Rapid purification and characterization of L-dopachrome-methyl ester tautomerase (macrophage-migration-inhibitory factor) from *Trichinella spiralis*, *Trichuris muris* and *Brugia pahangi*

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Macrophage-migration-inhibition factor (MIF) is an essential stimulator of mammalian T-lymphocyte-dependent adaptive immunity, hence MIF orthologues might be expressed by infectious organisms as an immunosubversive stratagem. Since MIF actively catalyses the tautomerization of the methyl ester of L-dopachrome (using dopachrome tautomerase), the occurrence of MIF orthologues in several parasitic helminths was investigated by assaying and characterizing such activity. Evidence of MIF orthologues (dopachrome tautomerase) was found in the soluble fraction of the nematodes *Trichinella spiralis* (stage 4 larvae) and *Trichuris muris* (adults), and the filarial nematode *Brugia pahangi* (adults). The MIF orthologues of *Tr. muris* (TmMIF) and *B. pahangi* (BpMIF) were purified to homogeneity using phenyl-agarose chromatography, that of *T. spiralis* (TsMIF) required a further step: cation-exchange FPLC. Retention time on reverse-phase HPLC and M_r on SDS/PAGE of the nematode MIFs were similar to those of human MIF. N-terminal sequences (19 residues) of TsMIF and TmMIF showed 47 and 36% identity, respectively, with human MIF. The N-terminal sequence of BpMIF (14 residues) was identical to that of an MIF orthologue in the genome of *B. malayi* (Swiss-Prot, P91850) and showed 43% identity to either human or TsMIF. TsMIF had 10-fold higher dopachrome tautomerase activity than MIF from the other sources. The enzyme activities of TsMIF, BpMIF and TmMIF were less sensitive to inhibition by haematin (I_{50}: $>15\,\mu$M, $>15\,\mu$M and $2.6\,\mu$M, respectively) than that of human MIF (I_{50} $0.2\,\mu$M). Significant dopachrome tautomerase or phenyl-agarose-purifiable MIF-like protein was not detected in the soluble fraction of the nematodes *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis*, the cestode *Hymenolepis diminuta*, or the trematodes *Schistosoma mansoni*, *S. japonicum* and *S. haematobium*, or the free-living nematode, *Caenorhabditis elegans*, which does contain an MIF-related gene.

INTRODUCTION

Macrophage-migration-inhibition factor (MIF) acts early in adaptive immunity to overcome glucocorticoid inhibition of T-lymphocyte activation by increasing expression of interleukins-1β, -6 and -8 and tumour necrosis factor-α by macrophages [1], and is an essential regulator of T-cell activation [2]. Although it was the first cytokine discovered [3] and the crystal structures of both rat and human MIF have been determined [4,5], its mode of action is largely unknown. The N-terminal proline residue is associated with catalysis of the tautomerization of D-dopachrome, D,L-dopachrome-methyl ester [6,7] or *p*-hydroxyphenylpyruvate [8], activities apparently unrelated to its effects on T-cell activation/macrophage migration [7]. Enzyme activity is sensitive to inhibition by free fatty acids in the 10 μM range [7] and haematin at submicromolar levels [9]. MIF may be partially purified with high efficiency using retention by phenyl-agarose at relatively low ionic strength [6].

In view of the importance of MIF in initiating adaptive immune responses, it was considered to be a likely candidate molecule to be expressed as an immunosubversive orthologue by pathogenic and parasitic organisms, and a putative example has been noted from genome analysis of the filarial nematode, *Brugia malayi* (Swiss-Prot, P91850). Therefore, we examined soluble extracts of several parasitic helminths for L-dopachrome-methyl ester (dopachrome) tautomerase activity, finding activity in three of nine species. The enzymes were purified, characterized and their N-terminal sequence determined, confirming their relationship with mammalian MIF.

