Enzymatically Inactive Macrophage Migration Inhibitory Factor Inhibits Monocyte Chemotaxis and Random Migration[†]

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Received June 14, 1999; Revised Manuscript Received July 26, 1999

ABSTRACT: Macrophage migration inhibitory factor (MIF) is a cytokine that was first described as an inhibitor of the random migration of monocytes and macrophages and has since been proposed to have a number of immune and catalytic functions. One of the functions assigned to MIF is that of a tautomerase that interconverts the enol and keto forms of phenylpyruvate and (*p*-hydroxyphenyl)pyruvate and converts D-dopachrome, a stereoisomer of naturally occurring L-dopachrome, to 5,6-dihydroxyindole-2-carboxylic acid. The physiological significance of the MIF enzymatic activity is unclear. The three-dimensional structure of MIF is strikingly similar to that of two microbial enzymes (4-oxalocrotonate tautomerase and 5-carboxymethyl-2-hydroxymuconate isomerase) that otherwise share little sequence identity with MIF. MIF and these two enzymes have an invariant N-terminal proline that serves as a catalytic base. Here we report a new biological function for MIF, as an inhibitor of monocyte chemoattractant protein 1- (MCP-1-) induced chemotaxis of human peripheral blood monocytes. We find that MIF inhibition of chemotaxis does not occur at the level of the CC chemokine receptor for MCP-1, CCR2, since MIF does not alter the binding of ^{125}I-MCP-1 to monocytes. The role of MIF enzymatic activity in inhibition of monocyte chemotaxis and random migration was studied with two MIF mutants in which the N-terminal proline was replaced with either a serine or a phenylalanine. Both mutants remain capable of inhibiting monocyte chemotaxis and random migration despite significantly reduced or no phenylpyruvate tautomerase activity. These data suggest that this enzymatic activity of MIF does not play a role in its migration inhibiting properties.

Macrophage migration inhibitory factor (MIF),[1] a widely expressed protein with a monomer molecular mass of 12.5 kDa, was among the first of the lymphocyte-derived cytokines to be described. MIF was originally discovered in the supernatants of antigen-activated lymphocytes as a factor that inhibited the random movement of monocytes or cultured macrophages (*1*−*3*). In recent years, a number of other biologic functions have been attributed to MIF, suggesting its potential role as a proinflammatory mediator (reviewed in ref *4*). The findings presented here assign a previously unknown biologic function to MIF: that of an inhibitor of monocyte chemotaxis. While MIF has been reported to inhibit random movement of monocytes and macrophages, this is the first report showing that MIF can inhibit the movement of monocytes under the influence of a chemotactic gradient.

MIF has been crystallized in its active trimeric configuration (*5*−*8*) and found to have three-dimensional homology to two bacterial enzymes, 4-oxalocrotonate tautomerase (4-OT) and 5-carboxymethyl-2-hydroxymuconate isomerase (CHMI), that otherwise share little sequence identity with MIF. Interestingly, MIF has also been found to have an enzymatic activity**.** MIF is capable of catalyzing the tautomerization of D-dopachrome (**1**, Scheme 1), the nonphysiological isomer of an intermediate in the biosynthesis of melanin, to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (*9*). MIF also catalyzes the reversible interconversion of the keto and enol forms of phenylpyruvate (**2**, Scheme 1) and (*p*-hydroxyphenyl)pyruvate (**3**, Scheme 1) (*10*). It is not

[†] This research was supported in part by the Texas Advanced Research Program (ARP-183) to C.P.W.

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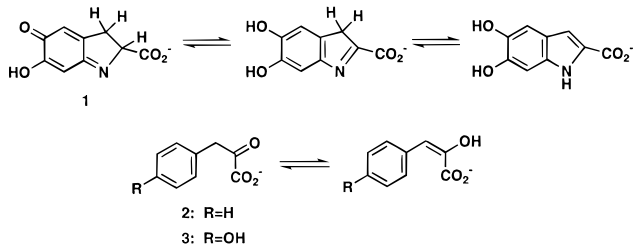
[1] Abbreviations: AP, ampicillin; BCA, bicinchoninic acid; BSA, bovine serum albumin; CHMI, 5-carboxymethyl-2-hydroxymuconate isomerase; DEAE, diethylaminoethyl; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; ESI-MS, electrospray ionization mass spectrometry; FACS, fluorescence-activated cell sorter; HBSS$^-$, Hank's balanced salt solution without cations; HPLC, high-pressure liquid chromatography; HSA, human serum alumin; IL-8, interleukin 8; IPTG, isopropyl β-D-thiogalactoside; LB, Luria−Bertani medium; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein 1; MIF, macrophage migration inhibitory factor; 4-OT, 4-oxalocrotonate tautomerase; PBMC, peripheral blood mononuclear cells; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TNFα, tumor necrosis factor α.

10.1021/bi991352p CCC: $18.00
Published on Web 09/04/1999

Scheme 1



known what physiological role, if any, these enzymatic reactions may play, and it has been postulated that the true physiologic substrate for MIF has not yet been discovered. However, these known enzymatic activities have been used experimentally to identify MIF's active site and to determine that the amino-terminal proline acts as the general base in both reactions (*11*−*14*) Intriguingly, 4-OT and CHMI also have an invariant N-terminal proline that serves as a catalytic base (*15*−*17*).

To determine whether MIF's biologic role as an inhibitor of monocyte chemotaxis and random migration is linked to its ability to act as a tautomerase, two MIF mutants with an altered N-terminal proline were generated, found to have little or no enzymatic activity, and then tested for retention of the ability to inhibit chemoattractant-induced monocyte movement. Despite diminished or absent enzymatic activity, the two mutants did not differ from wild-type MIF in their ability to inhibit monocyte chemotaxis and random migration.

