BIOLOGICAL AND ANTIGENIC SIMILARITIES OF MURINE INTERFERON-γ AND MACROPHAGE-ACTIVATING FACTOR

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The multiple biological activities present in semipurified lymphokine preparations have made it difficult to assign discrete biological functions to each lymphokine. As a result, the large number of identified lymphokine activities may actually reflect the manifestations of a few factors. While this research has also been hampered by the limited quantities of lymphokines available, hybridoma and recombinant DNA technologies have begun to help overcome these limitations.

Macrophage activation has been intensively investigated because it is generally agreed that activated macrophages play an essential role in the defense against microorganisms and in the immune response against neoplasia (1). Macrophage activation mediated by macrophage activation factor (MAF)[1] and gamma interferon (IFN-γ) has been characterized by similar morphologic, metabolic, and functional changes (2–6) including stimulation of nonspecific tumoricidal activities (5), induction of Ia antigen expression (7, 8), increased Fc receptor expression (9, 10), production of plasminogen activator (11), and production of hydrogen peroxide (12). Several investigators (5, 8, 12, 13) have postulated therefore that IFN-γ and MAF may be identical. In support, Schreiber et al. (14) have recently demonstrated biosynthetic and biochemical similarities of IFN-γ and MAF produced by a murine T cell hybridoma (24/G1). Both the antiviral and MAF activities of 24/G1 cultured supernatants were neutralized by anti-IFN-γ, but not anti-IFN-α and anti-IFN-β (15). Although previous investigations (15–17) have demonstrated neutralization of MAF activity with polyclonal anti-IFN-γ, definitive results could not be ascertained since these antisera were prepared from partially purified preparations and could contain antibodies that neutralize other lymphokine activities.

In the present report, murine IFN-γ produced by recombinant DNA techniques (18) (>99% pure) was tested for MAF activity; special attention was given

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[1] *Abbreviations used in this paper*: FUdR, 5-fluorodeoxyuridine; IFN, interferon; LPS, bacterial lipopolysaccharide; PBS, phosphate-buffered saline; PEC, murine peritoneal exudate cells; PMA, phorbol myristate acetate; Con A, concanavalin A; CMEM, complete minimal essential medium; EMC, encephalomyocarditis virus; IUdR, iododeoxyuridine; MAF, macrophage-activating factor; VSV, vesicular stomatitis virus.

to preclude the presence of endotoxin, which is known to activate macrophages (19, 20). In addition, rabbit antimurine IFN-γ was tested for neutralization of MAF activity from a variety of sources, including several cloned murine cytotoxic T lymphocyte lines. The results demonstrate that murine IFN-γ is able to activate tumoricidal activity in macrophages, and that MAF activity detected in culture supernatants which fail to demonstrate IFN-like antiviral activity can be nevertheless neutralized by antirecombinant murine IFN-γ antibody.

Materials and Methods

Animals. 8–12-wk-old male C57BL/6 mice and outbred Hartley guinea pigs were obtained from Charles River Breeding Laboratories, Wilmington, MA. New Zealand White female rabbits (2 kg, 6 mo old) were obtained from Elkhorn Rabbitry, San Jose, CA.

Cell Lines. The $B16F_{10}$ melanoma cell line, derived from a spontaneous melanoma of C57BL/6 mouse (21, 22), and the murine T cell hybridoma clone 3-57 were obtained from Dr. I. J. Fidler, National Institutes of Health. Murine interleukin 2-dependent cytotoxic T lymphocyte clones CTLL-R8, CTLL-R9, CTLL-R11, CTLL-R12 (originally obtained from the spleens of $CB6F_1$ mice immunized with leukemia RL-♂1) and EL4-17-2 (a subclone of a benzopyrene-induced murine EL4 thymoma) have been previously described (23, 24). RPMI 1788 (human B lymphoblastoid cell line), WISH (human amnion), A549 (human lung carcinoma) MDBK (bovine kidney), and murine L-929 (normal fibroblast) cells were obtained from American Type Culture Collection, Rockville, MD. The L_b strain of L-929 cells was obtained from Dr. D. Burke, University of Warwick, Coventry, United Kingdom.

IFN Preparations and IFN Assays. Murine IFN-α (sp act, 1.7×10^5 U/mg; lot 83002), murine IFN-β (sp act, 5.3×10^7 U/mg; lot 83005), rabbit IFN (sp act, 4.4×10^5 U/mg; lot 81004), and rat IFN (sp act, 3.8×10^6 U/mg; lot 82036) were obtained from Lee BioMolecular Co., San Diego, CA. Natural human IFN-γ (sp act, 2×10^5 U/mg), derived from phytohemagglutinin- and 4-phorbol-12-myristate-13-acetate (PMA)-stimulated peripheral blood mononuclear cells, was obtained from Dr. J. Vilcek, New York University, New York.

