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MACROPHAGE-ACTIVATING FACTOR PRODUCED BY A T CELL HYBRIDOMA: PHYSIOCHEMICAL AND BIOSYNTHETIC RESEMBLANCE TO γ-INTERFERON[1]

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Biochemical and biosynthetic evidence has been obtained which indicates that the macrophage activating factor (MAF) produced by the murine T cell hybridoma clone 24/G1, which primes macrophages for nonspecific tumoricidal activity, is a form of γ-interferon (IFNγ). MAF and antiviral activities were generated by the clone in proportional amounts under a variety of culture conditions. Production of both activities showed identical dependence on cell density even when production precipitously ceased at cell concentrations greater than 1.2×10^6 cells/ml. MAF and antiviral activities displayed identical sensitivities to pH and temperature and were indistinguishable on the basis of binding to insolubilized polynucleotides. Dye-ligand chromatography of a stimulated hybridoma supernatant on a column of Matrex Gel Red A resulted in the 1500-fold purification and 100% recovery of the MAF activity. A qualitatively identical elution profile was also obtained for the antiviral activity; however, only 32% of the original activity was recovered. When subjected to gel filtration on a high performance liquid chromatography system (HPLC), the MAF and antiviral activities present in the Matrex Red pool displayed identical elution profiles and exhibited an apparent m.w. of 50,000. This technique resulted in another 10-fold purification and 50% recovery of the two activities. On HPLC chromatofocusing the MAF activity in the Matrex Red pool could be resolved into seven chromatographically distinct species yet could not be resolved from the antiviral activity on either a qualitative or quantitative basis. These results thus provide quantitative molecular evidence to support the concept that IFNγ can act as a MAF.

Over the past few years, work from several laboratories has indicated that macrophages may play a role in host resistance to neoplastic disease (1–4). *In vitro* experiments have demonstrated that acquisition of nonspecific tumoricidal activity by murine macrophages results from a sequence of reactions involving at least two signals. The first signal is a lymphokine denoted macrophage-activating factor (MAF)[5] which primes the macrophage and makes it sensitive to the effects of a second signal which triggers the development of cytocidal activity (5–8).

The molecular identity of MAF has remained ill-defined. In particular, the relationship between MAF and interferon derived from T lymphocytes (IFNγ) has been the subject of intensive investigation. However, the resolution to this problem has not been forthcoming, primarily because neither molecule has, as yet, been purified to homogeneity. This task has proven to be difficult because of the limiting amounts of lymphokines which can be generated using conventional T cell sources such as normal splenic cell cultures. In an attempt to circumvent this obstacle we constructed murine T cell hybridomas (9, 10), and identified at least one which produced a lymphokine(s) capable of priming macrophages for nonspecific tumoricidal activity (11). A clone of this hybridoma (24/G1) was found to produce MAF in 10–25 times higher concentration than could be derived from mitogen-stimulated cultures of normal murine spleen cells (11). The hybridoma-derived MAF was indistinguishable from conventional MAF by a variety of biochemical and functional criteria including pH and temperature sensitivies, molecular weight, and a requirement for a second signal in order to trigger a full tumoricidal response.

The ability to reproducibly generate liter quantities of culture supernatants containing high concentrations of MAF has enabled us to examine the molecular nature of this lymphokine. We conclude on the basis of a variety of biochemical, biosynthetic, and functional data that the MAF activity produced by the T cell hybridoma clone 24/G1 is, in fact, IFNγ. In this report we present the partial purification of the MAF activity and demonstrate its resemblance to IFNγ by using high resolution biochemical techniques and by examining biosynthesis of the two activities at a clonal level. This conclusion will be supported elsewhere by immunochemical data, as well as by the demonstration that IFNγ made by recombinant DNA technology displays MAF-like properties including the quantitative ability to prime macrophages for nonspecific tumoricidal activity.[6]

MATERIALS AND METHODS

Media, supplements, and buffers. All media, supplements, and buffers used in these experiments were prepared from endotoxin-free stocks and were determined to be free of endotoxin using the *Limulus amebocyte* lysate assay (Sigma Chemical Co., St. Louis, MO). To destroy endotoxin that potentially might have been adherent to glass, all autoclaved glassware was baked for 3 hr at 180°C. RPMI-1640 and Eagle's minimum essential medium (MEM) were prepared from powdered stocks (Flow Laboratories, Inglewood,

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[5] Abbreviations used in this paper: Con A, concanavalin A; FCS, fetal calf serum; HKLM, heat-killed *Listeria monocytogenes*; HPLC, high performance liquid chromatography; IFNγ, γ-interferon; MAF, macrophage-activating factor; MEM, Eagle's minimum essential medium; Poly (I), polyinosinic acid; Poly (U), polyuridylic acid; VSV, vesicular stomatitis virus, DMEM, Dulbecco's modified Eagle Medium.
[6] Russel, S. W., R. D. Schreiber, A. Altman, and D. Katz. Macrophage activation: priming activity from a T cell hybridoma is attributable to gamma interferon, submitted for publication.

