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Immunology

Macrophage activation: Priming activity from a T-cell hybridoma is attributable to interferon-γ

(mononuclear phagocytes/tumor cell killing/macrophage-activating factor/lymphokine/association of antiviral and activating activities)

JUDITH L. PACE*, STEPHEN W. RUSSELL*, ROBERT D. SCHREIBER†, AMNON ALTMAN†, AND DAVID H. KATZ‡

*Department of Comparative and Experimental Pathology, University of Florida, Gainesville, Florida 32610; †Department of Immunology, Research Institute of Scripps Clinic, and ‡The Medical Biology Institute, La Jolla, California 92037

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ABSTRACT **Antiviral and macrophage-priming activities in the supernatant medium of a subclone of a concanavalin A-stimulated mouse T-cell hybridoma were investigated. The two activities were associated with a molecular weight of approximately 50,000 and could not be separated by various approaches. Both activities were eliminated by a highly specific neutralizing antibody against mouse interferon-γ, but not by antibody against interferon-α and -β. The ratio of priming to antiviral activity in the hybridoma culture supernate was indistinguishable from the ratio obtained with mouse interferon-γ prepared by recombinant DNA technology. It was concluded from these data that the priming activity in hybridoma culture supernates was attributable to interferon-γ and that this mediator is one form of the lymphokine macrophage-activating factor. Interferon-γ was greater than 800 times more efficient at priming mouse macrophages for tumor cell killing than was a mixture of interferon-α and -β. This finding contributes to growing awareness that type II interferon may have greater immunoregulatory potential than type I interferons.**

Macrophages can be activated to kill tumor cells *in vitro* by a nonspecific, extracellular mechanism that may be important in host defense against neoplastic cells *in vivo*. An important goal, therefore, is acquisition of better understanding of how the process of activation is regulated. Recently, it has been shown that under defined conditions the lymphokine macrophage-activating factor (MAF) does not render macrophages fully cytolytic (1–5). Instead, it heightens their responsiveness to a second signal(s) that then triggers the expression of killing—i.e., MAF "primes" macrophages to respond to a second, triggering stimulus.

A cloned hybridoma (24/G1) has been recognized that produces MAF, identified by its ability to prime mouse macrophages for tumor cell killing (6). The hybridoma-derived MAF was shown by various biochemical and functional criteria to be indistinguishable from conventionally prepared MAF (6). These supernates also contained antiviral activity attributed to interferon (IFN)-γ.

We report here that the antiviral and macrophage-priming activities produced by cells of a subclone of 24/G1 are not dissociable by various approaches. These results, together with data obtained by using recombinant mouse IFN-γ, support a conclusion that priming activity produced by this hybridoma is attributable to IFN-γ. We also report here that IFN-γ is much more potent as a macrophage priming agent than type I interferon is.

MATERIALS AND METHODS

Mice. Male C3H/HeN mice were obtained from Charles River Breeding Laboratories (Kingston, NY) and used at 6–9 weeks of age.

Tissue Culture Media and Reagents. Modified Eagle's minimal essential medium (Auto-Pow ME medium) was prepared from a powdered mix and supplemented with sodium bicarbonate at 2 mg/ml, 2 mM glutamine (all from Flow Laboratories), injectable penicillin G potassium at 100 units/ml, injectable streptomycin sulfate at 100 μg/ml (both from Pfizer), and 15 mM Hepes (Sigma) (H-ME medium). Fetal bovine serum was obtained from Sterile Systems (Logan, UT). Dulbecco's modified minimum essential medium (D-ME medium) supplemented with 5% fetal bovine serum, antibiotics, and 50 μM 2-mercaptoethanol was used to culture the mouse T-cell hybridoma. All tissue culture media and the serum used in this study were negative for endotoxin, as determined by assay (7) with *Limulus* amebocyte lysate (Associates of Cape Cod, Woods Hole, MA); assay sensitivity was 0.1 ng of endotoxin per ml.

