

MITOCHONDRIAL *N*-FORMYLMETHIONYL PROTEINS AS CHEMOATTRACTANTS FOR NEUTROPHILS*

BY HARVEY CARP‡

*From The Department of Pathology, Health Sciences Center, State University of New York at Stony Brook,
Stony Brook, New York 11794*

The accumulation of polymorphonuclear leukocytes (PMN)¹ plays an important role in the development and manifestations of the acute inflammatory response. Although the primary role of the neutrophil is destruction of invading microorganisms, potentially harmful substances may be released from neutrophils with subsequent damage to surrounding tissue (1).

PMN are thought to move from the vascular system and accumulate at sites of inflammation by the process of directed migration, or chemotaxis (2). A number of agents of both host and microbial origin have been shown to be capable of mediating this process (2). The extremely potent PMN chemoattractants released from *Escherichia coli* (3) have been partially characterized and were found to consist, mainly, of hydrophobic peptides with blocked N-terminal amino groups (4). Schiffmann et al. (5) proposed that the bacterial factors could consist of *N*-formylmethionine containing peptides derived from bacterial proteins, since prokaryotes initiate protein synthesis with *N*-formylmethionine. In support of this hypothesis, synthetic *N*-formylmethionine containing peptides were shown to be potent leukocyte attractants (5, 6).

In contrast to prokaryotes, the synthesis of proteins encoded in eukaryotic nuclear DNA is initiated with a nonformylated methionine residue (7). However, mitochondria, present in eukaryotic cells, are also capable of protein synthesis (8-10). Mitochondrial DNA directs the synthesis of a set of hydrophobic proteins, found in the mitochondrial respiratory complexes (8-10). The mitochondrial protein synthetic apparatus uses *N*-formylmethionine to initiate protein synthesis (8-10), in a manner similar to prokaryotes. Importantly, *N*-formylmethionine has been shown to be retained at the amino terminus of several purified mitochondrially synthesized proteins (11-13).

It therefore seemed possible that *N*-formylmethionyl mitochondrial proteins could be chemotactic for PMN, in a manner similar to bacterial and synthetic *N*-formylmethionine peptides. In this event, mitochondrial *N*-formylmethionine containing peptides released from degenerating mitochondria at sites of tissue damage might play a role in the accumulation of inflammatory cells observed at these sites.

* Supported by grant 5-T32-07088 from the U.S. Public Health Service.

‡ Postdoctoral fellow of the National Institute of Environmental Sciences.

¹ Abbreviations used in this paper: BOC-Phe-Leu-Phe-Leu-Phe, *N*-tert-butyloxycarbonyl-phenylalanine-leucine-phenylalanine-leucine-phenylalanine; complex II, succinate-coenzyme Q reductase; complex III, ubiquinone-cytochrome *c* reductase; CYOX, cytochrome oxidase; FMLP, *N*-formylmethionyl-leucyl-phenylalanine; HMS, 15 mM HEPES; 220 mM mannitol; 70 mM sucrose; MED 199, medium 199; MED 199S, MED 199 containing 20 mM HEPES, 2% bovine serum albumin, penicillin, and streptomycin; PMN, polymorphonuclear leukocytes.

We tested this possibility in an *in vitro* system. The present report demonstrates that disrupted human mitochondria stimulate PMN chemotaxis *in vitro*. Indirect evidence is presented to show that this chemotactic activity is mediated by mitochondrially derived *N*-formylmethionyl proteins. In addition, purified mitochondrial *N*-formylmethionyl proteins were found to be chemotactic for PMN *in vitro*, whereas nonformylated mitochondrial proteins, tested under the same conditions, were not chemotactic.

Materials and Methods

Preparation of Leukocytes. PMN were prepared from the heparinized peripheral blood of healthy volunteers by Ficoll-Hypaque and dextran sedimentation (Ficoll-Paque and Dextran T-500; Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) (14). Remaining erythrocytes were removed by hypotonic lysis. Typical PMN preparations contained >95% PMN, of which >95% excluded trypan blue.

Assay of Chemotactic Activity. Chemotaxis was measured by a modification of the leading front assay described by Zigmond and Hirsch (15). PMN were suspended at a density of 5×10^6 cells/ml in medium 199 (MED 199) (GIBCO Laboratories, Grand Island Biological Co., Grand Island, N. Y.) containing 20 mM HEPES, 2% bovine serum albumin (defatted), penicillin, and streptomycin, pH 7.4 (MED 199S). The leukocytes were placed in the upper compartment of a chemotaxis chamber. A 5.0- μ m micropore filter (Sartorius Membranfilter, Göttingen, West Germany) separated the upper and lower compartments. In all experiments, 100 μ l of the chemotactic stimulus (diluted in MED 199S) was added to the lower compartment. Leukocytes were allowed to migrate for 45 min at 37°C, after which the filters were removed and stained with hematoxylin. PMN migration was determined as the mean distance that the leading two cells had migrated into the filter (15). In all experiments, five fields were averaged for each of triplicate filters. Stock preparations of disrupted mitochondria or purified mitochondrial proteins (suspended in MED 199S containing 0.1% [vol/vol] Tween-80), as well as all other chemotactic factors, were diluted in MED 199S before testing chemotactic activity. In all cases, the final concentration of Tween-80 was not >0.006% (vol/vol). This concentration of detergent did not affect PMN migration under the present conditions. Controls, in which buffer alone (MED 199S, containing 0.006% [vol/vol] Tween-80) was present in the lower compartment of the chemotaxis chamber, were always run (buffer control). Chemoattractants used as positive controls included human serum activated with zymosan (16) (Sigma Chemical Co., St. Louis, Mo.) and the synthetic peptide *N*-formylmethionyl-leucyl-phenylalanine (FMLP); (Sigma Chemical Co.) (6). *N*-tert-butyloxycarbonyl-phenylalanine-leucine-phenylalanine-leucine-phenylalanine (BOC-Phe-Leu-Phe-Leu-Phe) was obtained from Peninsula Labs, Inc. (San Carlos, Calif.). When used, BOC-Phe-Leu-Phe-Leu-Phe (10^{-6} M) was present in both the upper and lower compartments of the chemotactic chamber (17). This agent alone did not affect PMN migration, under the present conditions.