CA) using USP sterile water (Travenol Laboratories, Inc., Deerfield, IL). Liquid Dulbecco's modified Eagle medium (DMEM) containing 4500 mg glucose/liter was purchased from M.A. Bioproducts (Walkersville, MD). Aseptically drawn fetal calf serum (FCS; Rehatuin F.S.) was obtained from Reheis Chemical Co. (Phoenix, AZ) and heat inactivated (1 hr, 56°C) before use. Other media supplements included: injectable penicillin G and streptomycin sulfate (Eli Lilly and Co., Indianapolis, IN), injectable sodium bicarbonate and sodium heparin (Gibco-Invenex Division, Chagrin Falls, OH), 1 M HEPES buffer solution, versene, sodium pyruvate, and L-glutamine (M.A. Bioproducts), 10 mM MEM nonessential amino acids and trypsin-EDTA (1X) (Gibco Laboratories, Grand Island, NY), gentamicin (Schering Corp., Kenilworth, NJ), Indomethacin (Sigma Chemical Co.) and concanavalin A (Con A; Miles-Yeda, Ltd., Rehovot, Israel).

Mice. C3HeB/FeJ, DBA/2, and (C3H/HeJxDBA/2)F1 mice were obtained from Jackson Laboratories (Bar Harbor, ME).

Construction of T cell hybridomas. Murine T cell hybridomas were constructed by fusion of alloantigen-activated T cell blasts with the BW5147 T lymphoma cell line, as described (9), using a tumor cell to T cell blast ratio of 1:2.5. Hybridomas were cloned by limiting dilution, as outlined elsewhere (10).

Production of culture supernatants containing MAF and IFN activities. The T cell hybridoma clone 24/G1 which we have previously reported to produce MAF and IFN activities in large quantities (11) was used as the predominant source of lymphokines in these experiments. Other hybridomas derived from different fusions were used in some experiments. Hybridoma cultures were maintained in DMEM containing 10% FCS, L-glutamine (2 mM), sodium pyruvate (1 mM), and gentamycin (50 μg/ml) and stimulated with 10 μg/ml Con A for 24 hr as described (11). Culture supernatants were clarified by centrifugation and filtration through 0.2 μM filters and stored at −20°C.

In some experiments conventional supernatants containing MAF and IFN were used. These preparations were produced by stimulation of cultures of normal murine splenic cells (5×10^6/ml) with 5 μg/ml Con A, as described (11).

Measurement of MAF activity. MAF was quantitated by measurement of its ability to induce nonspecific tumorilytic activity in proteose peptone-elicited C3HeB/FeJ macrophages toward ^{51}Cr-labeled P815 mastocytoma cells, as detailed elsewhere (11). Briefly, reactions were performed in triplicate in 96-well flat-bottom tissue culture plates in a total volume of 200 μl. Each well contained 2×10^5 adherent macrophages, serial dilutions of MAF, and an excess of a second signal (1×10^6 heat-killed *Listeria monocytogenes*, HKLM) to trigger the cytolytic event. After 4 hr the activating stimuli were removed by washing and 2.5×10^4 ^{51}Cr-labeled P815 were added to the wells. After incubation for 18 hr at 37°C in a humidified 5% CO_2 atmosphere, 100 μl of each culture supernatant was removed and analyzed for ^{51}Cr content. One U of MAF is defined as that amount which produces 50% maximal specific ^{51}Cr release in this assay.

Measurement of interferon activity. IFN was quantitated by measurement of its ability to protect murine L cells (clone 929) from infection by purified vesicular stomatitis virus (VSV) using a modified cytopathic effect assay described elsewhere (11). Briefly, 3×10^4 L cells were cultured for 24 hr in presence of serial dilutions of IFN. Reactions were performed in triplicate in a total volume of 200 μl. The resulting L cell monolayers were washed and then challenged with a 1:100,000 dilution of purified VSV. Plates were read when control wells which had not received IFN showed a complete cytopathic effect of the virus. One U of IFN is defined as that amount which provides the L cells with 50% protection from the cytopathic effect of the virus. In some experiments these values were normalized to the World Health Organization International Reference Standard of murine IFNβ (Research Resources Branch, NIAID, NIH).

Polynucleotide-Sepharose. One hundred mg of polyinosinic acid (Poly (I), Calbiochem-Behring, La Jolla, CA) was covalently coupled to 7.5 ml Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) using the method described by Wagner *et al.* (12). Polyuridylic acid [Poly(U)]-Sepharose was purchased from Pharmacia Fine Chemicals.

Chromatography of culture supernatants on Poly (I)-Sepharose. Two-ml samples of culture supernatants containing MAF and IFN activity were dialyzed against 1 liter of 10 mM Tris buffer, pH 7.5 (Sigma Chemical Co.) and then applied to a 1.0 × 12 cm column of Poly (I)-Sepharose at 23°C. The column was washed with three-column volumes of starting buffer and then eluted with buffer containing 1 M NaCl. Protein was quantitated by measurement of absorption of 280 nM. As a control, chromatography was also performed using unsubstituted Sepharose.