Recombinant murine IFN-γ was obtained from gene expression in the CHO (Chinese hamster ovary) cell line and in *Escherichia coli* as described (18). Murine IFN-γ (sp act, 4×10^6 U/mg) expressed in *E. coli* was used in these studies unless otherwise noted. It was purified to homogeneity as determined by high pressure liquid chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis and has an amino acid analysis indistinguishable from the natural material. A stock solution of 1×10^6 U/ml contained <0.1 ng/ml endotoxin as determined by the Limulus amebocyte lysate (LAL) test (25).

Recombinant human IFN-α (sp act, 2×10^8 U/mg), recombinant human IFN-α ADbgl (sp act, 4.5×10^7 U/mg), recombinant human IFN-β (1×10^8 U/mg), and recombinant human IFN-γ (sp act, 2×10^6 U/mg) were also tested for MAF activity. All of these materials were >95% estimated purity (as determined by methods described above).

IFN titers were determined by the inhibition of cytopathic effect in microtiter assays (26). The human IFN-β assay used encephalomyocarditis virus (EMC) and A549 cells. The human IFN-α assay was performed with vesicular stomatitis virus (VSV, Indiana strain) and MDBK cells. The human IFN-γ assay used VSV and WISH cells. Murine IFN was assayed with EMC and mouse L_b cells.

Lymphotoxin Assay. A semiautomated method to detect lymphotoxin activity, similar to that reported (27), was used. Cytotoxicity of murine L-929 cells was calculated by measuring crystal violet dye uptake with a Dynatech MR580 Microelisa autoreader (Dynatech Laboratories, Inc., Alexandria, VA). A unit of lymphotoxin activity is defined as the reciprocal of the lymphotoxin dilution killing 50% of the target cells (1×10^4 L-929 cells). In each assay an internal lymphotoxin standard was included. Human lymphotoxin used in these studies was derived from RPMI 1788 and had a specific activity of 1.5×10^7 U/mg (B. Aggarwal, unpublished results).

Rabbit Antimurine IFN-γ. The material used to immunize rabbits was recombinant murine IFN-γ, expressed in *E. coli* (18), of the same purity as previously described. The immunization schedule was as follows: On day 0, 200 μg protein (0.5 ml in complete Freund's adjuvant) was administered subcutaneously in six different sites. On day 10, 200 μg protein (in incomplete Freund's adjuvant) was administered intramuscularly. On day 20, 200 μg protein was administered intravenously. On day 24, the rabbit was bled.

Affinity Purification of Rabbit Antimurine IFN-γ. 12 mg of highly purified murine IFN-γ was coupled to ~2 ml affi-gel 10 resin (Bio-Rad Laboratories, Richmond, CA) as previously described (28). Coupling efficiency was determined to be 81%, based on the protein mass recovered after washing of the resin. 2 ml of hyperimmune rabbit antimurine IFN-γ antiserum was dialyzed vs. phosphate-buffered saline (PBS), pH 7.4, and then applied to the murine IFN-γ/affi-gel 10 resin, which had previously been equilibrated vs. the same buffer in a 1 × 10 cm econocolumn (Bio-Rad Laboratories). 97% of the total protein applied was recovered in 10-bed volumes of PBS; antibodies (3% total protein) specifically bound to the IFN were eluted in 2-bed volumes 0.1 M sodium citrate, pH 3.0. Eluate was immediately adjusted to pH 7.8 with one-half volume 1 M Tris, pH 8.5. By enzyme-linked immunoabsorbent assay (ELISA) analysis (29), all of the murine IFN-γ-binding capacity of the original hyperimmune antiserum sample was recovered in the specifically eluted pool; no binding activity was seen in the material that did not bind to the murine IFN-γ/affi-gel 10.

Neutralization of MAF Activity. The IFN-γ-neutralizing titer of rabbit antirecombinant murine IFN-γ serum was 200,000 U/ml. Neutralization of MAF activity was performed by the addition of 15 μl antiserum to 2 ml of MAF-containing samples. For neutralization of human IFN-α ADbgl, murine IFN-α, and IFN-β, 120 μl of antiserum was used. After incubation at 37°C for 2 h, the samples were placed directly in the MAF and antiviral assays.

Stimulation for MAF Production. The medium used to stimulate cells for MAF production was Eagle's minimum essential medium (MEM) supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM L-glutamine, twofold vitamin solution, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco Laboratories, Grand Island, NY) and 10% heat-inactivated fetal bovine sera (Sterile Systems, Inc., Logan, UT) (CMEM).

