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Separation of Immune Interferon and MIF[1] (40512)

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Immune interferon induced by the mitogen phytohemagglutinin (1) and macrophage migration inhibitory factor (MIF) (2, 3) are probably the earliest-described lymphokines. A plethora of lymphocyte-produced soluble factor activities (lymphokines) have subsequently been described (4). Immune interferon is usually induced in primed lymphocytes by specific antigen or in unprimed lymphocytes by T cell mitogens (1, 5–8). Immune interferon, whether induced by antigen or mitogen, is labile at pH 2 and antigenically distinct from virus induced (virus-type) interferons (9, 10). To date, it has not been possible to separate mouse immune interferon (also called type II interferon) and MIF (9). The question has arisen, then, as to whether MIF and mitogen-induced immune interferon might be different biological expressions of the same molecule(s). We present data here which show that MIF and immune interferon are distinct biological entities.

Materials and methods. Large scale production of immune interferon and macrophage migration inhibitory factor (MIF). Large scale production of mitogen-induced immune interferon using C57B1/6 mouse spleen cells, the T cell mitogen staphylococcal enterotoxin A, modified RPMI 1640 media, and the roller bottle tissue culture system was carried out exactly as described (11). In this system it requires approximately 200 mice to produce 10^7 units of immune interferon. The interferon used here was not neutralized by antibodies to mouse L cell interferon, which are specific for mouse fibroblast and leukocyte interferons (12). The antiviral activity of our interferon was destroyed at pH 2. The interferon was considered, therefore, as immune interferon.

Stepwise salt precipitation. Eleven hundred ml of the lymphokine preparation was concentrated by stepwise precipitation with ammonium sulfate as described (11). Briefly, solid ammonium sulfate was added with constant stirring to 55% saturation. After removal of the precipitate by centrifugation, the supernatant was further treated by addition of solid ammonium sulfate to 80% saturation. The precipitates were dissolved in distilled water to approximately one-twentieth the original volume. Supernatants and reconstituted precipitates were exhaustively dialyzed against phosphate–buffered saline (0.15 *M*, pH 7.2). All operations were carried out at 0–4°.

BSA-Affi-Gel 10 chromatography. Hydrophobic chromatography was performed on a bovine serum albumin (BSA)-Affi-Gel 10 column (0.9 × 12 cm) using exclusively lyophilized Affi-Gel 10 (11). Briefly, the 55–80% salt-precipitated immune interferon and MIF sample was dialyzed overnight at 4° against 0.05 *M* sodium acetate buffer, pH 5.0. The column, which had been equilibrated with 0.05 *M* sodium acetate, was loaded with 10 ml of the interferon at 4° by means of a peristaltic pump. The column was washed with the equilibrating buffer, followed by acetate–phosphate buffers of various pHs and salt concentrations (11). Fractions (2 ml) were pooled into 12 pools based on OD at 280 nm and on the interferon profile. The pools were tested for immune interferon and MIF activity.

Interferon assays. Interferon was assayed in microtiter plates as described (8). One unit of activity is that concentration required to reduce 40 PFU of vesicular stomatitis virus by 50%.

MIF assays. MIF was measured by a macrophage migration inhibition soft agar procedure, using peritoneal exudate (PE) cells from Swiss-Webster mice (Taconic Farms) exactly as described (6). Supernatants tested for MIF activity were incorporated into the agar at a final dilution of 1:5–1:80. Results are expressed as the percentage of migration

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inhibition as follows: percent migration inhibition = 100[1.0-(migration distance of PE cells with experimental sample/migration distance of PE cells with control sample)]. The percent migration inhibition is based on a mean of data from at least four replicate determinations with each sample. An average inhibition of 20% or more is considered as evidence for the presence of MIF.

Results and discussion. The interferon used in these studies was identified as immune interferon by its instability at pH 2 and by the failure of antibodies to virus-type interferon to neutralize its antiviral properties (11).

The stepwise salt precipitation data of Table I show that approximately 95% or more of the immune interferon was precipitated by 55–80% saturated ammonium sulfate, while the strongest MIF activity was found in the reconstituted precipitate of 55% saturated ammonium sulfate. Only 4% of the immune interferon was present in the 55% precipitate. MIF activity was not detected at a 1:5 dilution in the 55–80% saturated salt precipitate. Other mediator preparations have contained weak MIF activity in this latter fraction (Data not shown). Thus, the bulk of the biological activities of immune interferon and MIF were found in different fractions. This is suggestive evidence that the two lymphokines are different, but is not definitive since residual biological activity of both lymphokines was found, or occasionally found, in both reconstituted salt precipitates.

