Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 9233–9237, December 1987
Medical Sciences

Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines

(inflammation/monokine)

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Communicated by Daniel Steinberg, August 21, 1987

ABSTRACT **Stimulated human monocytes release several proteins thought to play a role in inflammation, including interleukin 1, tumor necrosis factor, and plasminogen activator. We have purified another proinflammatory protein that is chemotactic for human neutrophils from conditioned medium of lipopolysaccharide-stimulated monocytes. After a series of steps that included anion-exchange chromatography, gel filtration, and HPLC on cation-exchange and reverse-phase columns, an apparently pure protein was obtained that migrated as a single 7-kDa band on NaDodSO$_4$/polyacrylamide gels under reducing or nonreducing conditions. The amino acid composition of this monocyte-derived neutrophil chemotactic factor was different from that of interleukin 1 and tumor necrosis factor. N-terminal amino acid sequence of the first 42 residues was determined. This portion of the molecule has up to 56% sequence similarity with several proteins that may be involved in host responses to infection or tissue injury. It is identical to a portion of a sequence deduced from an mRNA induced by staphylococcal enterotoxin treatment of human leukocytes. At the optimal concentration of 10 nM, 50% of neutrophils added to chemotaxis assay wells migrated toward the pure attractant. Potency and efficacy are comparable to that of fMet-Leu-Phe, which is often used as a reference. In contrast to many attractants, the protein was not chemotactic for human monocytes.**

Accumulation of leukocytes at sites of inflammation is the result of many steps that include vasodilation, leukocyte adherence to vascular endothelium, and directed migration caused by elaboration of chemotactic factors at the initiating locus. The type of leukocyte in the inflammatory infiltrate differs according to the nature of the stimulus and the temporal stage of the response. Therefore, characteristics of a proinflammatory chemoattractant should include (*i*) production and release in response to the inflammatory stimulus and (*ii*) at least in some cases the capacity to attract specific types of leukocytes.

It was reported that interleukin 1 (IL-1), which is produced by monocytes in response to the inflammatory agent lipopolysaccharide (LPS), is chemotactic for human neutrophils and monocytes (1, 2). Thus, IL-1 appeared to fulfill the first of the above requirements for a proinflammatory attractant. However, we showed that highly purified or recombinant IL-1 has no chemotactic activity for neutrophils and that the neutrophil chemotactic activity in culture fluids of LPS-stimulated human monocytes could be separated from IL-1. The activity is due to a basic protein with a molecular mass of about 10 kDa determined by gel filtration (3).

We now report the purification to homogeneity of this monocyte-derived neutrophil chemotactic factor (MDNCF), present evidence that the molecule is distinct from monocyte IL-1 and tumor necrosis factor (TNF), and show that it has peptide sequence similarity to several other host-defense cytokines. MDNCF is potentially a mediator of a leukocyte-specific inflammatory response, since it is released by an inflammatory stimulus and has the selective capacity to attract neutrophils but not monocytes.

MATERIALS AND METHODS

Cell Culture. Human blood mononuclear cells were isolated by Ficoll/Hypaque density sedimentation from leukapheresis preparations obtained from healthy donors. Cells were washed three times with isotonic phosphate-buffered saline and resuspended in RPMI 1640 culture medium (Mediatech, Washington, DC) supplemented with 1% fetal calf serum and gentamycin at 50 μg/ml. The culture medium was free of detectable LPS. Cells were cultured at a concentration of 5×10^6 cells per ml in plastic tissue culture flasks with *Escherichia coli* LPS at 10 μg/ml (Difco). After incubation for 24 hr, cells were harvested, and cell-free conditioned medium was obtained by centrifugation at $400 \times g$ for 10 min.