Chromatography on Poly (U)-Sepharose. Twenty ml of culture supernatants were dialyzed against 10 mM Tris-HCl buffer, pH 7.5, and then applied to a 5-ml column of Poly (U)-Sepharose. The column was eluted with starting buffer and then eluted with buffer containing 1 M NaCl.

Chromatography of culture supernatants on Matrex Gel Red A. Eight hundred fifty ml of a stimulated culture supernatant of the T cell hybridoma clone 24/G1 was concentrated to 85 ml by ultrafiltration and then applied at 4°C to a 1.5 × 7.0-cm column of Matrex Gel Red A (Amicon Corp., Lexington, MA) equilibrated with 0.02 M phosphate-buffered physiological saline, pH 7.3, containing 1 mM PMSF and 25 mM EACA. The column was first washed with starting buffer and then with eight-column volumes of buffer containing 0.5 M NaCl. After reequilibration with the starting buffer, the column was then eluted with a linear gradient to a limit of 1 M ammonium sulfate.

HPLC gel filtration. Five hundred μl of the concentrated activity-containing pool from the Matrex Gel Red A column was injected into a 7.5 × 600 nM Bio-Sil TSK 250 HPLC gel filtration column (Bio-Rad Laboratories, Richmond, CA) connected to a high performance liquid chromatography (HPLC) system (Waters Associates, Milford, MA). The column was eluted at 23°C with 0.2 M ammonium formate buffer, pH 7.3, containing 0.1 M α-methyl-D-mannoside at a flow rate of 0.5 ml/min.

HPLC chromatofocusing. One hundred μl of the Matrex Gel Red A ammonium sulfate pool was diluted to 2 ml with 0.025 M *bis*-Tris-HCl buffer (Calbiochem-Behring), pH 7.1, and injected into a 7.5 × 300 mM Mono P column (Pharmacia Fine Chemicals), equilibrated in the same buffer and connected to a Waters HPLC system. After washing with starting buffer for 2 min at 23°C, the column was eluted at 0.5 ml/min with 74 ml of Polybuffer 74-HCl (Pharmacia Fine Chemicals), diluted 1:10, and adjusted to pH 5.0. At the completion of the isoelectric gradient, 2 ml of buffer containing 1 M NaCl was injected into the column and elution with Polybuffer continued for an additional 15 min. pH values of the fractions were determined and then adjusted to neutrality with 25 μl of 1 M HEPES buffer solution.

RESULTS

Correlation of MAF and IFN production by T cell hybridomas. Figure 1 depicts the production of MAF and IFN antiviral activity by 32 parental T cell hybridomas after stimulation with Con A. The results are expressed relative to the amount of each activity present in a standard mitogen-stimulated splenic cell culture supernatant (0.1). None of the cell cultures tested secreted either activity constitutively nor did any secrete one activity in absence of the other. Ten hybridomas produced significantly more (3–13 times) MAF activity than did conventional splenic cell cultures and 11 hybridomas produced more (3–16 times) IFN activity. Although a loose correlation existed between the two lymphokine activities for most of the hybridomas, two hybridomas secreted a disproportionately high amount of MAF activity and one produced an unusually high amount of antiviral activity.

A better correlation between the two activities was observed in culture supernatants from a T cell hybridoma clone (24/G1) (Fig. 2). Using a variety of experimental conditions which included different mitogen and FCS concentrations and different lengths

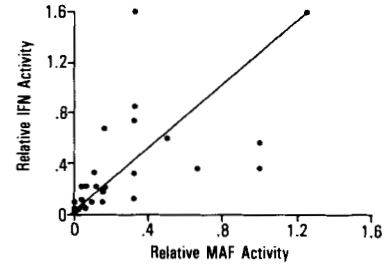

Figure 1. Correlation between MAF and IFN activities produced by various T cell hybridomas. Fifty-ml cultures of parental T cell hybridomas, produced by fusion of alloantigen activated T cell blasts with the BW5147 T lymphoma cell line, were stimulated with 10 μg/ml Con A for 24 hr. MAF activity and IFN antiviral activity were titrated relative to a standard supernatant of mitogen stimulated splenic cells. This standard was assigned a value of 0.1 for each activity.

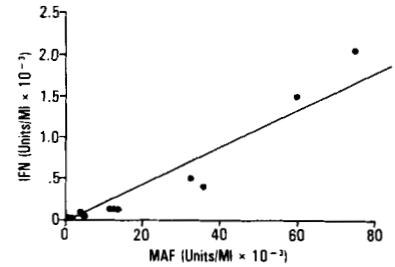

Figure 2. Correlation between MAF and IFN activities produced by the T cell hybridoma clone 24/G1. Cultures of the hybridoma clone were stimulated under a variety of culture conditions including 1) different Con A concentrations (2, 5, 10, 20, and 40 μg/ml), 2) different FCS concentrations (0, 0.5, and 5%), and, 3) different times of stimulation (0, 12, 24, 36, 48 hr).