EXPERIMENTAL PROCEDURES

General Methods and Reagents. Reagents for the PCR were obtained from Perkin-Elmer (Norwalk, CT). HPLC separations were performed on a Waters system using a Waters Protein Pak (DEAE-5PW) anion-exchange column and a Pharmacia Superose 12 (HR 10/30) gel-filtration column. The Econosil C18 reverse-phase HPLC column was obtained from Alltech. Unless otherwise noted, protein concentrations were determined with the commercially available bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co., Rockford, IL) and by the method of Waddell (*39*), and protein was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) under denaturing conditions on 12.5% gels (*40*). Enzymatic activity was determined by monitoring the ketonization of β-phenylpyruvate at 288 nm (*14*). DNA sequencing was done either at the University of Texas (Austin) Sequencing Facility or at Merck Research Laboratories (Rahway, NJ). Kinetic data were obtained on a Hewlett-Packard 8452A diode-array spectrophotometer and fitted by nonlinear regression data analysis with the Grafit program (Erithacus Software Ltd., Staines, U.K.) obtained from Sigma Chemical Co. Buffer and media were purchased from Gibco−BRL (Grand Island, NY) and Mediatech (Herndon, VA). QCL-1000, a kit for quantification of LPS in assay reagents, was purchased from Biowhittaker (Walkersville, MD). Filter plates were analyzed by use of the Tecan SpectraFluor Plus fluorescence reader. Unless specified, biochemicals were purchased from Sigma Chemical Co. The clone containing recombinant mouse MIF was obtained from Dr. Richard Bucala (The Picower Institute for Medical Research, Manhasset, NY).

Cloning of Human MIF. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed to generate cDNA from 0.1 ng of human pituitary gland poly (A)+ total mRNA (Clontech, Palo Alto, CA) with dT_{18} and MuLV reverse transcriptase in a 20 μL reaction mixture by the following protocol: 42 °C for 30 min, 99 °C for 5 min, 5 °C for 5 min. Subsequent PCR was performed for 35 cycles on the entire volume of cDNA in a 100 μL reaction mixture with the following primers: forward 5′-GCATTGCGCGGGTCTC-CTGGTCCTTCTG-3′ and reverse 5′-GCATTGGGCGGCGT-GCGACACAAGATC-3′. An additional 35 cycles of nested PCR was performed on 10 μL of the initial reaction mixture. The nested primers were from a previously published protocol (*18*): forward 5′-GCATCCATCATGCCGATGT-TCATCGTAAACAC-3′ and reverse 5′-GCATGGAAGCG-GATTCTCGGCGTCCTAGGC-3′. The PCR was performed on a PE 9600 (Perkin-Elmer) thermocycler as follows: an initial melting step of 95 °C for 1.75 min; 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and a soak step of 72 °C for 7 min. Oligonucleotides for the PCR and DNA sequencing were synthesized and provided deprotected and desalted by Operon Technologies (Alameda, CA). This strategy yielded a cDNA fragment of 363 bp as determined by 1.4% agarose gel electrophoresis (FMC, Rockland, ME). The target fragment was gel-purified using the Gene Clean II kit (Bio 101, La Jolla, CA) for further cloning.

The cDNA was polished with *Pfu* DNA polymerase to create blunt ends and subsequently cloned into the Stratagene pCR-Script Amp SK(+) cloning vector. The ligated vector was used to transform Epicurian XL-10 Gold Kan ultracompetent cells (Stratagene Corp., La Jolla, CA). Transformants were plated on LB plates containing 100 μg/mL ampicillin, 50 μg/mL tetracycline, 3 μg/mL IPTG, and 32 μM X-gal for blue/white selection. Single white colony isolates were grown in LB broth containing 100 μg/mL ampicillin, which was shaken at 200 rpm for 16 h at 37 °C. Plasmid was recovered by Qiagen QIAprep Spin miniprep kit and analyzed by 1.4% agarose gel electrophoresis, yielding a fragment of approximately 3000 bp. Digestion with *Nde*I and *Bam*HI restriction enzymes (New England Biolabs, Beverly, MA), followed by subsequent analysis by 1% agarose gel electrophoresis, yielded fragments of appropriate size. Sequencing on both strands by ABI automated sequencing system indicated the isolated plasmid contained the full-length MIF cDNA sequence (accession number M25639, GenBank).

Expression and Purification of Human MIF. The MIF insert was isolated by agarose gel electrophoresis, gel-purified by QIAquick gel extraction kit, and ligated into the *Nde*I/*Bam*HI-digested pET 11a(+) expression vector (Novagen, Inc, Madison, WI). Subsequent transformation of "One-Shot" TOP 10S′ cells (Invitrogen, Carlsbad, CA) and plating on MM+CA-A50 agar plates (Difco, Detroit, MI) generated colonies, 10 of which were randomly chosen for 3 mL overnight culture growth. Recovery and *Nde*I/*Bam*HI restriction analysis of the plasmid DNA from the overnight cultures indicated all colonies were positive for the full-length MIF sequence. Intact pET-11a(+) containing the MIF insert was used to transform *Escherichia coli* BL21(DE3) vector competent expression strain cells (Novagen, Inc, Madison, WI) in 800 μL of LB with 0.5% glucose. An aliquot (400 μL) was plated on MM+CA-A50 agar plates for long-term

maintenance, the balance being used to inoculate a 30 mL overnight culture at 37 °C. An aliquot (500 μL) of the overnight culture was used to inoculate 3 mL of fresh MM+CA-A50 medium, which was shaken for an additional 60 min at 37 °C. Induction with 1 mM IPTG was allowed to proceed for 4 h at 37 °C. A 1 mL aliquot of cell suspension was pelleted, resuspended in 150 μL of 1× SDS buffer with β-mercaptoethanol, boiled for 15 min, and pelleted to eliminate cell debris. Supernatant was analyzed by SDS−16% PAGE indicating expression of a 13 kDa protein corresponding to monomeric MIF.

A large scale-up (1 L) of human MIF recombinant cells was prepared to supply material for purification by column chromatography. Cells were pelleted, resuspended in 20 mM Tris-HCl, pH 7.4, and homogenized with a Dounce homogenizer. Homogenate was passed through a French press and spun at 140000*g* (35 000 rpm) for 30 min at 4 °C. The supernatant was loaded at 2 mL/min onto a freshly prepared Q-Sepharose anion exchange column, 2.5 cm i.d. × 5.0 cm height (Pharmacia, Piscataway, NJ), equilibrated with 20 mM Tris-HCl, pH 7.4. The flowthrough was collected and subsequently loaded at 2 mL/min onto a similarly prepared SP-Sepharose cation-exchange column (Pharmacia, Piscataway, NJ). Flowthrough fractions were collected during column load and postload wash with 20 mM Tris, pH 7.4. Protein concentration for each fraction was determined by A_{280} UV spectrophotometry, while fraction purity was assessed by Tris−glycine SDS−16% PAGE. Pure fractions of high concentration were loaded at 0.5 mL/min onto a Superdex 75 size-exclusion column, 2.5 cm i.d. × 70 cm height (Pharmacia, Piscataway, NJ), equilibrated with 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl. Fractions were collected and analyzed by UV A_{280} and SDS−PAGE. Two peaks eluted from the sizing column, the second corresponding to monomeric MIF. All chromatographic procedures were performed at 4 °C. This procedure yielded 46.3 mg of pure enymatically active MIF protein.

