

MOLECULAR CLONING OF A HUMAN MONOCYTE-DERIVED NEUTROPHIL CHEMOTACTIC FACTOR (MDNCF) AND THE INDUCTION OF MDNCF mRNA BY INTERLEUKIN 1 AND TUMOR NECROSIS FACTOR

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Activated monocytes produce a variety of cytokines that are involved in inflammation, such as IL-1, TNF, chemotactic factors, transforming growth factor β , platelet-derived growth factor, and IFN- α and - β (1). Chemotactic factors released at foci of injury or bacterial invasion are thought to mediate directed migration of leukocytes into inflammatory sites. Since the leukocyte composition of the inflammatory infiltrate depends on the temporal stage of the lesion (2) and the nature of the stimulus (3), it follows that some chemoattractants should be specific for a given type of leukocyte. We recently showed that LPS-stimulated monocytes produce a chemotactic factor that attracts neutrophils, but not monocytes (4). We purified this factor to homogeneity and described the NH₂-terminal sequence of the first 42 amino acids (5). We now report molecular cloning and sequencing of the full-length cDNA for this monocyte-derived neutrophil chemotactic factor (MDNCF)¹ and the deduced amino acid sequence of the entire molecule. Specific cDNA probes also enabled us to test the capacity of a number of cytokines to induce MDNCF mRNA expression in human PBMC. The stimulation of MDNCF mRNA expression by IL-1 and TNF suggests that the local pro-inflammatory action of these cytokines may be mediated by induction of chemotactic factor secretion.

Materials and Methods

cDNA Cloning of MDNCF and Nucleotide Sequence. Normal human PBMC were first fractionated by Ficoll-Hypaque and plastic adherent cells (> 90% nonspecific esterase-positive monocytes), were cultured in RPMI-1640 medium supplemented with 1% FCS and 10 μ g/ml LPS (Serotype 055:B55; Difco Laboratories Inc., Detroit, MI) for 6 h at 37°C. Total RNA

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¹ Abbreviation used in this paper: MDNCF, monocyte-derived neutrophil chemotactic factor.

was isolated by guanidine-isothiocyanate extraction and polyadenylated mRNA was purified by oligo-dT-cellulose chromatography. Double-stranded cDNA was synthesized from poly(A)⁺ RNA by the Amersham Corp., (Arlington Heights, IL) cDNA synthesis system, which is based on the method of Gubler and Hoffman (6). After treatment with Eco RI methylase and T4 DNA polymerase, the double-stranded DNA was fractionated by gel filtration, inserted into the Eco RI site of λ gt10 with Eco RI linkers, and packaged in vitro by means of Gigapack (Stratagene, San Diego, CA). 5×10^5 plaques were screened with mixtures of ³²P-labeled oligonucleotide probes (2×10^6 cpm/ml) by hybridizing nitrocellulose filters at 50°C and then lowering the temperature to 37°C. After hybridization, the filters were washed with $6 \times$ SSC for 15 min at 25°C three times and once for 1 min at 37°C. The filters were exposed at -80°C overnight to Kodak X-Omat x-ray film. Positive clones were recloned in a similar way and one strongly positive clone was expanded in *Escherichia coli* Hfl-C600 (termed r-MDNCF 2-1). The cDNA in r-MDNCF 2-1 was digested with Eco RI; 1.2-kb and 0.5-kb cDNA fragments were obtained. Southern blotting analysis of these rMDNCF cDNA restriction fragments using ³²P-labeled oligonucleotide probes indicated that the 0.5-kb fragment hybridized to the oligoprobes. Each cDNA fragment was subcloned into pUC19 plasmids and expanded in DH-5 host cells. DNA sequence analysis was performed by the dideoxy method of Sanger et al. (7) and the chemical cleavage method of Maxam and Gilbert (8).

