

Molecular cloning of a cDNA encoding a human macrophage migration inhibitory factor

(lymphokine/expression cloning/DNA sequence analysis/insertional mutational analysis)

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ABSTRACT A cDNA encoding a human macrophage migration inhibitory factor (MIF) was isolated, through functional expression cloning in COS-1 cells, from a cDNA library prepared from a lectin-stimulated T-cell hybridoma, T-CEMB. The 115-amino acid polypeptide encoded by the MIF cDNA (p7-1) was effectively released from the transfected COS-1 cells and yielded readily detectable MIF activity in the culture supernatant despite the apparent lack of a classical protein secretory sequence. Insertional mutational analysis and elution of MIF activity from polyacrylamide gel slices demonstrated that the M_r 12,000 protein with MIF activity released by the COS-1 cells is encoded by p7-1. The p7-1 cDNA hybridized with a 700-base mRNA expressed by Con-A-stimulated lymphocytes but not unstimulated lymphocytes. The availability of the MIF cDNA clone and recombinant MIF will facilitate the analysis of the role of this lymphokine in cell-mediated immunity, immunoregulation, and inflammation.

Lymphocytes secrete a large number of protein mediators, known as lymphokines, in response to antigenic or mitogenic stimulation. These lymphokines play an important role in immunoregulation, inflammation, and cellular immunity (1, 2). Migration inhibitory factor (MIF) for guinea pig macrophages was the first lymphokine to be discovered (3, 4). This factor originally was identified by its ability to prevent the migration of guinea pig macrophages out of capillary tubes *in vitro* (3, 4). Subsequently, the expression of MIF activity was found to correlate well with delayed hypersensitivity and cellular immunity in animal models and in humans (3-6). MIF activity has been detected in leukocyte culture supernatants of mice during allograft rejection (7, 8), in the synovia of patients with rheumatoid polyarthritis (9), and in a variety of chronic inflammatory loci (10). The expression MIF at sites of inflammation suggests a role for the mediator in regulating the function of macrophages in host defense.

The biochemical characterization of MIF activity has proved to be difficult largely because of the low levels of activity expressed by natural sources of the mediator. Our studies with human MIF, which also functions with guinea pig target cells, have shown that human MIF obtained from lectin-stimulated T cells or T-cell lines is heterogeneous, with at least three different species distinguishable by gel chromatography, isoelectric focusing, or sensitivity to enzymatic treatments (11). In addition, several other cytokines, including interferon γ (IFN- γ) and interleukin 4 (IL-4) can inhibit macrophage migration (12, 13). Thus the MIF activity present in activated T-cell supernatants is likely to result from the presence of multiple cytokines capable of influencing macrophage migration.

We have employed functional expression cloning in mammalian cells to identify novel lymphokines with MIF activity. Here we report the isolation of a cDNA encoding a protein that inhibits the migration of macrophages *in vitro*. The availability of recombinant MIF will facilitate the analysis of the role of this lymphokine in host defense and in inflammation.‡

MATERIALS AND METHODS

Cell Line. The T-CEMB cell line is a human T-cell hybridoma line generated by fusion of a hypoxanthine/aminopterin/thymidine-sensitive T-lymphoblastoid line (CEMWH4) with Con A-stimulated human peripheral blood T cells (14).

MIF Assay. The MIF assay (14, 15) employed human peripheral blood monocytes as indicator cells in an agarose-droplet assay system. The area of migration was calculated by the following formula: migration = (diameter of total area/diameter of agarose droplet)² - 1. The percentage of inhibition of each sample was derived as follows: % inhibition = 100 - (average migration of test samples/average migration of control samples) \times 100. Inhibition of 20% or greater was considered to be significant (11).