Site-Directed Mutagenesis of Mouse MIF. The two MIF mutants (P1F MIF and P1S MIF) were prepared with the plasmid containing the recombinant mouse MIF as the template. Restriction enzymes, T4 DNA ligase, and agarose were obtained from Promega Corp. (Madison, WI) or Gibco−BRL (Gaithersburg, MD). The mutations were made by the overlap extension polymerase chain reaction as described (*19*). The external PCR primers were oligonucleotides 5′-ATCTCGATCCCGCGAAAT-3′ (designated primer A) and 5′-CTCAGCTTCCTTTCGGGCTT-3′ (designated primer D). Primer A corresponded to the coding sequence of a region of the pET11b(+) vector ∼90 bp upstream from an *Nde*I restriction site, while primer D corresponds to the complementary sequence ∼12 bp downstream from a *Bam*HI restriction site. For the P1F mutant, the internal PCR primers were oligonucleotides 5′-GATGAACAT**AAA**CATATGTAT-3′ (primer B) and 5′-ATACATATG**TTT**ATGTTCATC-3′ (primer C). For the P1S mutant, the internal PCR primers were oligonucleotides 5′-GATGAACAT**AGA**CATATGTAT-3′ (primer B) and 5′-ATACATATG**TCT**ATGTTCATC-3′ (primer C). Oligonucleotides for the PCR and DNA sequencing were synthesized and provided deprotected and desalted by Oligos Etc. Inc. (Wilsonville, OR). In each set of primers, primer C contains a *Nde*I restriction site (underlined) followed by the codon for the desired mutation (boldface type). The subsequent nine bases correspond to the coding sequence of the gene for MIF. Primer B is the complementary primer. The PCRs were carried out in a Perkin-Elmer DNA Thermocycler 480 with template DNA, synthetic primers, and the PCR reagents supplied in the PCR reagent system following a previously described protocol (*15*). The template DNA was prepared with the Wizard Plus Minipreps DNA purification system as described (*15*). The AB and CD fragments were generated in two separate PCRs as described (*15*). Subsequently, the mutated DNA fragments for P1F MIF and P1S MIF were produced by performing the PCR on a mixture of the appropriate AB and CD fragments (5 μL each) with primers A and D. The mutated DNA fragments and the pET24a(+) vector were digested with *Nde*I and *Bam*HI restriction enzymes, purified, and ligated by T4 DNA ligase following a previously described protocol (*15*). The expression vector pET24a(+) and expression strain were obtained from Novagen, Inc. (Madison, WI). Aliquots were transformed into competent *E. coli* JM109 cells and grown on LB/Kn (100 μg/mL) plates at 37 °C. Single colonies were chosen at random and grown in liquid LB/Kn medium (50−100 μg/mL). The newly constructed plasmid was isolated and fully sequenced (both strands) in order to verify the mutation. Subsequently, the mutated plasmid was transformed in *E. coli* strain BL21(DE3)pLysS for protein expression.

Overexpression and Purification of Recombinant Mutant Mouse Proteins. A single colony of each expression strain was used to inoculate LB/kanamycin medium (50−100 μg/mL). The cultures were grown and the mutant proteins were expressed as described previously (*15*). Typically, 2 L of cells grown under these conditions yield ∼4 g of cells. P1F MIF and P1S MIF were purified to homogeneity by previously described protocols with the following modification (*14*). After cell lysis and centrifugation, the crude lysate was treated with protamine sulfate (20 mg/g of cell paste dissolved in 5−10 mL of the lysis buffer), allowed to stir for 10−15 min, and centrifuged (17000*g*, 30 min). Subsequently, the supernatant was loaded onto the Waters Protein Pak (DEAE-5PW) anion-exchange column and processed as described (*14*). The yield of purified protein (> 95% as assessed by SDS−PAGE) per liter is ∼25 mg of P1F MIF and ∼44 mg of P1S MIF. The native molecular masses of the purified proteins (P1F MIF and P1S M1F) were estimated by chromatography on a Superose 12 column and compared to that of wild-type MIF. The column was equilibrated with 20 mM sodium phosphate buffer (pH 7.3) at a flow rate of 0.4 mL/min and the effluent was monitored at 280 nm. Under these conditions, wild-type MIF eluted as two successive peaks (44 and 47 min), P1F MIF eluted as a single peak at 41 min, suggesting a trimeric configuration, and P1S MIF eluted as a single peak at 44 min.

Circular Dichroism Spectroscopy. Circular dichroism of mouse MIF, P1S MIF, and P1F MIF were measured in 20 mM Tris buffer (pH 7.4) containing 20 mM NaCl at a concentration of approximately 10 μM with a 1.0 mm optical path length. CD spectra were recorded on a Jasco J600 spectropolarimeter.

Mass Spectrometry. The masses of purified P1S MIF and P1F MIF proteins were determined by electrospray ionization mass spectrometry (ESI-MS) on a LCQ Finnigan octapole electrospray mass spectrometer. Samples for ESI-MS were

analyzed in a solution of 80% (v/v) acetonitrile in water made 0.05% in TFA. The samples were desalted on a Waters system using an Econosil C18 reverse-phase HPLC column (250 mm × 22 mm) in 500-μL portions (1−5 mg/mL), washing first with water/0.05% TFA for 10 min and eluting with a linear gradient (0−100% acetonitrile/0.05% aqueous TFA) over the next 45 min at a flow rate of 5 mL/min. The effluent was monitored at 214 nm and the major peaks were collected and analyzed by ESI-MS as described above. Typically, the unblocked and the methionine-blocked mutant elute as two successive peaks ∼40 min after the injection, with the unblocked mutant eluting first.

pH−Rate Profile of P1S MIF. The pH dependence of the rate of ketonization of the enol isomer of **2** by the P1S MIF mutant was determined in 50 mM sodium phosphate buffer (pH 4.8−7.8) at 24 °C. The rapid nonenzymatic rate of ketonization of the enol isomer above pH 7.8 or in other buffers precluded the accurate measurement of the enzymatic rate of ketonization. MIF was removed from the storage buffer, diluted into the buffer under study, and allowed to incubate for 15 min. The final concentration of MIF in the cuvette was either 12 μM (pH 4.8− 5.5) or 8 μM (pH 5.9− 7.8). The reactions were initiated by the addition of a quantity of the enol isomer of **2** (final concentration 10−150 μM) from a stock solution made up in ethanol. No significant inhibition of the enzymatic activity of MIF by ethanol is observed at concentrations ≤0.5% (v/v). The pH dependence of the kinetic parameters was fitted and analyzed as described previously (*12*).