T-Cell Hybridoma Culture Supernates. Derivation of the T-cell hybridoma clone 24/G1 has been described (6, 8, 9). Briefly, alloantigen-activated T-cell blasts were fused with T lymphoma BW5147. The hybrid cells were recloned in the laboratory of one of us (J.L.P.) by limiting dilution (0.5 cell per well; microscopic confirmation that each outgrowth came from a single cell). When used for lymphokine production, the cells were seeded at either 1×10^6 or 2×10^6/ml in serum-free medium and stimulated for 24 hr with concanavalin A at 5 μg/ml (Pharmacia). Lymphokine-rich culture supernates were recovered by centrifugation (500 × *g*, 20 min, 4°C). After filtration (0.45-μm pore diameter), they were stored at 4°C. Control culture supernates were similarly prepared, except that concanavalin A was added after the cells had been removed.

Peritoneal Macrophages. Peritoneal exudate cells (PEC) were harvested by lavage from mice that had been injected intraperitoneally 3 days previously with 1.5 ml of 10% protease peptone (Difco). Monolayers were prepared as described (5) by seeding 0.2 ml of the PEC suspension in H-ME medium/10% fetal bovine serum (2×10^5 macrophages, as determined by using Wright-stained cytocentrifuge preparations) into each flat-bottomed well of a 96-place tissue culture plate (Costar, no. 3596). The cells were incubated for 2 hr at 37°C in 5% CO_2/95% air, washed vigorously with H-ME medium to remove nonadherent cells, and used immediately.

Assay for Macrophage Priming or Activation. In this paper a fully activated macrophage is operationally defined as one that has acquired the ability to kill tumor cells. Macrophage priming is defined as partial activation to a noncytolytic state in which macrophages can be made to kill tumor cells by exposing them to a second, triggering signal. The 16-hr ^{51}Cr-release assay of macrophage-mediated cytolysis (5) that was used in these experiments has the capacity to detect and to distinguish between

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Abbreviations: MAF, macrophage-activating factor; IFN, interferon; PEC, peritoneal exudate cells; LPS, bacterial lipopolysaccharide from *Escherichia coli* 0111:B4; ME medium, minimal essential medium.

full activation and priming. Briefly, peritoneal macrophage monolayers were preincubated for 4 hr with 0.1 ml of various dilutions of hybridoma culture supernate, with or without bacterial lipopolysaccharide (LPS) at 3 ng/ml. The LPS, phenol-extracted and purified (lipid A-rich fraction II) from *Escherichia coli* 0111:B4 (10), was a gift from D. C. Morrison (Dept. of Microbiology, Emory Univ. School of Medicine, Atlanta, GA). ^{51}Cr-labeled (as sodium chromate, 250 μCi/mg of Cr; 1 Ci = 3.7×10^{10} Bq; Amersham) P815 mastocytoma cells (2×10^4) were then added in 0.1 ml. Sixteen hours later the uppermost 0.1 ml of supernate was removed from each well and assayed in an automatic gamma spectrometer. Samples were assayed in duplicate. Percentage specific ^{51}Cr release was calculated as described (5). Results are expressed in terms of cytolytic units, 1 unit of activity being defined as the amount of lymphokine, alone or with added LPS, that would cause 50% of the maximal specific ^{51}Cr release (5). In this assay, full activation to a cytolytic state would be detected in wells without added LPS, and priming by lymphokine—i.e., partial activation to a noncytolytic state—would be detected in the presence of the triggering dose of added LPS.

Antiviral (IFN) Assay. IFN was assayed by plaque-reduction in monolayers of vesicular stomatis virus (VSV)-infected mouse L-929 cells. The assay was performed as described (11), except that 6.4-mm-diameter flat-bottomed wells in 96-place tissue culture plates were used. IFN activity was expressed in terms of NIH reference units with mouse fibroblast IFN as the reference standard (1.4×10^5 NIH reference units/ml; specific activity, 2.7×10^5 reference units/mg of protein; Lee Biomolecular Research Laboratories, San Diego, CA; no. 20051). One unit of activity was defined as the amount of IFN that caused 50% reduction in the number of plaques.