Construction of pSP18-14 and In Vitro Transcription and Translation. pSP18 (Bethesda Research Laboratories, Gaithersburg, MD) (0.75 μ g) was digested with 5 U of Pst I and 5 U of Eco RI at 37°C for 90 min. The 0.3-kb Pst I-Eco RI fragment of MDNCF 2-1 was ligated into the digested pSP18 plasmid by the method of Maniatis et al. (9), and then transfected into JM 105-competent cells. Resultant colonies were confirmed for orientation of the 0.3-kb Pst I-Eco RI fragment by restriction endonuclease mapping. One isolate (termed pSP18-14) was linearized with Eco RI, and then purified DNA (1 μ g) was used as a template to synthesize RNA transcripts by SP6 RNA polymerase according to the method of Noma et al. (10). The size and yield of the RNA was determined by agarose gel electrophoresis to be 0.3 kb and 0.3 μ g, respectively. The transcript was translated into protein in a rabbit reticulocyte lysate in the presence of 0.1 nmol of [³H]leucine (120 Ci/mmol) at 30°C for 60 min according to the manufacturer's recommendation (Bethesda Research Laboratories).

Northern Blotting Analysis. 25-50 ng of the 0.3-kb Pst I-Eco RI fragment of MDNCF cDNA and chicken β -actin cDNA (Bethesda Research Laboratories) were labeled with deoxycytidine 5'- α -[³²P]triphosphate triethylammonium salt (3,000 Ci/mmol) by a random primer DNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). Unincorporated α -[³²P]dCTP was removed by nucleic acid chromatography system (NACS) column chromatography (Bethesda Research Laboratories). The specific activity of the resulting DNA was $\sim 5 \times 10^8$ cpm/ μ g. For Northern blotting analyses, whole human PBMC were stimulated with 10 μ g/ml LPS, 100 U/ml human rIL-1 α (endotoxin-free, purified; Dainippon Pharmaceutical Co. Ltd., Osaka, Japan), 100 U/ml human rTNF (endotoxin-free, purified; Dainippon Pharmaceutical Co. Ltd.), 100 U/ml human rIFN- α (endotoxin-free, purified, Hoffmann-La Roche, Inc., Nutley, NJ), 100 U/ml human rIFN- γ (endotoxin-free, purified, Genentech, Inc., South San Francisco, CA) or 100 U/ml human rIL-2 (endotoxin-free, purified; Hoffman-La Roche). Total RNA was isolated by guanidine-isothiocyanate extraction followed by cesium chloride density gradient centrifugation. Purified total RNA was electrophoresed in a 1% agarose gel containing 6.5% formaldehyde for ~ 5 h at 60 V, and then transferred to a nylon membrane. After transfer, the membrane was baked at 80°C for 2 h under vacuum and prehybridized at 42°C overnight. The prehybridization solution contained $5 \times$ SSC, 50% formamide, 0.1% Ficoll, 0.1% SDS, 10% dextran sulfate, 50 μ g/ml yeast tRNA, and 100 μ g/ml salmon sperm DNA in 50 mM sodium phosphate buffer, pH 6.5. Hybridization was performed at 42°C overnight in the above mixture supplemented with ³²P-labeled cDNA probe ($2-5 \times 10^8$ cpm/ μ g) at 10^6 cpm/ml. After hybridization, the membrane was washed twice with $0.1 \times$ SSC, 1% SDS at room temperature, then washed twice with $0.1 \times$ SSC, 1% SDS at 60°C. The membrane was exposed to x-ray film with an intensifying screen at -80°C.

Immunoprecipitation of MDNCF-expressed in Cell-free System. Rabbit polyclonal antibodies to

MDNCF were prepared by immunizing a rabbit with purified homogeneous MDNCF (5). Immunoprecipitation of radiolabeled MDNCF in the reticulocyte-lysate with polyclonal anti-MDNCF coupled to protein A-Sepharose 4B was carried out at 4°C after removal by absorption of nonspecifically bound molecules with normal rabbit IgG coupled to protein A-Sepharose.