Cell Preparation. Human peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood of healthy donors. Cells were separated in Lymphocyte Separation Medium (ICN Biomedicals, Inc, Aurora, OH) in conical centrifuge tubes with a Beckman benchtop centrifuge (≅ 500*g*, 25 °C, 30 min, no brake). Peripheral blood monocytes were isolated from leukopaks or buffy coats obtained from the New York Blood Center (New York) and purified by Ficoll-Hypaque centrifugation followed by rosetting with neuraminidase-treated sheep erythrocytes, as described (*20*). This procedure routinely yields cells that are 70−90% monocytes and 10−30% B lymphocytes, as determined by fluorescence-activated cell sorter (FACS) analysis. Recovered PBMC or monocytes were washed 3× with Hanks' balanced salt solution, without cations ($HBSS^-$, Gibco−BRL) and fluorometrically labeled by suspending at 5×10^6/mL in 2 μM calcein AM (Molecular Probes, Inc., Eugene, OR) in $HBSS^-$ + 0.1 mg/mL bovine serum albumin (BSA; Calbiochem, La Jolla, CA). The tube was shielded from ambient light and incubated for 30 min in a 37 °C dry heat oven. Following incubation, labeled cells were washed 2× with $HBSS^-$ + 0.1 mg/mL BSA and suspended in RPMI 1640 with 0.1 mg/mL human serum albumin (HSA, Armor Pharmaceutical Company, Kankakee, IL) or BSA at a final concentration of 2.5 to 5×10^6/mL.

Migration Assay. Monocyte migration inhibitory activities of recombinant murine and human wild-type and murine mutant MIF were analyzed by use of human peripheral blood mononuclear cells or T-cell depleted mononuclear cells in a modified Boyden chamber format. Calcein AM-labeled monocytes were suspended at 2.5 to 5×10^6/mL in RPMI 1640 medium, with L-glutamine (without phenol red) and 0.1 mg/mL human serum albumin or bovine serum albumin. An aliquot (200 μL) of cell suspension was added to wells of a U-bottom 96-well culture plate (Costar, Cambridge, MA) prewarmed to 37 °C. MIF in RPMI 1640 was added to the cell suspension to yield final concentrations of 1, 10, 100, and 1000 ng/mL. The culture plate was placed into the chamber of a temperature-controlled plate reader, mixed for 30 s, and incubated at 37 °C for 10−20 min. During the incubation, 28 μL of prewarmed human monocyte chemotactic protein 1 (MCP-1; Pepro Tech, Inc., Rocky Hill, NJ) at 10 or 25 ng/mL or RPMI 1640 with 0.1 mg/mL HSA was added to the bottom well of a ChemoTX plate (Neuro Probe Inc., Gaithersburg, MD; 3 mm well diameter, 5 μM filter pore size). The filter plate was carefully added to the base plate. Treated cell suspensions were removed from the incubator and 30 μL was added to each well of the filter plate. The assembled plate was incubated for 90 min at 37 °C in a humidified chamber with 5% CO_2. Following incubation, the cell suspension was aspirated from the surface of the filter and the filter was subsequently removed from the base plate and washed three times by adding 50 μL of 1× $HBSS^-$ to each filter segment. Between washes, a squeegee (NeuroProbe) was used to remove residual $HBSS^-$. The filter was air-dried and then read directly in the fluorescent plate reader, with excitation at 485 nm and emission at 535 nm. Chemotactic or random migration indeces are defined as average filter-bound fluorescence for a given set of wells divided by average fluorescence of filters in wells containing neither MCP-1 nor MIF. Titration of fluorescently labeled cells revealed that levels of fluorescence detected in this assay have a linear relationship to cell number (not shown).

CCR2 Binding Assay. Human PBMCs were isolated from human heparinized venous blood in lymphocyte separation medium and washed in $HBSS^-$. The cells were resuspended in binding buffer (50 mM Hepes, pH 7.2, 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.25% BSA) at 5×10^6 cells/mL. The cells were incubated with 1 μg/mL MIF for 30 min at 37 °C prior to addition to the plate. The binding assay was set up in a deep-well dish in a total volume of 150 μL/well consisting of 100 μL of the cell suspension and ∼25 000 cpm of ^{125}I-MCP-1 in the presence or absence of MIF or 100 nM nonlabeled MCP-1. The plate was incubated at room temperature for 1 h to allow binding. The cells were then filtered through a 0.45 μM filter plate via the multiscreen filtration system and washed twice with 25 mM Hepes buffer containing 500 mM NaCl. The filter plate was air-dried, 50 μL Microscint fluid was added per well, and the sealed plate was counted in a Packard Top count.

Statistical Analysis. In each case experimental data are described as the mean ± standard deviation of three or more replicate wells. Data were compared by a two-sample Student's *t*-test assuming equal variances, in the data analysis algorithm of Microsoft Excel software, with significance set at $P < 0.05$ and a hypothesized mean difference of zero relative to control fluorescence.

