MACROPHAGES SECRETE A NOVEL HEPARIN-BINDING PROTEIN WITH INFLAMMATORY AND NEUTROPHIL CHEMOKINETIC PROPERTIES

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Macrophages secrete a wide range of biologically active mediators in response to inflammatory stimuli (reviewed in reference 1). Some of these products, such as IL-1 and cachectin/TNF, have been well characterized (2, 3), whereas others remain poorly defined. Early studies suggested that during inflammation, secreted products of activated macrophages elicit a wide variety of tissue responses including neutrophil infiltration and activation (see references 4 and 5 for reviews). However, identification of the specific monocyte-derived endogenous mediators responsible for these effects has been difficult and has frequently led to conflicting results. Furthermore, many of the biological responses seen with crude or partially purified products of activated macrophages have not been reproduced with recombinant-derived cytokines, suggesting that some biologically important peptides have yet to be identified (6, 7).

During the purification and characterization of cachectin/TNF (8) and an inhibitor of Friend erythroleukemic cell differentiation (9, 10), a third protein was observed with interesting physical properties. Evidence is presented here that this protein, which appears as a doublet of ~8,000 daltons on SDS-polyacrylamide gels, forms multimers of varied molecular masses up to and exceeding 10^6 daltons as assessed by gel filtration. Partial NH_2-terminal amino acid sequence information reveals no significant homology with any previously described protein. The monokine is anionic under physiological conditions but is one of two

major heparin-binding proteins secreted by these cells. This monokine, which we term "macrophage inflammatory protein" (MIP),[1] induces neutrophil chemokinesis and, to a lesser extent, hydrogen peroxide production in vitro. In addition, MIP elicits a localized inflammatory response when administered subcutaneously.

Materials and Methods

Materials. Purified, recombinant human cachectin/TNF was obtained from Chiron Corp., Emeryville, CA. Purified, human rIL-1α was the generous gift of Dr. P. Lomedico (Hoffman-La Roche, Inc., Nutley, NJ). All chemicals were of the highest grades available from commercial suppliers.

Animals. C3H/HeN mice were obtained from Charles River Breeding Laboratories, Inc. (Kingston, NY). Mice of the endotoxin-resistant C3H/HeJ strain were obtained from Jackson Laboratories (Bar Harbor, ME).

Cell Culture. The mouse macrophage cell line RAW 264.7 and the cachectin/TNF–sensitive cell line L929 were obtained from American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 or DMEM (Gibco, Grand Island, NY), respectively. Both media were supplemented with 20 mM Hepes and 10% FCS (HyClone Laboratories, Sterile Systems, Inc., Logan, UT). For the production of stimulated RAW 264.7 supernatants, cells were grown in 150-mm tissue culture dishes (Falcon Labware, Becton, Dickinson & Co., Oxnard, CA) in RPMI plus 10% FCS until they reached confluency. The cells were washed five times in HBSS and the medium was replaced with serum-free RPMI supplemented with 1 μg/ml of LPS (LPS W, *E. coli* 0127:B8, Difco Laboratories, Inc., Detroit, MI). The cells were incubated at 37°C for 16–18 h and the supernatants filtered through 0.22-μm filters.

Purification of MIP. 1–5 liters of supernatant were concentrated 16–40-fold in a DC2 hollow fiber concentration system with a 10,000-dalton cutoff (Amicon Corp., Lexington, MA) and diafiltrated against 6 liters of 20 mM Tris buffer, pH 8.0, using the same device. Octyl glucoside was added to the concentrated, diafiltrated supernatant to a final concentration of 1% (wt/vol) and the mixture was applied to a Mono Q 10/10 (anion exchange [Pharmacia Fine Chemicals, Rahway, NJ]) column previously equilibrated with 20 mM Tris buffer, pH 8.0, and connected to a fast protein liquid chromatography (FPLC; Pharmacia Fine Chemicals) apparatus. A linear gradient of 114 ml (total vol) from 0 to 1 M NaCl in the same buffer (and a flow rate of 2 ml/min) was used for elution.

Samples of each fraction were subjected to SDS-PAGE in 10–15% or 10–18% linear gradient slab gels under reducing conditions. Molecular mass standards (Bethesda Research Laboratories, Bethesda, MD) were run in parallel. Fractions containing MIP eluted in the same region as cachectin/TNF and were easily recognized as a characteristic doublet of ~8,000 daltons.

