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Purification of guinea pig pH 3 migration inhibitory factor

(lymphokines/isoelectric focusing/hydrophobic affinity chromatography/NaDodSO$_4$/polyacrylamide gel electrophoresis/fluorography)

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ABSTRACT **Macrophage migration inhibitory factor (MIF) from the guinea pig was recently shown to reside in two discrete and separable proteins referred to as pH 3 MIF and pH 5 MIF. One subfraction of pH 3 MIF has now been purified to apparent homogeneity from supernatants of stimulated lymph node cells. To monitor purification, biosynthetically radiolabeled MIF was prepared. Sensitized lymphocytes were stimulated in the presence of [^{3}H]leucine by concanavalin A to produce radiolabeled mediators. MIF was purified ≈30,000-fold from the culture fluid by using gel filtration, sucrose density gradient electrophoresis, isoelectric focusing, and hydrophobic affinity chromatography. This procedure yielded a single ^{3}H-labeled polypeptide with an apparent M_r of 35,000 that coincides with MIF activity.**

When lymph node cells from animals exhibiting delayed type hypersensitivity are incubated with specific antigen or with certain mitogens, they elaborate a number of soluble mediators that have a wide range of biological activities (1). Among the best characterized of these is macrophage migration inhibitory factor (MIF), a mediator that inhibits the migration of macrophages out of capillary tubes (2, 3). Recently, guinea pig MIF was shown to be separable into two distinct species. One species, pH 3 MIF, has an isoelectric point of 3.0–4.5 and a M_r of 65,000 and is neuraminidase sensitive and trypsin resistant. The other species, pH 5 MIF, is trypsin sensitive and neuraminidase resistant and has an apparent M_r of 25,000–40,000 (4).

The close correlation of MIF with manifestations of cellular immunity has been well established (5, 6), and the biochemical isolation and purification of this mediator has been the subject of many studies. Some obstacles to purifying lymphocyte mediators include the heterogeneity of these substances, the presence of biochemically closely related components, and the lack of quantitative and linear assays. The very small quantities of individual mediators in lymph node cell supernatants also pose a serious problem. For example, after several purification steps, the low protein concentration in our MIF preparations could no longer be quantified or characterized by using conventional methods. We therefore incorporated [^{3}H]leucine into the products of lymph node cells (7, 8). To purify MIF, the ^{3}H-labeled supernatants were subjected to a series of chromatographic procedures. The various fractions were assayed for MIF activity and, in parallel, were analyzed for protein composition by using NaDodSO$_4$ electrophoresis and fluorography. This approach enabled us to purify to apparent homogeneity a species of active guinea pig pH 3 MIF with a pI of 3.0–3.6.

MATERIALS AND METHODS

Production of Radiolabeled Lymph Node Cell Supernatants. To produce metabolically labeled supernatants, lymph node cells from purified protein derivative sensitized male Hartley guinea pigs were prepared as described (6). These were suspended at 4.8×10^7 ml in leucine-free minimum essential medium containing penicillin at 100 units/ml (GIBCO), streptomycin at 100 μg/ml, 1% L-glutamine, and L-[4,5-^{3}H]leucine at 250 μCi/ml (1 mCi/ml; 1 Ci = 3.7×10^{10} becquerels; Amersham). Concanavalin (Con A) at 20 μg/ml was added to one-half of the cell suspension (175 ml), which was cultured at 37°C for 24 hr. The other half, the control cell suspension, was cultured without Con A. After culture, the cells were removed by centrifugation, and Con A was added to the control supernatants. The supernatants were made 1 mM in diisopropyl phosphofluoridate, concentrated to 1–2 ml by vacuum dialysis, and clarified by centrifugation (30,000 × *g* for 15 min).

Fractionation of ^{3}H-Labeled MIF. The concentrated radiolabeled MIF-containing and control supernatants were chromatographed on precalibrated Sephadex G-100 columns (2.5 × 100 cm) as described (9). From each of the two columns, the fraction corresponding to the M_r 25,000–70,000 was collected, concentrated to 1.8 ml by vacuum dialysis, and dialyzed against 0.02 M Tris·acetate, pH 8.6/5% sucrose.