Isolation of mRNA and Construction of cDNA Library. Total RNA was extracted (16) from T-CEMB cells that had been stimulated with phytohemagglutinin (PHA, 1%) and phorbol 12-myristate 13-acetate (PMA, 10 ng/ml) for 18 hr. Five micrograms of mRNA prepared by oligo(dT)-cellulose chromatography (17) was used to synthesize double-stranded cDNA as described (18). The double-stranded DNA was blunted and ligated to a 5-fold excess of synthetic semi-*Xho* adapters (19). The semi-*Xho*-adapted cDNA was size-fractionated by agarose gel electrophoresis. Gel containing DNA of \geq 500 base pairs was excised and the cDNA fragments were isolated from the gel slice by adherence to glass powder (20). The COS-1 cell expression vector pXM was linearized and ligated to equimolar amounts of the semi-*Xho*-adapted cDNA. The ligation reaction was used to transform *Escherichia coli* strain HB101, thus generating a library of \approx 60,000 ampicillin-resistant colonies.

DNA Preparation and COS-1 Cell Transfection. Bacterial colonies from the library were replica-plated onto nitrocellulose filters. Colonies from each filter were scraped into Luria broth and plasmid DNA was isolated. Each primary DNA sample was prepared from a pool of 200-500 colonies. Five micrograms of each plasmid DNA was used to transfect COS-1 cells (simian virus 40-transformed monkey kidney cell line) as described (21, 22). Culture supernatant fluid from

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Abbreviations: MIF, migration inhibitory factor; IFN, interferon; IL, interleukin; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. M25639).

each dish of transfected COS-1 cells was harvested 72 hr after transfection and examined for MIF activity.

Protein Analysis. COS-1 cells transfected with recombinant DNA of MIF-positive clones were incubated with 0.5 mCi of [³⁵S]methionine (1 mCi = 37 MBq) for 4 hr at 37°C. Radio-labeled supernatant was subjected to NaDodSO₄/PAGE (15%). After electrophoresis, the gel was immersed in EN³HANCE (New England Nuclear) dried, and exposed to x-ray film. To investigate whether MIF activity in COS supernatant could be recovered after NaDodSO₄/PAGE, the polypeptide of interest was excised from the polyacrylamide gel and electroeluted at 6 W for 2 hr in elution buffer containing 50 mM NH₄HCO₃ and 200 ng of human serum albumin per ml. The latter was added to prevent nonspecific sticking. In parallel, polypeptides with the same apparent molecular weight from supernatant of mock-transfected COS cells were also excised and subjected to electroelution. The eluant was reconstituted with medium and examined for MIF activity.

DNA Sequence Analysis. The nucleotide sequence of the cDNA insert of p7-1 was determined as described (18, 19) by generating ordered sets of overlapping fragments by BAL-31 nuclease digestion and subcloning into M13 vectors. Single-stranded DNA was prepared, and the nucleotide sequence was determined by the dideoxynucleotide chain-termination procedure (23).

Generation of Insertional Mutants of the MIF Clone. Although the MIF cDNA clone p7-1 contained a single *Pst* I site, the *Pst* I sites in the adapters flanking the insert rendered this site unsuitable for mutational analysis of the MIF cDNA. Therefore, we removed the two flanking *Pst* I sites by treating the p7-1 insert (isolated after partial digestion of the plasmid with *Pst* I) with T4 DNA polymerase, then ligating *Eco*RI adapters to the resulting flush ends. This adapted fragment was subcloned into the single *Eco*RI site of a derivative of pXM designated pXMT4, which has no *Pst* I sites. A clone, p7-1-24, with the cDNA in the correct orientation was selected for mutational analysis.

Two insertional mutants of MIF were constructed. The first mutant was generated by inserting a 14-base oligodeoxynucleotide (5'-TGTAATTACATGCA-3') at the single *Pst* I site of p7-1-24. This sequence was designed such that the MIF coding region could be interrupted by a termination codon (TAA) regardless of the orientation of insertion. The second insertional mutant was constructed by inserting a 99-base oligodeoxynucleotide into the *Pst* I site of p7-1-24. The sequence was designed to add 33 amino acids to the MIF

coding region when inserted in either orientation into the *Pst* I site of p7-1-24. Clones containing the 14-base oligonucleotide or the 99-base oligonucleotide were identified by hybridization with ³²P-labeled 14-base oligomer or 99-base oligomer.