Peak MIP-containing fractions (as assessed by SDS-PAGE and silver staining) were pooled, concentrated, and fractionated on a high-performance gel filtration column (Superose 12; Pharmacia Fine Chemicals) previously equilibrated with 100 mM ammonium acetate. MIP was recovered in the void volume of the column and was >95% pure as judged by SDS-PAGE and silver staining. MIP purified in this manner contained ~0.2 ng LPS/μg MIP.

Heparin Chromatography. Heparin-conjugated Sepharose (Pharmacia Fine Chemicals) was used to assay the ability of MIP to bind heparin. A C10/20 column (Pharmacia Fine Chemicals) packed with 8 ml of gel was attached to FPLC and equilibrated with 20 mM Tris, pH 8.0. 2 ml of 12-fold–concentrated and diafiltrated RAW 264.7 supernatant (as above) was applied to the column, and a linear gradient of 0–2 M NaCl in the same buffer was used for elution.

Chromatofocusing. 8 μg of a peak MIP-containing fraction from Mono Q chromatog-

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[1] *Abbreviations used in this paper:* FPLC, fast protein liquid chromatography; GBSS, Gey's balanced salt solution; MIP, macrophage inflammatory protein; PMN, polymorphonuclear leukocyte.

raphy was equilibrated in 25 mM bis-Tris plus 10% betaine (wt/vol), pH 7.1, and applied to a Mono P column previously equilibrated in the same buffer. Protein was eluted with a linear gradient of Polybuffer 74 (Pharmacia Fine Chemicals) (1:10 in double-distilled water) with 10% betaine, pH 4.0, resulting in a descending pH gradient ranging from 7–4.

Protein Assay. Protein content was measured by the Bradford assay (11) using BSA as a standard (Bio-Rad Laboratories, Richmond, CA).

Endotoxin Assay. Endotoxin levels were determined using a chromogenic limulus assay (Whittaker M.A. Bioproducts, Walkersville, MD) according to the instructions of the manufacturer.

Protein Sequencing. Purified MIP was sequenced by the Rockefeller University protein-sequencing facility. The Dayhoff protein sequence bank was searched for homologous amino acid sequences using the computer program P-fast-D.

In Vivo Inflammatory Activity. Polymorphonuclear leukocyte (PMN) infiltration was evaluated using footpad injections, according to Granstein et al. (12). Briefly, female C3H/HeJ mice (6–12 wks) were lightly anesthetized with phenobarbital (25 mg/kg body weight, i.p.). Animals were randomized to receive a subcutaneous footpad injection of 0.05 ml containing either 10^{-10} mol N-FMLP; 10, 100, or 1,000 ng of human rIL-1α or recombinant human cachectin/TNF; or 1, 10, 100, or 1,000 ng of murine MIP (purified as above) in RPMI 1640 with 0.1% FCS. RPMI 1640 with 0.1% FCS alone served as a control. In some cases, mice received the test substance in one hindlimb footpad and the control carrier in the contralateral hindlimb. In other cases, a randomized block design was used. Mice were sacrificed 4 h after injection and hindlimbs were fixed in 10% buffered formalin. Hindlimbs were decalcified, embedded in paraffin, and thin sections of footpads were stained with hematoxylin and eosin.

In Vitro PMN Migration Assays. Heparinized venous blood was obtained from healthy volunteers. Leukocytes containing >95% PMNs, as judged by cell sorter analysis, were isolated by Ficoll-Hypaque density gradient centrifugation and dextran sedimentation (13). Residual erythrocytes were removed by lysis with hypotonic saline. Cells were resuspended in Gey's balanced salt solution (GBSS; pH 7.4) and 2% BSA to a final concentration of 2.5×10^6 cells/ml.

In vitro chemokinesis was assayed using a modification of the technique described by Boyden (14). Bottom wells of blind well chambers were filled with 25 μl of chemoattractant (FMLP [10^{-8} M], MIP, or buffer) and the top wells filled with 45 μl of GBSS/BSA containing 1.1×10^4 PMNs. The two wells were separated by a cellulose nitrate membrane with a 3-μM pore size (SM 11302; Sartorius Balances, Westbury, NY). Chambers were incubated at 37°C in a humidified 5% CO_2, 95% room air chamber for 45 min. Membranes were removed and stained according to a previously described protocol (15). The number of PMNs migrating into the membrane was counted every 10 μM up to 130 μM using an automated Optomax Imaging system (Optomax, Inc., Hollis, NH). Migration was quantitated in three randomly selected fields for each membrane with each sample tested in triplicate. Chemokinesis was defined as the mean distance migrated into the membrane (expressed as %) compared to GBSS alone (0%) and that of the positive FMLP control (100%).