RESULTS

MIF Inhibits Monocyte Chemotaxis and Random Migration. We tested MIF for its ability to inhibit directed migration of monocytes stimulated by a gradient of monocyte chemotactic protein 1 (MCP-1) in a modified Boyden chamber assay. In the presence of MIF (1−100 ng/mL,

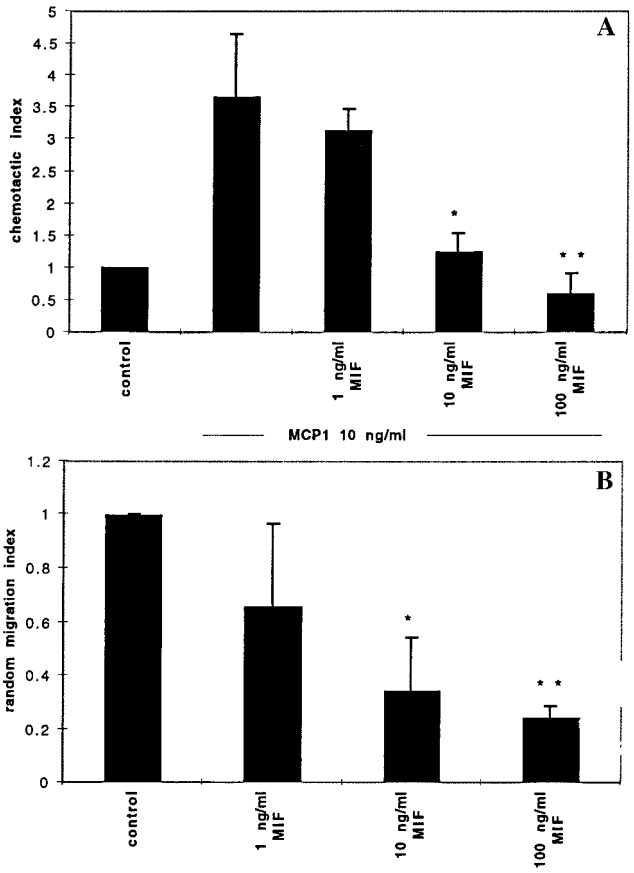


FIGURE 1: MIF inhibits chemotaxis of human peripheral blood monocytes to MCP-1. (A) Effect of wild-type MIF on monocyte chemotaxis to MCP-1. (B) Effect of wild-type MIF on random migration of monocytes. Human peripheral blood monocytes were isolated and fluorescently labeled with calcein AM. Cells [(2−5) $\times$ 10^6 /mL] were incubated with or without human recombinant MIF (1−100 ng/mL) for 10 min at 37 °C and pipetted onto 5 μm pore filters resting on a solution of MCP-1 (10 ng/mL) for chemotaxis, or buffer, for random migration. Cells were incubated for 90 min at 37 °C before filters were removed, and filter-bound fluorescence was quantified by use of a fluorescent plate reader. Chemotactic or random migration indices are defined as filter-associated fluorescence relative to control wells lacking MIF and MCP-1. This experiment is representative of five repeats. Single asterisk indicates $P < 0.05$, double asterisk indicates $P < 0.01$ compared with positive control.

Figure 1A), there was a dose-dependent inhibition of the chemotactic response to MCP-1. This effect occurred when either human T-cell depleted monocytes or human peripheral blood mononuclear cells were examined and could be inhibited either with human or mouse recombinant MIF (data not shown). Since lipopolysaccharide (LPS) is capable of inhibiting monocyte chemotaxis at high concentrations (>0.5 ng/mL, data not shown), we tested recombinant human and murine MIF preparations as well as MIF P1 mutants for LPS levels by the *Limulus* amebocyte lysate assay. LPS levels in all of the wild-type and mutant MIF preparations used are far below levels required to inhibit chemotaxis (<0.05 pg/mL LPS per ng/mL MIF). Moreover, polymyxin B (10 μg/mL), a polycationic molecule known to bind and inactivate LPS, failed to prevent migration inhibition by MIF (data not shown). Therefore, it is clear that MIF's chemotaxis inhibitory properties cannot be attributed to contaminating LPS.

Leukocyte migration has classically been separated into three categories: random migration, which is cellular locomotion occurring in the absence of any known stimulus; chemotaxis, or directed movement under the influence of a chemoattractant gradient; and chemokinesis, or increased random movement of a cell under the influence of an activating substance that is not present in a gradient (*21*). Previously published work on MIF has shown inhibition of random movement of monocytes (*1*−*3*, *18*). In our hands, human recombinant MIF was also capable of inhibiting random migration of monocytes when coincubated with monocytes in the upper chamber of the modified Boyden chamber assay (Figure 1B).

Production, Expression, and Characterization of MIF Mutants. To assess whether enzymatic activity is required for chemotaxis inhibitory activity, two P1 MIF mutants were constructed. Pro-1 is a critical residue in the enzymatic reaction (*12*, *13*). The P1S and P1F mutants of murine MIF were constructed by overlap extension PCR, expressed in *E. coli* strain BL21(DE3)pLysS, and purified to homogeneity (as judged by SDS−PAGE) by a modification of previously described procedures (*12*, *14*, *22*). The yield of each mutant protein was comparable to that typically obtained for the wild-type murine MIF. The CD spectra of the two mutants were indistinguishable from that obtained for wild-type MIF, suggesting that no gross conformational changes had occurred. Mass spectral analysis of the predominant HPLC peak obtained for the P1S MIF mutant generated one signal corresponding to a molecular weight of 12 363, which is the expected weight of the 114-amino acid species beginning with a serine. Thus, the N-terminal methionine was post-translationally cleaved in *E. coli*. Mass spectral analysis of the predominant HPLC peak obtained for the P1F MIF mutant generated one signal corresponding to a molecular weight of 12 553, which is the expected weight of a 115-amino acid species beginning with a methionine. Hence, the N-terminal methionine for this mutant was not posttranslationally cleaved in *E. coli*. There was no mass spectral evidence for the presence of a formyl group, indicating that the methionine on the P1F MIF mutant was posttranslationally deformylated.

The kinetic properties of the enzymatic activity of MIF and the two P1 mutants were measured by monitoring the ketonization of the enol isomer of β-phenylpyruvate. A comparison of the kinetic parameters measured for the enzymatic activity of the P1S MIF mutant ($K_m = 440$ μM; $k_{cat} = 5$ s^{-1}; $k_{cat}/K_m = 1.1 \times 10^4$ M^{-1} s^{-1}) to those measured for the wild-type MIF ($K_m = 220$ μM; $k_{cat} = 215$ s^{-1}; $k_{cat}/K_m = 9.8 \times 10^5$ M^{-1} s^{-1}) showed a 2-fold increase in K_m and a 43-fold decrease in k_{cat}. The value of k_{cat}/K_m decreases 89-fold. In contrast, the P1F MIF mutant had no detectable enzymatic activity at a concentration of 18 μM. At higher concentrations (up to 140 μM), the mutant still did not exhibit detectable enzymatic activity, but there was considerable interference with the assay due to the high concentration of protein.