Gel Filtration on Sephadex G-100. Sephadex G-100 (Pharmacia) columns (bed size, 2.5×80 cm) were equilibrated and maintained endotoxin-free at 4°C under ascending flow of 0.02 M Hepes, pH 7.2/0.1 M α-methyl D-mannoside/1 M NaCl/0.02% sodium azide. The columns were calibrated by using bovine serum albumin (67,000), ovalbumin (45,000), chymotrypsinogen A (23,000), and cytochrome *c* (12,500) markers. Fractionation of supernates began with the concentration (approximately 50×) of 250 ml of supernate on a YM-10 ultrafiltration membrane (Amicon). Columns were operated at a flow rate of 25 ml/hr. Fractions (3 ml each) were collected in sterile polypropylene tubes. In all tubes in which either or both activities were detected in qualitative screening assays, antiviral or macrophage priming/activating activity, or both, was quantified.

Heat-Stability Studies. Three-milliliter samples of control or lymphokine-rich culture supernates were incubated for various periods of time, as indicated in the text, at either 56°C or 80°C in a constant-temperature bath with circulator. At each time point a sample tube was removed, rapidly cooled in an ice bath, and then assayed for residual macrophage priming/activating and antiviral activities.

Neutralization Studies. Sheep antiserum to a mixture of mouse type I IFNs (α, 20%; β, 80%) (Newcastle disease virus-induced, L-cell origin; no. G-024-501-568) and the appropriate control serum (no. G-025-501-568) were obtained from the Research Resources Branch, National Institute of Allergy and Infectious Diseases (Bethesda, MD). Rabbit globulin against mouse type II IFN (γ or immune) was a gift from H. M. Johnson (Dept. of Microbiology, Univ. of Texas Medical Branch, Galveston, TX). It was produced by stimulating spleen cells with staphylococcal enterotoxin A and was processed as described (12). Normal rabbit globulin prepared in a similar fashion was used as the control. The immune globulin had no detectable activity against mouse migration inhibitory factor, macrophage chemotactic factor, or granulocyte/macrophage colony-stimulating factor but did contain small amounts of activity against mouse lymphotoxin (H. M. Johnson, personal communication).

Each of the antisera described above was titered against both type I and type II IFNs. Little or no crossreactivity could be detected when antiserum made against one type of IFN was assayed against the other. The dilution of each antiserum that would just neutralize the antiviral activity of the kind of IFN against which it had been made was then used to determine what effect that amount of antibody would have on macrophage priming activity that was contained in 24/G1.5 supernates. Samples were incubated with either the diluted antibody or similarly diluted control for 3 hr at 37°C, with occasional agitation. Assay of antiviral or macrophage priming/activating activities remaining in the treated supernates followed.

Recombinant IFN-γ. The recombinant mouse IFN-γ used in these studies was generously supplied by Genentech (South San Francisco, CA). The basic approach used in producing the IFN-γ by recombinant DNA technology has been described (13). Details for the mouse include the introduction of the appropriate cDNA by transfection into transformed monkey cells of the line COS-7, after which culture supernates were assayed for evidence of antiviral activity (P. W. Gray and D. V. Goeddel, personal communication). The supernate used in these studies, produced in the manner described, contained 1,000 units/ml.

RESULTS

Macrophage Priming/Activating and Antiviral Activities in Supernates. Cells of T-cell hybridoma clone 24/G1 did not produce macrophage priming, activating, or antiviral activities constitutively (Table 1). Stimulation of the cells with concanavalin A did induce the production of lymphokine, however. Direct activation of macrophages to a cytolytic level was not found. By contrast, macrophages treated with supernatant culture medium and LPS (3 ng/ml) did kill. Therefore, the mitogen-induced lymphokine had a priming rather than a full activating effect. LPS was not required to obtain an antiviral effect and did not augment it. Also, the amounts of the two activities produced were dependent on cell concentration; a doubling of cell number from 1×10^6/ml to 2×10^6/ml increased each activity by the same amount, approximately 4-fold.