Murine spleen cells were first purified by Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) gradients. The monocytes were cultured in 10 ml of CMEM (5×10^6/ml) in 15-ml plastic centrifuge tubes (Corning Glass Works, Corning, NY). After 48 h incubation at 37°C in the presence of 5 μg/ml concanavalin A (Con A) (Sigma Chemical Co., St. Louis, MO), all supernatants were sterilized by passage through a 0.22 μM filter (Millex-GS; Millipore Corp., Bedford, MA) and used immediately. Control supernatants were obtained from cells incubated without mitogen but reconstituted to the initial concentration of mitogen in the stimulated cultures after incubation.

RPMI 1788, EL4-17-2, hybridoma clone 3-57, or the murine cytotoxic T cell clones were seeded with 1.5×10^6 cells into 25-cm^2 plastic tissue culture flasks (Corning Glass Works). They were cultured as described above except that, in addition to Con A, 10 ng/ml PMA (Sigma Chemical Co.), which is known to enhance IFN-γ production (30), was included.

Human platelet residues (American Red Cross, Needham, MA), bovine and ovine spleens (Armour and Company, Dixon, CA), and guinea pig spleen cells were purified by Ficoll-Paque and stimulated with Con A as described above.

Labeling of Tumor Cells. T-75-cm^2 tissue culture flasks (Corning Glass Works) containing $2–3 \times 10^6$ $B16F_{10}$ tumor cells in the log phase of growth were labeled with 0.20 μCi/ml media [^{125}I]iododeoxyuridine ([^{125}I]IUdR) (sp act, 100 mCi/μM; New England Nuclear, Boston, MA) in the presence of 10^{-5} M 5-fluorodeoxyuridine (FUdR) (Sigma Chemical Co.). The amount of radioactivity after 24 h was usually 4,000 cpm/10^4 cells. Before use, the labeled cells were washed three times.

We have observed that the presence of the DNA inhibitor FUdR increases the uptake of label and increases the sensitivity of the MAF assay (unpublished observations). Previous reports have demonstrated that treatment of tumor cells with metabolic inhibitors in-

creases target cells' sensitivity to cytolysis by mediators such as lymphotoxin (31), tumor necrosis factor (32), and cytolysis by natural killer cells (33).

Macrophage Activation Assay. The macrophage activation assay was similar to that described by Fidler et al. (34) with some modifications. Peritoneal exudate cells (PEC) were obtained from mice that were injected intraperitoneally 5 d previously with 1.5 ml thioglycollate (Difco Laboratories, Detroit, MI). The PEC were harvested by injection of 10 ml cold Ca^{++}- and Mg^{++}-free Hank's balanced salt solution (HBSS) (Gibco Laboratories), washed twice, and adjusted to 1×10^6 cells/ml in warm MEM. 200 μl were distributed in each well of a 96-well flat-bottomed microtiter plate (Corning Glass Works). After incubation in a humidified atmosphere (37°C, 5% CO_2) for 1 h, the plates were washed with warm MEM and aspirated to remove residual nonadherent cells. 200 μl of the samples to be tested for MAF activity were added in replicates of six in the presence of 5 ng/ml lipopolysaccharide (LPS). Significant MAF activity in this assay requires 18 h exposure of PEC to activating agents. Hence, the PEC monolayers were activated for 24 h and washed in warm MEM before use. At this time, 1×10^4 [^{125}I]IUdR-B16F$_{10}$ tumor cells in 200 μl were distributed to each well (effector cell/target cell ratio, 20:1). After 24–48 h of macrophage-tumor cell contact, 100 μl of the supernatant was collected and counted for isotope release in an automatic gamma counter (model 28150; Micromedic Systems, Huntsville, AL). Each assay contained tumor cells alone to determine background cytotoxicity (usually 0–5%). In addition, all plates contained 5 μg/ml LPS (Sigma Chemical Co.) in CMEM as a positive control. All experiments were repeated at least three times and statistics were performed by the Student's *t* test. Results are presented as the percent specific tumor cytotoxicity (± SEM) which was determined by: [(total test supernatant cpm − total control supernatant cpm)/(total cpm plated − total control supernatant cpm)] × 100. The SEM of six replicates did not exceed 5%.

Results

Length of Exposure Requirement. To determine the exposure time period required for 'conventional MAF' to stimulate PEC to express MAF activity, PEC was treated with Con A-conditioned media for several time periods (Table I). Significant MAF activity was observed only when Con A-conditioned media (which contained 300 U/ml IFN-γ) was exposed to PEC for 18 h; MAF activity was increased by exposure to PEC for 24 h. No significant MAF activity was

TABLE I

Activation of Peritoneal Exudate Cells (PEC): Length of Exposure Requirement

Length of exposure	Percent cytotoxicity by treatment with:		
	Con A-conditioned media	Con A control	LPS
h			
4	6	0	14
8	5	3	13
18	20	6	39
24	54	6	55

Supernatants derived from murine spleen cells cultured for 48 h in the presence of 5 μg/ml Con A (Con A-conditioned media) or cultured cell supernatants adjusted to 5 μg/ml Con A at the end of culture (Con A control), and 5 μg/ml LPS were used to treat murine PEC for the time period indicated. PEC were then washed and incubated with [I^{125}]IUdR-labeled B16F$_{10}$ tumor cells for 24 h. Results, determined by the amount of label released, represent the mean percent cytotoxicity of six replicates.

observed by treatment with the Con A-conditioned media control (which contained <6 U/ml IFN-γ). As a positive assay control, treatments of 5 μg/ml LPS were used and demonstrated similar exposure requirements for expression of MAF activity.