HPLC Gel Filtration. HPLC gel filtration of culture supernatants from PBMC was performed at room temperature on a 7.5 × 600 mm TSK 3000 column equilibrated with Dulbecco's PBS, pH 7.4 1-ml fractions were collected at flow rate of 1 ml/min. The column was calibrated with BSA (mol wt 67,000), OVA (mol wt 43,000), and cytochrome *c* (mol wt 12,500). Neutrophil chemotactic activity was measured in a multiwell chemotaxis chamber as previously described (11).

Results

cDNA Cloning for MDNCF. The computer-assisted sequence homology test (Word Search by University of Wisconsin Genetic Computer Group, Madison, WI) of the NH₂-terminal sequence of MDNCF derived from LPS-stimulated PBMC showed that MDNCF has a unique protein sequence (Fig. 1 A). Therefore, cDNA cloning of MDNCF was initiated and oligo nucleotide probes (Fig. 1 B) based on the MDNCF amino acid sequence were synthesized for the screening of a cDNA library. The cDNA library was constructed by insertion of cDNA synthesized from normal human monocyte polyadenylated mRNA obtained after stimulation of the monocytes with 10 µg/ml LPS for 6 h, into the Eco RI site of the phage vector λgt10. 5 × 10⁵ individual plaques were screened for hybridization with ³²P-labeled oligonucleotide probes. Although 13 putative positive clones were obtained in the first screening, only one clone was positive in the second screening (termed r-MDNCF 2-1). The phage DNA in r-MDNCF 2-1 was digested by Eco RI and 1.2- and 0.5-kb cDNA fragments were obtained from agarose gel electrophoresis. Each Eco RI DNA fragment was subcloned into the pUC19 plasmid (termed p-MDNCF 2-1, 1.2, and p-MDNCF 2-1, 0.5). The cDNA was sequenced by the dideoxy method of Sanger et al. (7) and the chemical cleavage method of Maxam and Gilbert (8). Fig. 1 C shows restriction endonuclease maps of the cDNA insert of MDNCF 2-1 and the strategy for the nucleotide sequencing. Fig. 1 D shows the complete nucleotide and deduced amino acid sequence of MDNCF. There is a 101-base 5' untranslated region, a 297-base coding region, and a long 1.2-kb 3' untranslated region. The initial AUG codon begins an open reading frame that encodes a 99-amino acid polypeptide. No other AUG-initiated open reading frame of sufficient length to code for MDNCF was found in the cDNA sequence. The 3' untranslated region contains a nucleotide sequence TATTTATT beginning 609 bases from the 5' region that is identical to a conserved sequence present in the mRNA of many inflammatory mediators such as TNF mRNA (12). The NH₂-terminal end of the deduced amino acid sequence is highly hydrophobic and this NH₂-terminal hydrophobic region is consistent with a typical signal peptide sequence (13). Based on the NH₂-terminal amino acid sequence of purified MDNCF, the mature form of the protein starts with serine at residue 28. The deduced amino acid sequence shows complete identity with the first 42 residues of purified MDNCF as determined by the Edman degradation method (5). The reading frame terminates with serine at residue 99, which would make a deduced mature protein of 72 amino acids and a calculated molecular mass of 8 kD. Purified natural MDNCF fits this deduction, since the molecular mass determined by SDS-PAGE was ~8 kD, and the amino acid composition corresponds to that of the deduced protein. The