Table 1. Macrophage priming/activating and antiviral activities in culture supernates from T-cell hybridoma 24/G1

	Priming/activating†		Antiviral‡	
Cells, no./ml*	With LPS§	No LPS	With LPS§	No LPS
	Unstimulated			
1×10^6	0	0	0	0
2×10^6	0	0	0	0
	Stimulated with concanavalin A			
1×10^6	1,450	0	379	379
2×10^6	5,882	0	1,575	1,412

* T-cell hybridoma 24/G1 cells were seeded into serum-free D-ME medium containing 50 μM 2-mercaptoethanol in 25-cm^2 tissue culture flasks (7 ml per flask) and cultured for 24 hr with or without concanavalin A (5 μg/ml). Unstimulated cells were cultured under similar conditions for a similar time but without mitogen. Concanavalin A (5 μg/ml) was then added to supernates after cells had been removed.

† Cytolytic units/ml, as determined by assay of macrophage-mediated ^{51}Cr release from P815 mastocytoma cells (5).

‡ NIH reference units/ml of IFN activity, as measured in a plaque-reduction assay (11) modified for 6.4-mm-diameter flat-bottomed wells.

§ Fraction II (3 ng/ml) of phenol-extracted *E. coli* 0111:B4 (10).

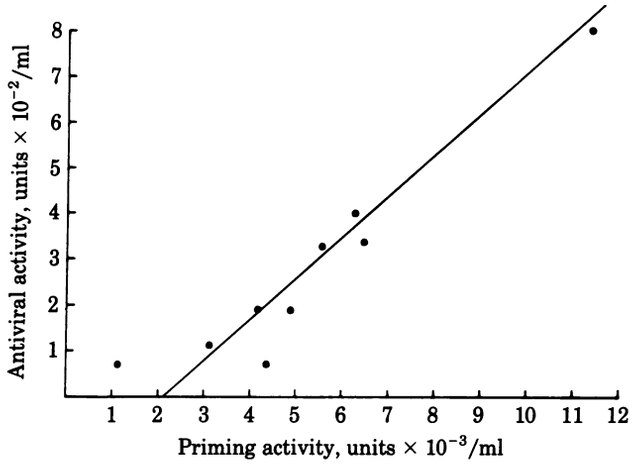

FIG. 1. Comparison of macrophage priming and antiviral activities in supernates of 24/G1 subclones after cells of each had been stimulated for 24 hr in serum-free medium with concanavalin A (5 μg/ml). The line of best fit was obtained by linear regression analysis. The coefficient of correlation (r) is 0.949.

Quantification of Antiviral and Priming Activities Produced by Subclones of 24/G1. No subclone produced one of the activities without the other (Fig. 1). In fact, production levels of the two activities paralleled each other with a high degree of correlation (r = 0.949). In multiple experiments, subclone number 5 (24/G1.5) was consistently the best producer. The remainder of this work therefore was based on the activities produced by this subclone.

Sensitivity of Activities to Heat in Serum-Free Medium. Incubation at 56°C caused the concomitant loss of both activities (Fig. 2; r = 0.975). Both activities were eliminated completely by heating for 5 min at 80°C (data not shown).

Molecular Sieve Chromatography. The molecular weight of the material associated with each activity was estimated by Sephadex G-100 chromatography. Two separate batches of concentrated (50×) supernate were fractionated, each on a different column but bed volumes were similar. Activity in individual 3-ml fractions was quantified by both the antiviral and macrophage priming assays. In each of the two fractionations, single symmetrical peaks were obtained. In each case the elution profiles for the two activities were superimposed. The mean (± SEM) estimated molecular weight from five separate assays was 51,117 ± 1,341 for macrophage priming activity and 51,402 ± 1,292 for the antiviral activity.