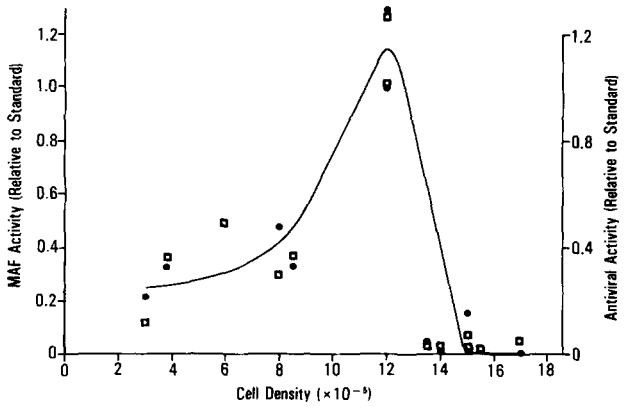

Figure 3. Influence of hybridoma cell culture density on production of MAF and IFN activities. Cultures of clone 24/G1 were grown to different cell densities and stimulated with 10 μg/ml Con A. MAF (●) and antiviral (■) activities were titrated relative to a standard hybridoma supernatant. This was assigned a value of 1.0.

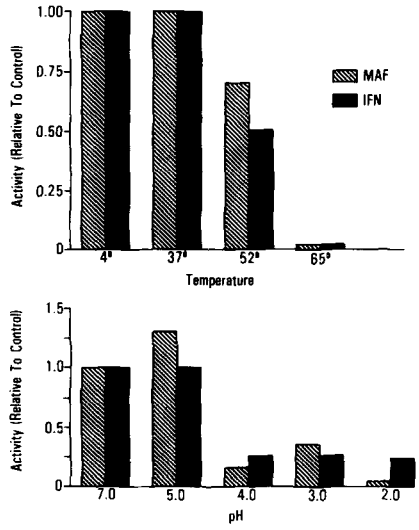

Figure 4. Comparison of heat and pH sensitivities of the MAF and antiviral activities produced by T cell hybridoma clone 24/G1. Heat sensitivity was determined by incubation of 5-ml samples of a stimulated culture supernatant for 1 hr at the designated temperatures. Sensitivity to pH was investigated by dialysis of 5-ml samples for 18 hr at 4°C against 1 liter of physiological saline buffered to the designated pH values. Samples were then neutralized by dialysis against 0.1 M phosphate buffer, pH 7.4, for 4 hr and then against phosphate-buffered saline.

of time for stimulation, a close correlation always was found between MAF and antiviral activity even when the changes in culture conditions resulted in a 20-fold difference in the amount of each activity produced.

Figure 3 demonstrates that the amount of MAF and antiviral activity secreted by the hybridoma clone increased in proportion to the cell density of the culture up to 1.0–1.2 × 10^6 cells/ml. However, at higher cell densities, production of either activity declined precipitously. This result was obtained despite the fact that the cell culture was capable of growing to 2.0–2.4 × 10^6 cells/ml. No significant MAF or antiviral activities were secreted by cultures with cell densities greater than 1.4 × 10^6 cells/ml even when higher Con A concentrations were used or when longer stimulation periods were employed.

Comparison of temperature and pH sensitivities of hybridoma-derived MAF and IFN activities. Figure 4 demonstrates that the MAF and IFN activities present in the hybridoma culture supernatant shared identical sensitivities to temperature and pH. Both activities were stable to incubation in medium containing 5% FCS for 1 hr at 4°C or 37°C but were destroyed when held at 65°C for 1 hr. Some loss of activity (25–50%) was observed when the supernatant was exposed to 52°C or 56°C (not shown) but this sensitivity was variable. Both activities were stable to dialysis to pH 7.0 and 5.0 but were diminished by 80% after treatment at or below pH 4.0.

Adsorption of MAF and IFN activities by Polynucleotide-Sepharose columns. Several studies have indicated that all murine IFN species bind reversibly to polynucleotide-containing Sepharose columns (13, 14). This behavior was determined to be somewhat unique because most other proteins present in the IFN samples were found to pass through the column unretarded. Table I demonstrates that both the MAF and IFN activities present in the hybridoma supernatant behave in an identical fashion on polynucleotide columns. When applied to a Polyinosine-Sepharose column under low ionic strength conditions, only 12% of the recovered antiviral activity and 23% of the recovered MAF activity eluted in the unretarded fractions. This is in contrast to the elution of 88% of the total protein. When the column was eluted with 1 M NaCl, the desorbed fractions contained 88% of the recovered antiviral activity, 77% of the MAF activity, but only 12% of the total protein. Figure 5 demonstrates that elution profiles of the two activities obtained on a poly (U)-Sepharose column were virtually superimposable even when each fraction was analyzed for MAF or antiviral activity. Similar results (data not shown) were obtained on Poly (I)-Sepharose columns using lymphokine rich supernatants produced from normal murine splenic cells: only 22% of the recovered antiviral activity and 21% of the MAF activity was found in the breakthrough fractions while 78% of the antiviral activity and 79% of the MAF activity were found in the desorbed fractions. That adsorption of the activities required column-bound polynucleotides was confirmed by chromatography on control columns of unsubstituted Sepharose. Under these conditions, the majority of hybridoma-derived antiviral activity (88%) and MAF activity (69%) passed through the columns unretarded.