The reconstituted 55–80% saturated ammonium sulfate precipitate of Table I was fractionated on a hydrophobic BSA-Affi-Gel 10 column (Fig. 1) in an effort to determine the relationship of possible residual MIF activity to immune interferon. Fractions were pooled, based on OD and interferon profiles, and tested for immune interferon and MIF activity. The results are presented in Table II. Immune interferon is heterogeneous and eluted from the column in three peaks. The first peak (P1) was the breakthrough or unbound interferon peak, while the second (P8 and P9) and third (P11 and P12) were bound by the column and eluted under the indicated conditions of pH and salt concentration of Fig. 1.

The results from the column clearly differentiated immune interferon and MIF activities. MIF appeared to be heterogeneous with activity in pools 1, 6, 7, and 11 while absent from pools 2, 3, 4, 5, 8, 9, 10, and 12. Pools 8, 9, and 12 had substantial immune interferon activity, but lacked MIF activity. MIF activity was most active in pools 6 and 7, which lacked immune interferon activity. The MIF activity in pools 6 and 7 appears to have been eluted from the column by buffer A treatment, since the MIF in pool 7 appears to come from the tail of the previous peak, rather than from buffer B treatment. Pool 11 had the highest concentration of immune interferon, but a corresponding increase in MIF activity was not observed. A repeat of the column elution experiment resulted in a sim-

TABLE I. DISTRIBUTION OF IMMUNE INTERFERON AND MIF ACTIVITIES IN A LARGE SCALE LYMPHOKINE PREPARATION AFTER STEPWISE FRACTIONATION WITH AMMONIUM SULFATE.

Fraction	Lymphokine concentration		% inhibition	Total number of units of interferon (Log_{10} units)
	Immune interferon (Log_{10} units/ml)	MIF Dilution		
Starting material	3.3 (1100)[a]	1:5[b]	37	6.4
		1:20	<20	
55% saturated ammonium sulfate precipitate	3.3 (50)	1:20	43	5.0
		1:80	22	
55–80% saturated ammonium sulfate precipitate	4.6 (63)	1:5	<20	6.4
55–80% ammonium sulfate supernatant	1.0	not tested		4.0

[a] Values in parentheses represent original or reconstituted volumes in ml. Details of the salt precipitation are presented in the Materials and Methods.

[b] Samples were tested at 1:5 to 1:80 dilutions. Data are presented to indicate the highest dilution that resulted in 20% or more inhibition of migration. Less than 20% inhibition of macrophage migration is considered negative for MIF activity.