The MIF-containing fraction and the control fractions from the Sephadex G-100 columns were then subjected to electrophoresis in a model 212 density gradient apparatus (Instrumentation Specialities, Lincoln, NE) to yield 10 fractions, which were concentrated to 1 ml each as described (3). Portions (60–70 μl) of each fraction were assayed for MIF activity. The fractions having MIF activity (fractions 4 and 5) contained trichloroacetic acid-precipitable material at 35,000–45,000 cpm/μl. These fractions were combined, concentrated, and subjected to isoelectric focusing in a sucrose density gradient, as were the corresponding fractions from the control culture (4). Eleven fractions, 40 ml each, were collected with a pH range from 2.0 to 6.5. The amount of ^{3}H-labeled protein varied between that in fraction 3 ≈1000 cpm/μl, which was further purified, and that in fraction 8 (14,000 cpm/μl). All fractions were dialyzed against 20 mM Tris·HCl, pH 8.0/20 mM NaCl/5.3 mM $MgCl_2$/0.3 mM EDTA/0.1 mM dithiothreitol (Tris/saline/Mg buffer) by using dialysis tubing with a cutoff of M_r 12,000 (Fisher). The fractions were concentrated to 4.0 ml. A sample (0.2–0.4 ml) of each fraction was assayed for MIF activity and 0.2-ml samples were analyzed on NaDodSO$_4$ gels. Alternatively 3.4 ml of fraction 3 was subjected to gel filtration on Sephadex G-75 or chromatography on phenyl-Sepharose.

Fraction 3 from the isoelectric focusing was filtered over Sephadex G-75 (1.5 × 300 cm; particle size 40–120 μm, Pharmacia). The column was equilibrated with Tris/saline/Mg buffer containing bovine serum albumin at 0.06 mg/ml and precalibrated by using 1.75 ml of guinea pig serum and 5 mg of ovalbumin (Pharmacia, Uppsala, Sweden). Nine 13-ml fractions were collected; each was concentrated to 2.3 ml. From each

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Abbreviations: MIF, migration inhibitory factor; Con A, concanavalin A.

fraction, 2.0 ml was assayed for MIF activity, and 0.3 ml was used for $NaDodSO_4$ electrophoretic analysis.

Alternatively fraction 3 from the isoelectric focusing was dialyzed against Tris/saline/Mg buffer, made 40% in ammonium sulfate, and applied to a phenyl-Sepharose column (4 × 1 cm; Pharmacia). The column was washed with 15 ml of wash buffer (Tris/saline mg buffer/40% ammonium sulfate). A linear gradient was then applied that consisted of a further 100 ml of the wash buffer and 100 ml of Tris/saline/Mg buffer/50% ethylene glycol. Seven 15-ml fractions were collected; each was concentrated to 2.3 ml. From each fraction, 2.0 ml was assayed for MIF activity, and 0.3 ml was analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis.

Assay for MIF. MIF activity was assayed by diluting fractions to 2 ml with essential medium/penicillin/streptomycin/15% guinea pig serum as described (4). The fractions were placed in duplicate 1-ml Mackaness chambers together with capillaries containing packed peritoneal macrophages. Migration of macrophages out of capillaries was measured at 18 hr, and MIF activity was calculated as percent inhibition of migration of the active fraction relative to that of a parallel control fraction.

To quantitate recovery and degree of purification of MIF, one unit of MIF was defined as the amount of MIF that gives 20% inhibition of migration in this assay. To determine the number of MIF units in a given fraction, serial dilutions were assayed.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Fractions were prepared for electrophoresis by addition of $NaDodSO_4$ to 2%, incubation at 100°C for 5 min, and dialysis against 1% $NaDodSO_4$/2% mercaptoethanol/0.033 M Tris base/0.022 M phosphate, pH 7.0. The modified method of Laemmli (10) was used to separate the polypeptide chains using 10–14% polyacrylamide slab gels as described (11). The standard proteins myosin, β-galactosidase, phosphorylase a, albumin, creatine kinase, carbonic anhydrase, and soybean trypsin inhibitor indicated M_r of 200,000, 130,000, 94,000, 68,000, 40,000, 29,000, and 22,000, respectively.

RESULTS

Fractionation of ^{3}H-Labeled Lymph Node Supernatants by Gel Filtration and Electrophoresis. On culturing sensitized guinea pig lymph node cells with the mitogen Con A in medium containing [^{3}H]leucine, a variety of radiolabeled polypeptides are released. The secreted radiolabeled products from Con A-stimulated lymph node cells were subjected to a series of fractionation procedures designed to concentrate MIF activity. Secreted radiolabeled products of unstimulated lymph node cells were fractionated in parallel. The first two purification steps, which are procedures well established for unlabeled MIF were Sephadex G-100 gel filtration (step 1) and electrophoresis (step 2). The yield of MIF activity after step 2 was 70–80% (i.e., 35-fold purification). The [^{3}H]leucine-labeled polypeptides in the active fractions were examined by using $NaDodSO_4$/polyacrylamide gel electrophoresis and fluorography (12). Fluorographs of active step 1 and step 2 fractions showed a multitude of ^{3}H-labeled polypeptides (data not shown).