Dye ligand chromatography of MAF and IFN activities. Kniep *et al.* (15) have reported that MAF bound to a particular insolubilized dye column (Matrex Gel Red A), while most other proteins

TABLE I

Binding of IFN and MAF by polynucleotides

Column	IFN (%)	MAF (%)
Poly (I)-Sepharose		
Breakthrough[a]	12	23
Desorbed[a]	88	77
Recovery	113	150
Sepharose		
Breakthrough[a]	88	69
Desorbed[a]	12	31
Recovery	68	161

[a] Expressed as the percentage of the recovered activities.

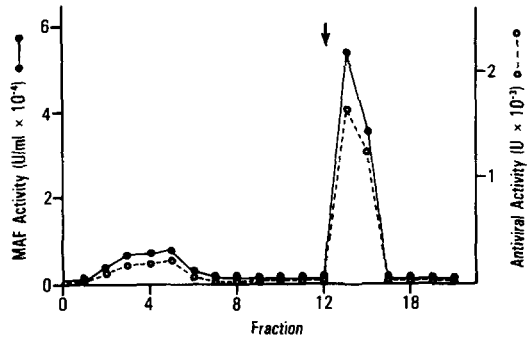

Figure 5. Poly (U)-Sepharose chromatography of hybridoma-derived MAF and antiviral activities. Twenty ml of a Con A-stimulated culture supernatant of the T cell hybridoma clone 24/G1 was subjected to chromatography on a 5-ml column of Poly (U)-Sepharose equilibrated in 0.01 M Tris-HCl, pH 7.5. Flow rate was 25 ml/hr and 5 ml fractions were collected. After washing, the column was eluted with 0.01 M Tris-HCl, pH 7.5, buffer containing 1 M NaCl (indicated by the *vertical arrow*).

in stimulated T cell culture supernatants did not. Based on this observation, we attempted to study the dose-dependent absorption of the MAF and antiviral activities by this resin. Four-ml samples of the hybridoma supernatant were incubated with increasing amounts of packed Matrex Gel Red A and the resulting supernatant titrated for residual MAF and antiviral activities. As a control, the total protein content of the absorbed supernatants was also measured. Figure 6 demonstrates that a concomitant dose dependent absorption of MAF and IFN activities occurred. Fifty percent of either activity was absorbed by 6 μl of packed gel and 30 μl of gel was sufficient to remove 90–100% of the activities. This result is clearly distinct from the absorption of total protein where only 3% of the protein was removed by 30 μl of gel.

We next utilized the Matrex Gel Red A on a preparative scale. Eight hundred fifty ml of a hybridoma supernatant was concentrated 10-fold and then subjected to dye-ligand chromatography on a 12-ml column of Matrex Red (Fig. 7 and Table II). Ninety percent of the applied protein emerged from the column unretarded and an additional 2.8% eluted with 0.5 M NaCl. These

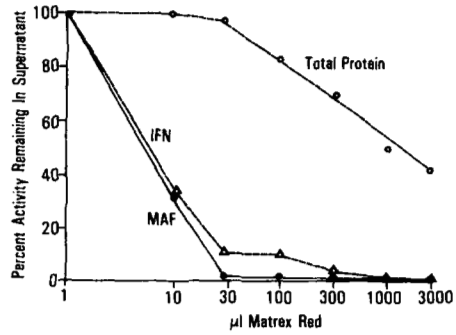

Figure 6. Dose-dependent absorption of MAF and antiviral activity by Matrex Gel Red A. Varying amounts of packed Matrex Gel Red A were incubated 1 hr at 4°C with 4-ml samples of a stimulated clone 24/G1 supernatant. The gel was removed by centrifugation and filtration of the samples through 0.2-μ sterile filters. Protein was determined by Folin analysis.

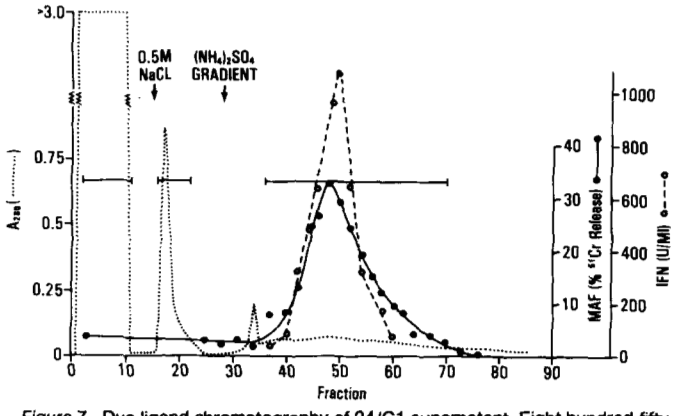

Figure 7. Dye-ligand chromatography of 24/G1 supernatant. Eight hundred-fifty ml of a stimulated hybridoma supernatant was concentrated 10-fold and subjected to chromatography on a 1.5 × 7.0-cm column of Matrex Gel Red A at 4°C as described in *Materials and Methods*. Flow rate was 24 ml/hr and 10-ml fractions were collected. *Horizontal bars* represent column pools.