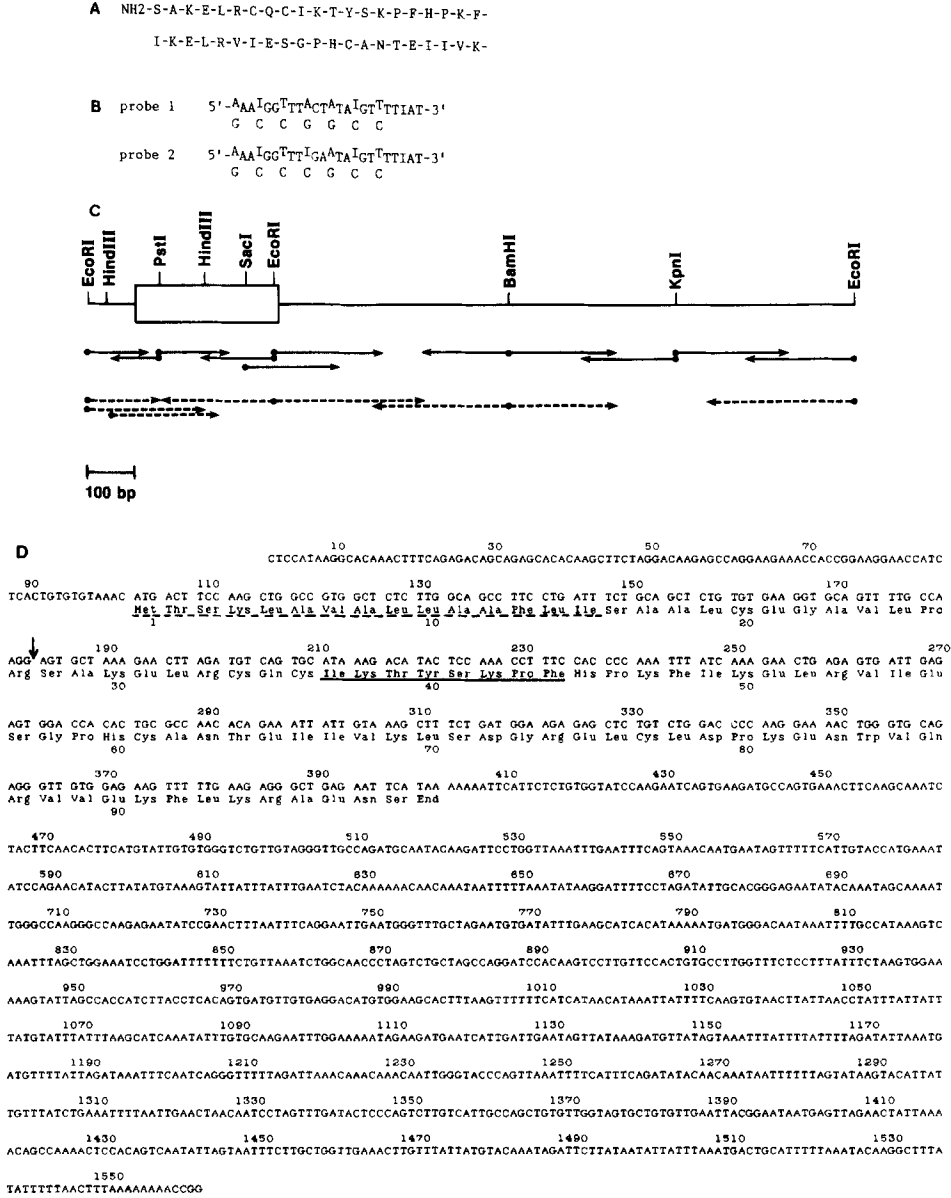


FIGURE 1. Nucleotide sequence and deduced amino acid sequence of MDNCF. (A) Amino acid sequence of natural MDNCF purified from conditioned medium of LPS-stimulated PBMC (5). (B) Oligoprobes used for screening of the cDNA library. (C) Restriction endonuclease cleavage maps of the cDNA insert of pMDNCF 2-1. Solid arrows indicates the regions determined by dideoxy method. Broken arrows indicates the regions determined by chemical reaction method. (D) Nucleotide sequence and deduced amino acid sequence of the plasmid pMDNCF 2-1 cDNA insert. The hydrophobic core of the predicted signal sequence is underlined with a broken line. The amino acid sequence from which oligonucleotide probes were synthesized for the cDNA cloning is underlined with a solid line. The arrow indicates the site for the NH₂ terminal of the mature form of MDNCF. These sequence data have been submitted to the EMBL/GenBank Data Libraries under accession number Y00787.