The pK_a of serine is 1.4 units less than that of proline (*23*). This difference suggests that serine in the P1S mutant would be a weaker base than proline in the wild-type MIF. To determine how much this lower basicity contributes to the decrease in the reaction rate, the pH dependence of the kinetic parameters for the P1S MIF was measured. The pH−rate profile for k_{cat}/K_m vs pH shows an ascending limb with a slope of 1 (Figure 2). A nonlinear least-squares fit of the

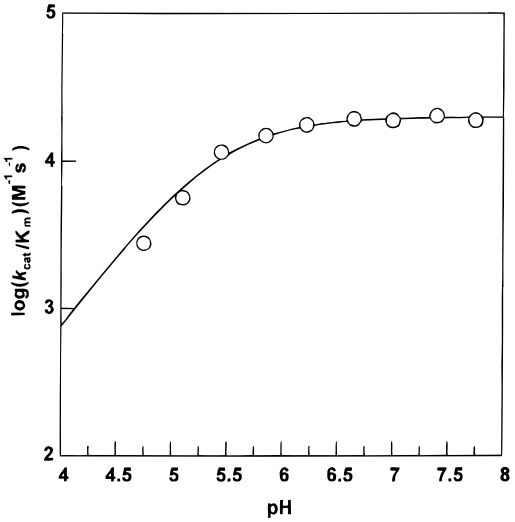


FIGURE 2: pH dependence of log (k_{cat}/K_m) for the P1S mutant MIF. The pH dependence of log (k_{cat}/K_m) is shown for the P1S mutant MIF. The curve is generated from a nonlinear least-squares fit of the data to eq 1 as described in the text. The pK_a value is reported in the text.

pH dependence of k_{cat}/K_m to

$$k_{cat}/K_m = \frac{(k_{cat}/K_m)^{max}}{1 + [H^+]/K_{HE}} \quad (1)$$

where K_{HE} corresponds to the ionization constant for the free enzyme gives a pK_a value of 5.4 ± 0.1. The plot of k_{cat} vs pH also shows an ascending limb with a slope of 1 (data not shown). A nonlinear least-squares fit of the pH dependence of k_{cat} to

$$k_{cat} = \frac{(k_{cat})^{max}}{1 + [H^+]/K_{HES}} \quad (2)$$

where K_{HES} corresponds to the ionization constant for the enzyme−substrate complex gives a pK_a value of 5.5 ± 0.1. The pH dependence of the enzymatic activity of P1S MIF could not be accurately determined at pH values greater than 7.8 because of the rapid nonenzymatic ketonization of **2**.

MIF Mutants Retain the Ability To Inhibit Monocyte Chemotaxis and Random Migration. The P1F MIF mutant effectively inhibited monocyte chemotaxis (Figure 3A) and random migration (Figure 3B) despite an absence of phenylpyruvate tautomerase activity. Similar inhibition of random monocyte migration and chemotaxis to MCP-1 occur with the P1S MIF mutant (not shown). Both of the Pro-1 MIF mutants blocked chemotaxis and random migration at comparable concentrations to wild-type MIF. Since P1F MIF is completely enzymatically inactive and the P1S MIF mutant is nearly inactive, these data suggest that migration inhibition by MIF is not dependent on phenylpyruvate tautomerase enzymatic activity. Mass spectrometric analysis showed the P1F MIF mutant was unchanged after incubation with monocytes, suggesting that the cells are unable to cleave the N-terminal methionine.

MIF Does Not Compete with MCP-1 at the Level of the CCR2 Receptor. The mechanism of MIF inhibition of chemotaxis is not known. To address the possibility that MIF acts by competing with MCP-1 for its receptor, mouse MIF

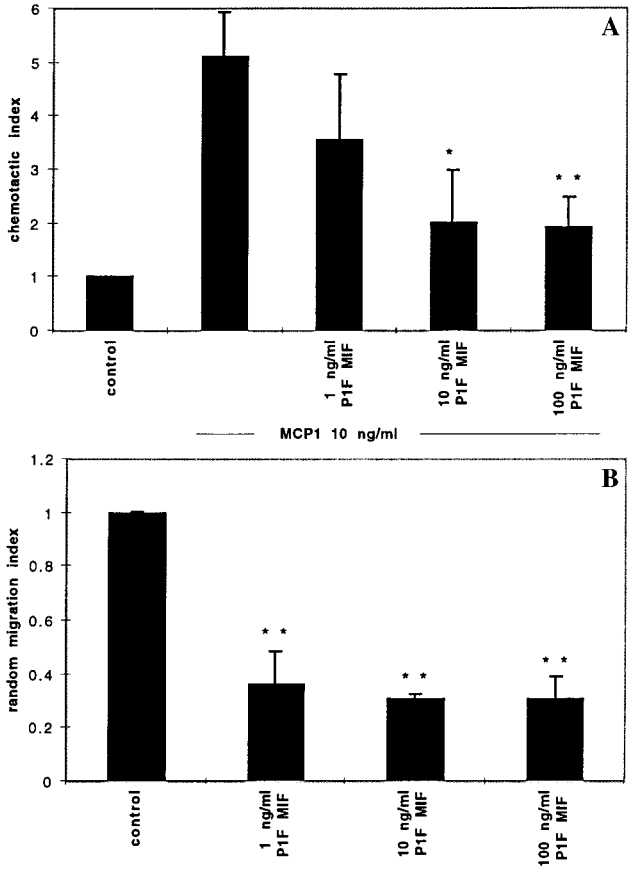


FIGURE 3: P1F MIF mutant retains monocyte migration inhibitory activity. (A) Effect of MIF mutant on monocyte chemotaxis to MCP-1. (B) Effect of MIF mutant on random migration of monocytes. Human peripheral blood monocytes were isolated and fluorescently labeled with calcein AM. Cells [(2−5) × 10^6/mL] were incubated with or without mutant P1F MIF (1−100 ng/mL) for 10 min at 37 °C and pipetted onto 5 μm pore filters resting on a solution of MCP-1 (10 or 25 ng/mL). Cells were incubated for 90 min at 37 °C before filters were removed, and filter-bound fluorescence was quantified by use of a fluorescent plate reader. Chemotactic or random migration indices are defined as filter-associated fluorescence relative to control wells lacking MIF and MCP-1. This experiment is representative of five repeats, and is also representative of experiments with the P1S MIF mutant. Single asterisk indicates $P < 0.05$, double asterisk indicates $P < 0.01$ compared with positive control.

was added to an assay measuring interaction of MCP-1 with CCR2. Addition of MIF did not alter the interaction of MCP-1 with its receptor, CCR2, as measured by binding of ^{125}I-MCP-1 binding to monocytes (Figure 4). Nonradioactive MCP-1 competed 100% of binding in this assay. This result shows that MIF does not inhibit chemotaxis by competing with MCP-1 for binding to CCR2. This experiment does not rule out the possibility that MIF interacts with CCR2 at a distinct site from MCP-1.