RNA Analysis. Twenty micrograms of total cellular RNA from PHA/PMA-stimulated or unstimulated T-CEMB cells, Con A-stimulated or unstimulated human peripheral blood lymphocytes, or CEM cells was electrophoresed in a 1.2% agarose gel containing 2.2 M formaldehyde (24). The formaldehyde-denatured RNA was transferred to a nylon filter (Zetabind; Cuno). cDNA probe was prepared by cleaving the cDNA inserts from the vector with restriction endonuclease *Xho* I. The inserts were labeled with ³²P by random priming (25). The nylon filter was prehybridized (4 hr at 43°C) and then hybridized (16 hr at 43°C) with ³²P-labeled cDNA probe in 6× SSC/0.5% NaDodSO₄/0.1% Ficoll/0.1% polyvinylpyrrolidone/0.1% bovine serum albumin containing denatured salmon sperm DNA at 100 μg/ml. (SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.) After hybridization, the filter was washed twice with 10 mM sodium phosphate/0.1% NaDodSO₄/1 mM EDTA/1× SSC for 30 min at room temperature and twice with 10 mM sodium phosphate/0.1% NaDodSO₄/1 mM EDTA/0.2× SSC for 15 min at 68°C, dried, and exposed to x-ray film.

RESULTS

Identification and Isolation of a Human MIF cDNA Clone by Expression. mRNA from PHA/PMA-stimulated T-CEMB cells (14), which produce MIF without concomitant production of significant levels of IFN-γ, was used to construct a cDNA library comprising ≈60,000 clones with the COS cell expression vector pXM. This library was screened by transfecting pools of plasmid DNAs, each containing 200–500 clones, into COS-1 cells as described (19); the resulting supernatants were tested for MIF activity. Our initial screen of 100 pools yielded several samples with various levels of MIF activity (Table 1). Each of these samples was tested five times, using cells from different donors to minimize the chance of obtaining falsely positive samples. Pool 44, which yielded the highest reproducible level of MIF activity, was selected and subdivided into smaller pools. These were retested for their ability to induce MIF expression when introduced into COS cells. This process was repeated until a single clone (p7-1) was identified that consistently yielded the highest level of MIF activity when transfected into COS cells.

Table 1. MIF activity in supernatants from COS-1 cells transfected with DNAs from the T-CEMB cDNA expression library

Transfected DNA	Sample	MIF activity, % inhibition				
		Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
200–500 clones, undiluted	2	–9	17	31	28	–3
	10	–1	10	33	7	10
	23	–23	–3	47	–22	18
	39	59	69	50	34	34
	43	23	21	4	27	40
	44	52	71	37	36	40
	Mock	–7	–11	3	–4	–21
20–43 clones, 10-fold dilution	39-32b	16	26	–6	5	17
	44-11a	28	11	0	–7	5
	44-11b	30	34	15	26	36
	Mock	3	2	–5	–9	2
	Single clone, 20-fold dilution	4411b7-1*	39	26	39	24
	Mock	4	3	–7	–11	5

Of the 100 supernatants from the COS-1 cell transfections of the primary pools, sample 44 showed the best overall MIF activity. The samples were then subdivided to contain fewer clones until individual clones were isolated.

*Plasmid p7-1.

A 5-fold dilution of the conditioned medium from this transfection also inhibited the migration of guinea pig and mouse peritoneal macrophages by 38% and 31%, respectively, consistent with the previous finding of non-species specificity of MIF (5, 6).