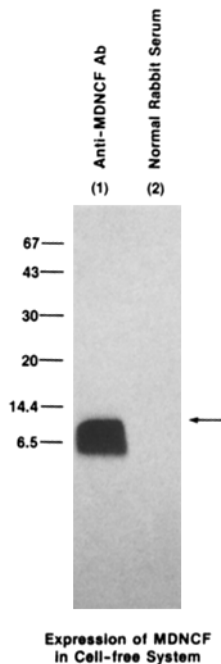


FIGURE 2. Translation of MDNCF mRNA in reticulocyte lysate system.

deduced amino acid sequence of MDNCF contains no *N*-glycosylation site (asparagine-X-threonine or asparagine-X-serine).

Expression of MDNCF Protein Using an In Vitro Transcription-Translation System. Plasmid pSP18 containing the 0.3-kb Pst I-Eco RI fragment coding for most of the MDNCF protein (amino acid residues 17-96) was transcribed by SP6 RNA polymerase, followed by translation of the in vitro-synthesized mRNA with a reticulocyte lysate and immunoprecipitation of the translated protein by rabbit anti-MDNCF antibody. As shown in Fig. 2, significant amounts of radiolabeled 10-kD MDNCF could be precipitated by antibody, but not by normal rabbit serum. However, we could not detect chemotactic activity using this cell-free translated material.

Amino Acid Sequence Homology With Other Cytokines. After establishing the entire cDNA sequence, we found striking sequence similarity between MDNCF and human platelet basic protein (PBP) (14) and PF4 (15) as well as *v-src*-inducible protein (9E3) (16), IFN- γ inducible protein (IP 10- γ) (17), and a growth-regulated gene product in transformed cells (*gro*) (18) as shown in Fig. 3. The positions of four cysteines (at residues 34, 36, 61, and 77) are identical among these proteins. The greatest similarity was observed between MDNCF and 9E3 (50 identities out of 98 amino acid possible matches, and 59% in nucleotide matches of the coding region). The similarity in protein structure was also established by a hydrophobicity output test between MDNCF and 9E3, as shown in Fig. 3 B.

Induction of MDNCF mRNA in Human PBMC by LPS, IL-1, and TNF. Total RNA was prepared from LPS-stimulated PBMC, and Northern blotting analysis was performed. As shown in Fig. 4 a, LPS induced high levels of mRNA for MDNCF (1.8 kb) at 1 h, which reached a maximum at 3 h. The high level of mRNA persisted

A
 100
 Hu.F maraalsaaP SnprLLrVAL LLLLlVaaGr raag. asVA tELRCQClq. TIQGIHFkNi QSVnVksPGP HCaqtEVIAT Lkn.GRkaCL nPasFIVkKI
 Md.G HtsKLAVAl LaaFLlSaAl CEGaVlPrSA nELRCQClkT VSKPFIHKfI kElrVlEsGP HCantEIlVk LsD.GReICL DPkenWVqFV
 9E.H HNgKlG AV LaLLLVsaAl sGrVlVnHG nELRCQClST hskfIHkSI QdVklTSPG HCRnVEIlAT LkD.GReVCL DptAPWVdLI
 Pp.I sstk gqtrnlAG KPeSldsDly aELRCmClkT tSg.IHFkNI QSLVlGAGt HcnqVEIlAT LkD.GRkICL DFDaFRlKkI
 Pp.J MNctalIlc clIFltlsg. eaEed gDLqClCVkT tSg.VfPrhI tSLEVIkaGP HCptaqIlAT Lkn.GRkICL DlgAPlykKI
 Ip.J MN.XL-VAL L.LLV A.L .EG-----LA -ELRCQClkT TSQPihPK-I QSLVlP-GP HC---VEIlAT MRRkGeKtCL nPESkaIKNI
 Consensus -----MN.XL-VAL L.LLV A.L .EG-----LA -ELRCQClkT TSQPihPK-I QSLVlP-GP HC---VEIlAT LkD.GRkICL DP-APWVkKI