Anion-Exchange Chromatography and Gel Filtration. Three liters of conditioned medium was concentrated to 300 ml by Amicon hollow-fiber ultrafiltration (H1P5-20, molecular weight cutoff 5000), dialyzed in the same apparatus against 0.02 M Tris·HCl, pH 8.0/0.05 M NaCl, and then applied on a column of DEAE-Sepharose Fast Flow (Pharmacia). The pass-through fraction was collected, concentrated to 4 ml by an Amicon Diaflo membrane (YM5, molecular weight cutoff 5000), then applied to a 2.6×83 cm Sephacryl S-200 column equilibrated with phosphate-buffered saline at pH 7.0. Flow rate was 1 ml/min; fraction volumes were 9 ml. The column was calibrated with bovine serum albumin (68 kDa), ovalbumin (43 kDa), cytochrome *c* (12.5 kDa), and aprotinin (6.5 kDa).

Cation-Exchange HPLC. The pool of fractions with chemotactic activity eluted from Sephacryl S-200 was dialyzed overnight at 4°C against starting buffer (20 mM Mops, pH 6.5/0.2 M NaCl), and applied to a 0.6×15 cm IEX-535 CM column (Toyo Soda, Tokyo) at room temperature. The limit buffer was 20 mM Mops, pH 6.5/0.4 M NaCl. A linear

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Abbreviations: β-TG, β-thromboglobulin; IL-1, interleukin 1; LPS, lipopolysaccharide; MDNCF, monocyte-derived neutrophil chemotactic factor; TNF, tumor necrosis factor.

gradient was programmed at a flow rate of 1.0 ml/min; 2.0-ml fractions were collected.

Reverse-Phase HPLC. To the pool of active fractions eluted from the cation exchange column, trifluoroacetic acid was added to a concentration of 0.01%. The sample was applied to a 0.5 × 25 cm Hi-Pore reverse-phase column (Bio-Rad) equilibrated with a starting solvent of 0.01% trifluoroacetic acid in water. A linear gradient was programmed, with a limit buffer of 60% (vol/vol) acetonitrile in water containing 0.01% trifluoroacetic acid. Flow rate was 1.0 ml/min; 1.0-ml fractions were collected.

NaDodSO$_4$/PAGE. Electrophoresis was carried out on a vertical slab gel of 15% acrylamide with a discontinuous Tris glycine buffer system (4). Samples, as well as a solution of molecular weight standards, were mixed with equal volumes of double-strength sample buffer [20% (vol/vol) glycerol, 6% (wt/vol) NaDodSO$_4$, with or without 10% (vol/vol) 2-mercaptoethanol], boiled, and applied to the gel. After electrophoresis at 10 mA for 4 hr, the gel was stained with Coomassie brilliant blue R-250 (Eastman Kodak).

Amino Acid Composition and Sequence Analysis. After a 24-hr hydrolysis in 6 M HCl *in vacuo* at 106°C, amino acid composition was determined on both a Beckman system 6300 and a Waters Picotag system. N-terminal sequence of native and carboxymethylated (5) protein was determined by Edman degradation on an automated Applied Biosystems 470A sequencer equipped with an on-line 120 phenylthiohydantoin analyzer (Applied Biosystems, Foster City, CA).

Chemotaxis Assay. Neutrophil (6) and monocyte (7) chemotaxis was assayed in multiwell chambers. The number of neutrophils or monocytes per well was 5 × 10^4. Cells that had migrated were counted with an image analyzer (8). The reference chemoattractant fMet-Leu-Phe (Peninsula Laboratories, Belmont, CA) was dissolved in ethanol at a concentration of 1 mM and diluted for assay.