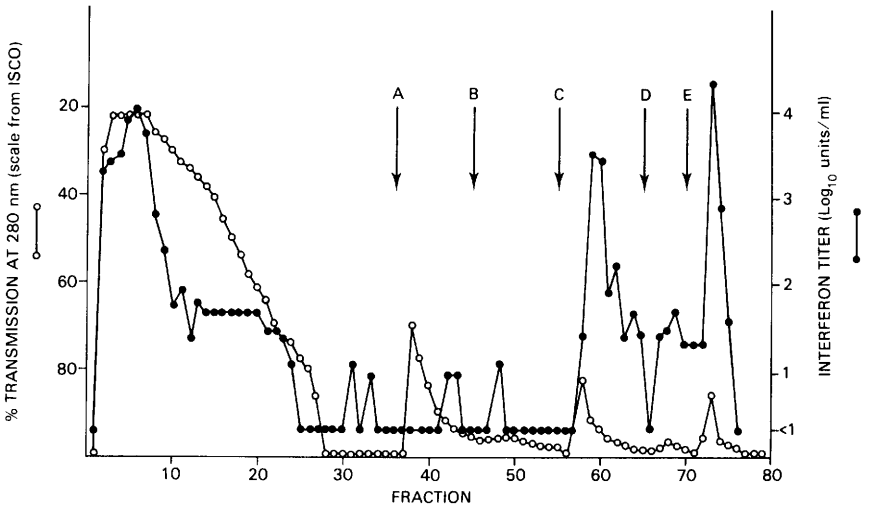

FIG. 1. Stepwise elution profile of immune interferon and protein from a BSA-Affi-Gel 10 column. The 55–80% ammonium sulfate precipitate of Table I was loaded and eluted from the column as indicated in the Materials and methods. The pools are based on OD at 280 nm and on the interferon profile. Elution was carried out with the following buffers: A. 0.05 *M* sodium acetate, pH 5.0, plus 0.02 *M* sodium phosphate, pH 7.0, mixed to a pH of 5.2; B. Acetate–phosphate, pH 5.4; C. 0.08 *M* NaCl in acetate–phosphate, pH 5.4; D. 0.08 *M* NaCl in acetate–phosphate, pH 6.0; E. 1 *M* NaCl in 0.02 *M* sodium phosphate, pH 7.0.

TABLE II. DISTRIBUTION OF IMMUNE INTERFERON AND MIF ACTIVITY IN POOLED FRACTIONS FROM STEPWISE ELUTIONS OF BSA-AFFI-GEL 10 COLUMN[a]

		Activity		
			MIF	
Pool (P)	Fractions	Immune interferon (units/ml)	Dilution[b]	% Inhibition
1	2–14	2500	1:5[c]	31
2	15–30	23	1:5	<20
3	31–36	<10	1:5	<20
4	38	<10	1:5	<20
5	39–42	<10	1:5	<20
6	48–52	<10	1:20[c]	62
7	57	<10	1:80	33
8	58–59	1500	1:5	<20
9	60–63	725	1:5	<20
10	66–69	26	1:5	<20
11	73	10000	1:10	41
			1:20	<20
12	74–75	465	1:5	<20

[a] Fractions from stepwise elutions of Fig. 1 were pooled according to OD values and interferon profile. Interferon and MIF activities were determined as described in the Materials and methods.

[b] All samples were tested at a 1:5 dilution. Some of those with MIF activity were further tested at 1:5 to 1:80 dilutions. Data are presented to indicate the highest dilution that resulted in 20% or more inhibition of migration. Less than 20% inhibition of macrophage migration is considered negative for MIF activity.

[c] Highest dilution tested.

ilar elution profile for immune interferon and MIF activities. The salt precipitation data of Table I and the elution profiles of immune interferon and MIF from the BSA-Affi-Gel 10 column, then, show both qualitatively and quantitatively that these two lymphokines are different. The relatively high MIF activity of pools 6 and 7 of Table II could be the result of concentration by hydrophobic chromatography (Fig. 1). The chromatographic separation of MIF from a possible inhibitor must also be considered.

Separate rechromatography of eluted immune interferon peaks resulted in the elution of single peaks in the same positions as were originally observed (11). Thus the heterogeneity of immune interferon by hydrophobic affinity chromatography apparently represents different species of immune interferon and not artifacts of the procedure.

The interferon profiles from a BSA-Affi-Gel 10 column using a continuous pH and salt gradient elution procedure and a stepwise discontinuous gradient procedure both demonstrated three major peaks of interferon (11). Normally discontinuous gradient elutions are not as suitable for chromatographic studies because of the potential for the formation of minor secondary peaks by homogeneous substances (11). Closely related substances, however, can sometimes be separated by this procedure. Attempts to separate immune interferon and MIF on a column eluted with a continuous pH gradient were not suc-

cessful. The discontinuous gradient method did separate them. Thus, the discontinuous gradient method presented here is superior to that of a continuous gradient for the separation of immune interferon and MIF.

To our knowledge this is the first report of the physical separation of the biological activities associated with immune interferon and MIF activities. The methodology should be useful to show the physical relationship of other lymphokines.

Summary. A preparation of mitogen-induced immune interferon and MIF was subjected to sequential salt precipitation with first 55% and then 80% saturated ammonium sulfate. At least 95% of the interferon activity was found in the reconstituted 55–80% precipitate, while MIF activity was found in both precipitates, but most active in the reconstituted 55% salt precipitate. The immune interferon and MIF activities of the 55–80% reconstituted salt precipitate were shown to be different by BSA-Affi-Gel 10 affinity chromatography. Both lymphokines were also shown to be heterogeneous by the above techniques.

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