TABLE II

MAF and IFN yields from Matrex Red column

Material	Protein		MAF		IFN	
	mg	% Recovery	U × 10^{-5}	% Recovery	I U × 10^{-4}	% Recovery
Starting	2791		42.5		13.6	
Breakthrough	2521	90.3	1.9	4.4	0.2	1.5
NaCl Pool	79	2.8	1.7	4.1	0.1	0.7
$(NH_4)_2SO_4$ Pool	2	0.07	45.4	107.0	4.3	31.6

fractions contained little MAF or antiviral activity. Elution of the column with an ammonium sulfate gradient effected the concomitant desorption of both MAF and IFN activities. When protease inhibitors (such as PMSF and ε-amino caproic acid) were incorporated into the gradient buffer, 100% of the applied MAF activity and only 0.07% of the total protein was recovered. This constituted a 1500-fold purification of the activity. Only one-third to one-half of the antiviral activity was present in the ammonium sulfate pool. The remainder of this activity was not recovered. The MAF-containing pool was concentrated to 1.6 ml and exhibited stability at 4°C for at least 1 week or indefinitely at −70°C.

HPLC gel filtration of Matrex Red Purified MAF and IFN. A portion (0.5 ml) of the concentrated pool from the dye column was subjected to gel filtration analysis using HPLC. Figure 8 shows that the elution profiles of the MAF activity and the antiviral activity were virtually superimposable on one another but were distinct from that of the other proteins present in the pool. On the basis of marker analysis, both activities displayed a m.w. of 50,000. Table III demonstrates that pool 5 from the column contained 43% of the applied MAF activity and 67% of the applied antiviral activity but only 10% of the protein. Thus, another 5- to 10-fold purification had been achieved. Overall yields of both activities (54% for MAF and 77% for IFN) were not affected when samples of the six-column pools were mixed, indicating that separation of two or more interdependent factors had not occurred.

HPLC chromatofocusing of Matrex Red Purified MAF and IFN. Although the experiments presented thus far indicated that the MAF activity present in the hybridoma supernatant was indistinguishable from a portion of the IFN antiviral activity, they could not be considered conclusive because the biochemical techniques involved were capalbe of only relatively low resolution. Such techniques would fail to resolve families of closely related

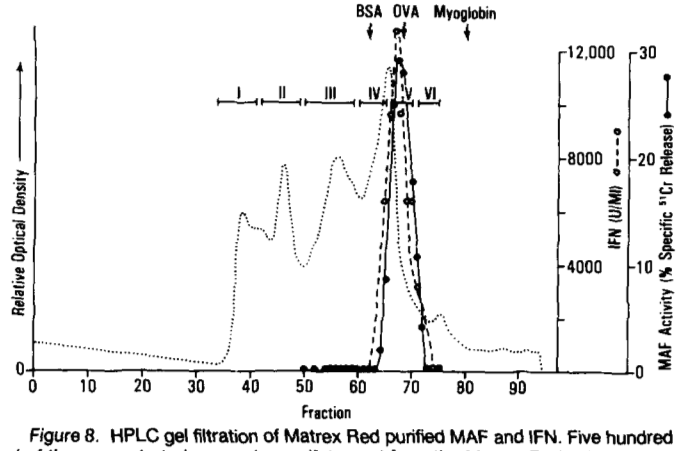

Figure 8. HPLC gel filtration of Matrex Red purified MAF and IFN. Five hundred μl of the concentrated ammonium sulfate pool from the Matrex Red column were injected to a 7.5 × 600-mm BioSil TSK-250 column and eluted at 0.2 ml/min at 23°C with 0.2 M ammonium formate buffer, pH 7.3, as described in *Materials and Methods*. Fraction size was 250 μl. *Horizontal bars* represent column pools.

TABLE III

MAF and IFN recoveries after HPLC gel filtration

HPLC pool	Protein recovery		MAF recovery		IFN recovery	
	μg	%	U × 10^{-3}	%	I U × 10^{-2}	%
Unfractionated	1584		750.0		150.0	
1	120	7.6	0.5	0.07	0	0.0
2	320	20.0	1.5	0.20	0	0.0
3	450	28.4	3.7	0.50	0.1	0.4
4	345	21.8	48.0	6.4	4.8	3.2
5	162	10.2	320.0	42.7	100.0	66.7
6	87	5.5	30.0	4.0	10.0	6.7
Total	1484.5	93.7	403.7	53.9	114.9	77

but distinct proteins such as the immunoglobulins or the complement proteins C3 and C5. We, therefore, subjected the Matrex Gel Red A MAF pool to the biocheical separation technique which offered the highest resolution available: chromatofocusing on an HPLC system. Seventy-five μl of the pool was diluted to 2 ml with starting buffer and injected into a Mono P column. Elution was accomplished by generation of an isoelectric gradient between the values of pH 7.0 and 5.0. When the limit pH was reached, the column was stripped with buffer containing 1 M NaCl. Figure 9 shows that the proteins in the pool were resolved into a number of distinct species. A substantial quantity of the proteins had isoelectric points below 5.0 and were eluted from the column only after application of buffer which contained 1 M NaCl. In other experiments (not shown), nonspecific adsorption of most proteins to the chromatofocusing resin was not observed and elution at the protein's isoelectric point was found to be quantitative. MAF activity eluted over a relatively broad range in a complex pattern that centered around an isoelectric point of 5.8. Some MAF activity also eluted with 1 M NaCl at the end of the gradient indicating that MAF behaved in heterogeneous fashion on this column. The elution profile of the antiviral activity was virtually identical to that of the MAF activity, both quantitatively as well as qualitatively. When pools were made from the fractions and titrated in the respective biological assays, nearly identical recoveries were obtained for each activity in each pool (Table IV). The overall recovery of the MAF activity (76%) was almost quantitative and was in close agreement to the recovery of the antiviral activity (85%).