DISCUSSION

In recent years, multiple biologic roles have been assigned to MIF in addition to its initially discovered role as a regulator of the random movement of monocytes and macrophages. A growing body of evidence shows that MIF plays a role in the systemic stress response mediated by glucocorticoid hormones via the hypothalamic−pituitary−adrenal axis (reviewed in ref *24*). While MIF is a cytokine formed by macrophages and T cells, it has also been found

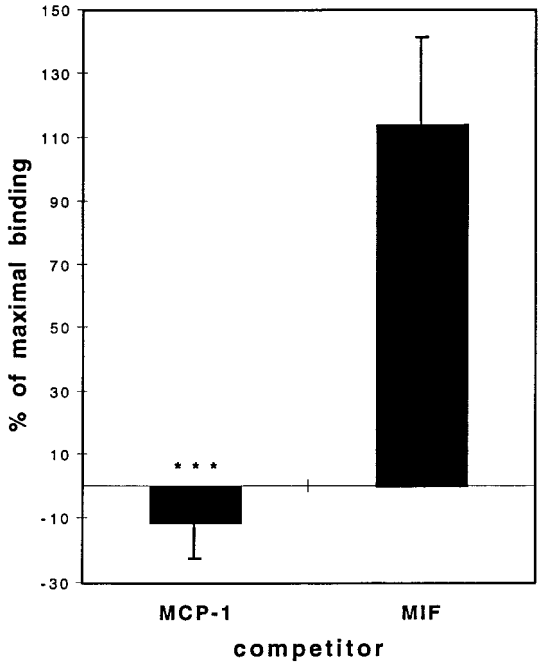
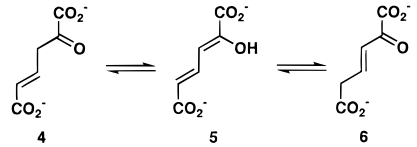


FIGURE 4: MIF does not compete with MCP-1 at the level of the CCR2 receptor. Peripheral blood monocytes were incubated with ^{125}I-MCP-1 in the presence or absence of competing unlabeled MCP-1 (100 nM) or MIF (1 μg/mL) for 60 min, filtered, and analyzed for cell-associated counts. Binding of ^{125}I-MCP-1 in the absence of competing protein is defined as 100%. This experiment is an average of two repeats. Triple asterisk indicates $P < 0.001$ compared with maximal binding.

to be an abundant preformed constituent of the anterior pituitary gland (*25*, *26*). Following induction of endotoxic shock in mice, MIF protein levels in the pituitary fall while circulating levels show a concomitant rise, as do pituitary mRNA levels (*26*). Thus, increased production and release of MIF from the pituitary is part of the response to the stress of an infectious challenge. MIF has been shown to override the suppressive effects of the synthetic glucocorticoid dexamethasone on monocyte and T-cell cytokine production and T-cell proliferation (*27*, *28*). Since glucocorticoids and MIF are both released during the stress response, and MIF is capable of antagonizing the effects of glucocorticoids, these studies suggest MIF may be an endogenous counterregulator of glucocorticoid stress hormones.

Studies with animals suggest a role for MIF in the inflammatory response. Antagonism of MIF by neutralizing antibodies has been shown to decrease symptoms in mouse models of Gram-positive and Gram-negative sepsis (*26*, *29*), delayed-type hypersensitivity (*30*), and collagen-induced arthritis (*31*) and anti-glomerular basement membrane glomerulonephritis in rats (*32*). Moreover, neutralizing anti-MIF antibodies lowered the amount of TNFα and IL-8 secreted by alveolar cells from patients with adult respiratory distress syndrome, a condition of excessive acute pulmonary inflammatory injury (*33*). MIF has been shown to exacerbate the outcome of sepsis, increasing lethality in mice injected with LPS (*26*). A recently developed MIF knockout mouse shows enhanced resistance to *P. aeruginosa* and resistance to endotoxin-induced lethal shock relative to wild-type controls (*34*). In addition, MIF has been shown to modestly prime human neutrophils for superoxide production (*13*). While these data implicate MIF as a proinflammatory mediator, in at least one location MIF appears to play an antiinflammatory role. MIF in the aqueous humor of the anterior chamber of the eye suppresses natural killer-cell-mediated lysis of corneal epithelial cells, thus contributing to immune privilege in the eye (*35*).

Scheme 2



We have identified a new biologic activity for MIF: as an inhibitor of monocyte chemotaxis. This property is closely related to the original biologic function attributed to MIF, codified in its name, as an inhibitor of the random migration of monocytes and macrophages. We find the migration inhibitory property extends to directed cell movement elicited by a chemotactic gradient of MCP-1. The inhibition of chemotaxis is nearly complete at concentrations comparable to those needed for inhibition of random migration.

It is not known how MIF inhibits chemotaxis of monocytes. MIF did not compete with MCP-1 for the CCR2 receptor, so its inhibitory effect on chemotaxis must occur by some other mechanism. While it is possible that MIF binds to and alters CCR2 function at some distinct binding site, preliminary results indicate MIF does not alter the CCR2-mediated intracellular calcium flux in response to MCP-1. Since MIF also blocks random monocyte migration, its effect may be on some universal aspect of cellular movement rather than on receptors for chemotactic agents.

Recent observations that MIF has enzymatic activity raise the question of whether any of the biological roles attributed to MIF are mediated through this enzymatic reaction (*10*−*14*). Thus far, this question has been investigated by examining the biological activity of P1 MIF mutants that are reportedly devoid of enzymatic activity. One study concluded that the glucocorticoid counterregulatory activity of MIF could be separated from its enzymatic activity on the basis of experiments conducted with a P1S MIF mutant (*11*). Another study reported that the low level of neutrophil priming activity observed for MIF is significantly lower when Pro-1 is replaced with a glycine (P1G MIF) (*13*). Neither mutant had detectable D-dopachrome tautomerase activity and it was further reported that the P1G mutant did not catalyze the enolization of (*p*-hydroxyphenyl)pyruvate (*11*, *13*).