Isolation of Subcellular Organelles. Mitochondria were prepared from cultured human cells by a modification of the digitonin washing technique described by Greenawalt (18). Aseptic conditions were maintained throughout the isolation procedure. All steps were performed in the cold, unless otherwise noted. Washed cells (5×10^9) were diluted in 3 vol of 100 mM sucrose (containing 20 mM HEPES, penicillin, and streptomycin, pH 7.4) and homogenized in a tight-fitting Teflon-on-glass Dounce homogenizer (Arthur H. Thomas Co., Philadelphia, Pa.). Immediately thereafter, mannitol was added (final concentration of mannitol, 220 mM; sucrose, 70 mM; HEPES, 15 mM, pH 7.4 [HMS]) and unbroken cells were pelleted by centrifugation at 1,000 *g* for 10 min. Homogenization was repeated until >70% of the nuclei were released, as determined by phase-contrast microscopy. Mitochondria were pelleted from the combined supernatants by centrifugation at 5,100 *g* for 10 min. The resulting mitochondrial pellet was washed three times with HMS (5,100 *g* for 10 min) and suspended at a protein concentration of 100 mg/ml in the same buffer. 1 vol of the foregoing mitochondrial suspension was added to an equal volume of 1.2% (wt/vol) digitonin (Calbiochem-Behring Corp., American Hoechst Corp., San Diego Calif.) dissolved in HMS and stirred for 15 min on ice, after which the digitonin-treated mitochondria were diluted by the addition of 3 vol of HMS, homogenized by

hand, and centrifuged at 10,000 *g* for 10 min. The resulting mitochondrial pellet was then washed three times with HMS (10,000 *g* for 10 min), suspended in MED 199S, and used immediately thereafter in experiments in which nondisrupted mitochondria were used. For experiments in which disrupted mitochondria were used, the washed mitochondrial pellet was suspended at a protein concentration of 200 mg/ml in MED 199S (containing 0.1% (vol/vol) Tween-80) and sonicated (Branson Sonifier, Branson Sonic Power Co., Danbury, Conn., maximum power setting) for seven cycles of 2 min, in the cold. Disrupted mitochondria thereby obtained were stored at -80°C before use. Lysosomes were isolated from HeLa cells by the technique described by Van Dijk et al. (19). Isolated lysosomes were suspended in MED 199S and sonicated (Branson Sonifier, maximum power setting) for three cycles of 2 min, in the cold, before use. A biochemical assessment of the purity of the isolated subcellular, organelles was obtained by the method described by Leighton et al. (20), assuming 22% microsomal, 20% mitochondrial, and 1.0% lysosomal protein in the initial homogenate (21). Cytochrome oxidase (22), glucose-6-phosphatase (23), and acid phosphatase (24) were used as mitochondrial, microsomal, and lysosomal markers, respectively (21). Typical digitonin-washed mitochondrial preparations contained >90% mitochondrial protein and not >5% microsomal and 2% lysosomal contamination. Typical lysosomal preparations contained not >10% mitochondrial contamination.

Cell Culture. HeLa cells (Flow Laboratories, Inc., Rockville, Md.) were suspension cultured in Eagle's minimum essential medium with Spinner Salts (GIBCO Laboratories) containing penicillin, streptomycin, and 5% calf serum. WI-38 (Flow Laboratories) and human foreskin fibroblasts (a gift of Dr. L. Taichmann, State University of New York at Stony Brook) were cultured in Eagle's minimum essential medium with Earle's Salts (GIBCO Laboratories) containing penicillin, streptomycin, and 10% fetal calf serum, in roller bottles. All sera were heat inactivated at 56°C for 30 min, before use. In all cases, serum-containing medium was removed, and cells were washed with serum-free medium and maintained in serum-free culture for at least 8 h before harvesting. HeLa cells were harvested by centrifugation (1,000 *g* for 15 min); WI-38 and foreskin fibroblasts were harvested by scraping with a rubber policeman. After harvesting, all cell types were washed four times with at least 50 vol of phosphate-buffered saline (containing penicillin and streptomycin, pH 7.4) before being used for isolation of mitochondria or lysosomes. All cell cultures were checked (Hoecht's staining) and found to be free of mycoplasma contamination.