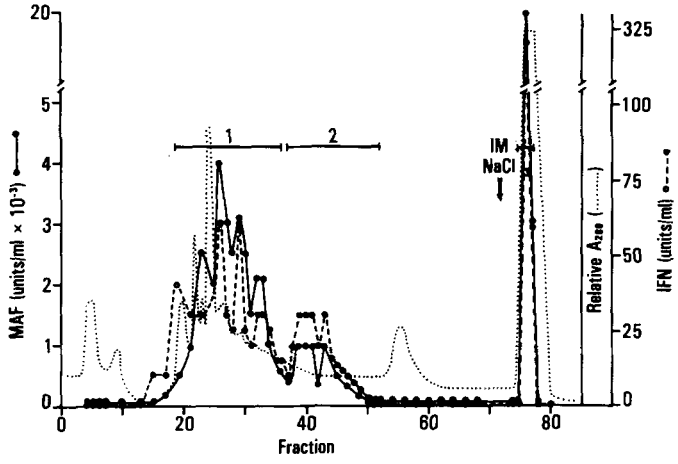

Figure 9. HPLC chromatofocusing of Matrex Red purified MAF and IFN. Seventy-five μl of the concentrated ammonium sulfate pool from the Matrex Red column was diluted to 2 ml with 0.025 M *bis*-Tris-HCl, pH 7.1, and injected into a 7.5 × 300-mm Mono P column at 23°C. Elution was performed at 0.5 ml/min with Polybuffer 74 diluted 1:10, pH 5.0. Fraction size was 1 ml. At the end of the pH gradient, 2 ml of buffer containing 1 M NaCl was injected into the column and elution was contained for an additional 15 min.

TABLE IV

Recovery of MAF and IFN activity after HPLC chromatofocusing

Pool	MAF		IFN	
	U × 10^{-3}	% Recovered	U × 10^{-2}	% Recovered
1	115	30.0	54	45.0
2	51	13.3	8	6.7
3	128	33.3	40	33.3
1–3 Calculated	294	76.6	102	85
1–3 Titrated	307	80.0	N.D.	ND[a]
Unfractionated	384		120	

[a] ND, not determined.

DISCUSSION

The data presented in this report provide compelling molecular evidence that the activity produced by the T cell hybridoma clone 24/G1 which primes macrophages for nonspecific tumoricidal activity is IFNγ. This conclusion is supported by the results of both biosynthetic and biochemical investigations. Production of MAF by the hybridoma clone was always found to be proportional to the production of antiviral activity under a variety of culture conditions. When the unexpected observation was made that production of MAF activity ceased at hybridoma cell concentrations which were only one-half of saturation density, the identical situation was also found to be true for the antiviral activity. Both activities displayed quantitatively identical sensitivities to pH and temperature even when experimental conditions were employed which resulted in only the partial loss of either activity (such as incubation at 52°C). Both activities bound to polynucleotide columns or to columns composed of the insolubilized dye, Matrex Gel Red A, under conditions where almost all (88–99%) the other proteins present in the culture supernatant, did not. Both activities displayed virtually identical elution profiles and recoveries on HPLC separations based on either molecular weight or isoelectric points. Most significantly, despite the fact that the HPLC chromatofocusing step resolved the MAF activity into seven chromatographically distinct species, it was unable to separate the MAF and antiviral activities on either a qualitative or quantitative basis.

Although these results lend strong support to the conclusion that IFNγ can act as a MAF, they do not indicate that it is the only molecular species which can display MAF activity. The studies performed here were largely confined to the activity produced by a particular clone (24/G1) of a T cell hybridoma; parenthetically, at an early point in our studies with this clone we were unable to detect antiviral activity in its supernatants which nevertheless displayed significant macrophage-activating properties. Only later, as the clone stabilized did it become obvious that both antiviral and MAF activities were produced concomitantly. Although we have previously shown that the MAF activity derived from this clone was similar to MAF produced by a heterogeneous population of normal murine splenic cells (11), the possibilities must still be considered that distinct T cell populations can produce, under defined conditions, distinct MAF molecules or that different MAF molecules are required to induce killing of different target cells. Such may be the case for a lymphokine produced by a subclone of a continuous T cell line, denoted EL-4 (Farrar) which was found to be devoid of antiviral activity but which could prime macrophages for nonspecific tumorilytic activity toward the fibrosarcoma cell line TU5 (16). This activity displayed properties which distinguished it from classical IFNγ including stability to pH 2, a significantly lower apparent m.w. (24,000), and a lack of inhibition by polyvalent rabbit antisera directed to murine IFNγ. However, this factor has yet to be found in supernatants of stimulated murine splenic cell cultures.