EXPERIMENTAL

Organisms

Adult *B. pahangi* were harvested from the peritoneal cavity of gerbils (*Meriones unguiculatus*) infected at least 3 months previously. Worms were washed in Hank's saline at 37 °C. *Caenorhabditis elegans* (mixed stages) were cultured on standard NGM agarose plated with *Escherichia coli* OP50 auxotrophic for uracil, harvested and washed with PBS. Adult *Heligmosomoides polygyrus* were obtained from male CFLP mice infected with 400 third-stage larvae after 15 days. Worms were washed 10 times in Hank's saline and once in PBS. *Hymenolepis diminuta* were prepared as follows. Ten cystocercoids obtained from infected *Tenebrio molitor* were used to infect five Wistar rats by gavage. After 6 weeks, worms were removed from the small intestine and washed in PBS. Adult *Nippostrongylus brasiliensis* were obtained from the intestine of 6 week old Wistar rats infected 10 days previously with 2000 third-stage larvae and washed in PBS. Adult *Schistosoma haematobium* worms of a Kenyan strain were recovered from hamsters, and both adult *S. japonicum* of a

Abbreviations used: MIF, macrophage-migration-inhibitory factor; BpMIF, TmMIF and TsMIF, MIF orthologues from *Brugia pahangi*, *Trichuris muris* and *Trichinella spiralis* respectively; dopachrome, L-dopachrome-methyl ester.

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Chinese strain and adult *S. mansoni* worms of a Puerto Rican strain were recovered from TO mice. Recovery was by perfusion of the mesenteric system with citrate-saline, which was also used to extensively wash the worms by repeated sedimentation [10]. *S. mansoni* egg antigen was provided by the World Health Organization (UNDP/TDR). Adult *Trichuris muris* were obtained from AKR mice 42 days after infection with the E/N (the Edinburgh strain of *Tr. muris*) isolate. Worms were removed from the large intestine with fine forceps and thoroughly washed in RPMI 1640. *Trichinella spiralis* infective larvae were obtained by pepsin digestion of minced skeletal muscle of mice infected at least 1 month earlier [11]. Larvae were washed thoroughly in serum-free RPMI 1640. All worm/larval samples were snap-frozen immediately after preparation and stored at −70 °C until further examination.

Testing for occurrence of dopachrome tautomerase (MIF) in helminths

Buffers used: A, 2 mM EDTA/0.15 M NaCl/0.1 mM dithiothreitol/25 μM PMSF/1 μM leupeptin/1 μM pepstatin A/20 mM Na^+-phosphate (pH 7.0); B, buffer A without the protease inhibitors; C, 40 % (v/v) ethylene glycol/1 mM EDTA/20 mM K^+-phosphate (pH 6.8); and D, 30 % (v/v) ethylene glycol/50 mM Na^+-acetate (pH 5.5). Unless otherwise specified, manipulations were carried out at 0–4 °C.

Soluble extracts were collected as follows. Adult *B. pahangi* (0.2 g), *H. polygyrus* (1.2 g), *Hy. diminuta* (1.8 g), *N. brasiliensis* (0.8 g), *S. haematobium* (0.25 g), *S. japonicum* (0.35 g), *S. mansoni* (0.3 g), *Tr. muris* (0.77 g) and mixed stages of *C. elegans* (0.3 g) were homogenized in approximately 6 vols. of buffer A by hand using a 2 ml Dounce homogenizer and ultracentrifuged (100 000 ***g*** for 1.5 h). The bulk of GSH transferases were subsequently removed from these extracts, except that from *C. elegans*, by passage through a 2 ml column of S-linked GSH–agarose equilibrated in buffer A. They were then snap-frozen and stored at −20 °C until required. *T. spiralis* larvae (0.8 g wet weight) were partially thawed in 2 vols. of buffer A and powdered using a pestle and mortar cooled with liquid nitrogen. After further dilution with buffer A (5 vols.), the soluble fraction was obtained by ultracentrifugation as above. GSH transferase removal was not required. *S. mansoni* egg antigen (10 mg) was dissolved in 1 ml of buffer A.

MIF orthologues (if detected by dopachrome tautomerase activity) were then purified using a column of phenyl-agarose (1 × 5 cm) equilibrated in buffer A. The column was washed with 1 column vol. of buffer A followed by 5 vols. of buffer B. Bound protein was then eluted in 0.5 ml fractions with buffer C. Contaminants remaining tightly bound were removed by washing at room temperature with 50 % (v/v) ethylene glycol, followed by 6 M guanidinium HCl before re-equilibration with buffer A. Extracts apparently lacking MIF dopachrome tautomerase activity were also applied to phenyl-agarose under the same conditions in case similar proteins lacking dopachrome tautomerase activity could be purified. Dopachrome tautomerase activity was assayed at all stages of purification as described previously [9]. The active fractions obtained upon phenyl-agarose chromatography of the *T. spiralis* extract were transferred into buffer D and fractionated by cation-exchange FPLC at room temperature [6,9].