Chemokinetic data are expressed as the mean ± SEM of the percent control chemokinetic response. A one-way analysis of variance was used to compare the response to MIP with that of GBSS alone in the bottom well of the Boyden chamber.

Induction of Hydrogen Peroxide Release. The ability of MIP to elicit the release of H_2O_2 from adherent human PMNs or monocytes was tested by the method recently described in detail (16). In brief, PMNs and mononuclear leukocytes in heparinized or citrated blood were isolated in Neutrophil Isolation Medium (Packard Instrument Co. Inc., Downer's Grove, IL), washed, and plated separately at 1.5×10^4 PMNs or 2×10^5 mononuclear cells per well in flat-bottomed, 6-mm diameter polystyrene tissue culture wells that had previously been coated with FCS and extensively washed. The assay mixture contained 2.4 nmol scopoletin, 0.5 μg horseradish peroxidase, 1 mM sodium azide, and the indicated test agents in a final volume of 0.13 ml of Krebs-Ringer phosphate buffer
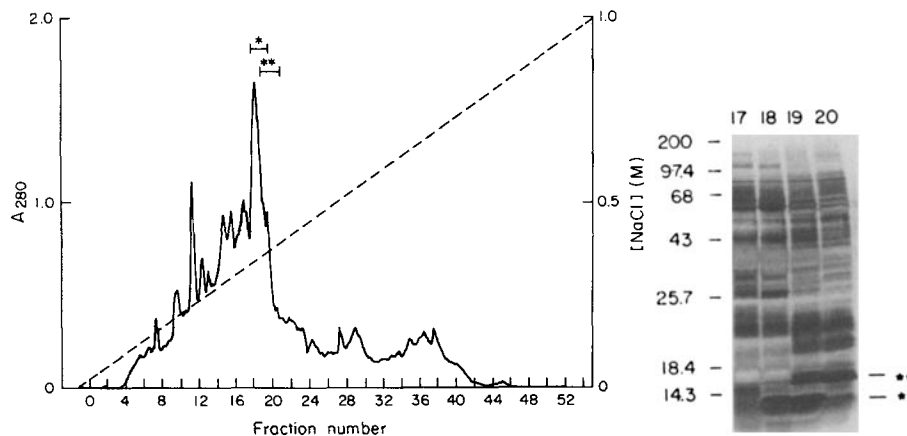
FIGURE 1. Fractionation of concentrated, diafiltrated supernatant from RAW 264.7 on Mono Q. 2 liters of supernatant were concentrated twenty times and diafiltrated against 6 liters of 20 mM Tris-HCl, pH 8.0. The concentrated supernatant was applied to Mono Q and eluted with a linear gradient of 0–1 M NaCl in the same buffer. MIP (*) eluted slightly before cachectin/TNF (**) at ~0.37 M NaCl. The inset shows a 10–15% SDS-PAGE gel to which 50 μl from the indicated fractions have been applied.

with glucose at 37°C. Loss of fluorescence of scopoletin due to oxidation by H_2O_2 was recorded at 15- or 30-min intervals in a plate-reading fluorometer and converted to nmol H_2O_2 by microcomputer (17).

IL-1 and Cachectin/TNF Bioassays. MIP was assayed for IL-1 activity by its ability to stimulate C3H/HeJ thymocytes to undergo blastogenesis in the presence of suboptimal quantities of phytohemagglutinin, as previously described (18).

Cachectin/TNF activity was assayed by its ability to kill actinomycin D–treated L929 murine fibroblasts (19). Approximately 50,000 L929 cells were plated in each well of a 96-well plate (Falcon Labware, Becton, Dickinson & Co.) in DME containing 1 μg/ml actinomycin D and increasing quantities of MIP. After 14–16 h, the chromogen (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was added and the cells incubated for an additional 4 h. Cell viability was assessed by the ability of the cells to reduce the chromogen during this time period by a modification of the method of Mosmann (20). The medium was aspirated and the cells were lysed with 0.04 N hydrochloric acid in isopropanol. After addition of 1 vol double-distilled water, the extent of chromogen reduction was assayed by reading the plates at OD 570/690 using an automated ELISA plate reader, and values were compared to a standard curve obtained with recombinant human cachectin/TNF. Cachectin/TNF was also assayed by the suppression of lipoprotein lipase on 3T3-L1 cells as previously described (8). The ability of MIP to induce cachectin/TNF in primary cultures of macrophages was assessed by eliciting macrophages with an i.p. injection of 2 ml of sterile thioglycollate broth (Difco Laboratories Inc.) and collecting the cells 4–6 d later by peritoneal lavage. The cells were washed and resuspended in serum-free RPMI and plated at 10^6 cells/well in 24-well tissue culture plates. Test substances (LPS, 0.001–1 μg/ml; MIP, 1 μg/ml) were added and the cells incubated at 37°C for 18 h. The cell-free supernatants were collected and assayed for cachectin/TNF activity by cytotoxicity on L929 cells as described above.