Special Reagents and Assays. Purified bovine heart mitochondrial cytochrome oxidase (CYOX) (22), ATPase (25), ubiquinone-cytochrome *c* reductase (complex III) (26), and succinate-coenzyme *Q* reductase (complex II) (27) were gifts of Dr. R. Capaldi, University of Oregon. Stock preparations of all mitochondrial proteins were extensively dialyzed against MED 199S (containing 0.1% [vol/vol] Tween-80) before use. In some experiments, cytochrome oxidase enzymatic activity was inhibited by treatment with NaN_3 before dialysis. The immunoglobulin fraction of specific rabbit antibodies directed against bovine mitochondrial CYOX and ATPase were prepared, assayed, and tested for specificity as previously described (28). Antibodies directed against bovine mitochondrial CYOX and ATPase cross react immunologically with the respective human enzymes (28). Rabbit antibodies directed against the third and fifth components of human complement, as well as control rabbit immunoglobulin, were obtained from Accurate Chemical & Scientific Corp. (Westbury, N. Y.). The immunoglobulin fraction of all of the foregoing antisera were used in chemotaxis experiments, following dialysis against MED 199 and heating at 56°C for 30 min. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.) with ovalbumin as standard.

Results

Chemotactic Activity of Disrupted Mitochondria. To study the effect of mitochondrial proteins on neutrophil migration in vitro, mitochondria were isolated from three cultured human cell types. Mitochondria isolated from HeLa cells, WI-38, and human foreskin fibroblasts were disrupted and tested for PMN-chemotactic activity, as described in Materials and Methods. As shown in Table I, disrupted mitochondria from all of the cell types tested stimulated PMN migration in vitro. The most active

TABLE I
Chemotactic Activity of Disrupted Mitochondria

Chemotactic factor		Migration into filter of leading cell front
Source	Protein*	PMN chemotaxis‡
	μg	μm
Buffer control	—	39 \pm 1.7
HeLa-Mito§	100	94 \pm 7.3
HeLa-Mito§	10	64 \pm 3.0
HeLa-Mito§	1.0	42 \pm 2.0
WI-38-Mito§	100	130 \pm 7.2
WI-38-Mito§	10	70 \pm 5.1
WI-38-Mito§	1.0	40 \pm 3.0
Foreskin-Mito§	100	123 \pm 8.9
Foreskin-Mito§	10	69 \pm 5.3
Foreskin-Mito§	1.0	58 \pm 2.6
Foreskin-Mito§	0.1	40 \pm 2.0

* Amount of mitochondrial protein added to the stimulus compartment of the chemotaxis chamber.

‡ Chemotactic activity is expressed as the mean \pm 1 SE of at least three experiments, each performed in triplicate.

§ Mitochondria isolated from HeLa cells (HeLa-Mito), human foreskin fibroblasts (foreskin-Mito), and WI-38 fibroblasts (WI-38-Mito) were disrupted before chemotactic assay, as described in Materials and Methods.

|| $P < 0.05$ compared with buffer control (Student's *t* test).

preparation (foreskin fibroblast) stimulated migration when 1.0 μg of mitochondrial protein was present in the stimulus compartment of the chemotaxis chamber (see Table I).

In the foregoing experiments, disruption of the isolated mitochondria, before chemotactic testing, was necessary to demonstrate significant chemotactic activity. However, after incubation of nondisrupted HeLa cell mitochondria with a lysosomal lysate (from the same cells) for 1 h at 37°C in MED 199S (pH 7.0), significant chemotactic activity was detected (microns migrated leading front PMN: buffer control = 42 \pm 2.1 μm ; HeLa-mitochondria, nondisrupted [100 μg] 47 \pm 1.9 μm ; HeLa-mitochondria, nondisrupted [100 μg] + HeLa lysosomes [20 μg] 71 \pm 2.7 μm ; mean of three experiments \pm 1 SE). Lysosomes alone were not chemotactic and boiled lysosomes did not activate nondisrupted mitochondria (data not shown).

Characterization of Mitochondrial Chemotactic Activity. To date, mitochondrially synthesized *N*-formylmethionyl proteins have been identified as components of mitochondrial CYOX, ATPase, and complex III (8-13). Therefore, specific antibodies directed against mitochondrial CYOX and ATPase were tested for their ability to block the chemotactic activity of disrupted mitochondria in vitro. To assure maximum inhibition of chemotaxis, disrupted mitochondria were preincubated with a combination of specific anti-CYOX and ATPase antibodies. After 15 min at 37°C, the remaining chemotactic activity was measured as before. As shown in Table II, such treatment significantly decreased the chemotactic activity of disrupted mitochondria. (Pretreatment with anti-CYOX or anti-ATPase, separately, also resulted in a decrease in chemotactic activity [data not shown]). Control rabbit immunoglobulin (Ig) was without effect (Table II). Furthermore, anti-CYOX and anti-ATPase antibodies were

TABLE II
Characterization of Mitochondrial Chemotactic Activity

Chemotactic factor*	Migration into filter of leading front PMN‡				
	Treatment of chemotactic factor§				
	None	Anti-CYOX + anti- ATPase	Anti-C ₃	Anti-C ₅	Ig
	μm	μm	μm	μm	μm
HeLa-Mito	87 ± 5.2	60 ± 3.1¶	89 ± 6.0	92 ± 4.9	89 ± 2.2
WI-38-Mito	118 ± 5.0	78 ± 4.7¶	122 ± 3.9	115 ± 2.9	115 ± 4.0
Foreskin-Mito	101 ± 3.9	73 ± 4.8¶	96 ± 3.1	105 ± 4.0	106 ± 3.5
Activated serum	130 ± 4.0	NT**	NT	55 ± 2.4¶	NT
FMLP	119 ± 3.1	124 ± 2.2	NT	NT	NT
Buffer control	37 ± 2.0	43 ± 2.9	39 ± 3.0	40 ± 1.7	36 ± 2.2

* Concentrations: activated (zymosan) human serum, 10% (vol/vol); FMLP, 10^{-8} M; HeLa, WI-38, and foreskin fibroblast mitochondria, 100 μg (protein). In all cases chemotactic factors were present only in the stimulus compartment of the chemotaxis chamber.

