# EVIDENCE FOR A GAMMA-INTERFERON RECEPTOR THAT REGULATES MACROPHAGE TUMORICIDAL ACTIVITY

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Antigen- or mitogen-stimulated T lymphocytes produce factors that can induce a number of functional and biochemical modifications in macrophage populations. These modifications include increases in endocytic, biosynthetic, secretory, and effector cell functions, as well as changes in membrane physiology and composition (1–4). This process has been called macrophage activation and the lymphokines that induce these effects are known as macrophage-activating factors (MAF).[1]

Over the past three years, work from several laboratories has indicated that gamma-interferon (IFN-γ), the IFN produced by T lymphocytes, represents one type of MAF. Purified or partially purified preparations of IFN-γ produced by normal T cells, T cell hybridomas, or by recombinant DNA technology have been found to prime macrophages for nonspecific tumoricidal activity (5–11), induce or enhance intracellular cytocidal reactions (12, 13), and increase expression of Ia or DR antigens on the cell surface (14–16). Using monoclonal antibodies to murine recombinant IFN-γ, we have recently shown that IFN-γ represents the major and possibly only lymphokine produced by concanavalin A (Con A)–stimulated normal murine splenic cells or the 24/G1 murine T cell hybridoma that can induce tumoricidal activity or Ia expression in macrophages.[2]

These observations have prompted an analysis of the interaction of IFN-γ with macrophage populations. It is the purpose of this report to describe this interaction on a functional and biochemical basis and to demonstrate the existence of a IFN-γ receptor on macrophages. This receptor is capable of binding either

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[1] *Abbreviations used in this paper:* Con A, concanavalin A; DME, Dulbecco's modified Eagle's medium containing 4,500 mg glucose/liter; EPM, elicited peritoneal macrophage; FCS, fetal calf serum; HKLM, heat-killed *Listeria monocytogenes*; HPLC, high pressure liquid chromatography; IFN, interferon; IL-2, interleukin 2; $K_a$, association equilibrium constant; $K_d$, dissociation equilibrium constant; MAF, macrophage-activating factor; PBS, phosphate-buffered physiological saline; PEC, peritoneal exudate cells; PMN, polymorphonuclear leukocyte.

[2] Schreiber, R. D., L. J. Hicks, A. Celada, and P. Gray. 1984. Monoclonal antibodies to IFN which modulate macrophage activation by lymphokines. Manuscript in preparation.

natural or recombinant murine IFN-γ in a specific and saturable manner. Receptor engagement is required for the initiation of macrophage activation.

## 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 h at 180°C. RPMI-1640 and Eagle's minimal essential medium were prepared from powdered stocks (Flow Laboratories, Inglewood, CA) using USP sterile water (Travenol Laboratories, Inc., Deerfield, IL). Liquid Dulbecco's modified Eagle's medium containing 4,500 mg glucose/liter (DME) was purchased from M.A. Bioproducts, Walkersville, MD. Aseptically drawn fetal calf serum (FCS, Rehatuin FS) was obtained from Reheis Chemical Co. (Phoenix, AZ) and heat inactivated (1 h, 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 nonessential amino acids and trypsin-EDTA (1x) (Gibco Laboratories, Grand Island, NY), gentamicin (Scheering Corp., Kenilworth, NJ), indomethacin, trizma base, trizma hydrochloride, β-mercaptoethanol, ethanolamine, and saponin (Sigma Chemical Co.), sodium chloride, sodium mono- and dibasic-phosphate (Fisher Scientific Co., Fairlawn, NJ), bovine serum albumin, Fraction V (United States Biochemical Co., Cleveland, OH), soybean trypsin inhibitor (Calbiochem-Behring Corp., La Jolla, CA) and concanavalin A (Con A, Miles-Yeda, Ltd., Rehovot, Israel).

*Animals.* C3HeB/FeJ, CBA/CaJ, C57BL/6J, DBA/2J, SJL, and C3H/HeJ mice, and Lewis rats were obtained from the breeding colony at the Research Institute of Scripps Clinic. A/J, C3D2F1/J, and Swiss (BKL) were obtained from The Jackson Laboratory, Bar Harbor, ME. Swiss (NCS) mice were obtained from the breeding colony at The Rockefeller University, New York, NY. Armenian hamsters were obtained from Cambridge Diagnostics, Cambridge, MA and guinea pigs from Crest Caviary, Raymond, CA.

*Lymphokine Preparations.* Lymphokine-containing supernatants were prepared by Con A stimulation of cultures of the murine T cell hybridoma 24/G1 or normal murine splenic cells as described previously (5).

*Recombinant Gamma Interferon.* *E. coli*–derived murine IFN-γ was produced as previously described (17). A preparation (lot number 1551/43) was used that had been purified to a specific antiviral activity of $7.2 \times 10^6$ IRU/mg. IFN was stored at 4°C in 10 mM Tris-HCl, 0.5 M NaCl buffer, pH 8.0 containing 0.1% β-mercaptoethanol.