Results

Purification of MIP. When supernatants of stimulated RAW 264.7 were fractionated by Mono Q (anion exchange) chromatography, MIP was apparent as a distinctive doublet of ~8,000 daltons on SDS-PAGE after silver staining (Fig. 1); it reproducibly eluted within a fraction or two of cachectin/TNF at

~0.37 M NaCl and appeared to be produced in approximately the same quantities as judged by silver staining.

Chromatofocusing revealed that Mono Q–purified MIP eluted at a slightly more acidic pH than cachectin/TNF. This corresponded to a pI of 4.6 (not shown).

To further purify MIP, advantage was taken of its tendency to aggregate. The aggregation of MIP was observed to occur during the concentration of the crude material and before diafiltration or fractionation on Mono Q (data not shown). When fractionated by gel filtration in PBS, MIP formed multimers of various molecular masses ranging from ~20,000 daltons to material eluting in the void volume ($\geq 2 \times 10^6$ daltons; data not shown). In 100 mM ammonium acetate this tendency was exaggerated and the majority of the protein eluted in high molecular mass fractions (Fig. 2). Under these conditions, MIP of >95% purity, as judged by SDS-PAGE and silver staining, was obtained. A heparin-binding protein that cochromatographs on Mono Q and Superose 12 and comigrates on SDS-PAGE with MIP from RAW 264.7 cells is recovered from supernatants of cultured thioglycollate-elicited primary C3H/HeN murine macrophages in response to LPS treatment in vitro (data not shown).

Partial NH$_2$-terminal amino acid sequence data of purified MIP (Fig. 3) showed a single major sequence although sequences of two separate batches showed a consistent minor peak at three positions out of the first 31 residues. The sequence data revealed no significant homologies with any previously described protein.

Affinity of MIP for Heparin. During the purification of MIP, an affinity was noted for heparin (Fig. 4). When 2 ml of 12-fold–concentrated RAW 264.7 supernatant was applied to the column and eluted with a linear gradient of 0–2 M NaCl, MIP was one of two major proteins detected by SDS-PAGE and silver staining, eluting at ~0.7 M NaCl.

Determination of Biological Activities. At concentrations as high as 20 μg/ml, MIP did not stimulate blastogenesis of C3H/HeJ thymocytes; human rIL-1α, on the other hand, was active in this assay at concentrations as low as 10 pg/ml (data not shown). Similarly, MIP did not kill L929 cells in the presence of actinomycin D even at concentrations of 1 μg/ml whereas recombinant human cachectin/TNF was able to induce killing at concentrations as low as 15 pg/ml (data not shown). Further, MIP did not induce down-regulation of lipoprotein lipase in 3T3-L1 cells (data not shown). At 1 μg/ml, MIP did not induce cachectin/TNF production by primary thioglycollate-elicited mouse macrophages in the presence of 10 μg/ml polymyxin B (data not shown).