‡ Chemotactic activity is expressed as the mean \pm 1 SE of at least three experiments, each performed in triplicate.

§ 5 μl of anti-C₅ (25 mg/ml), anti-C₃ (25 mg/ml), anti-CYOX + anti-ATPase (25 mg/ml), or rabbit Ig (25 mg/ml) was incubated with 100 μl of chemotactic factor for 15 min at 37°C before chemotactic assay.

|| Mitochondria isolated from HeLa cells (HeLa-Mito), foreskin fibroblasts (foreskin-Mito), and WI-38 fibroblasts (WI-38-Mito) were disrupted before chemotactic assay, as described in Materials and Methods.

¶ $P < 0.05$, compared with untreated control sample (Student's *t* test).

** Not tested.

not chemotactic alone (Table II) nor did they affect the PMN chemotactic response to FMLP (Table II).

The inhibition of mitochondrially derived PMN-chemotactic activity by specific antibodies directed against two mitochondrially synthesized proteins, suggests that these proteins may have been responsible for at least part of this activity. In addition, antigenic fragments of partially degraded or incompletely synthesized mitochondrial proteins may have also played a role. These conclusions are also consistent with the inability of antibodies directed against the third (C₃) and fifth (C₅) components of complement to inhibit mitochondrially derived chemotactic activity (see Table II). This suggests that complement components were not involved under the present conditions.

Chemotactic Activity of Purified Mitochondrial Proteins. We further tested the hypothesis that mitochondrially synthesized, *N*-formylmethionyl proteins are chemotactic for PMN in experiments using purified bovine heart mitochondrial proteins in place of disrupted mitochondria. Both formylated and nonformylated mitochondrial proteins were tested. Mitochondrial CYOX contains seven protein subunits (22). Subunits I–III are synthesized by the mitochondria (8–10, 12, 13, 29) and subunits I and II have been shown to contain *N*-formylmethionine residues (12, 13). The remaining subunits of CYOX (subunits IV–VII) are synthesized in the cytoplasm and do not contain *N*-formylmethionine (8–10, 12, 13, 29). As shown in Fig. 1, cytochrome oxidase (holoenzyme) and subunits I–II (mitochondrially synthesized) all stimulated PMN migration in vitro (see Fig. 1A), whereas cytochrome oxidase subunits IV–VI (cytoplasmically synthesized) did not (see Fig. 1B). Inhibition of cytochrome oxidase

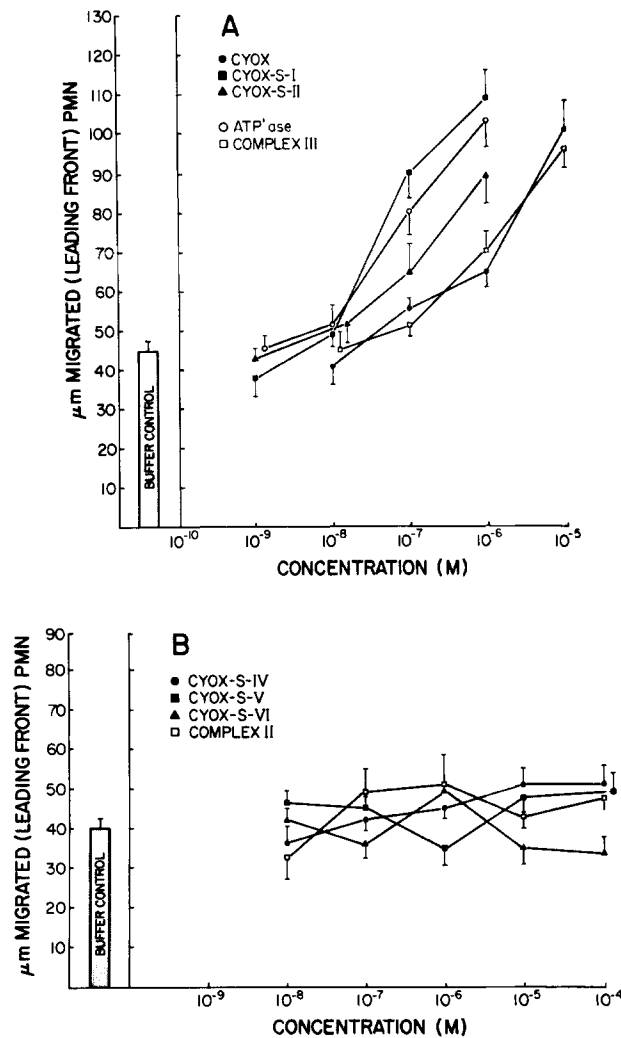


FIG. 1. Chemotactic activity of purified mitochondrial proteins. Chemotactic activity is plotted as the migration (in microns) into the filter of the leading front PMN. Each point represents the mean \pm 1 SE of at least three experiments, each performed in triplicate. The abscissa represents the concentration of chemotactic factor present in the stimulus compartment of the chemotactic chamber. Panel A shows the chemotactic activity of *N*-formylmethionine containing mitochondrial proteins. Panel B shows the activity of mitochondrial proteins not containing *N*-formylmethionine. CYOX_I, CYOX_{II}, CYOX_{IV}, CYOX_V, CYOX_{VI}: CYOX subunits I, II, IV, V, and VI, respectively.