101
 Hu.F IeKMLnsdks N
 Md.G VeKFLkraen S
 9E.H VkaLMakaql NSdapl
 Pp.I VqKkladeSa d
 Pp.J IKKlLes
 Ip.J lKavskemSk rSp
 Consensus VKKLL---S- NS---

B Hydrophobicity Output

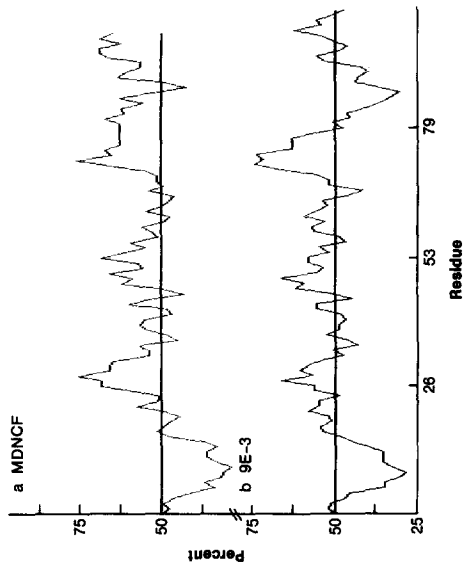


FIGURE 3. (A) Sequence similarity with other cytokines. Hu.F, human gro; Md.G, MDNCF; 9E.H, 9E3; Pp.I, PBP; Pp.J, PF4; Ip.J, Ip 10-γ. Capital letters show the conserved amino acids. (B) Hydrophobicity output test between MDNCF and 9E3.