Although free of the above-mentioned IL-1- and cachectin/TNF-like activities, MIP induced a localized inflammatory response at 4 h when injected subcutaneously into the footpads of C3H/HeJ mice. Maximal inflammation occurred when 100 ng of MIP were administered and was characterized primarily by a PMN infiltration (Fig. 5B). The control response to an injection of carrier is shown in Fig. 5A. The degree of neutrophil infiltration seen with MIP was not as marked as that seen when 10^{-10} mol of FMLP were administered (Fig. 5D). However, the degree of neutrophil infiltration was comparable to that observed with 10 ng of recombinant cachectin/TNF. Human rIL-1 elicited no inflammatory responses at 4 h when administered at these doses (data not shown). MIP
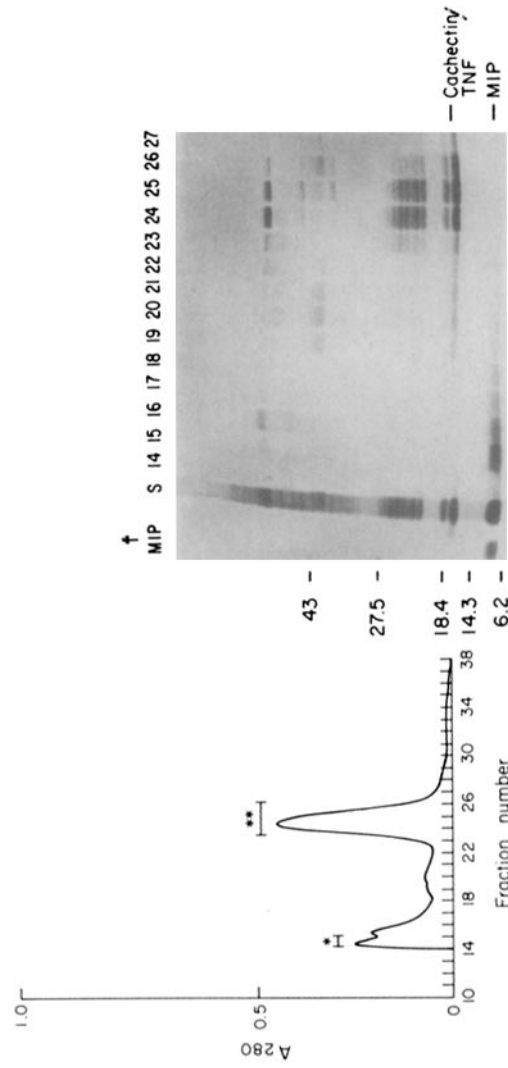
FIGURE 2. Peak fractions from Mono Q were concentrated to 200 μl and applied to a Superose 12 column equilibrated with 100 μM ammonium acetate. MIP (*) eluted in the void volume, well separated from cachectin/ TNF (**). The insert shows a 10–18% SDS-PAGE gel to which 50 μl from the indicated fractions have been applied. The dagger indicates purified MIP used as a marker.

(1)   (10)

Ala-Pro-Tyr-Gly-Ala-Asp-Thr-Pro-Thr-Ala-X-X-Phe-Ser-Tyr-Ser-Arg-Lys-Ile-

    (Met)   (Pro)   (Thr)

FIGURE 3. Amino acid sequence for the first 31 positions of MIP. Residues in parentheses indicate positions at which minor peaks were obtained in two different sequence runs on different batches of material.
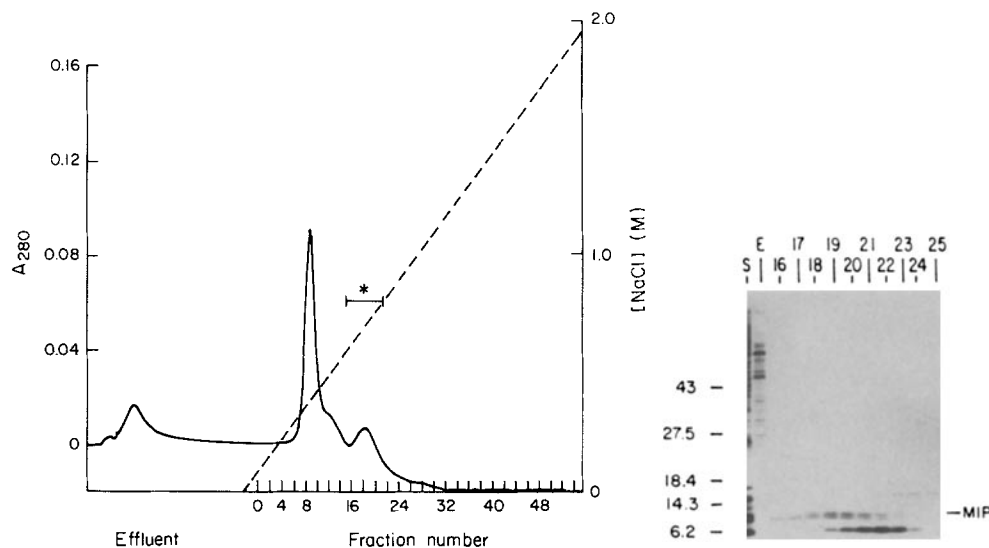
FIGURE 4. Binding of MIP to heparin. 2 ml of 12-fold-concentrated and diafiltrated RAW 264.7 supernatant were applied to a heparin–Sepharose column and eluted with a linear gradient of 0–2 M NaCl. Two major peaks were observed; MIP (*) eluted in the second peak, corresponding to 0.6–0.75 M NaCl. The insert shows a 10–18% SDS-PAGE gel to which 50 μl from the indicated fractions have been applied.