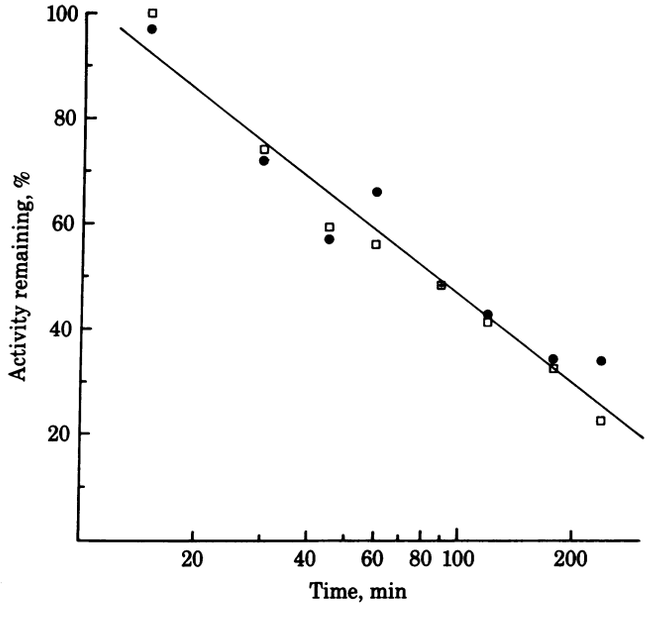

FIG. 2. Incubation of serum-free supernate from subclone 24/G1.5 at 56°C. The macrophage priming (□) and antiviral (●) activities remaining at various times after the samples came to temperature have been plotted. The line of best fit for all of the points was obtained by linear regression analysis. The coefficient of correlation (r) is 0.975.

Table 2. Effect on antiviral and macrophage priming activities of antibody against IFN-α/IFN-β or IFN-γ

Material tested	Activity in supernates: Priming*	Activity in supernates: Antiviral†
24/G1.5, incubated with:		
Anti-IFN-γ control‡	10,530	120
Anti-IFN-γ	<100	0
Anti-IFN-α/IFN-β control‡	12,820	125
Anti-IFN-α/IFN-β	14,090	110
IFN-α/IFN-β incubated with:		
Anti-IFN-γ control‡	<100	100
Anti-IFN-γ	<100	86
Anti-IFN-α/IFN-β control‡	<100	130
Anti-IFN-α/IFN-β	<100	0

* Cytolytic units/ml measured in a 16-hr ^{51}Cr-release assay using P815 mastocytoma cells as targets (5).
† NIH reference units/ml of IFN activity, as measured in a plaque reduction assay (11) modified for 6.4-mm-diameter flat-bottomed wells.
‡ Normal serum processed similarly to relevant antiserum.

Effect of Anti-IFN Antibodies. To this point we had been unable to separate the macrophage priming and antiviral activities in supernates of 24/G1.5 by any of the means tried. These results suggested that priming activity was being mediated by IFN-γ. To test this hypothesis, experiments were performed with antibody made against either type I (mixture of IFN-α and IFN-β) or type II (IFN-γ). Antibody against IFN-γ eliminated both the macrophage priming and antiviral effects of the 24/G1.5 supernate (Table 2). By contrast, anti-IFN-α/IFN-β had little or no effect on either activity, although the antiviral activity of the mixture IFN-α/IFN-β was neutralized by this reagent completely and consistently. Repeated analyses showed that any slight inhibitory effects obtained when one kind

Table 3. Ratios of priming and antiviral activities associated with subclone 24/G1.5 supernate and recombinant IFN-γ

Source of activity and treatment	Priming activity,* units/ml	Antiviral activity,† units/ml	Ratio of activities, priming/antiviral
24/G1.5 + medium	2,230	58	38
Recombinant IFN-γ + medium	3,430	83	41
24/G1.5 + control serum‡	2,310	52	44
Recombinant IFN-γ + control serum‡	3,590	79	45
24/G1.5 + anti-IFN-γ	0	0	—
Recombinant IFN-γ + anti-IFN-γ	0	0	—

* Cytolytic units/ml, measured in a 16-hr ^{51}Cr-release assay with P815 mastocytoma cells as targets (5).
† NIH reference units/ml for IFN activity determined in a plaque-reduction assay (11) modified for 6.4-mm-diameter flat-bottomed wells.
‡ Normal serum processed similarly to relevant antiserum.

Table 4. Comparison of macrophage priming efficiencies of IFN-α/IFN-β and IFN-γ (24/G1.5)

IFN type	Activity		Cytolytic units/ antiviral unit
	Priming*	Antiviral†	
IFN-γ (24/G1.5)	19,230	280	68
IFN-α/IFN-β	11,628	140,000	0.08

* Cytolytic units/ml measured in a 16-hr ^{51}Cr-release assay with P815 mastocytoma cells as targets (5).