In the past, a great deal of uncertainty has developed as to the relationship between MAF and IFNγ. Reports exist which conclude that the two activities arise from distinct molecular entities. Two other murine T cell hybridomas have been identified which produced MAF activity in the apparent absence of antiviral activity (17–19). However, one of these hybridomas produced MAF only in limited amounts (17, 18) and, therefore, the question arises as to whether the antiviral activity was missed due to a difference in sensitivity of the MAF and IFN assays. Neither study attempted fractionation or mixing experiments of the hybridoma

supernatants to examine the possibility that expression of the IFN antiviral activity was blocked due to the presence of a secondary inhibitory activity. Kniep *et al.* (15) reported that the MAF activity from a normal T cell culture supernatant did not bind to polynucleotide-Sepharose columns but that the antiviral activity did. This observation could not be confirmed in our studies using either the hybridoma-derived MAF or conventional MAF preparations or in studies by others (20). In contrast to these studies, other reports have indicated that MAF and antiviral activities were indistinguishable from one another (20–23). This conclusion was based on either low resolution biochemical techniques such as conventional gel filtration or sensitivities to pH or temperature or on biosynthetic approaches utilizing cultures consisting of either normal, heterogeneous T lymphocytes or T cell clones. However, these same techniques would fail to resolve two closely related but distinct molecular entities such as the complement proteins C3 and C5. These two proteins are synthesized by the same cells, are identical in molecular weight and polypeptide chain composition, and display identical sensitivities to heat and pH (reviewed in Reference 24).

The experiments performed in this current study have produced some of the first quantitative molecular evidence to support a role for IFNγ in activation of macrophages for tumor cell killing. These studies were only possible due to recent advances in cell culture and biochemical technologies namely: 1) the ability to construct murine T cell hybridomas which could be used to examine lymphokine production on a clonal basis, and 2) the availability of new biochemical separatory media such as HPLC columns designed specifically for protein separations which could add a new dimension to lymphokine purification. The results of these studies have confirmed the recognized physiochemical properties of MAF or IFNγ including pH and heat sensitivities, molecular weight, and isoelectric points (11, 15, 25–28). They have, in addition, shown that the two activities are qualitatively and quantitatively identical to one another even when separations were attempted utilizing high resolution biochemical techniques. These techniques have allowed for a 10,000- to 20,000-fold purification of the MAF activity while still preserving over 40% of the original activity. They have also clearly demonstrated that while charge heterogeneity exists for the molecular species which act as MAF, the virtually identical heterogeneity exists for the molecules which display antiviral activity. The recovery of each activity after such a chromatofocusing step was nearly quantitative (80–85%). It is difficult to conceive of any possibility other than that of the molecular identity of MAF and IFNγ to explain such a result. To date, only one other recent study has attempted the biochemical separation of MAF and IFNγ using a variety of sequential steps (20). This report also concluded that MAF and IFNγ activities were inseparable. This conclusion will also be supported by work to be published elsewhere[6] which shows that IFNγ produced by recombinant DNA technology, can display MAF activity, that MAF and IFNγ are inhibited by a highly specific polyclonal rabbit antiserum to murine IFNγ, and that IFNα or IFNβ display 10^2 to 10^3 times less macrophage-activating properties than IFNγ.

The results of the Matrex Gel Red A chromatography experiment suggest that not all species of IFNγ may display MAF activity. This conclusion is based on the observation that 100% of the MAF activity, but only one-third to one-half of the antiviral activity, was recovered after dye-ligand chromatography. However, once this separation had occurred, the residual antiviral activity present in the MAF pool was not dissociable from MAF. It is unlikely that the different functional forms represent distinct gene products, because only one gene has been identified for IFNγ (29).

There are a number of possible explanations for the aforementioned results. These include the chromatographic removal or concentration of unrecognized inhibitors of either the MAF or antiviral activities or the possibility that the MAF and antiviral activities are effected by distinct domains on IFNγ which differ in their chemical stability. It is also possible that minor modifications to the molecular structure of IFNγ such as limited proteolysis could allow for the expression of distinct biological activities in the absence of gross physiochemical alterations. Thus, while all forms of IFNγ may possess antiviral activity, perhaps only a certain form(s) can display MAF activity. Conversion of one form to another could, however, be under genetic regulation due to the production of an enzyme responsible for the conversion or to glycosidation of the molecule which might influence its convertibility. This hypothesis is consistent with the observation that while MAF and antiviral activities produced by a T cell clone always correlated with one another, they showed a considerable degree of variation when different T cell lines or clones are used. Such a hypothesis may also explain the interrelationship of IFNγ with migration inhibition factor and the macrophage Ia$^+$ recruiting factor (30–31). Thus, distinct forms of IFNγ, which arise post-synthetically, may be required for regulation of a variety of macrophage biological activities. Work is currently underway to examine these possibilities.

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