Isoelectric Focusing. Fractions purified through step 2 were subjected to isoelectric focusing. As previously shown with unlabeled material, two peaks of MIF activity are found on isoelectric focusing (Fig. 1*C*). Fractions 3–5 contain pH 3 MIF and fractions 9–11 contain pH 5 MIF. The [^{3}H]labeled polypeptides in all fractions from isoelectric focusing were examined by using $NaDodSO_4$/polyacrylamide gel electrophoresis and fluorographed. Fluorographs of nonstimulated cells and of mitogen-stimulated cells are shown in Fig. 1 *A* and *B*, respectively.

A number of radiolabeled components are visible in the pH 5 MIF-containing fraction. Only a few ^{3}H-labeled polypeptides are found in the pH 3 MIF-containing fractions. Comparison of Fig. 1 *A* and *B* shows that polypeptides in the pH 3 MIF range are synthesized exclusively by Con A-stimulated lymph node cells. Note that, in the acidic region (pH 3.0–4.6), only one component, apparent M_r 38,000, is common to all MIF activity-containing fractions (Fig. 1). Therefore, we initially considered this component to be a candidate for pH 3 MIF. This component is the only detectable molecular species in fraction 3 (pH 3.0–3.6) that contains consistently strong MIF activity, leading us to concentrate our efforts to purify MIF on this fraction.

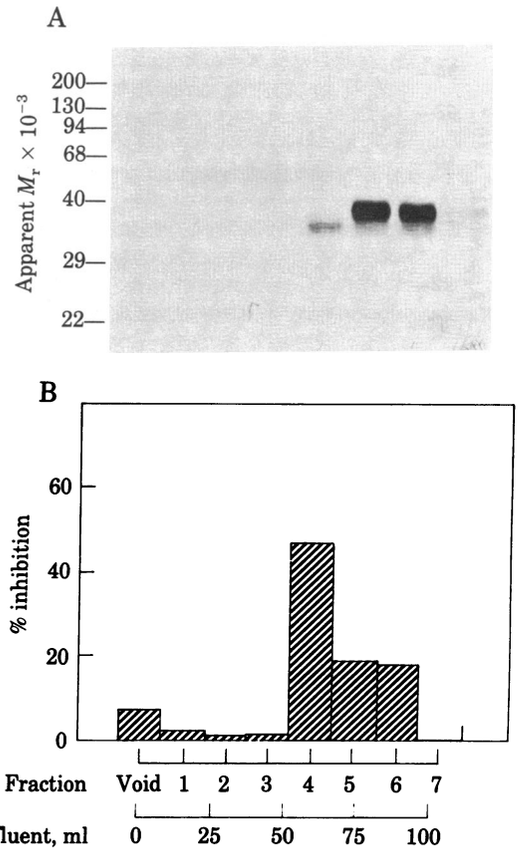

FIG. 1. Isoelectric focusing of step 2-purified material in a pH gradient from 2.0 to 6.2, representing one of seven experiments. (*C*) MIF activity (% inhibition) is indicated by the bars. The solid line indicates the pH gradient. Isoelectric focusing fractions of material from mitogen-stimulated cells were assayed for MIF activity using the corresponding isoelectric focusing fractions from unstimulated cells as control. (*A* and *B*) Fluorographs after $NaDodSO_4$/polyacrylamide gel electrophoresis of aliquots of the same fractions from nonstimulated and mitogen-stimulated cells, respectively. Note that, in fraction 3 (MIF activity = 48%), only one band, apparent M_r 38,000, is present (arrow); only this fraction was further purified.

Chromatography of Fraction 3 from Isoelectric Focusing on Sephadex G-75. Fraction 3 from isoelectric focusing was fractionated on Sephadex G-75 to determine which ^{3}H-labeled polypeptide(s) coincides with MIF activity. Seven fractions were tested for MIF activity and, in parallel, were analyzed for composition by $NaDodSO_4$/polyacrylamide gel electrophoresis and fluorography (Fig. 2). The 38,000 M_r component is present in fractions 2 and 3. In fractions 3 and 4, a faster migrating component, M_r 35,000, is also present. MIF activity is found in fractions 3 and 4 but not in fraction 2. This finding suggests that MIF activity is associated with the 35,000 M_r component.