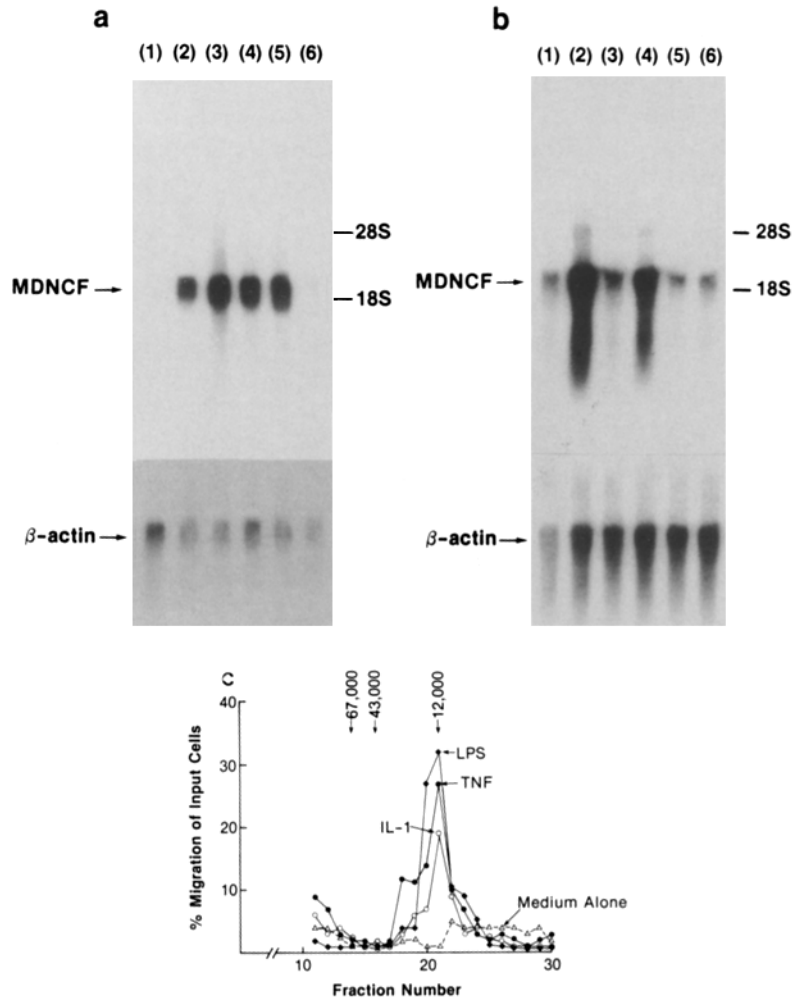


FIGURE 4. (a) Northern blotting analysis of mRNA induction in LPS stimulated PBMC after 0 h (lane 1), 1 h (lane 2), 3 h (lane 3), 6 h (lane 4), 16 h (lane 5). Lane 6 represents the mRNA in PBMC cultured for 16 h without LPS. (b) Expression of MDNCF mRNA in PBMC at 4 h after stimulation with medium alone (lane 1), IL-1 (lane 2), IL-2 (lane 3), TNF (lane 4), IFN- γ (lane 5), and IFN- α (lane 6). (c) HPLC gel filtration analysis of LPS, IL-1 and TNF-induced neutrophil chemotactic activity. 5×10^6 PBMC/ml in RPMI-1640 medium supplemented with 1% FCS were cultured for 16 h in the presence of 10 μ g/ml LPS, 100 U/ml IL-1, or 100 U/ml TNF. The culture supernatants were concentrated 20-fold by Amicon Corp. (Danvers, MA) diaflow membranes (YM 5) and 250 μ l of each sample was applied to HPLC gel filtration column. The chemotactic activity was measured at 1:4 dilution.

as long as 16 h. Small amounts of MDNCF mRNA were detected in nonstimulated PBMC at the end of the culture period. This was not due to endotoxin contamination because we did not observe any induction of IL-1 α or β mRNA under the same culture conditions. The induction of MDNCF mRNA preceded the appearance of MDNCF activity in culture media (4). We also tested cytokines, including IL-1, IL-

2, TNF, IFN- α , and IFN- γ that activate human monocytes for their capacity to induce MDNCF mRNA.

As shown in Fig. 4 *b*, both IL-1 and TNF induced similarly high levels of MDNCF mRNA. The time course of MDNCF mRNA induction by IL-1 and TNF was similar to that obtained with LPS (data not shown). Neutrophil chemotactic activity was detected in both IL-1- and TNF-stimulated PBMC culture media. The activity was eluted from HPLC gel filtration column in the same location as LPS-induced MDNCF (Fig. 4 *c*).

Discussion

We have cloned the cDNA that encodes for human MDNCF. The deduced amino acid sequence of the open reading frame was compared with the previously determined NH₂-terminal amino acid sequence of purified MDNCF. This shows that the NH₂ terminal of mature MDNCF corresponds to serine at residue 28 of the deduced amino acid sequence. We have recently chemically synthesized a polypeptide corresponding to this mature MDNCF polypeptide (residues 28 to 99), and this product has potent neutrophil chemotactic activity (Appella, E., S. Tanaka, K. Matsushima, and T. Yoshima, unpublished observation). The result confirms that the cDNA encodes for MDNCF. Experiments are in progress to express MDNCF in mammalian cells and *E. coli*.

Recently, Schmidt and Weissmann (19) reported a cDNA sequence that is identical to that of MDNCF, except for minor differences. Our cDNA is 11 bases longer at the 5' region and about 100 bases shorter at the 3' regions. In the 3' noncoding region we identified the bases at positions 650 and 1258 as G and A, respectively, in contrast to the published A and T. Schmidt and Weissmann obtained their cDNA from mRNA that was increased 10-fold above control levels when human PBMC were stimulated with staphylococcal enterotoxin, a potent lymphocyte mitogen. In vitro translation of cDNA-derived mRNA in a reticulocyte lysate yielded a peptide with a molecular mass of \sim 11 kD, a value consistent with the length of the open reading frame of the cDNA. Since this discovery was based on cDNA cloning, the authors were unable to attribute a function to the peptide or to know the size of the natural cellular product.