Macrophage Activation by IFN-γ. Greater than 99% pure recombinant murine IFN-γ, expressed in *E. coli* (18), from 0.1 to 100 U/ml was used to stimulate MAF activity in PEC (Fig. 1). Significant levels of MAF activity were observed after 24 h of PEC-tumor cell contact in the presence of 100 U/ml, 25 U/ml, or 10 U/ml of IFN-γ ($P < 0.05$). When low levels of LPS (5 ng/ml) were included, significant MAF activity was detected with as little as 1 U/ml IFN-γ. PEC treated with only 5 ng/ml LPS showed only background levels of cytotoxicity, 0% in this experiment. The positive controls (PEC treated with 5 μg/ml LPS) demonstrated 23% cytotoxicity.

Since the IFN-γ used for investigation was derived from *E. coli* and since LPS is known to stimulate macrophages, various concentrations of LPS were tested for MAF activity (Fig. 2). Significant levels of MAF activity were detected with LPS treatments, but only when concentrations exceeded 400 ng/ml. This agrees with previous reports (35, 36), where 500 ng/ml LPS was required to induce significant tumor cytotoxicity.

Kinetics of Macrophage Activation. A kinetic study was performed using treat-

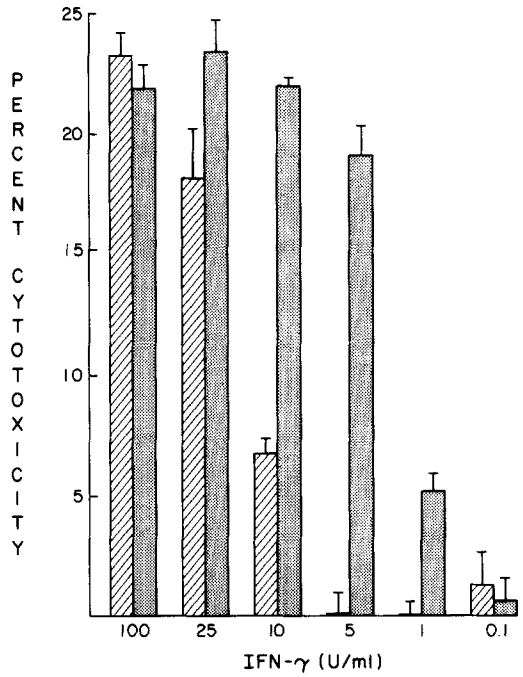

FIGURE 1. Macrophage activation by recombinant murine IFN-γ. PEC were activated by exposure to IFN-γ with (dotted bars) or without (hatched bars) the presence of 5 ng/ml LPS for 24 h, washed, and incubated with [I^{125}]IUdR-labeled $B16F_{10}$ tumor cells for 24 h. No significant activation of PEC resulted after treatment with 5 ng/ml LPS alone. Results, determined by the amount of I^{125} label released, represent the mean percent cytotoxicity (± SEM) of six replicates.

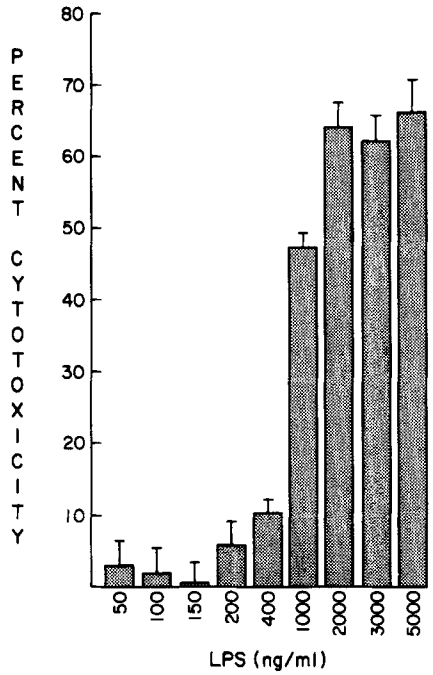

FIGURE 2. Dose response of LPS in the MAF assay. PEC were activated by exposure to the indicated concentrations of LPS, washed, and incubated with [I^{125}]IUdR-labeled $B16F_{10}$ tumor cells for 48 h. Results, determined by the amount of label released, represent the mean percent cytotoxicity (±SEM) of six replicates.