RESULTS

Purification of Human MDNCF. Three liters of conditioned medium from LPS-stimulated mononuclear cells was concentrated by hollow-fiber ultrafiltration to 300 ml. After equilibration of the concentrated conditioned medium with starting buffer and application to a column of DEAE-Sepharose Fast Flow, chemotactic activity was recovered in the pass-through fractions. As shown in Table 1, this was the first major purification step. The pool of active fractions was concentrated and applied to a Sephacryl S-200 column. Neutrophil chemotactic activity eluted in the 5- to 16-kDa range, with an ≈9-fold increase in specific activity. It was then concentrated, equilibrated with a buffer at pH 6.5 containing 0.2 M NaCl and applied to a CM-HPLC column. As shown in Fig. 1, neutrophil chemotactic activity eluted in a sharp peak that coeluted with the major A_{280} peak. This step resulted in a 12-fold increase in specific activity. Because several bands were detected by NaDodSO$_4$/PAGE, further purification of pooled fractions (time, 56–60 min) was carried out by reverse-phase HPLC. Fig. 2 shows that neutrophil chemotactic activity eluted with a single A_{226} peak. When this fraction was analyzed by NaDodSO$_4$/PAGE one band was found at 7 kDa, under either reducing (Fig. 3) or nonreducing conditions. The yield from 3 liters of conditioned medium was ≈110 μg of apparently homogeneous neutrophil chemotactic factor. Specific activity was 1600 times that of the starting material; recovery was ≈12%.

Table 1. Purification scheme for human MDNCF

	Total protein, mg	Total NCA, units	Specific activity, units/mg
Crude supernatant	1400	99,000	72
Concentrated and dialyzed supernatant	1000	57,000	57
DEAE-Sepharose	110	41,000	380
Sephacryl S-200	4	14,000	3,500
CM-HPLC	0.26	11,000	42,000
Reverse-phase HPLC	0.11	12,000	113,000

Protein concentration was determined by dye protein assay with bovine serum albumin as standard. A chemotactic activity concentration of 1 unit/ml was defined as the reciprocal of the dilution at which 50% of maximal chemotactic response was obtained. NCA, neutrophil chemotactic activity.

Amino Acid Analysis of MDNCF. Table 2 shows the amino acid composition of purified MDNCF. Of note is the absence of methionine. Amino acid composition was compared with IL-1 and TNF, two monocyte products that have been reported to be chemotactic for neutrophils. Table 2 shows

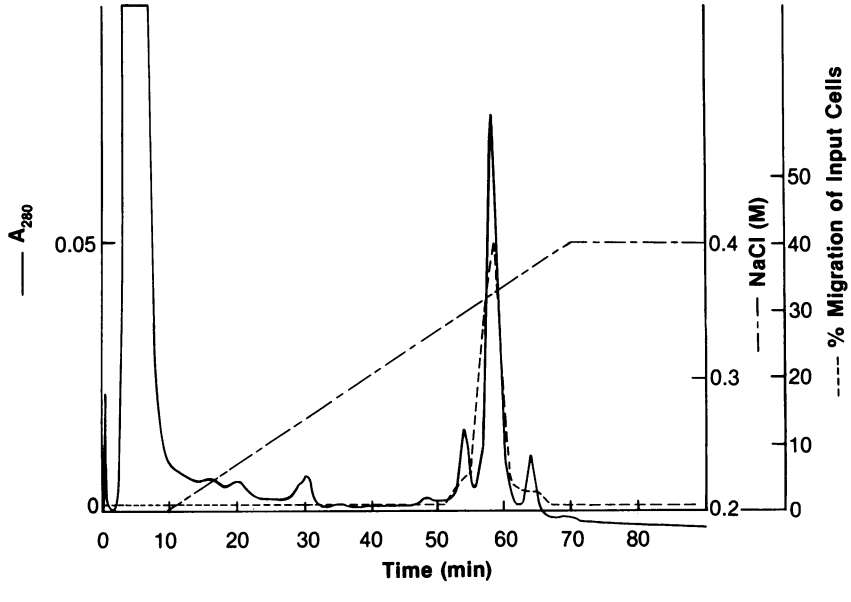

FIG. 1. Neutrophil chemotactic activity eluted from cation-exchange HPLC column. —, A_{280}; – – –, neutrophil chemotactic activity; ———, NaCl gradient.