The striking similarity of the amino acid sequence of MDNCF with several known proteins that are believed to be involved in inflammation and cell growth, particularly with a *v-src*-induced protein (9E3) sequence, suggests MDNCF may have other as yet unidentified biological activities. 9E3 has been cloned by identifying the predominant mRNA induced after oncogenic transformation of chicken fibroblasts by *v-src*. Although at present the function of this protein is unknown, this molecule may function as an autocrine factor that promotes the growth of fibroblasts and is involved in the neoplastic transformation of fibroblasts by *v-src* (16). Therefore, we tested whether *v-src*-transformed NIH 3T3 cells express a higher level of murine MDNCF mRNA than that observed in normal NIH 3T3 cells. Although human MDNCF cDNA probe detected \sim 2 kb mRNA in murine NIH 3T3 cells, we did not see any increase in the level of this mRNA in *v-src*-transformed cells. We have also tested whether or not tyrosine kinase activators induce MDNCF mRNA, because *src* protein is well known to have tyrosine kinase activity and to be closely associated with

growth factor stimulation. We could not detect any induction of MDNCF mRNA in human normal dermal fibroblasts by epidermal growth factor, whereas we observed the rapid, dramatic induction of MDNCF mRNA in fibroblasts by IL-1 and TNF (data not shown).

The induction of MDNCF activity by IL-1 and TNF in human PBMC and fibroblasts could account for the considerable *in vivo* neutrophil attractive capacity of IL-1 despite lack of chemotactic activity *in vitro*. Since multiple steps are involved in chemoattraction of neutrophils from the blood stream to inflammatory sites, such as adhesion of neutrophils to the endothelial lining of blood vessels, infiltration of neutrophils through the endothelial lining, and movement to the inflammatory site, it is possible that multiple mediators are involved in these steps (20). It has been reported that IL-1 and TNF increase the adhesion of neutrophils to endothelium *in vitro* through the induction of adhesive molecules, and it is now clear that IL-1 and TNF induce MDNCF by PBMC and dermal fibroblasts (20). Therefore, IL-1 and TNF generate inflammatory infiltrates *in vivo* by inducing both adhesive and chemotactic molecules.

The time course of the induction of MDNCF by LPS, IL-1, and TNF *in vitro* is comparable to the time course of the *in vivo* accumulation of neutrophils at sites of injected IL-1, TNF, or LPS (20). It has been reported that FMLP induces neutrophil accumulation at 30 min to 1 h, but IL-1 induces intradermal neutrophil accumulation at 1-3 h. Therefore, the time lag between FMLP that is directly chemotactic and IL-1 may be due to the induction of MDNCF production by IL-1. This idea is supported by data showing that the induction of intradermal neutrophil accumulation by IL-1 could be blocked by a protein synthesis inhibitor, cycloheximide (20).

The cloning of cDNA for MDNCF has provided the basis for both recombinant and chemical synthesis of the molecule. This will enable us to make considerable progress defining MDNCF sites of synthesis, receptors, and contribution to acute and chronic inflammation.

Summary

The cDNA coding for human monocyte-derived neutrophil-specific chemotactic factor (MDNCF) was cloned from LPS-stimulated human monocyte mRNA. The cDNA sequence codes for a polypeptide consisting of 99 amino acids, including a putative signal sequence. Comparison of the deduced amino acid sequence with the NH₂-terminal amino acid sequence of natural MDNCF shows that the mature functional protein comprises 72 amino acids, beginning with serine at residue 28. The deduced amino acid sequence shows striking similarity to several platelet-derived factors, a *v-src*-induced protein, a growth-regulated gene product (*gro*), and an IFN- γ inducible protein. The availability of the MDNCF cDNA enabled us to use it as a probe to identify inducers of MDNCF mRNA expression in human PBMC. MDNCF mRNA was increased >10-fold within 1 h after stimulation with LPS, IL-1, or TNF, but not by IFN- γ , IFN- α , or IL-2. Furthermore, we also determined that LPS, IL-1, and TNF stimulated the mononuclear cells to produce biologically active MDNCF. This observation may account for the *in vivo* capacity of IL-1 and TNF to induce neutrophil infiltrates.

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