The observation that the P1S and P1G mutants of MIF lacked enzymatic activity was intriguing because it contrasts with the results of studies on Pro-1 mutants of 4-oxalocrotonate tautomerase (4-OT), a structurally homologous protein that also utilizes Pro-1 as its catalytic general base (*15*). 4-OT, a bacterial isomerase, catalyzes the conversion of 2-oxo-4-hexenedioate (**4**, Scheme 2) to its conjugated isomer, 2-oxo-3-hexenedioate (**6**, Scheme 2) (*11*) through the dienol intermediate 2-hydroxy-2,4-hexadienedioate (**5**, Scheme 2) (*16*). Both the P1G and P1S 4-OT mutants have comparable enzymatic activities, which are less than that of the wild type. The k_{cat} values are ~77-fold less than those determined for the wild type, and the k_{cat}/K_m values are 20-fold less than those determined for the wild type (*16*).[2]

In view of the similarities between 4-OT and MIF, it was not clear why the P1S and P1G mutants of 4-OT would have

some enzymatic activity and the same mutants of MIF would have no enzymatic activity. A closer examination of the assays used to measure the enzymatic activity of two P1 MIF mutants indicated that a rigorous kinetic analysis had not been carried out on either of these two mutants (*11*, *13*). Moreover, the activity of P1S MIF was measured by the dopachrome assay, which does not appear to be highly quantitative as the activity is reported as either present or absent (*11*). These observations raise further questions about whether the P1S mutant retains some enzymatic activity and whether the residual activity is responsible for the observed biological activity.

To address these issues and further examine the link between the enzymatic activity of MIF and its biological properties, two amino-terminal mouse MIF mutants (P1S and P1F) were constructed and tested for phenylpyruvate tautomerase activity. We find that the P1S MIF mutant is enzymatically active, although certainly not as active as wild-type MIF, whereas the P1F MIF mutant has no enzymatic activity.

The P1S MIF mutant was isolated primarily with an unblocked amino terminus, while the P1F MIF mutant was isolated with a methionine-blocked amino terminus. These observations are consistent with previous studies concerning the posttranslational processing of the initiating *N*-formylmethionine. The formyl and the methionine groups are removed in two successive enzyme-catalyzed reactions (*36*). After the deformylation reaction, the methionine is removed by methionine aminopeptidase (*37*). There is a correlation between the removal of the methionine and the amino acid in the penultimate position (*36*). If Ala, Gly, Pro, Ser, Thr, or Val is in the penultimate position, then it is likely that the initiating methionine will be removed. If Phe occupies this position, then the methionine is not likely to be removed (*37*).

The kinetic properties of the P1S MIF mutant show that the predominant effect of the replacement of the rigid secondary amine proline with the more flexible primary amine, serine, is on k_{cat}, and, in turn, the value of k_{cat}/K_m. The observed decrease may result from a structural perturbation, the slower release of product (as compared to wild type), the lower basicity of the primary amine as compared to the secondary amine, the increased flexibility of serine as compared to proline, or a combination of these factors. A structural perturbation cannot be ruled out in the absence of an in-depth structural analysis. However, on the basis of CD and gel filtration, it appears that the gross structural integrity of P1S MIF is intact and comparable to that of wild type. A slower release of product for the mutant also can not be ruled out in the absence of an extensive kinetic analysis. The lower basicity of the primary amine in the P1S MIF mutant, as compared to the secondary amine of Pro-1 of wild type, is indicated in the pH−rate profile by the lower pK_a value of the general base (5.4 for P1S vs 6.0 for the wild type) (*12*). The 0.6 unit difference would predict a 4-fold difference in the rate of catalysis (*15*). The additional 11-fold decrease in catalysis may be due to the increased flexibility of Ser-1 (compared to Pro-1) resulting in the suboptimal positioning of the general base, as well as the effects of any minor structural perturbations combined with the slower release of product (*15*).

The methionine-blocked P1F MIF mutant has no detectable activity although it has an N-terminal group that could act as a general base. Because a major structural perturbation is not indicated by CD or gel-filtration analysis, two factors may explain why this amino group does not act as a general base. The presence of the extra amino acid may push the amino-terminal group of methionine out of the hydrophobic pocket so that it does not have a significantly low pK_a to act as a general base. Alternatively, the large phenyl side chain of the mutant may limit the mobility of the amino-terminal group or completely block the active site (*14*).

Although serine or phenylalanine substitution of the catalytic N-terminal proline renders MIF enzymatically inactive (P1F MIF) or nearly so (P1S MIF), the two P1 mutants exhibited comparable abilities to inhibit monocyte chemotaxis and random migration to that of enzymatically intact wild-type MIF. Therefore, the physiological relevance of the MIF enzymatic activity remains to be elucidated. Another immunoregulatory mediator with both biologic and enzymatic activity is cyclophilin, which is the target for the immunosuppressant drug cyclosporin. In addition to its biologic role as an immunosuppressant, it can function as a peptidyl-prolyl *cis*−*trans* isomerase. However, a cyclosporin analogue, MeAla6-CsA, inhibits cyclophilin's enzymatic activity but has no immunosuppressive effects. Therefore, like MIF, a connection has not been established between cyclophilin's biologic and enzymatic activities (*38*).

While a MIF receptor has been postulated (*4*), no such receptor has been definitively characterized. One interesting experiment will be to test the two microbial enzymes (4-oxalocrotonate tautomerase and 5-carboxymethyl-2-hydroxymuconate isomerase) that share three-dimensional structure but little sequence identity with MIF for their ability to inhibit monocyte migration. Such an analysis will indicate whether migration inhibition activity is related to three-dimensional structure or to specific sequence.

ACKNOWLEDGMENT

We are grateful to Dr. J. Kozarich for helpful discussion, to Dr. R. Bucala for providing us with the clone containing recombinant mouse MIF, to Dr. Barbara Leiting, Kelly Pryor, and Frank Marsilio for purifying recombinant human MIF protein, and to the University of Texas (Austin) analytical instrumentation service core supported by Center Grant ES 07784. Thanks to Drs. R. Vosatka and P. Detmers for critical reading of the manuscript.

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BI991352P