Identification of the MIF Polypeptide. The polypeptide encoded by the cDNA of p7-1 was identified in metabolic labeling experiments. NaDodSO₄/PAGE of polypeptides secreted by COS-1 cells transfected with p7-1 DNA revealed a polypeptide of $M_r \approx 12,000$ (Fig. 1, lane 2) which was absent from the mock-transfected control (lane 1). The region of the gel containing the M_r 12,000 polypeptide was excised and electroeluted. Strong MIF activity was found in the eluant (Fig. 2). The molecular weight of the MIF-specific polypeptide is in agreement with the molecular weight of MIF produced by several lymphoid cell lines (26–28). No MIF activity was detected from the same molecular weight region of gel lanes containing proteins of mock-transfected cells (Fig. 2).

Sequence Analysis of the cDNA Insert of Plasmid p7-1. The sequence of the p7-1 cDNA insert contains an open reading frame of 345 nucleotides, beginning with the ATG codon at nucleotides 51–53 (Fig. 3). The ATG is followed by 114 codons downstream by a TAA termination triplet at nucleotides 396–398. The 345 nucleotides encode a 115-amino acid polypeptide with a calculated molecular weight of 12,650, which is in agreement with the apparent molecular weight of the MIF-specific protein band observed in metabolic labeling experiments (Fig. 1, lane 2).

Although MIF is thought to be a secreted protein, the predicted sequence does not contain a stretch of hydrophobic amino acids that resembles conventional secretory leader sequences (29), either at the N terminus or internally. A very hydrophobic sequence, characteristic of a protein signal peptide, is also lacking in IL-1 α and IL-1 β (30), acidic and basic fibroblast growth factors (31, 32), endothelial cell growth factor (33), and MIF-related proteins (9). The apparent absence of a leader sequence suggests that the mechanism of secretion of this MIF is distinct from that of typical secretory proteins, which involves passage through the Golgi apparatus and endoplasmic reticulum (34). Cells may export

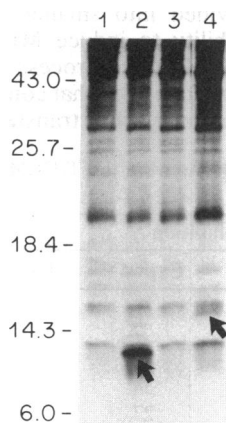


FIG. 1. Polypeptides secreted by transfected COS-1 cells. [³⁵S]-Methionine-labeled supernatants (20 μ l per lane) were analyzed by NaDodSO₄/PAGE and the labeled proteins were visualized by fluorography. Shown are supernatants from mock-transfected cells; (lane 1), from cells transfected with DNA of p7-1 (lane 2), from cells transfected with DNA of p7-1-24B2 (its cDNA insert contains a stop codon at the *Pst* I site) (lane 3), and from cells transfected with DNA of p7-1-24232 (its cDNA insert contains a 99-base oligomer at the *Pst* I site) (lane 4). The molecular weight ($M_r \times 10^{-3}$) of standard proteins in the same gel is indicated at left. Arrows indicate the M_r 12,000 polypeptide present only in lane 2 (p7-1-transfected) and the M_r 15,500 polypeptide present only in lane 4 (p7-1-24232-transfected).

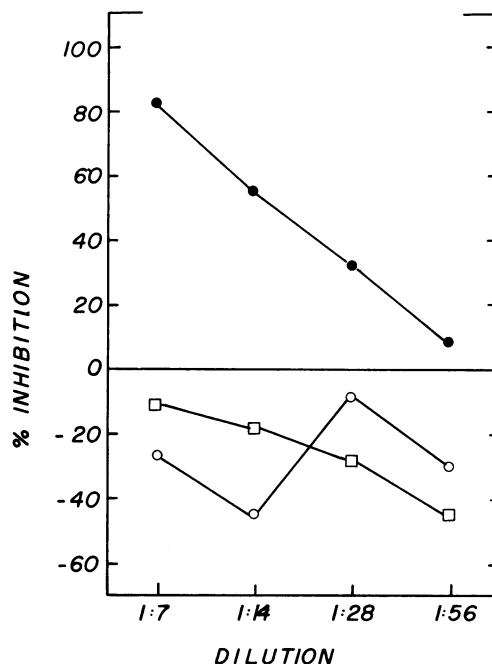


FIG. 2. MIF activity of proteins obtained by electroelution. Supernatants (300 μ l) from COS-1 cells transfected with p7-1 DNA or mock-transfected were loaded onto the same polyacrylamide gel and were subjected to NaDodSO₄/PAGE. Regions corresponding to $M_r \approx 12,000$ were excised from both. MIF activity of the M_r 12,000 regions (\bullet , p7-1; \circ , mock) was examined after electroelution. As an additional control, elution buffer (\square) was included in the same assay.