Protein purity was determined by reverse-phase HPLC using an Aquapore OD 300 column, 2.1 × 220 mm, flow rate 0.13 ml/min, monitoring at both 214 and 280 nm. Solvents used were: A, 0.09 % (v/v) trifluoracetic acid in water; and B, 0.06 % (v/v) trifluoroacetic acid in acetonitrile. Initial conditions were 16 % solvent B. The elution gradient comprised a linear increase of solvent B to 37 % at 10 min, then to 59 % at 50 min, followed by a wash to 66 % solvent B and re-equilibration. Purified fractions were also analysed by SDS/PAGE using 15 % (w/v) polyacrylamide. Concentration of purified proteins was estimated from A_{214} following reverse-phase HPLC using A_{214} 1.0 = 0.1 mg.ml^{-1}. Primary protein sequence was determined by automated Edman degradation of HPLC-purified samples.

RESULTS

Dopachrome tautomerase activity in helminth extracts

To investigate the presence of MIF orthologues in helminths, soluble extracts of nine species were initially examined for

Table 1 Characterization of MIF (dopachrome tautomerase) from various helminth species

MIF from *H. polygyrus*, *N. brasiliensis*, *Hy. diminuta*, *S. haematobium*, *S. japonicum*, *S. mansoni* and *C. elegans* was not detected by dopachrome tautomerase activity (i.e. < 0.2 μmol/min per g of wet weight) or by SDS/PAGE (< 0.8 μg per g of wet weight).

Organism	Yield (μg of MIF‡ per g of wet weight)	Specific activity* (μmol/min/mg)	Haematin I_{50} (μM)
H. sapiens	360	0.2	
(Liver)†	22		
B. pahangi	36	177	> 15
T. spiralis	44	3540	> 15
Tr. muris	35	277	2.6

* Dopachrome tautomerase activity of purified protein at 30 °C.
† Calculated from [9].
‡ Estmated from A_{214} of HPLC-purified protein.

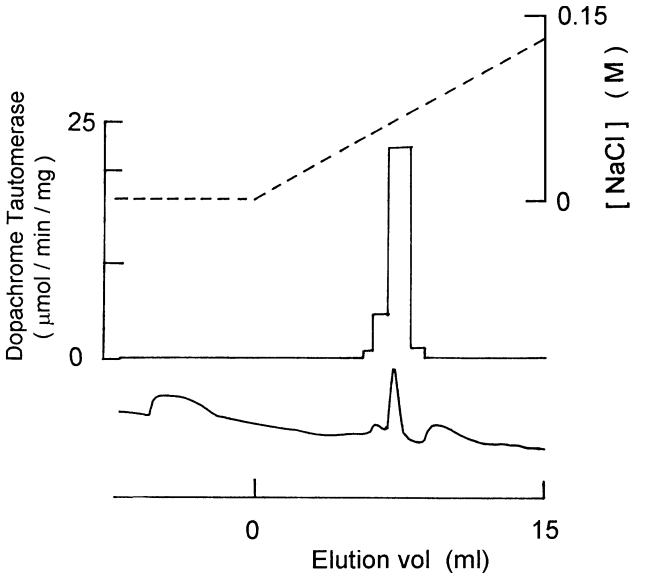

Figure 1 Purification of TsMIF

TsMIF was separated from bulk soluble proteins of *T. spiralis* by phenyl-agarose chromatography at 0–4 °C as described in the text. The partially pure eluate was then transferred into buffer D using Sephadex G-25 (PD-10), and fractionated at room temperature by cation-exchange FPLC (Mono S HR 5/5) at 0.35 ml/min. TsMIF was identified by assay of dopachrome tautomerase activity.