Affinity Chromatography of Fraction 3 from Isoelectric Focusing on Phenyl-Sepharose. In a further attempt to separate the 35,000 M_r component from the 38,000 M_r component and

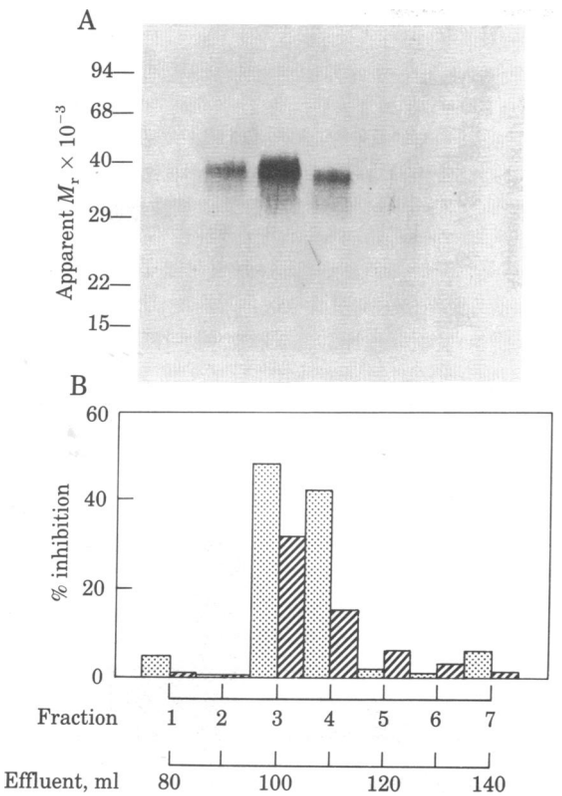

FIG. 2. Gel filtration on Sephadex G-75 of fraction 3 from isoelectric focusing, representing one of three experiments (*B*). MIF activity ▨, of 1.6 ml of each fraction; ▨, activity of 0.8 ml. (*A*) Fluorograph after $NaDodSO_4$/polyacrylamide gel electrophoresis of aliquots of the same fractions from the mitogen-stimulated cells. The 38,000 M_r component is present in fraction 3 and, at a lower concentration, in fraction 2 and absent in fraction 4. A 35,000 M_r component in fraction 4 presumably contributes to the intensity of the band in fraction 3. MIF activity is found in the fractions 3 and, at a somewhat lower level, in fraction 4. Thus, MIF activity seems to correlate with the 35,000 M_r component.

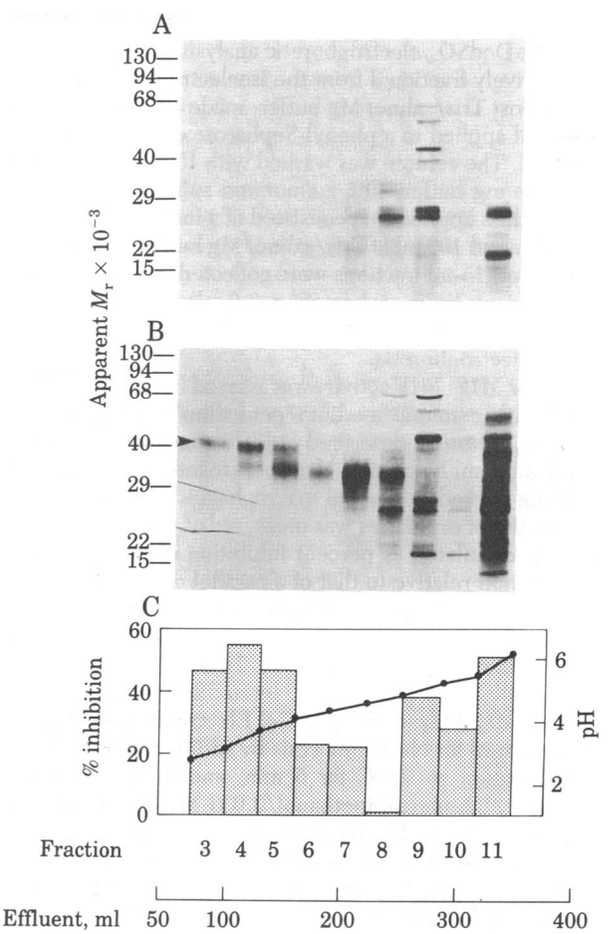

FIG. 3. Affinity chromatography on phenyl-Sepharose of fraction 3 from isoelectric focusing, representing one of two experiments. (*B*) MIF activity. (*A*) Fluorograph after $NaDodSO_4$/polyacrylamide gel electrophoresis of aliquots of the same fractions of mitogen-stimulated material. Note that a component with M_r 35,000 coincides with MIF activity.

for further purification, we subjected fraction 3 from the isoelectric focusing step to affinity chromatography on phenyl-Sepharose (13). The components were eluted from the column with a linear gradient of decreasing ammonium sulfate and increasing ethylene glycol. The M_r 38,000 component eluted in fractions 5 and 6 (Fig. 3). Coordination of the MIF activity with components in the fluorographs shows that the 35,000 M_r component (fraction 4) and not the 38,000 M_r component contains MIF activity.