enzymatic activity with NaN₃ did not affect chemotactic activity (data not shown). Mitochondrial ATPase and complex III, both of which contain mitochondrially synthesized subunits (8–11), also stimulated PMN migration (Fig. 1 A). Furthermore, mitochondrial complex II, which does not contain mitochondrially synthesized subunits (8–10), did not stimulate PMN migration (see Fig. 1 B). The results in Fig. 1 demonstrate that mitochondrially synthesized *N*-formylmethionyl proteins are capable of stimulating PMN chemotaxis *in vitro*, whereas cytoplasmically synthesized, nonformylated mitochondrial proteins, tested under the same conditions, did not stimulate PMN migration.

Inhibition of Mitochondrial Chemotactic Activity by BOC-Phe-Leu-Phe-Leu-Phe. We further tested the notion that the PMN chemotactic activity of mitochondrially synthesized proteins is mediated through the presence of an *N*-formylmethionine residue by using the formyl peptide antagonist BOC-Phe-Leu-Phe-Leu-Phe (17, 30). BOC-Phe-Leu-Phe-Leu-Phe is thought to inhibit the PMN response to *N*-formylmethionine containing peptides by competitively inhibiting the binding of these factors to specific cell surface receptors (2, 17, 30). As shown in Table III, when BOC-Phe-Leu-Phe-Leu-Phe was present in both compartments of the chemotaxis chamber, the PMN chemotactic response to purified mitochondrial proteins or disrupted mitochondria was significantly reduced. Under the conditions in Table III, BOC-Phe-Leu-Phe-Leu-Phe alone did not affect PMN migration to zymosan-activated serum (see Table III), whereas the PMN chemotactic response to FMLP was significantly decreased (see Table III). The findings using BOC-Phe-Leu-Phe-Leu-Phe suggest that mitochondrial PMN chemoattractants interact with neutrophil *N*-formylmethionyl peptide receptors (2, 17, 30) and imply that the chemotactic activity of disrupted mitochondria and purified mitochondrial proteins is mediated, at least in part, through *N*-formylmethionine residues present in these proteins.

Disrupted Mitochondria and Purified Mitochondrial Proteins Stimulate PMN Directed Migra-

TABLE III
Effect of BOC-Phe-Leu-Phe-Leu-Phe on Mitochondrially Derived Chemotactic Activity

Chemotactic factor*	PMN chemotaxis‡	
	Without BOC-Phe-Leu-Phe-Leu-Phe	With BOC-Phe-Leu-Phe-Leu-Phe§
	μm	
Buffer control	44 \pm 3.5	45 \pm 2.2
CYOX	99 \pm 5.2	56 \pm 3.2 ¶
CYOX _I	109 \pm 5.7	61 \pm 4.5 ¶
CYOX _{II}	89 \pm 5.0	59 \pm 4.0 ¶
ATPase	102 \pm 4.3	70 \pm 3.7 ¶
HeLa-Mito**	85 \pm 5.0	64 \pm 3.8 ¶
WI-38-Mito**	94 \pm 5.4	70 \pm 5.0 ¶
Foreskin-Mito**	120 \pm 3.8	79 \pm 6.1 ¶
FMLP	115 \pm 3.8	67 \pm 3.1 ¶
Activated serum	125 \pm 3.4	129 \pm 3.7

* Concentrations: CYOX, 10^{-5} M; CYOX subunit I (CYOX_I), 10^{-6} M; CYOX subunit II (CYOX_{II}), 10^{-6} M; activated (zymosan) human serum, 10% (vol/vol); FMLP, 10^{-6} M; HeLa WI-38, and foreskin fibroblast mitochondria, 100 μg (protein). In all cases, chemotactic factors were present only in the stimulus compartment of the chemotaxis chamber.

§ BOC-Phe-Leu-Phe-Leu-Phe (10^{-6} M) was present in both upper and lower compartments of the chemotaxis chamber.

‡ Chemotactic activity is expressed as the mean \pm 1 SE of at least three experiments, each performed in triplicate.

|| $P < 0.05$, compared with buffer control (Student's *t* test).

¶ $P < 0.05$, compared with sample without BOC-Phe-Leu-Phe-Leu-Phe (Student's *t* test).

** Mitochondria isolated from HeLa cells (HeLa-Mito), foreskin fibroblasts (foreskin-Mito), and WI-38 fibroblasts (WI-38-Mito) were disrupted before chemotaxis assay, as described in Materials and Methods.

TABLE IV
Stimulation of Directed Cell Migration by Disrupted Mitochondria and Purified Mitochondrial Protein

Contents of chamber compartment*		Migration into filter of leading front PMN‡
Upper	Lower	
Buffer + PMN	Buffer	37 ± 1.2
HeLa-Mito§ + PMN	HeLa-Mito§	65 ± 2.2
Buffer + PMN	HeLa-Mito§	85 ± 3.0 ¶
CYOX + PMN	CYOX	59 ± 2.9
Buffer + PMN	CYOX	87 ± 3.6 **

* Concentrations: CYOX, 10^{-5} M; HeLa-Mito, 100 µg (protein).

‡ Chemotactic activity is expressed as the mean ± 1 SE of at least three experiments, each performed in triplicate.