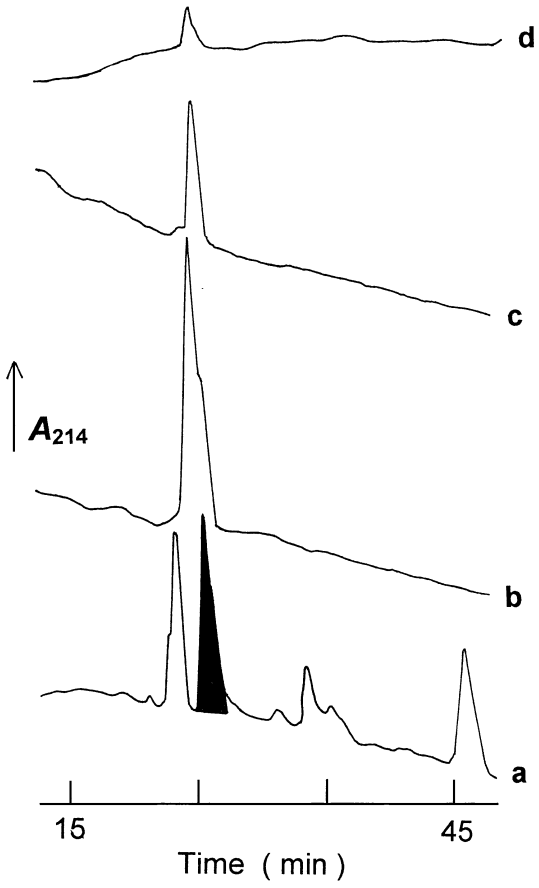

Figure 2 Analysis of nematode MIFs by reverse-phase HPLC

Dopachrome tautomerase (MIF) was purified from soluble extracts of nematodes by phenyl-agarose chromatography and samples were analysed by reverse-phase HPLC as described in the text. (**a**) *T. spiralis* (TsMIF is the shaded peak, the only peak present in the FPLC-purified material, results not shown), (**b**) *Tr. muris*, (**c**) *B. pahangi* and (**d**) human liver MIF obtained as described in [9].

dopachrome tautomerase activity. Each extract was also applied to a column of phenyl-Sepharose and the retained material eluted with phosphate-buffered ethylene glycol, which is used to purify and concentrate mammalian MIF proteins [6,9]. The results (Table 1) showed significant activity in *T. spiralis*, *Tr. muris* and *B. pahangi*, but none in the other species examined. In case an MIF orthologue that lacked dopachrome tautomerase activity had been purified by phenyl-Sepharose chromatography, the eluates were analysed by SDS/PAGE. A polypeptide of similar M_r to mammalian MIF (12 kDa) was seen in samples purified from *T. spiralis*, *Tr. muris* and *B. pahangi*, but no similar material was seen in the eluates from phenyl-agarose chromatography of extracts from the other species. Remarkably, the MIF orthologues in the eluates from *Tr. muris* and *B. pahangi* appeared to be virtually free from contaminating proteins after this single chromatographic step, whereas that from *T. spiralis* contained several minor contaminants. To purify the *T. spiralis* MIF orthologue (TsMIF), the eluate was fractionated by cation-exchange FPLC (Figure 1). Each MIF orthologue gave a single, characteristically asymmetric, peak upon reverse-phase HPLC with similar retention times (Figure 2). The yield of MIF orthologues per g of wet tissue (Table 1) was comparable with that from human liver [9].

Hs	P	M	F	I	V	N	T	N	V	P	R	A	S	V	P	D	G	F	L
Rn	P	M	F	I	V	N	T	N	V	P	R	A	S	V	P	E	G	F	L
Bm	P	Y	F	T	I	D	T	N	I	P	Q	N	S	I	S	S	A	F	L
Bp	P	Y	F	T	I	D	T	N	I	P	Q	N	S	I					
Ts	P	I	F	T	L	N	T	N	I	K	A	T	D	V	P	S	D	F	L
Tm	P	I	F	T	F	A	S	N	V	P	A	D	T	I	T	G	F	F	L
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19

Figure 3 Comparison of N-terminal sequences of nematode and mammalian MIFs

Primary sequence of MIF proteins purified from *B. pahangi* (Bp), *T. spiralis* (Ts) and *Tr. muris* (Tm) was obtained by automated Edman degradation. Sequences are compared with MIF from *Homo sapiens* (Hs; Swiss-Prot, P14174), *Rattus norvegicus* or *Mus musculus* (Rn; Swiss-Prot, P30904) and *Brugia malayi* (Bm; Swiss-Prot, P91850).

The dopachrome tautomerase specific activity of TsMIF was much higher than that of the other nematode or mammalian MIFs (Table 1). The nematode enzymes, particularly TsMIF and *B. pahangi* MIF (BpMIF), were less sensitive to inhibition by haematin than human MIF. The dopachrome tautomerase activity of murine MIF was also checked for relative sensitivity to haematin, and showed a similar sensitivity and co-operativity to that of human MIF, but the mode of inhibition was competitive with respect to dopachrome (compared with non-competitive with human MIF [9]).