ments of 25 U/ml and 100 U/ml recombinant murine IFN-γ and 5 μg/ml LPS (Fig. 3). Conventional MAF containing supernatants from C57BL/6 spleen cells treated with 5 μg/ml Con A for 48 h were included with their controls (spleen cells cultured without Con A but reconstituted to the initial concentration of Con A). Significant levels of MAF activity were first detected after 24 h of PEC-tumor interaction. PEC treated with 100 U/ml IFN-γ displayed 14.9 ± 0.9% cytotoxicity ($P < 0.001$) and 5 μg/ml LPS displayed 8.6 ± 1.5% cytotoxicity ($P < 0.010$). Highest levels of MAF activity were observed at 72 h. PEC treated with IFN-γ at 100 U/ml, 25 U/ml, 5 μg/ml LPS, or the Con A-stimulated spleen cell supernatant all displayed significant levels of MAF activity. IFN-γ treatments of 100 U/ml displayed the highest percentage of cytotoxicity (60.8 ± 4.8%). The Con A-stimulated spleen cell supernatant displayed 28.5 ± 1.9% cytotoxicity while its control was not significant. The background cytotoxicity of PEC treated with media alone was only 4% (not significant).

Neutralization of Macrophage Activation. The IFN-γ titer of the Con A-treated spleen cell supernatant tested was 60 U/ml (Fig. 3). The level of cytotoxicity observed for this supernatant was between the levels of cytotoxicity after treatment of PEC with 25 and 100 U/ml of IFN-γ. Since the amount of cytotoxicity in the Con A-treated spleen cell supernatant could be explained solely by the presence of IFN-γ, neutralization experiments were performed to determine whether MAF activity could be detected in the absence of IFN-γ antiviral activity.

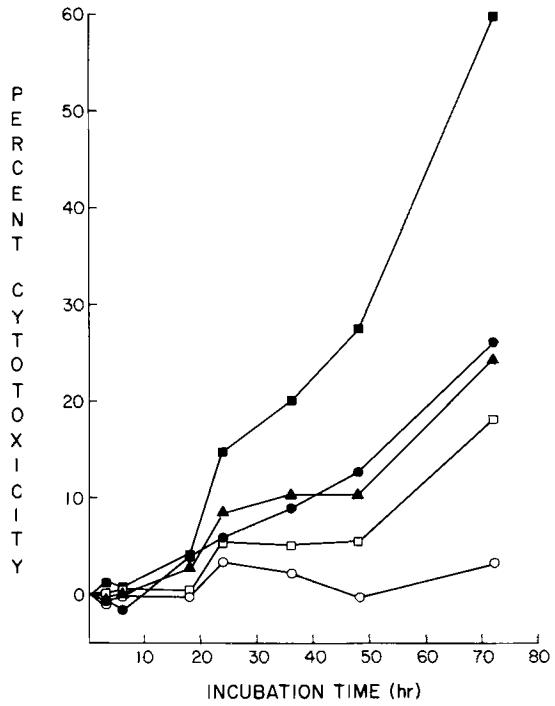

FIGURE 3. Kinetics of macrophage activation. Treatments of 25 U/ml (□) and 100 U/ml (■) recombinant murine IFN-γ, supernatants from 48-h-cultured Con A-treated spleen cells (●), and controls (cells incubated without Con A but reconstituted to the initial concentration of Con A (○), in the presence of 5 ng/ml LPS were used for PEC activation. Treatments of 5 μg/ml LPS (▲) were included as positive assay controls. Con A-treated spleen cell supernatants contained 60 U/ml IFN-γ and their controls displayed <4 U/ml IFN-γ. PEC were activated for 24 h, washed, and incubated with [I^{125}]IUdR-labeled $B16F_{10}$ cells for the time periods displayed. Results, as determined by the amount of label released, represent the mean percent cytotoxicity (±SEM) of six replicates.

Murine spleen cells were incubated with Con A (as described in Materials and Methods) to generate MAF-containing supernatants and were used immediately upon collection. Supernatants were diluted 1:4 to 1:256 and divided into two portions. One portion was treated with rabbit anti-IFN-γ. After appropriate incubation, all samples were tested in the MAF and IFN-γ assay. The results of the antiviral assay (not shown) confirmed antiviral activity in the amount of 90 U/ml IFN-γ in the Con A-treated spleen cell supernatant (1:4 dilution). The same supernatant treated with anti-IFN-γ displayed <4 U/ml. Similarly, each dilution of the supernatant tested with the antibody-treated counterpart was in agreement, suggesting that the antiviral activity of IFN-γ was neutralized. High levels of MAF activity were found with all dilutions of the Con A-treated spleen cell supernatants (Fig. 4). The highest dilution used (1:256), which represents 1.4 U/ml IFN-γ, gave significant levels of MAF activity (17.3 ± 2.4%, $P < 0.001$). When Con A-treated spleen cell supernatants were treated with anti-IFN-γ, no significant MAF activity remained compared with the antibody control. PEC treated with 5 μg/ml LPS (positive assay control) demonstrated