§ Mitochondria disrupted before chemotactic assay, as described in Materials and Methods.

|| $P < 0.05$, compared with migration with buffer alone in both compartments (Student's *t* test).

¶ $P < 0.05$, compared with migration with HeLa-Mito in both compartments (Student's *t* test).

** $P < 0.05$, compared with migration with CYOX in both compartments (Student's *t* test).

tion (Chemotaxis). To determine whether disrupted mitochondria or purified mitochondrial proteins stimulated directed PMN migration (chemotaxis) or only increased random PMN migration, experiments were performed in which active material was placed in various compartments of the chemotactic chamber (15). As shown in Table IV, there was significantly less PMN migration when disrupted HeLa cell mitochondria or CYOX were present at equal concentrations in both compartments of the chemotactic chamber, as opposed to when PMN were exposed to a gradient of stimulus (i.e., mitochondria or CYOX were present only in the lower compartment). However, both of these combinations produced significantly more cell migration than exposure of PMN to buffer alone (Table IV). Similar results were obtained with WI-38 and foreskin mitochondria, as well as with mitochondrial ATPase. These results suggest that disrupted mitochondria and purified mitochondrial proteins are capable of stimulating directed PMN migration (chemotaxis), as well as random migration (15).

Discussion

Previous studies have demonstrated that a number of agents of both host and microbial origin possess potent PMN chemotactic activity (2). These include complement fragments (16, 31), oxidized lipids (32), connective tissue breakdown products (33), as well as factors derived from macrophages (34) and bacterial cells (3, 4). All of these agents are believed to play an important role in the accumulation of PMN at sites of inflammation *in vivo*. In addition to other factors, *N*-formylmethionine containing proteins released from degenerating mitochondria at sites of tissue injury may also play a role in the accumulation of inflammatory cells at such sites.

In this context it is important to note that mitochondrial degeneration is a sensitive indicator of cellular damage (35). In fact, mitochondrial enzymes have been reported

to be present, in small amounts, in the circulation following injury to myocardial or hepatic tissue in man (36). Although the tissue concentration of released mitochondrial proteins *in vivo* is not known, based on the results of the present study it can be estimated that the quantity of mitochondrially synthesized protein present in 250 mg of cardiac muscle (29) would give significant *in vitro* chemotactic activity.

Degradative enzymes present in tissue lysosomes have been shown to be capable of disrupting mitochondrial structure and function *in vitro* (37, 38). Our results suggest that lysosomally mediated mitochondrial disruption also results in the release of PMN chemoattractants from mitochondria. Oxygen radicals and lysosomal granule enzymes derived from PMN (1), initially attracted to an inflammatory site by nonmitochondrially derived chemotactic factors, may also aid in the disruption of mitochondria, with resultant release of mitochondrial PMN chemoattractants. Furthermore, if released mitochondrial *N*-formylmethionine-containing proteins are capable of stimulating PMN degranulation in a manner similar to synthetic *N*-formylmethionyl peptides (6), they could serve as an amplification loop for further generation of chemotactic factor. Although native mitochondrial proteins were found to be chemotactic, partially degraded fragments (as well as incompletely synthesized fragments) produced as a result of mitochondrial degradation, may also attract PMN. Additional studies are necessary to clarify the conditions leading to the release of mitochondrial *N*-formylmethionyl proteins and the effects of the released proteins on PMN, as well as other inflammatory cells.

In the present study, purified bovine heart mitochondrial proteins were shown to be chemotactic for neutrophils. The analogous human proteins were not tested. However, when examined, bovine and human mitochondrial proteins have been found to be closely related in structure (22, 29, 39). In fact, there is a 72% sequence homology between bovine CYOX subunit II used in the present study and the analogous human protein (39). It is also interesting to note that the amino terminal sequence of bovine CYOX subunit I begins with the amino acid sequence *N*-formylmethionine-phenylalanine (13). This is identical to the sequence of a known synthetic PMN chemotactic peptide (6). Further studies using purified human mitochondrial proteins are necessary to extend the present results.

Disrupted mitochondria isolated from cultured human cells were found to be chemotactic for PMN. Although cultured cells are known to acquire characteristics *in vitro* not displayed by these cells *in vivo*, two of the cell types used (WI-38 and foreskin fibroblasts) were low passage, diploid cells. Furthermore, a preliminary experiment suggested that mitochondria isolated from fresh dog heart were also chemotactic for PMN *in vitro* (H. Carp, unpublished observation). However, additional studies using mitochondria isolated from fresh human tissue are necessary to confirm our results obtained using cultured cells.

Complement components are extremely potent PMN chemotactic agents (2, 31). Although a previous study (40) showed that human heart mitochondrial membranes interact with the complement system *in vitro*, chemotactic activity was not tested. However, under the conditions of the present study, complement components were not present in significant concentrations (i.e., serum-free chemotaxis medium and at least 8 h serum-free cell culture before mitochondrial isolation). In addition, anti-C₃ and anti-C₅ antibodies did not inhibit mitochondrial chemotactic activity. Although complement components play an important role in the accumulation of PMN *in vivo*

(2, 31, 41), under the present in vitro conditions complement did not appear to be involved.

Finally, in addition to mammalian cells, other eukaryotic cells, including fungi and protozoa (several of which are pathogenic for man [42]) also contain mitochondria (8-10). *N*-formylmethionine containing proteins released from these organisms might also attract inflammatory cells.