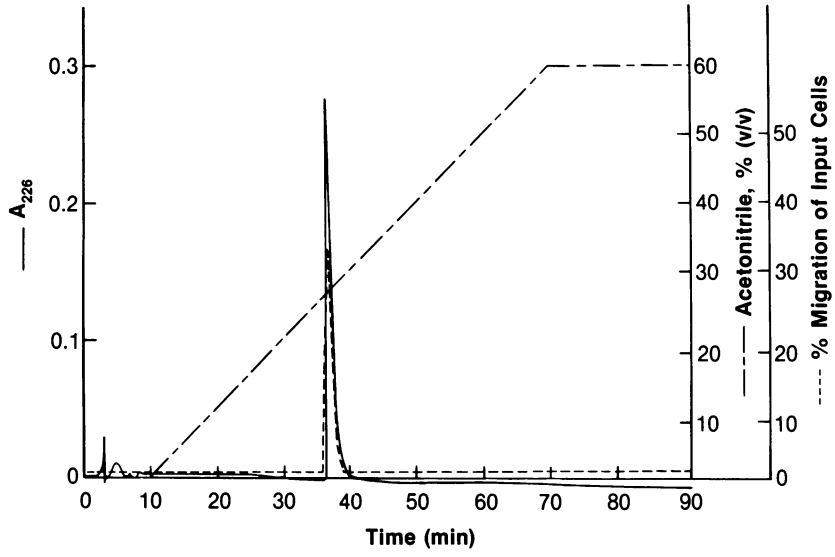

FIG. 2. Neutrophil chemotactic activity obtained by reverse-phase HPLC. —, A_{226}; ---, neutrophil chemotactic activity; ———, acetonitrile gradient.

that the amino acid composition of MDNCF is different from that of IL-1 and TNF.

N-Terminal Amino Acid Sequence of MDNCF. N-terminal amino acid sequence of the first 42 residues is shown in Fig. 4. This sequence has not been listed in the genetic sequence data bank (GenBank, National Institutes of Health Computer Facility) and is not similar to the sequence of IL-1 or TNF. To our knowledge, no protein with this sequence has been reported in the literature. However, the sequence of the first 42 residues of MDNCF is identical to a portion of a deduced sequence coded by an mRNA (3-10C) induced by staphylococcal enterotoxin A treatment of human leukocytes (15). Furthermore, the N terminus of MDNCF exhibits up to 56% sequence similarity with portions of several proteins, the relevant sequences of which are shown in Fig. 4. Platelet factor 4 (13) and β-thromboglobulin (β-TG) (12) are derived from human platelets. Chicken embryo fibroblasts produce 9E3 in response to infection with Rous sarcoma virus (11). The monocyte-like cell line, U937, produces γIP-10 when stimulated by γ interferon (14). In addition to the high degree of sequence similarity, the positions of the three cysteines are identical for the five proteins.

Chemotactic Activity of MDNCF for Neutrophils and Monocytes. The potency and efficacy of purified MDNCF for neutrophils are shown in Fig. 5. Fifty-four percent of neutrophils added to the assay wells migrated at the optimal MDNCF concentration of 10 nM. Migration at the optimal concentration of fMet-Leu-Phe in the same assay was 38%.

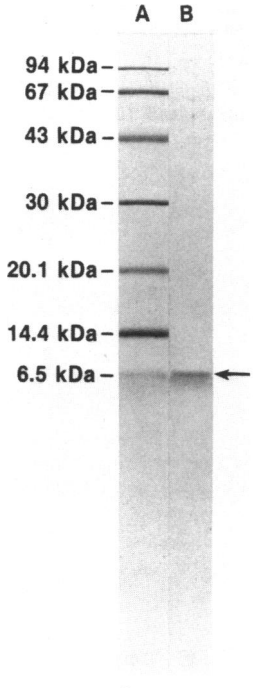

FIG. 3. $NaDodSO_4$/PAGE of purified MDNCF. About 1 μg of purified material from reverse-phase HPLC was applied to a 15% polyacrylamide gel under reducing conditions. Molecular weight standards were applied in lane A. $NaDodSO_4$/PAGE position of purified material is indicated by an arrow in lane B.