induced chemokinesis in human neutrophils in vitro. Data presented in Fig. 6 represent the mean percent of the control migratory response of five experiments. Values are presented as the percent increase in neutrophil migration relative to the negative GBSS and positive FMLP control (10^{-8} M). At concentrations ≥ 100 ng/ml, MIP elicited a significant increase in neutrophil migration ($p < 0.01$ by analysis of variance). Inclusion of polymyxin B (10 μg/ml) had no effect on MIP-induced chemokinesis. Further, LPS at concentrations of 10–1,000 ng/ml was not active in this assay (data not shown).

Recombinant cachectin/TNF, but not rIL-1β, triggers a delayed but significant respiratory burst in human PMN, provided the cells are adherent to a surface coated with serum or extracellular matrix proteins (16). Similarly, MIP at ≥ 1 μg/ml triggered adherent PMN to release H_2O_2 in four experiments, one of which is illustrated in Fig. 7. Compared to cachectin/TNF tested at the same time, the response to MIP was more delayed (60-min lag vs. 15-min lag for cachectin/TNF), and the maximal sustained rate was smaller (1.2 nmol/min per 10^6 PMN vs. 3.0 for cachectin/TNF and 2.1 for PMA). However, the duration of the response was greater (2.5 h compared to 1 h for cachectin/TNF) and the total amount of H_2O_2 released was similar. Because MIP binds heparin, experiments were also performed with PMN isolated from citrated rather than hepa-
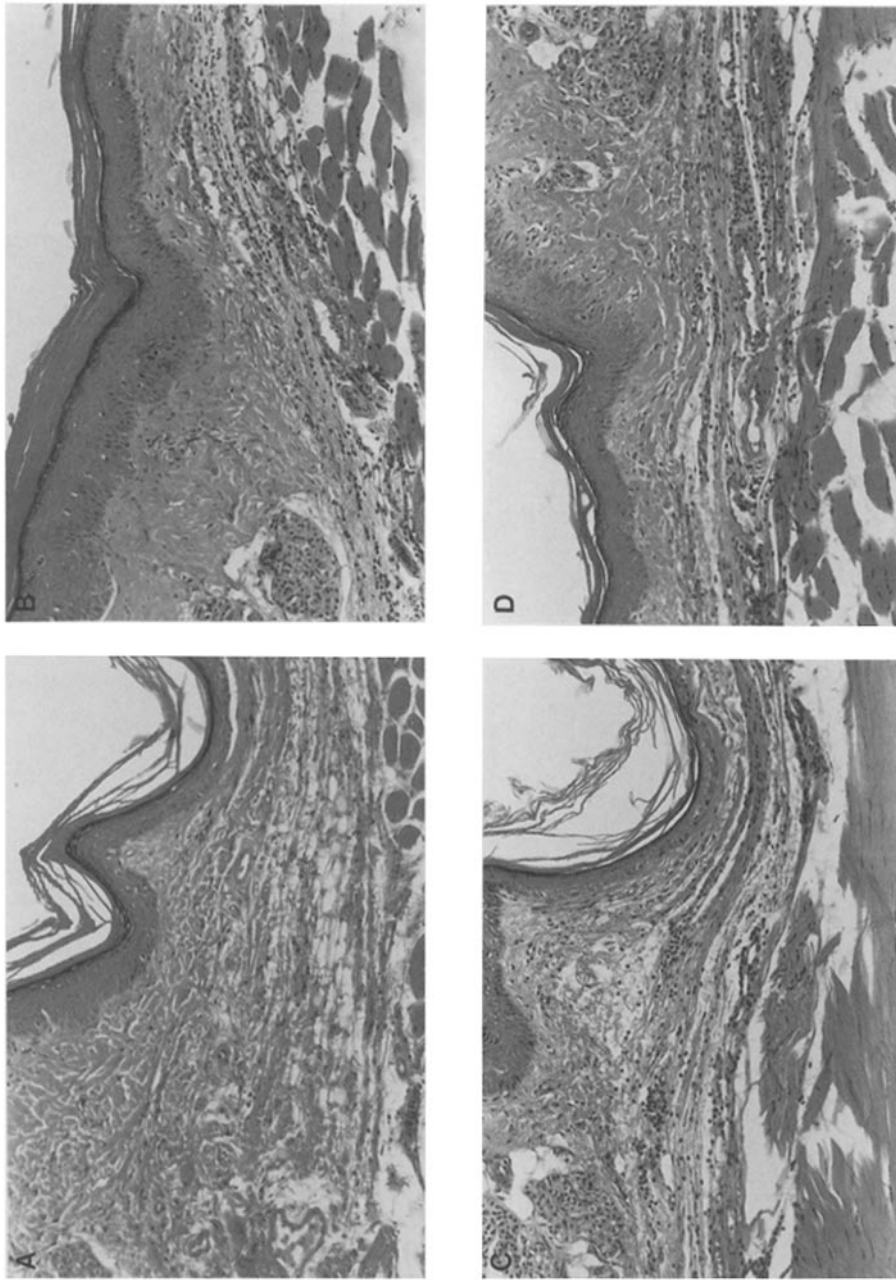
FIGURE 5. Photomicrographs of C3H/HeJ footpads 4 h after subcutaneous injection of monokines. Tissues were fixed in buffered formalin and stained with hematoxylin eosin. (A) Sham infection reflecting normal histology. (B) 100 ng MIP: moderate infiltration of neutrophils and mast cells. (C) 100 ng cachectin/TNF: moderate infiltration of neutrophils. (D) 10^{-10} mol FMLP: extensive infiltration with some focal necrosis. ×400.
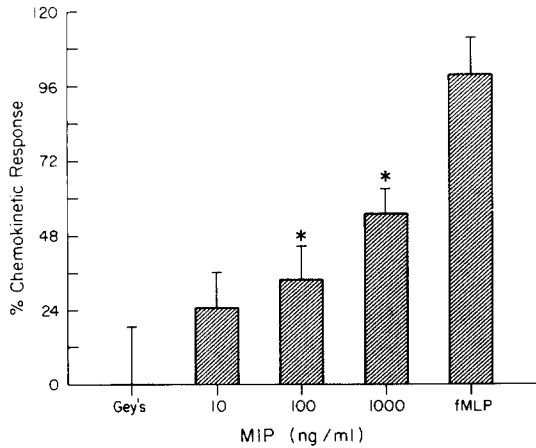
FIGURE 6. Induction of neutrophil chemokinesis by MIP. Data represent the mean percent of the control migratory response of five experiments. Values are presented as the percent increase in neutrophil migration relative to the negative GBSS and the positive FMLP control (10^{-8} M). (*) Concentrations of MIP showing significant increases in the percent chemokinetic response over GBSS alone ($p < 0.01$ by analysis of variance).
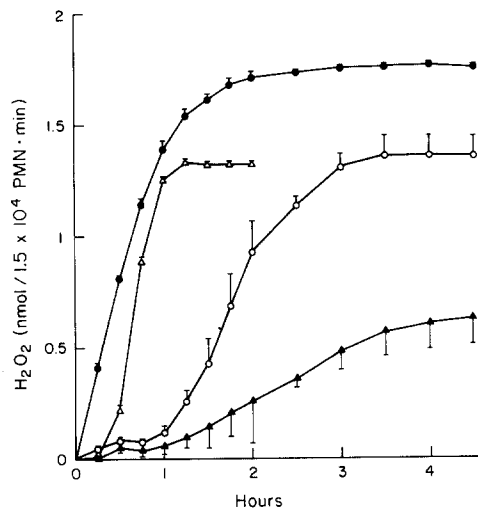