Many questions remain, and further studies are necessary to determine whether the release of mitochondrial *N*-formylmethionyl proteins plays a role in mediating the accumulation of inflammatory cells observed at sites of tissue damage in vivo. However, we believe our in vitro results support this possibility and render it worthy of further study.

Summary

Mitochondria synthesize several hydrophobic proteins. Like bacteria, mitochondria initiate protein synthesis with an *N*-formylmethionine residue. Because *N*-formylmethionyl peptides have been found to be chemotactic for polymorphonuclear leukocytes (PMN), mitochondria isolated from cultured human cells and purified bovine mitochondrial proteins were tested for PMN chemotactic activity in vitro. Isolated mitochondria, following disruption, stimulated PMN migration in vitro. Nondisrupted mitochondria were not chemotactic. However, intact mitochondria that had been incubated with a lysosomal lysate did stimulate PMN migration. Antibodies directed against two mitochondrial enzymes, cytochrome oxidase and ATPase, (both of which contain mitochondrially synthesized subunits) but not anti-C₃ or anti-C₅ decreased mitochondrially derived chemotactic activity. In addition, purified bovine mitochondrial *N*-formylmethionyl proteins stimulated PMN migration in vitro, whereas nonformylated mitochondrial proteins did not. Furthermore, the chemotactic activity of purified mitochondrial proteins and disrupted mitochondria was decreased by the formyl peptide antagonist butyloxycarbonyl-phenylalanine-leucine-phenylalanine-leucine-phenylalanine. Finally, disrupted mitochondria and purified mitochondrial proteins stimulated PMN-directed migration (chemotaxis), according to accepted criteria. In addition to other chemotactic factors, release of *N*-formylmethionyl proteins from mitochondria at sites of tissue damage, may play a role in the accumulation of inflammatory cells at these sites.

I am grateful to Dr. Aaron Janoff for his very special contributions to this study. His support and advice made this study possible. I would like to thank Dr. R. Capaldi (University of Oregon) for a gift of purified mitochondrial proteins and Dr. L. Taichmann (State University of New York at Stony Brook) for help in obtaining cultured human foreskin fibroblasts. Dr. Phillippe Laurent also provided helpful suggestions.

Received for publication 11 August 1981.

References

1. Janoff, A., R. White, H. Carp, S. Harel, R. Dearing, and D. Lee. 1979. Lung injury induced by leukocytic proteases. *Am. J. Pathol.* **97**:111.
2. Schiffmann, E., and J. Gallin, 1979. Biochemistry of phagocyte chemotaxis. *Curr. Top. Cell. Regul.* **15**:203.

3. Ward, P., I. Lepow, and L. Newman. 1968. Bacterial factors chemotactic for polymorphonuclear leukocytes. *Am. J. Pathol.* **52**:725.
4. Schiffmann, E., H. Showell, B. Corcoran, P. Ward, E. Smith, and E. Becker. 1975. The isolation and partial characterization of neutrophil chemotactic factors from *Escherichia coli*. *J. Immunol.* **114**:1831.
5. Schiffmann, E., B. Corcoran, S. Wahl. 1975. N-formylmethionyl peptides as chemoattractants for leukocytes. *Proc. Nat. Acad. Sci. U. S. A.* **72**:1059.
6. Showell, H., R. Freer, S. Zigmond, E. Schiffmann, S. Aswanikumar, B. Corcoran, and E. Becker. 1976. The structure-activity relations of synthetic peptides as chemotactic factors and inducers of lysosomal enzyme secretion for neutrophils. *J. Exp. Med.* **143**:1154.
7. Jackson, R., and T. Hunter. 1970. Role of methionine in the initiation of haemoglobin synthesis. *Nature (Lond.)* **227**:672.
8. Mahler, H. R. 1979. Mitochondrial biogenesis: evolution and regulation. In *Extrachromosomal DNA*. D. Cummings, P. Borst, I. Dawid, S. Weissman, and C. Fox, editors. Academic Press, Inc., New York. 11-34.
9. Beutow, D., and W. Wood. 1978. The mitochondrial translation system. *Subcell. Biochem.* **5**:1.
10. Schatz, G., and T. Mason. 1974. The biosynthesis of mitochondrial proteins. *Annu. Rev. Biochem.* **43**:51.
11. Von Jagow, G., H. Schagger, W. Engel, W. Machleidt, I. Machleidt, and H. Kolb. 1978. Beef heart complex III: isolation and characterization of cytochrome *b*. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **91**:121.
12. Steffens, G., and G. Buse. 1976. Studies on cytochrome-*c*-oxidase. I. Purification and characterization of the enzyme from bovine heart and identification of peptide chains in the complex. *Hoppe-Seyler's Z. Physiol. Chem.* **357**:1125.
13. Tanaka, M., K. Yasunobu, Y. Wei, and T. King. 1981. The complete amino acid sequence of bovine heart cytochrome oxidase subunit VI. *J. Biol. Chem.* **256**:4832.
14. Boyum, A. 1974. Separation of blood leukocytes, granulocytes and lymphocytes. *Tissue Antigens.* **4**:269.
15. Zigmond, S., and J. Hirsch. 1973. Leukocyte locomotion and chemotaxis. New method for evaluation and demonstration of cell-derived chemotactic factor. *J. Exp. Med.* **137**:387.
16. Gallin, J., R. Clark, and M. Frank. 1975. Kinetic analysis of chemotactic factor generation in human serum via activation of the classical and alternate complement pathways. *Clin. Immunol. Immunopathol.* **3**:334.
17. Aswanikumar, S., B. Corcoran, and E. Schiffmann. 1977. Peptides with agonist and antagonist chemotactic activity. In *Peptides*. M. Goodman and J. Meienhoffer, editors. John Wiley & Sons, New York. 141.
18. Greenawalt, J. 1974. The isolation of outer and inner mitochondrial membranes. *Methods Enzymol.* **31**:310.
19. Van Dijk, W., P. Roholl, D. Reijngoud, and J. Tager. 1976. A simple procedure for the isolation of lysosomes from normal rat liver. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **62**:177.
20. Leighton, F., B. Poole, H. Beaufay, P. Baudhuin, J. Coffey, J. Fowler, and C. deDuve. 1968. The large-scale separation of peroxisomes, mitochondria, and lysosomes from the livers of rats injected with Triton WR-1339. Improved isolation procedures, automated analysis, biochemical and morphological properties of fractions. *J. Cell Biol.* **37**:482.
21. de Duve, C. 1971. Tissue fractionation. Past and present. *J. Cell Biol.* **50**:20.
22. Downer, N., N. Robinson, and R. Capaldi. 1976. Characterization of a seventh different subunit of beef heart cytochrome oxidase. Similarities between the beef heart enzyme and that from other species. *Biochemistry.* **15**:2030.
23. Barrett, A., and M. Heath. 1977. Lysosomal enzymes. In *Lysosomes: A Laboratory*