† NIH reference units/ml for IFN activity as measured in a plaque-reduction assay (11) modified for 6.4-mm-diameter flat-bottomed wells.

of anti-IFN was tested on IFN of the opposite type—e.g., anti-type I on type II IFN—apparently were vagaries of the assay because they were not reproducible.

Comparison of Priming Activities Produced by Subclone 24/G1.5 and Recombinant IFN-γ. To establish further that the priming activity in the supernates of subclone 24/G1.5 was due to IFN-γ, in the same experiment we compared the ratio of priming to antiviral activity for 24/G1.5 supernate and recombinant mouse IFN-γ. The ratios were similar, and the activities of both the hybridoma-produced and recombinant IFNs could be neutralized by antibody against native IFN-γ (Table 3).

Comparison of Macrophage Priming Efficiencies of IFN-α/IFN-β and IFN-γ (24/G1.5). There were between 10^4 and 1.5×10^4 units of macrophage priming activity in the hybridoma supernate (Table 2); the amount of this activity that was detectable in the IFN-α/IFN-β mixture was below the level that was quantifiable. Yet, the amounts of antiviral activity in the two IFN preparations were similar. These data suggested that the priming efficiencies of the two types of IFN might differ. This hypothesis was tested directly. As suspected, IFN-γ was more efficient, per unit of antiviral activity, at priming macrophages than was the mixture (approximately 850 times) (Table 4).

DISCUSSION

We conclude from these data that IFN-γ can prime murine macrophages for tumoricidal activity. This conclusion is based on the following criteria. (*i*) Correlation was always found between the amounts of antiviral and macrophage priming activities in the supernates of T-cell hybridoma subclones. (*ii*) The molecular weight associated with each activity was the same, as estimated by molecular sieve chromatography, and was within the range of molecular weights that has been reported for mouse IFN-γ (14). (*iii*) A highly specific polyclonal antibody made against mouse IFN-γ neutralized both antiviral and macrophage priming activities, whereas an antibody made against a mixture of IFN-α and IFN-β had no effect. (*iv*) The ratio between the priming and antiviral activities in the hybridoma culture supernate was indistinguishable from that determined for recombinant mouse IFN-γ. Recent reports by others showing that the priming and antiviral activities in culture supernates of mixed spleen cell populations are associated (15), or even copurify (16), further support our conclusion.

Previous work has shown that type I IFN can activate macrophages to express cytotoxicity against tumor cells (17, 18). In all instances, relatively large amounts (measured in antiviral units) were required to effect activation. We have shown here that large amounts of type I IFN are also needed to effect priming. By comparison, very little IFN-γ was needed, showing that type II was much more effective as a priming agent compared to a mixture of type I IFNs. While this manuscript was in preparation, Roberts and Vasil (16) made a similar comparison using IFN-γ derived from concanavalin A-stimulated spleen cells. Their IFN-γ was approximately 1,000 times more effective at activating macrophages than IFN-β was. This value is in excellent agreement with the one derived here, 850 times, by using hybridoma-produced IFN-γ. Similar conclusions regarding the relative immunomodulatory activity of the two IFN types have been reached by other investigators using different assays (19–24). Taken together these findings support the growing impression that the immunoregulatory functions of IFN-γ may be more important than its antiviral function.

Finally, in light of the findings reported here, one is led to ask whether or not IFN and MAF are identical. Certainly, attention has previously been called to the striking functional and physicochemical similarities that exist between these two mediators (15, 25), and the fact that they copurify (ref. 16; unpublished data). Furthermore, Kelso *et al.* (26) have not been able to identify T-cell clones that produce antiviral or activating activity independently of each other. However, on the basis of what is reported here, we cannot conclude that MAF is IFN, especially in view of recent claims that, under some circumstances, activating and antiviral activities can be separated (27–30). We can conclude, however, that IFN-γ is at least one form of MAF, as it has been defined operationally. Resolution of the question as to whether or not there are other MAFs awaits the completion of biochemical studies.

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