MIF by a mechanism similar to that of IL-1 α , which at present remains unclear. However, it has been reported that IL-1 β is not anchored on the plasma membrane and that its secretion occurs by a novel mechanism that does not use a secretory leader sequence or the classical secretory pathway (34).

Analysis of the DNA sequence revealed several other interesting features. The cDNA encodes two potential asparagine-linked glycosylation sites at amino acids 73–75 (Asn-Arg-Ser) and 100–112 (Asn-Asn-Ser) (35). The presence of two potential asparagine-glycosylation sites has been reported in numerous cytokines (18, 19, 36–38). There are three cysteine residues, located at positions 57, 60, and 81. We have often observed that MIF loses its biological activity upon storage. The presence of three cysteine residues may account at least in part, for this observation. Indeed, IFN- β Ser-17 (39), which was obtained through site-specific mutagenesis and differed from IFN- β Cys-17 (40) only by the substitution of serine for one of the three cysteine residues, was found to be far more stable to long-term storage than IFN- β Cys-17 (39).

We compared the nucleotide sequence of p7-1 cDNA with the nucleotide sequences recorded in GenBank (May 25, 1988) and found that the p7-1 cDNA shares no sequence homology with IFN- γ or IL-4, both of which have been reported (12, 13) to exhibit MIF-like activity. It also shares no sequence homology with other cytokines or with the two cDNAs encoding two MIF-related proteins, MRP-8 and MRP-14 (9).

Insertional Mutational Analysis of the MIF cDNA. The relatively efficient secretion of the M_r 12,000 protein with MIF activity from p7-1-transfected COS cells, despite the lack of a clear signal peptide in the coding sequence, raised the possibility that the molecularly cloned protein was not MIF but an inducer of endogenous MIF expression by the COS cells. To test this possibility, we constructed two insertional mutations of the coding region of the p7-1 cDNA.

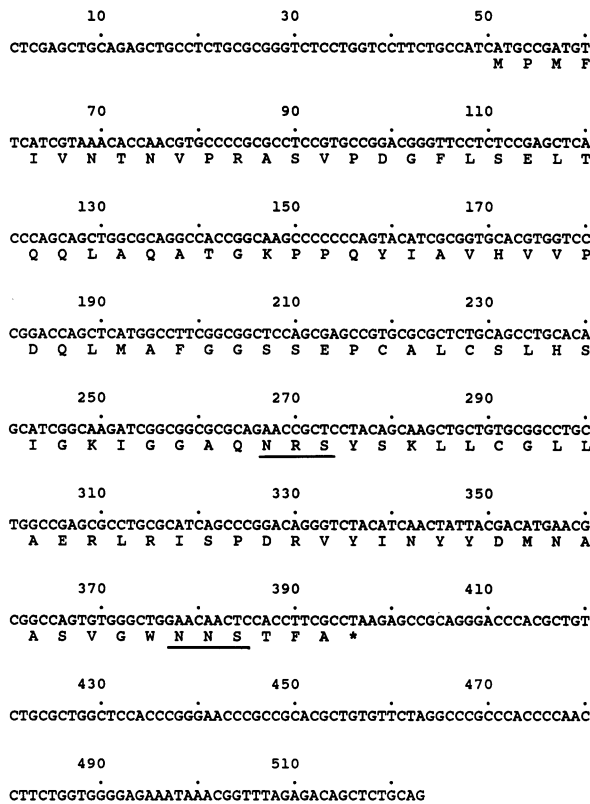


FIG. 3. Nucleotide sequence and predicted amino acid sequence of human MIF cDNA clone p7-1. The consensus sequences for asparagine-linked glycosylation sites are underlined. Amino acids are designated by the single-letter code, and an asterisk indicates the termination codon.