FIGURE 7. Stimulation of H_2O_2 release by MIP. Human PMN incubated in serum-coated microtest plate wells were treated at time 0 with PMA (100 ng/ml; ●); recombinant cachectin/TNF (10 ng/ml; △), MIP (1,000 ng/ml; ○); or an equivalent volume of buffer alone (control; ▲). H_2O_2 release was monitored over the next 4.5 h (2 h for cachectin/TNF). Values are means ± SEM for triplicates in 1 of 4 similar experiments.

rinized blood and gave similar results. Neither MIP (not shown) nor cachectin/TNF (16) triggered H_2O_2 release from monocytes.

Discussion

In the present communication we describe the isolation of a novel monokine that we term MIP. This monokine bears no significant sequence homology to any previously described protein yet shares some of the overlapping properties typical of inflammatory mediators such as cachectin/TNF and IL-1.

MIP was isolated on the basis of its interesting physical properties. Although MIP migrates as a doublet of ~8,000 daltons on SDS-PAGE, it readily forms high molecular mass aggregates in excess of 2×10^6 daltons as judged by gel filtration. It is not clear at the present time why there is a doublet observed on SDS-PAGE. Partial amino acid sequence data show one major sequence with minor peaks at three positions. It is possible that these two bands represent allelic forms of the same protein, alternative splicing of the same gene product or

partial proteolytic digestion. The binding of MIP to heparin under conditions where the protein is anionic suggests a specific interaction. This is further emphasized by the observation that MIP is one of two major macrophage-secreted proteins that bind to heparin at high salt concentrations. It is possible that MIP may play a role in the coordination of the inflammatory activities of macrophages, mast cells, and neutrophils. MIP may also interact with basement membrane proteoglycans during inflammation. We are currently investigating the nature of the other major protein that binds to heparin under these conditions.

The findings presented here are consistent with the suggestion that both cachectin/TNF and MIP are capable of inducing an inflammatory response. Cachectin/TNF has previously been shown to induce neutrophil chemokinesis (21, 22) as well as activation (16, 23–25). In the present study, MIP was shown also to be capable of inducing neutrophil chemokinesis. In addition, at the doses used here, cachectin/TNF and MIP each elicited similar degrees of inflammation in vivo. Although others have shown that rIL-1α can induce an inflammatory reaction in vivo (12), no such effect was found here; it is possible that the human rIL-1α used here is less active in this regard than the murine IL-1α used by Granstein et al. (12).

MIP consistently induced only low and variable levels of hydrogen peroxide production by human PMNs. Whether this low level of response is due to the aggregation of the protein during purification or reflects its actual biological activity is not clear at the present time. It is also possible that the small and delayed response is due to an undetected contaminant. It is not known for any of the bioactivities reported here whether the protein deaggregates under the conditions used or what is the active form. It is therefore difficult to assign a specific activity on a molar basis for these activities.

It is unlikely that these effects of MIP administration are due to cachectin/TNF contamination as there was no cachectin/TNF bioactivity detected in the L929 assay at the doses used. Further, MIP did not induce primary thioglycollate-elicited macrophages to produce cachectin/TNF. Endotoxin contamination was ruled out as an explanation as the chemokinetic effect was not affected by the presence of polymyxin B; and LPS itself, at concentrations greater than were present in the assay, had no effect. The inflammation induced by MIP was observed in the endotoxin-resistant C3H/HeJ mouse strain using preparations with low levels of endotoxin contamination (0.2 ng LPS/μg MIP).

It is of interest that several monokines secreted by macrophages share overlapping properties. Cachectin/TNF and IL-1 have a number of bioactivities in common (1); it now appears that a third monokine, MIP, is capable of inducing some of the inflammatory reactions elicited by cachectin/TNF. It will be of interest to see how the production of these various monokines is controlled in vivo as well as whether they synergize in their various bioactivities. Recently, Yoshimura et al. (7) provided evidence for a chemotactic factor from human peripheral blood mononuclear cells of a molecular mass of ~10,000 daltons by gel filtration and a pI of 8.0–8.5. As their basic protein has not yet been fully characterized, its relationship to the acidic one described here is not known.

Additional biological roles for MIP require further investigation. Preliminary

data indicate that several cell types bear receptors for this monokine. The present results suggest that this novel heparin-binding protein is produced in sizable amounts after endotoxin stimulation and may play a role in the cascade of host responses to endotoxemia and inflammation. Investigations are under way to further define the biological activities of this monokine as well as its interactions with other macrophage products.

Summary

We report the identification and purification of a new inflammatory monokine synthesized by the macrophage tumor cell line RAW 264.7 in response to endotoxin. This monokine, which we term "macrophage inflammatory protein" (MIP), is a doublet with an apparent molecular mass of ~8,000 daltons on SDS-PAGE but forms aggregates of $>2 \times 10^6$ daltons as assessed by gel filtration. Partial NH_2-terminal amino acid sequence data reveal no significant homology with any previously described protein. Although the monokine is anionic under physiological conditions, it is one of two major macrophage-secreted proteins that bind to heparin at high salt concentrations.

At 100 ng/ml or greater, MIP is chemokinetic for human polymorphonuclear cells and triggers hydrogen peroxide production. Subcutaneous injection of 10 ng or greater of MIP into footpads of C3H/HeJ mice elicits an inflammatory response, characterized by neutrophil infiltration. These findings suggest that MIP is an endogenous mediator that may play a role in the host responses that occur during endotoxemia and other inflammatory events.

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