Primary sequence

To confirm that the presumed nematode MIFs were indeed distinct from host MIF, purified samples were subjected to protein sequencing. Primary sequence was obtained for BpMIF to residue 14, and for TsMIF and *Tr. muris* MIF (TmMIF) to residue 19, all of which were clearly distinct from known murine (host) MIF (Figure 3). The sequence of BpMIF was identical to that of BmMIF obtained from genomic sequencing. Human MIF showed 47 % identity with TsMIF (9 out of 19 residues), 43 % identity with BpMIF (6/14) and 36 % identity with TmMIF (7/19). Relationships between these short nematode MIF sequences were not obviously closer than those between nematode and mammal; for example, 42 % identity of TsMIF and TmMIF (8/19). Residues 1, 3, 8, 18 and 19 are conserved, whereas only TsMIF lacks a proline residue at position 10 (substituted by a lysine residue).

DISCUSSION

MIF (dopachrome tautomerase) was readily detected in soluble extracts of *T. spiralis*, *Tr. muris* and *B. pahangi*, and the method could presumably be used to investigate the production of MIF by other helminth species. Initial attempts to purify TsMIF using phenyl-agarose chromatography in buffer A lacking NaCl as described for mammalian MIF [6,9] were unsuccessful. The inclusion of 0.15 M saline to increase hydrophobic interaction resulted in retention of TsMIF by the matrix. Surprisingly, this less stringent procedure was at least as effective a purification step as that used for the mammalian MIFs and, remarkably, the TmMIF and BpMIF were essentially purified to homogeneity under these conditions. Note, however, that these samples had additionally been passed through glutathione–agarose and frozen. Whether TmMIF or BpMIF are retained by phenyl-agarose in the absence of 0.15 M saline remains to be tested.

Purification of the dopachrome tautomerase activity from the three nematodes corresponded with the purification of protein of similar mobility in SDS/PAGE and retention time on reverse-phase HPLC to that of mammalian MIF. Whereas the immuno-

modulatory properties of the MIF orthologues remain to be determined, their catalytic, structural and physical properties are sufficiently similar to mammalian MIF to justify their preliminary designation as MIF orthologues. The possibility that the purified MIF orthologues arose as a result of contamination with host material is unlikely for the following reasons. *T. spiralis* larvae are prepared by extensive digestion of cyst-infested muscle with a high concentration of pepsin, followed by repeated washing. *B. pahangi* adults, which were simply washed free of host material, yielded only an MIF orthologue of identical N-terminal sequence to that present in the genome of *B. malayi*. Furthermore, it seems very unlikely that it would be possible to isolate a hitherto unknown MIF from traces of host material without also finding the known host MIF.

No dopachrome tautomerase activity was detected in extracts from the other helminth species examined, nor was any protein of similar mobility to MIF on SDS/PAGE retained by phenyl-agarose from these extracts. Therefore if an MIF orthologue occurs in these species, it must either be present at a much lower concentration, or have significantly different physical and catalytic properties to the characterized MIF proteins, such as the MIF-related microbial enzymes: 4-oxalocrotonate tautomerase, 5-carboxymethyl-2-hydroxymuconate isomerase and chorismate mutase [12,13].

A thorough analysis of MIF-related sequences in bacteria, helminths and mammals noted just 12 invariant residues, mainly occurring near the isomerase active site [14]. Mammalian MIFs and parasitic helminth MIFs also have conserved cysteinyl residues (at positions 56 and 59), which have been implicated in redox reactions [15] and as having immunological properties [16]. The MIF-like protein of the free-living helminth, *C. elegans*, lacks these cysteines [14] and, in our study, was not readily detectable by dopachrome tautomerase activity or retention by phenyl-agarose. Thus, the properties of the TsMIF, TmMIF and BpMIF are more similar to the immunomodulatory mammalian MIFs than to those of *C. elegans*.

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The occurrence of MIF orthologues in parasitic helminths is likely to be part of an immunosubversive or immunosuppressive strategy, and the simple purification described for helminth-derived MIFs will facilitate study of their possible cytokine/hormonal effects in comparison with their mammalian counterparts.

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