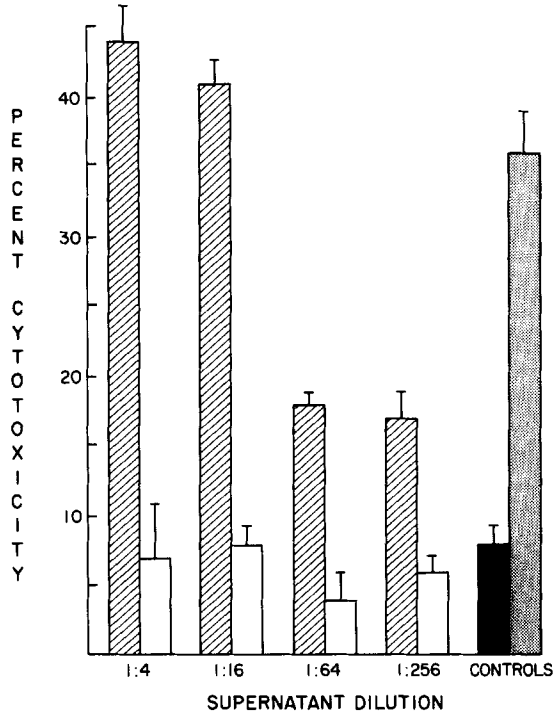

FIGURE 4. Neutralization of MAF activity with rabbit antiserum to recombinant murine IFN-γ. Con A-treated spleen cell supernatants (hatched bars) and the same supernatants treated with antirecombinant murine IFN-γ (□) were used to activate PEC. Antibody alone (■) and 5 μg/ml LPS treatments (dotted bar) were included as controls. Diluted Con A-treated spleen cell supernatants (1:4) contained 90 U/ml IFN-γ. PEC were activated for 24 h, washed, and incubated with [I^{125}]IUdR-labeled $B16F_{10}$ cells for 48 h. Results, as determined by the amount of label released, represent the mean percent cytotoxicity (±SEM) of six replicates.

significant levels of MAF activity (36.0 ± 4.1%, $P < 0.001$). Antimurine IFN-γ had no effect on LPS stimulation of MAF activity.

The antimurine IFN-γ antibodies used in these neutralization experiments were generated in rabbits immunized with purified recombinant murine IFN-γ. A possibility that neutralization of MAF activity was due to the presence of antibody specificities against non-IFN-γ molecules was considered. Therefore, the polyclonal antibodies were purified by affinity chromatography. Complete neutralization of MAF activity found in Con A-stimulated murine spleen cell supernatants was observed when the polyclonal or affinity-purified antirecombinant murine IFN-γ antibodies were used. No neutralization of MAF activity occurred when the unbound antibodies (flow through from affinity chromatography purification) was used. This indicated that neutralization of MAF activity was specific for the antirecombinant murine IFN-γ antibodies.

Macrophage Activation by Supernatants of Murine T Lymphocyte Lines. Three of four cloned murine interleukin 2-dependent cytotoxic T lymphocyte lines that were tested for MAF production are displayed in Fig. 5. All three cell lines (CTLL-R9, CTLL-R11, and CTLL-R12) produced MAF activity constitutively, and MAF activity was increased when the cells were stimulated with Con A and

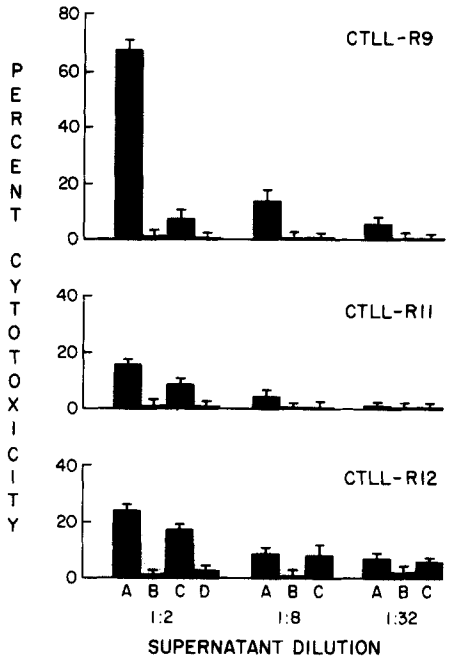

FIGURE 5. Neutralization of MAF activity produced by cloned T lymphocyte lines. Supernatants from three cloned interleukin 2-dependent murine T lymphocyte lines stimulated with Con A and PMA for 48 h (*A*), stimulated cell supernatants treated with 400 neutralizing units of antirecombinant IFN-γ (*B*), unstimulated cell supernatants (*C*), and unstimulated cell supernatants treated with antirecombinant IFN-γ (*D*) were used. The IFN-γ activities of stimulated cell supernatants from CTLL-R9, CTLL-R11, and CTLL-R12 were 80, 10, and 10 U/ml, respectively. All unstimulated and antibody-treated supernatants contained <4 U/ml IFN-γ. PEC were activated for 24 h, washed, and incubated with [I^{125}]IUdR-labeled $B16F_{10}$ cells for 48 h. Results, as determined by the amount of label released, represent the mean percent cytotoxicity (±SEM) of six replicates.