Table 2. Amino acid composition of human MDNCF, IL-1, and TNF

Amino acid	Residues, no. per molecule		
	MDNCF	IL-1*	TNF†
Asp + Agn	4.7	17	12
Thr	1.9	6	6
Ser	4.6	14	13
Glu + Gln	10.3	23	20
Pro	4.1	8	10
Gly	2.4	8	11
Ala	3.1	5	13
Val	4.5	11	13
Met	0.0	6	0
Ile	4.2	5	8
Leu	6.3	15	18
Tyr	0.9	4	7
Phe	3.0	9	4
His	1.9	1	3
Lys	8.9	15	6
Arg	4.8	3	9
Cys	‡	2	2
Trp	ND	1	2

ND, not determined.
*Deduced amino acid composition from human IL-1β cDNA (9).
†Deduced amino acid composition from human TNF cDNA (10).
‡A quantitative estimate of cysteine was not made. Three carboxymethylated cysteines were detected by N-terminal sequence analysis of the first 42 residues. A minimum molecular mass, calculated by rounding off the number of residues per molecule to integers and assuming 4 cysteines per molecule (see *Discussion*), is ≈9300.

	10	20	30	40
MDNCF	S A K E L R C Q C I K T Y S K P F H P K F I K E L R V I E S G P H C A N T E I I V K			
9E3	C Q C I S T H S K F I H P K S I Q D V K L T P S G P H C K N V E I I A T			
β-TG	C M C I K T T S G I H P K N I Q S L E V I G K G T H C N Q V E V I A T			
PF-4	C L C V K T T S Q V R P R H I T S L E V I K A G P H C P T A Q L I A T			
γIP-10	C T C I S I S N Q P V N P R S L E K L E I I P A S Q F C P R V E I I A T			

FIG. 4. N-terminal amino acid sequence of the first 42 residues of reduced and carboxymethylated human MDNCF. Partial sequences of similar proteins are shown. Sequence refs. are as follows: ref. 11 for 9E3, ref. 12 for β-TG, ref. 13 for platelet factor 4, and ref. 14 for γIP-10. Homologous portions of MDNCF and 9E3 sequences are boxed.

No significant monocyte migration was observed over an MDNCF concentration range of 0.1–30 nM in that experiment and at concentrations up to 0.2 μM in a second experiment. Thus, MDNCF attracted neutrophils but not monocytes. A few migrated eosinophils were also seen, but quantitative studies on eosinophil or basophil migration have not yet been made.

DISCUSSION

In our study (3) on conditioned medium from LPS-stimulated monocytes, we separated IL-1 activity from polymorphonuclear leukocyte chemotactic activity. Purification of the polymorphonuclear leukocyte chemotactic activity to homogeneity has enabled us to compare amino acid composition of MDNCF and IL-1, which shows that the two proteins are totally different (Table 2). MDNCF is also different from TNF, another product of stimulated monocytes. Ming *et al.* (16) reported that human recombinant TNF was chemotactic for neutrophils and monocytes *in vitro*. We have confirmed these results for neutrophils. However, TNF does not account for a significant amount of chemotactic activity in conditioned medium from LPS-stimulated monocytes, since the major chemotactic activity is considerably lower in molecular size than TNF (3).

To our knowledge, the amino acid sequence of the first 42 residues of the N terminus of MDNCF has not been reported for any other protein. However, the N terminus of MDNCF has up to 56% sequence similarity with the 36-residue portions of the proteins shown in Fig. 4. Of further interest is the fact that β-TG has a total of four half-cystine residues (three of which are shown in Fig. 4) that form two intrachain disulfide bridges (12). Since the positions of the four cysteines for each of the four MDNCF-related proteins are identical, it is probable that MDNCF and these proteins have similar conformation, by virtue of a pair of intrachain cystines.