Thus, by a combination of Sephadex G-100 gel filtration, electrophoresis, isoelectric focusing, and phenyl-Sepharose affinity chromatography, one species of MIF has been purified to apparent homogeneity. The final purification is ≈30,000-fold, and the yield is approximately 7% (Table 1).

DISCUSSION

We have shown that a polypeptide from supernatants of mitogen-stimulated guinea pig lymph node cells of apparent M_r 35,000 and coinciding with MIF activity can be purified to apparent homogeneity. This component is found in the most acidic fraction of the pH 3 MIF peak and has a pI of 3.0–3.6. It could be consistently correlated with MIF activity by using two independent fractionation procedures, Sephadex G-75 gel filtration and hydrophobic affinity chromatography. To this date, however, it cannot be excluded that other biological activities are associated with this substance.

Previously, several investigators have reported the purification of MIF. Sorg and Bloom (7) and Sorg (8) have purified a radiolabeled polypeptide that has MIF activity, a K_d of 0.15,

Table 1. Summary of purification of pH 3 MIF

Step	Total units*	Protein,† mg	Specific activity, units/mg	Purification	Overall recovery,‡ %
1. Culture fluid	60	10	6	1	100
2. Sephadex G-100 chromatography	50	1.2	42	7	80–90
3. Electrophoresis	45	0.2	225	37	80
4. Isoelectric focusing (fraction 3)	8–10	5×10^{-4}	18,000	3,000	15
5. Phenyl-Sepharose chromatography	4–6	2.5×10^{-5}	180,000	30,000	7

* For definition, see *Materials and Methods*.

† Protein content was assessed by the method of Lowry (18) (steps 1–3) and by quantitation of trichloroacetic acid-precipitable radioactivity (steps 3 and 4). For step 5, the value given is an estimate based on relative intensity of components in the fluorographs.

‡ Note that steps 4 and 5 refer to only one species of MIF (pH 3 MIF) whereas steps 1–3 refer to a mixture of both species.

and an isoelectric point of 5.2 in guinea pig and mouse lymph node cell supernatants. However, it is not quite clear that this component is homogeneous. Also, Block *et al.* (14) have reported the purification of human MIF by Sephadex G-100 gel filtration and isotachophoresis. These authors found a component (which stains with Coomasie blue) that has an apparent M_r of 25,000 and an isoelectric point of 3.5–4.0, which they considered to be human MIF. However, this component is clearly contaminated with two unrelated proteins, thus making the identification of MIF impossible.

Of the large group of tissue hormones having M_r >12,000, only α-interferon (15) and colony-stimulating factor (16) have been purified to homogeneity. These substances are available in large amounts. However, in the studies described here, radiolabeling made it possible to detect a M_r 35,000 component present in minute quantities in lymph node cell supernatants that consistently coincides with MIF activity.

It is of note that pH 3 MIF under nondenaturing conditions is found with proteins of apparent M_r 50,000–70,000 (4). However, when these fractions are subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis, no components larger than M_r 38,000 can be detected. Although we cannot exclude a M_r 70,000 component that lacks leucine, these findings suggest that at least part of pH 3 MIF exists under physiological conditions as a dimer. This assumption is strongly supported by the fact that nonglycosylated pH 3 MIF produced in the presence of tunicamycin chromatographs together with proteins of apparent M_r ≈30,000 (unpublished observations). These findings may indicate that glycosylation of PH 3 MIF is necessary for dimerization and that unglycosylated pH 3 MIF remains therefore in the monomeric form.

Contrary to a report by others, we find no evidence that guinea pig MIF is a multiple aggregate of a common subunit with M_r 15,000 (17). Our studies indicate that the smallest unit of pH 3 MIF has a M_r of ≈35,000.

Further understanding of the products of activated lymphocytes and their mode of action is dependent on their purification to homogeneity. Only when larger amounts of homogeneous lymphocyte mediators from cell lines or hybridomas are available will we have enough material to permit analysis of their roles in cell-to-cell interaction, regulation of the immune response, and defense against infection and tumor cells. With this information, manipulation of the events underlying cellular immunity in clinical situations might be possible.

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