PMA. Supernatants from CTLL-R9 displayed the highest level of MAF (and IFN-γ) activity. CTLL-R8 did not demonstrate MAF (or IFN-γ) production, even after stimulation (data not shown). While all of the cell lines are able to produce IFN-γ (37), no antiviral activity (<4 U/ml) was detected in unstimulated cell line supernatants that displayed MAF activity. This suggested that low levels of IFN-γ, below the sensitivity of the antiviral assay, were produced. When supernatants obtained from stimulated or unstimulated cell lines were treated with anti-IFN-γ, complete abrogation of MAF activity resulted.

Discussion

Many reagents such as LPS (16,20), muramyl dipeptide (38), PMA (39), and ionophores (39–41) have been found to activate macrophages. The possibility of lymphokines participating in macrophage activation was first reported some 13 years ago when it was demonstrated that macrophages obtained from mice undergoing tumor rejection had tumoricidal capabilities (42). Using purified recombinant IFN-γ and affinity-purified antibodies, we have demonstrated that murine IFN-γ has potent macrophage activation activity in the cytotoxicity assay

employed. The possibility that macrophage activation was due to contaminating LPS in IFN-γ preparations can be excluded since these preparations contained <0.1 ng/ml LPS. Furthermore, the level of LPS required for MAF activity in this assay is 1,000-fold greater.

In support of these findings, recombinant murine IFN-γ obtained from cultured supernatants of COS-7 monkey kidney cells has recently been shown to display MAF activity (16, 43). Although IFN-γ from the COS-7 cells was not purified, the possibility of macrophage activation by contaminating lymphokines and LPS can be excluded.

Nathan et al. (12) have recently demonstrated human monocyte activation after treatment with purified recombinant human IFN-γ expressed in *E. coli* (Genentech, Inc.). Significant enhancement of hydrogen peroxide secretion and induction of toxoplasmacidal activity occurred in a dose-dependent manner. Furthermore, both activities induced by recombinant IFN-γ could be abrogated with neutralizing monoclonal antibody to IFN-γ. Thus, IFN-γ appears to activate macrophages in both the murine and human systems.

Our results and those previously reported (44, 45) demonstrate that 1 U/ml IFN-γ is sufficient to render murine macrophages tumoricidal. Similarly, low levels of IFN-γ (0.1–1 U/ml) can significantly augment hydrogen peroxide release and induce toxoplasmacidal activity of macrophages (12). The fact that the MAF assay appears to be 5–10-fold more sensitive than the standard antiviral assay for IFN-γ may be of importance in investigations where macrophage activation is observed with no detection of antiviral activity (46). Additionally, small molecular weight factors reported to induce macrophage activation (47, 48) may actually be breakdown products of IFN-γ. We have preliminary evidence that fragments of IFN-γ can induce macrophage activation and that the domains for antiviral activity and MAF activity may be distinct (manuscript in preparation).

Macrophage activation is thought to occur by at least a two signal process (49, 50). The first signal is thought to prime the macrophage to make it receptive to a second triggering signal, as a result of which the macrophage develops full cytotoxic activity. Our data demonstrate that IFN-γ alone is fully able to activate macrophages. However, we have also presented evidence that IFN-γ/MAF activity may be augmented with small amounts of LPS, as has been demonstrated previously (35, 36). Other, less well-defined substances can also apparently activate or modulate the activation of macrophages, including proteases (51), lipoproteins (52), tumor cell products (53), and prostaglandins (54, 55).

Table II represents a summary of preparations of murine origin tested for MAF activity. All of the materials tested that displayed antiviral activity possessed MAF activity. Further, neutralization experiments performed with rabbit anti-recombinant murine IFN-γ showed complete abrogation of MAF activity. In addition to recombinant IFN-γ, murine IFN-α, and IFN-β were also found to have MAF activity. This agrees with Roberts et al. (44) and Pace et al. (15), who have demonstrated that at least 100-fold more murine IFN-α/β than IFN-γ was required to demonstrate the same amount of MAF activity. The MAF (and antiviral) activity of murine IFN-α and IFN-β was not neutralized by antimurine IFN-γ.