Sequence similarity of β-TG and platelet factor 4 led to the suggestion that these two platelet proteins arose by duplication of an ancestral gene (12). Holt *et al.* (17) surmised that β-TG, platelet factor 4, and γIP-10 comprise a family of molecules involved in inflammation and immune regulation. The β-TG precursor, CTAPIII (connective tissue activating protein) could promote wound healing since it stimulates replication of connective tissue cells (for review, see ref. 18), platelet factor 4 is reported to be a chemoattractant (19), and γIP-10 is an interferon-induced product of the U937 monocyte-like cell line (14). MDNCF belongs not only structurally but also functionally to this family of similar proteins involved in inflammation, since it is generated in response to an inflammatory stimulus and has a known proinflammatory

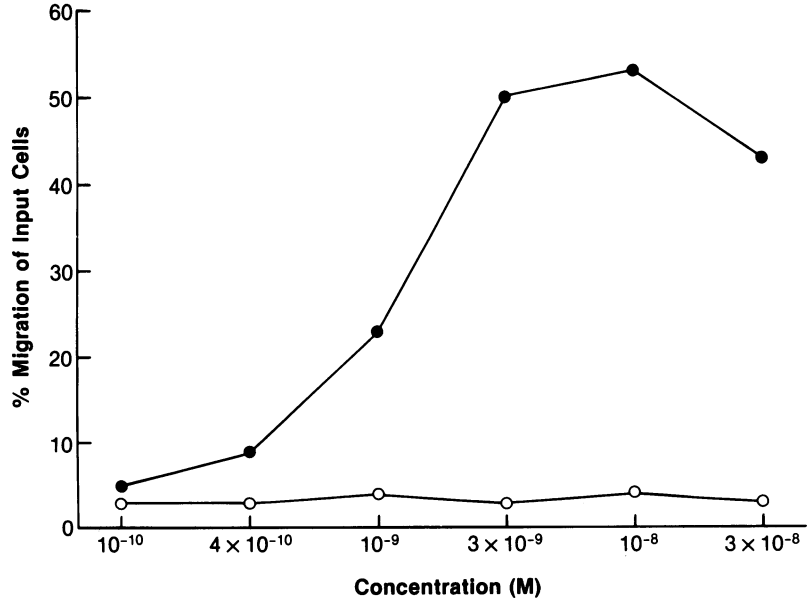

FIG. 5. Chemotactic activity of purified MDNCF for human neutrophils and monocytes. ●, Neutrophils; ○, monocytes. Pure protein obtained by reverse-phase HPLC was used for this assay.

function (chemotaxis). The ancestral gene from which these proteins were later derived may have originated in the primitive macrophage.

Addition of LPS to human blood monocytes caused release of MDNCF (3). It will be important to determine what other perturbations of the monocyte plasma membrane or receptors are stimuli for its production. Schmid and Weissmann (15) characterized two cDNA clones that were induced when human blood leukocytes were stimulated with staphylococcal enterotoxin A, a lymphocyte mitogen that is a potent inducer of interferon γ. A portion of the deduced protein sequence of one of the cDNAs is identical to the sequence for the first 42 residues of MDNCF, and *in vitro* translation in a reticulocyte lysate system yielded a protein similar in molecular mass to that of MDNCF (11 kDa). No studies of the chemical or biological function of the protein were reported (15). The mRNA for the protein was also induced by staphylococcal enterotoxin A in the monocyte-like U937 cell line, which suggests that this lymphocyte mitogen may also act directly on blood monocytes.

Of great interest is the fact that MDNCF exhibits leukocyte selectivity, in that it fails to attract monocytes at concentrations up to 0.2 μM. This is in contrast to a number of well-studied attractants, such as fMet-Leu-Phe, leukotriene B_4, and the serum complement fragment C5a, which do not exhibit such selectivity. Neutrophils, the dominant leukocytes in many inflammatory reactions, play a vital role in host defense. However, neutrophils and their products cause pathological changes in many diseases, such as rheumatoid arthritis and idiopathic pulmonary fibrosis. Our objective is to determine if MDNCF is a proinflammatory mediator that is released by tissue macrophages in response to inflammatory stimuli, leading to neutrophil-rich leukocyte accumulation in host defense and disease.

We thank Pauline Fratantoni for organizing the large number of cytapheresis collections required for the production of MDNCF. T.Y. was supported by the Japanese Overseas Cancer Fellowship of the Foundation for Promotion of Cancer Research.

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