- Handbook, J. Dingle, editor. Elsevier North-Holland Biomedical Press. Amsterdam. 2nd edition. 19.
24. de Duve, C., B. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans. 1955. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem. J.* **60**: 604.
 25. Berden, J., and M. Voorn-Brouwer. 1978. Studies on the ATPase complex from beef-heart mitochondria. *Biochim. Biophys. Acta.* **501**:424.
 26. Rieske, J. 1967. Preparation and properties of reduced coenzyme Q-cytochrome *c* reductase (Complex III of the respiratory chain). *Methods Enzymol.* **10**:239.
 27. Davis, K., and Y. Hatefi. 1971. Succinate dehydrogenase. I. Purification, molecular properties, and substructure. *Biochemistry.* **10**:2509.
 28. Hackenbrock, C., R. Chau, K. Hannon, and L. Hochli. 1979. Localization of enzymes in mitochondrial membranes with ferritin conjugates of affinity purified antibodies. *Methods Enzymol.* **56**:683.
 29. Hare, J., E. Ching, and G. Attardi. 1980. Isolation, subunit composition, and site of synthesis of human cytochrome oxidase. *Biochemistry.* **19**:2023.
 30. Naccache, P., M. Volpi, H. Showell, E. Becker, and R. Sha'afri. 1979. Chemotactic factor-induced release of membrane calcium in rabbit neutrophils. *Science (Wash. D. C.)*. **203**:461.
 31. Fernandez, H., P. Henson, A. Otani, and T. Hugli. 1978. Chemotactic response to human C_{3(a)} and C_{5(a)} anaphylatoxins. Evaluation of C_{3a} and C_{5a} leukotaxis *in vitro* and under simulated *in vivo* conditions. *J. Immunol.* **120**:109.
 32. Goetzl, E., J. Woods, and R. Corman. 1977. Stimulation of human eosinophil and neutrophil polymorphonuclear leukocyte chemotaxis and random migration by 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid. *J. Clin. Invest.* **59**:179.
 33. Senior, R. M., G. L. Griffin, and R. P. Mecham. 1980. Chemotactic activity of elastin-derived peptides. *J. Clin. Inv.* **66**:859.
 34. Kazmierowski, J. A., J. I. Gallin, H. Y. Reynolds. 1977. Mechanism for the inflammatory response in primate lungs. Demonstration and partial characterization of an alveolar macrophage-derived chemotactic factor with preferential activity for polymorphonuclear leukocytes. *J. Clin. Invest.* **59**:273.
 35. Trump, B., and F. Ginn. 1969. The pathogenesis of subcellular reaction to lethal injury. In *Methods and Achievements in Experimental Pathology*. E. Bajusz and G. Jasmin, editors. Yearbook Medical Publishers, Inc. Chicago. **IV**:1.
 36. Rej, R. 1980. An immunochemical procedure for determination of mitochondrial aspartate aminotransferase in human serum. *Clin. Chem.* **26**:1694.
 37. Mellors, A., A. Tappel, P. Sawant, and I. Desai. 1967. Mitochondrial swelling and uncoupling of oxidative phosphorylation by lysosomes. *Biochim. Biophys. Acta.* **143**:299.
 38. Allan, R., and E. Welman. 1980. Proteolysis of isolated mitochondria by myocardial lysosomal enzymes. *Biochem. J.* **190**:139.
 39. Barrell, B., A. Bankier, and J. Drouin. 1979. A different genetic code in human mitochondria. *Nature (Lond.)*. **282**:189.
 40. Giclas, P., R. Pinkard, and M. Olson. 1979. *In vitro* activation of complement by isolated human heart subcellular membranes. *J. Immunol.* **122**:146.
 41. Ward, P., and J. Hill. 1972. Biologic role of complement products: Complement derived leukotactic activity extractable from lesions of immunologic vasculitis. *J. Immunol.* **108**:1137.
 42. Robbins, S. 1974. *Pathologic Basis of Disease*. W. B. Saunders Company. Philadelphia. 442.