makes it clear that the above simple explanation cannot be correct. Goldstein et al have shown that human blood neutrophils produce appreciable O_2^- when stimulated with C5a in the absence of cytochalasin B, circumstances in which there is either negligible or no secretion of granule enzymes.[14] More to the point in the present context, we have found that F-Met-Leu-Phe in the absence of cytochalasin B can cause the generation of significant amounts of O_2^- production from rabbit polymorphonuclear leukocytes in the absence of detectable secretion of granule enzymes (Table 1). In other experiments, small amounts of granule enzymes were secreted by cells challenged with F-Met-Leu-Phe in the absence of cytochalasin B but always less than the amount of O_2^-, measured as percent of maximum in each instance. Not only can O_2^- production occur in the absence of granule enzyme secretion, but also, when A23187 is the stimulus, granule enzyme secretion can occur in the absence of detectable O_2^- production, as shown in Text-figure 6. This finding is in accord with that of Zabucchi and Romeo that oxygen consumption and exocytosis from polymorphonuclear leukocytes stimulated by A23187 can be separated.[17] Thus, under one or another circumstance, O_2^- generation and granule enzyme secretion can be completely dissociated from each other, indicating that the O_2^- generation cannot be due to the stimulation of granule secretion.

If the O_2^- generation induced by the peptide chemotactic factors is not due to degranulation or to exactly the same processes involved in degranulation, then what causes it? The tentative general hypothesis we wish to offer is that an increase in intracellular free Ca^{2+} is the proximate cause of the production of O_2^-. When external Ca^{2+} is present, the increase in free intracellular Ca^{2+} may arise from an influx of Na^+ and/or Ca^{2+} into the neutrophil that is stimulated by the peptides and by A23187. In the absence of external Ca^{2+} the chemotactic factor plus cytochalasin B stimulates a transmembrane influx of Na^+ which, by changing bound to free intracellular calcium, may be the trigger for the production of O_2^-. This hypothesis can explain the effect of external Ca^{2+} in promoting the generation of O_2^- by the chemotactic peptides and by A23187 and the great enhancement of O_2^- production when cytochalasin B acts with these agents (see below).

The hypothesis is based on and supported by our prior work showing that F-Met-Leu-Phe induces an influx of Na^+ and Ca^{2+} into the neutro-

phil [23]; in the presence of cytochalasin B the chemotactic peptide induces a much larger influx of the same cations.[24] The degree of this influx is directly related to the resulting granule secretion. The Ca^{2+} ionophore A23187 induces an influx of Ca^{2+} into a variety of cells and tissues.[25] In accord with these findings, we have shown that A23187 in the presence of external Ca^{2+} causes an influx of Ca^{2+} into the neutrophil and that this influx is greatly enhanced both in rate and extent by the presence of cytochalasin B.[24] The degree of enzyme release is linearly related to the influx of Ca^{2+}.[24] The hypothesis that cytochalasin B enhances the generation of O_2^- by enhancing Ca^{2+} influx induced by chemotactic factors is not in conflict with, but in addition to, the concept of Root and Metcalf that cytochalasin B prevents the internalization of oxygen-consuming membrane regions in phagocytosis.[26]

Thus, as an explanation of how stimulation of the same receptor can induce locomotion and granule release, we have suggested that chemotactic factors alone stimulate a small, localized, superficial influx of Na^+ and/or Ca^{2+} or, alternatively, a graded release of calcium from the membrane which is necessary for locomotion (see Reference 27); granule enzyme secretion is caused by a larger, more generalized influx of Na^+ and/or Ca^{2+} caused by the concomitant presence of cytochalasin B or an appropriate surface.[25] By an extension of this hypothesis, O_2^- production would also be engendered by a large influx of Na^+ and/or Ca^{2+} into the neutrophil in the presence of external Ca^{2+} and an influx of Na^+ in the absence of external Ca^{2+}.

As shown here for rabbit peritoneal neutrophils, F-Met-Leu-Phe can induce superoxide formation in the absence of detectable lysosomal enzyme secretion and, as Bass et al [28] demonstrated, the same synthetic chemotactic peptides can stimulate locomotion of human neutrophils in the absence of detectable superoxide production or activation of the hexose monophosphate shunt pathway. The general, tentative explanation we wish to offer for the ability of the same receptors to independently stimulate these various neutrophil responses has two main features: The first is that peptide–receptor interaction causes several more or less independent changes in membrane function which raise free intracellular calcium in differing ways. The second is that triggering of the various neutrophil functions by intracellular calcium differs both in the levels needed and in where the Ca^{2+} must be located. More specifically, the synthetic peptides induce an influx of Na^+ and Ca^{2+} which is seemingly independent of the displacement of membrane Ca^{2+} produced by the same agents.[23,29] Cytochalasin B greatly enhances the stimulated influx of Na^+ and Ca^{2+} but has no effect on either the displacement of

membrane Ca^{2+} or the activation of the "Na^+, K^+" ATPase induced by the enzyme.[30] Our present hypothesis is that the stimulation of locomotion induced by chemotactic peptides is triggered by the displacement of membrane Ca^{2+}, whereas the lysosomal enzyme release and superoxide formation are caused by the influx of Ca^{2+}. The displacement of membrane Ca^{2+} is apparently a more sensitive response to receptor occupancy than is the increased influx of the two cations.[23,30] Thus, on the basis of the hypothesis just presented, stimulated locomotion would be expected to be a more sensitive response to peptide stimulation than either lysosomal enzyme release or O_2^- production, as Bass et al have shown.[28]

Higher concentrations of peptide (Table 1) or A23187 (Text-figure 6) are required for O_2^- production than for lysosomal enzyme release. These findings suggest that higher levels of free intracellular Ca^{2+} are required for O_2^- production than lysosomal enzyme secretion. Whether this putative difference reflects a difference in the intracellular localization of the free Ca^{2+} needed for the two responses or from differing quantitative requirements is unknown.

On the basis of the explanation just offered, it may be expected that agents other than chemotactic factors may stimulate one or more of the functions induced by chemotactic factors but not others. Similarly, other chemotactic factors, acting possibly at other receptors, may show a different pattern of induced activities, as has been found.[31]

The hypothesis that increasing the Ca^{2+} level intracellularly is the proximate cause of O_2^- generation causes no difficulty if one accepts the hypothesis that the origin of the superoxide is activation of an NADPH oxidase in a cytoplasmic granule.[21,22] However, the weight of present opinion is that O_2^- arises from the activity of a membrane-bound NADH or NADPH oxidase (reviewed in Reference 30). If one accepts the latter view, then one must envisage that the initiating membrane interaction leads to a transmembrane transport of cations; the resulting increased intracellular Ca^{2+} level in some (not necessarily direct) fashion induces an activation of a membrane oxidase. Although this concept may seem unduly complex, if it turns out to be true, it would be merely another example of nature not obeying our initial notions of simplicity.

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