The first mutant, p7-1-24B2, was generated by inserting a 14-mer into the unique *Pst* I site so that a termination codon disrupted the open reading frame. The second mutant, p7-1-24232, was created by insertion of a 99-mer, at the same site, that extended the open reading frame by 33 amino acids regardless of the orientation of the insertion. Each of these plasmids was tested for ability to induce secretion of the M_r 12,000 protein observed with the original p7-1 plasmid as well as MIF activity when transfected into COS cells. Neither the M_r 12,000 protein (Fig. 1, lane 3) nor MIF activity (Fig. 4) was detected in the supernatants from the COS cells transfected with the truncated form of MIF (p7-1-24B2). Transfection of COS cells with the mutant having the extended coding region also failed to yield detectable levels of MIF activity (Fig. 4) or M_r 12,000 protein. However, the COS cell supernatant contained a novel species of apparent $M_r \approx 15,500$, consistent with the expected size of the extended coding region of the insertional mutant p7-1-24232 (Fig. 1, lane 4). The results from these experiments further support the conclusion that the induced M_r 12,000 species from p7-1-transfected COS cells is encoded by the cDNA insert of p7-1 plasmid and that this protein has MIF activity.

Expression of MIF mRNA. We examined mRNA from several cell sources for ability to hybridize with the cDNA insert of p7-1. Blot analysis revealed that the T-cell line CEM, the T-cell hybridoma line T-CEMB, and lectin-stimulated human peripheral blood lymphocytes synthesized readily detectable levels of mRNA that hybridized with the MIF clone (Fig. 5). However, the messenger was not detected in RNA samples from unstimulated lymphocytes despite prolonged autoradiographic exposure (Fig. 5, lane 1). The presence of the RNA transcript in activated human lymphocytes

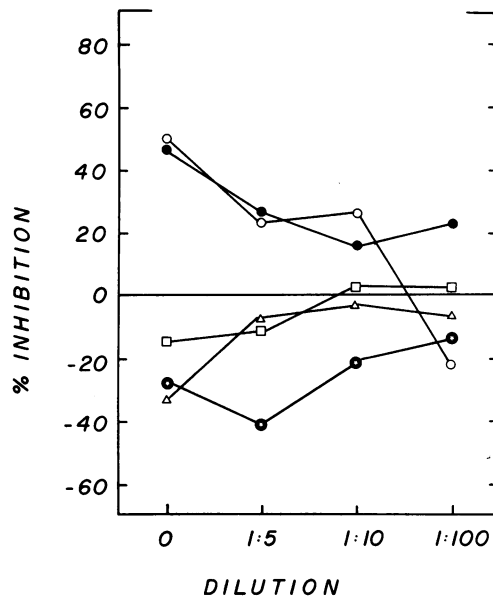


FIG. 4. MIF activity of supernatants from COS-1 cells transfected with p7-1 (●), p7-1-24 (○), p7-1-24B2 (△), or p7-1-24232 (●) or mock-transfected (□). p7-1-24B2 and p7-1-24232 were constructed as insertional mutants of p7-1 containing 14-mer or 99-mer, respectively.

(Fig. 5, lane 4) suggests that the human MIF gene is expressed and that MIF is a product of activated lymphocytes.

DISCUSSION

We have identified and isolated a cDNA clone that encodes a biologically active human MIF by detection of the functional polypeptide produced by mammalian cells. The protein encoded by the MIF cDNA has a molecular weight of $\approx 12,000$ and its bioactivity is recovered even after NaDodSO₄/PAGE.