TABLE II

*Summary of Murine Factors Tested for Neutralization of Murine MAF Activity**

Source	Nontreated		Anti-IFN-γ treated‡	
	Murine MAF activity	Murine IFN activity	Murine MAF activity	Murine IFN activity
		U/ml		*U/ml*
Natural IFN-α	15	10,000	14	10,000
Natural IFN-β	17	10,000	18	10,000
Recombinant IFN-γ				
Expressed in *E. coli*	40	100	0	<16
Expressed in CHO	72	500	0	<16
Leukocyte supernatants	44	90	6	<30
Clone EL4-17-2 thymoma superna	3	<30	4	<30
tant	0	<16	ND	ND
Clone 3-57 hybridoma supernatant				
Cloned T cell line supernatants	7	<4	ND	ND
CTLL-R8	50	80	0	<4
CTLL-R9	30	10	0	<4
CTLL-R11	38	10	0	<4
CTLL-R12				

* Results presented are a summary of several different experiments. MAF activity is expressed as percent specific cytotoxicity. ND, not determined.

‡ Supernatants were treated with antimurine recombinant IFN-γ as described in Materials and Methods.

There appears to be a species specificity for expression of MAF activity in the murine assay (Table III). Recombinant human IFN-α, IFN-β, and IFN-γ, and stimulated leukocyte supernatants obtained from human, bovine, ovine, and guinea pigs did not display MAF activity towards murine macrophages. One of the purified recombinant human hybrid IFN-α, IFN-α ADbgl (56), displayed significant levels of murine-directed MAF activity. This hybrid IFN has been reported to be the only human IFN to display antiviral (57) and antitumor properties (58) in murine systems. Interestingly, the MAF (and antiviral) activity of human IFN-α ADbgl was not affected by anti-IFN-γ.

In conclusion, murine IFN-γ appears to have potent macrophage activation capabilities. This MAF activity, as well as that induced from a variety of sources, is totally neutralized by affinity-purified antibody to recombinant murine IFN-γ. Collectively, these data strongly argue for the biological and antigenic similarity, if not identity, of these lymphokines. However, it is important to note that total abrogation of MAF activity by anti-IFN-γ does not exclude the possibility that MAF other than IFN exist. Obviously, any antibody molecule has the capacity to bind to either the molecule containing the determinant(s) to which it was raised or to a nonidentical molecule containing either the identical or a similar determinant(s). Thus, the anti-IFN-γ used in these studies might cross-react with a non-IFN-γ protein that had MAF activity. Obtaining a monoclonal antimurine IFN-γ will by useful; however, further studies involving peptide mapping and sequencing will be required to prove identity.

TABLE III

*Summary of Nonmurine Factors Tested for Murine MAF Activity**

Source	Murine MAF activity	Murine IFN activity
		U/ml
Human		
Recombinant IFN-α ADbgl (500 U/ml)	30	500‡
Recombinant IFN-α A (25,000 U/ml)	3	<6
Recombinant IFN-β (50,000 U/ml)	6	<6
Recombinant IFN-γ (50,000 U/ml)	0	<6
Natural IFN-γ (50,000 U/ml)	0	<6
Stimulated RPMI 1788 supernatant (1:2)	0	<6
Stimulated leukocyte supernatant (1:2)	1	<6
Human lymphotoxin (1,000 U/ml)	0	<6
Recombinant human growth hormone (100 μg/ml)	4	<6
Other		
Rat IFN (500 U/ml)	0	<30
Rabbit IFN (500 U/ml)	2	<30
Stimulated leukocyte supernatants (1:2):		
Guinea pig	0	<30
Ovine	1	<30
Bovine	0	<30

* Results presented are a summary of several different experiments. MAF activity is expressed as percent specific cytotoxicity.

‡ After treatment with anti-murine IFN-γ as described in Materials and Methods, human IFN-α ADbgl demonstrated 31% cytotoxicity and 500 U/ml murine antiviral activity.

Summary

Murine peritoneal exudate cells (PEC) treated with murine recombinant interferon-γ (IFN-γ) (>99% estimated purity), or concanavalin A-stimulated spleen cell supernatants developed tumoricidal properties (macrophage activation factor [MAF] activity). MAF activity was found to occur with treatments of 10 U/ml IFN-γ, and at levels as low as 1 U/ml IFN-γ if a second signal (5 ng/ml endotoxin) was present in the MAF assay. Endotoxin (lipopolysaccharide [LPS]) alone at these levels failed to induce MAF; induction of MAF was observed at 1,000-fold greater levels. The ability of IFN-γ to stimulate murine PEC was species specific. Various sources of materials that displayed MAF activity, including supernatants from interleukin 2-dependent cloned cytotoxic murine T lymphocyte lines that did not display detectable antiviral activity, were neutralized by antibody raised and affinity purified against recombinant IFN-γ. Thus, IFN-γ, although never detectable by antiviral assays, appears to be present in many lymphokine preparations and has potent macrophage activation capability.

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