Insertion of a termination codon into the coding region of the MIF cDNA disrupted the production of the M_r 12,000 protein and MIF activity by transfected COS cells. Extension of the open reading frame of MIF-cDNA by 33 amino acids produced a polypeptide of $M_r \approx 15,500$, consistent with the expected size of the extended coding region of the insertional mutant. The results of insertional mutational analysis of MIF cDNA further support the finding that the M_r 12,000 MIF is encoded by the p7-1 cDNA. The definitive proof will require the purification and the determination of the amino acid sequence of the M_r 12,000 MIF protein.

The apparent molecular weight of the MIF-specific polypeptide obtained by NaDodSO₄/PAGE corresponds well with the number of amino acids encoded by the p7-1 cDNA

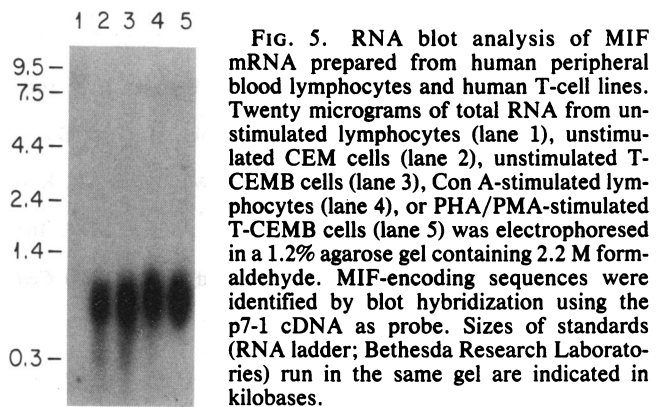


FIG. 5. RNA blot analysis of MIF mRNA prepared from human peripheral blood lymphocytes and human T-cell lines. Twenty micrograms of total RNA from unstimulated lymphocytes (lane 1), unstimulated CEM cells (lane 2), unstimulated T-CEMB cells (lane 3), Con A-stimulated lymphocytes (lane 4), or PHA/PMA-stimulated T-CEMB cells (lane 5) was electrophoresed in a 1.2% agarose gel containing 2.2 M formaldehyde. MIF-encoding sequences were identified by blot hybridization using the p7-1 cDNA as probe. Sizes of standards (RNA ladder; Bethesda Research Laboratories) run in the same gel are indicated in kilobases.

and is in agreement with the molecular weight of MIF produced by several lymphoid cell lines (26–28). However, the undenatured MIF species elaborated by lectin-stimulated lymphocytes have molecular weights of 25,000–68,000 (11, 41, 42). It is possible that the native MIF exists as a dimer or multimer of individual subunits. Indeed, evidence for subunit structure in human MIF was reported by Possanza *et al.* (26). Furthermore, glycosylation may also affect molecular weight estimates. Consistent with this explanation is the presence of two potential asparagine-glycosylation sites in the predicted protein sequence, at amino acids 73–75 and 110–112. In addition, human MIF elaborated by mitogen-stimulated peripheral blood lymphocytes has been found to be heterogeneous, comprising at least three molecular species (11).

The MIF encoded by p7-1 cDNA is an independent molecular entity and shares no sequence homology with other cytokines, including IFN- γ and IL-4, both of which are capable of inhibiting migration of macrophages (12, 13). In this regard, it is of note that tumor necrosis factor and bacterial lipopolysaccharide have also been observed to inhibit the migration of macrophages (data not shown), supporting the notion that MIF activity in culture supernatant of mononuclear cells or at the site of inflammation may result from the inhibitory activity of multiple cytokines or from the combined effect of cytokines and bacterial products. It also suggests independently evolving genes whose dissimilar protein products share some similar biological activities.

In preliminary experiments, we have observed that supernatant derived from p7-1-DNA-transfected, but not mock-transfected, COS-1 cells induced human monocyte-derived macrophages to express IL-1 β and to up-regulate HLA-DR gene expression (data not shown), suggesting that the p7-1 MIF is involved in the activation of macrophages. The availability of recombinant MIF will enable us to analyze its role in immunoregulation, delayed hypersensitivity and cellular immunity, and to delineate its involvement in chronic inflammatory processes.

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