*High Pressure Liquid Chromatography (HPLC) Gel Filtration of Recombinant IFN-γ.* 180 μl of the purified recombinant IFN-γ was injected into a 7.5 × 600 mm Bio-Sil TSK250 HPLC gel filtration column (Bio-Rad Laboratories, Richmond, CA) connected to a Waters HPLC system (Waters Associates, Milford, MA). The column was eluted at 23°C with 0.02 M phosphate-buffered physiological saline (PBS), pH 7.2, at a flow rate of 1 ml/min.

*Preparation of Iodinated IFN-γ.* The two-peak HPLC fractions that displayed a molecular mass of 32,000 daltons were pooled and used for radiolabeling. 14 μg of IFN in 100 μl was incubated with 1 mCi $^{125}$I Bolton-Hunter reagent (18) in a "V"-shaped glass reaction vial (ICN Chemicals, Radioisotope Division, Irvine, CA) for 2 h at 4°C. Protein-associated and free $^{125}$I were separated by centrifugation through tubes containing BioGel P-6 as described by Fishelson et al. (19). Plastic tubes (10 × 75 mm, Falcon Labware, Oxnard, CA) pierced at the bottom with a needle were plugged with scrubbed nylon fibers (Fenwal Laboratories, Deerfield, IL) and packed with BioGel P-6, 100 to 200 mesh (Bio-Rad Laboratories) equilibrated in PBS. The tube was then placed inside a 12 × 75–mm plastic tube, centrifuged to dryness for 3 min at 1,000 rpm in an Adams Sero-Fuge (Clay Adams, Inc., New York, NY) and the inside tube transferred to a new 12 × 75–mm tube. The

labeled IFN was applied to the top of the BioGel P-6 and the tubes were centrifuged as above. Protein-bound, but not free, $^{125}I$ was eluted from the gel by this procedure and collected at the bottom of the 12 × 75 mm tubes. The P-6 tube was washed once with 100 μl of buffer and the two eluted volumes were combined. Recovery of IFN was essentially 100% as assessed with the quantitative MAF assay. Typical preparations were labeled to a specific activity of 7.6 μCi/μg. $^{125}I$-IFN-γ was stored at 4°C and retained biological activity for at least 2 wk.

*Alpha and Beta Interferons.* These murine IFN were purchased from Lee Biomolecular Laboratories (San Diego, CA). IFN-α had a specific activity of $5 \times 10^5$ IRU/mg, and IFN-β of $1.5 \times 10^7$ IRU/mg.

*Peritoneal Macrophages.* Peritoneal exudate cells (PEC) were harvested as described previously by lavage from mice that had been injected intraperitoneally 3 d previously with 1.5 ml of 10% protease peptone (Difco Laboratories, Detroit, MI) (5). Rats, hamsters, and guinea pigs were injected with 5% casein 5 d before the PEC were harvested. Macrophage monolayers were prepared by seeding the PEC suspension into flat-bottom 96-well tissue culture plates (Costar, No. 3696, Cambridge, MA) or 6-well tissue culture plates (Costar, No. 3506). The cells were adhered for 2 h at 37°C and the plates washed vigorously to remove nonadherent cells.

*Mouse Bone Marrow–derived Macrophages.* Macrophages derived from bone marrow cultures were obtained according to the method of Meerpohl et al. (20) with some modifications. Mice were killed by cervical dislocation and both femurs were dissected free of adherent tissue. The ends of the bones were cut off and the marrow tissue eluted by irrigation with PBS. Cells were suspended by vigorous pipetting and washed by centrifugation. $10^7$ cells were cultured in a nontissue culture plastic 150-mm petri dish (Lab-Tek 4030, Miles Laboratories, Inc., Naperville, IL) in 50 ml of DME containing 2 mM L-glutamine, 1 mM Na pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, 20% heat-inactivated horse serum, and 30% L cell–conditioned medium. The cell suspensions were incubated at 37°C in a humidified 5% $CO_2$ atmosphere. After 6–9 d, macrophages that were loosely adherent to the dishes were harvested with cold PBS and used.

*Human Cells.* Human peripheral blood monocytes, lymphocytes, and polymorphonuclear leukocytes (PMN) were purified from heparinized, normal venous blood by discontinuous density gradient centrifugation on Ficoll-Urografin (21). The upper layer contained a mixture of monocytes and lymphocytes, while 98% of PMN were present in the lower layer. Erythrocytes were obtained at the bottom of the tube. Monocytes were separated from lymphocytes by adherence onto autologous serum–treated plastic plates according to the method of Fischer et al. (22). The purity of all cell preparations was >98% as assessed by cytocentrifugation and Giemsa-peroxidase staining (23).

*Preparation of Membranes from Bone Marrow–derived Macrophages.* Membranes were prepared according to the procedure of Gabel et al. (24). In brief, cells were disrupted with a sonicator and the cell lysates centrifuged at 850 *g* for 10 min. The resulting low speed supernatant was then recentrifuged at 40,000 *g* for 30 min. Membrane pellets were resuspended by homogenization and washed three times. Membrane preparations were stored at −70°C.

*Cultured Cell Lines.* The human histiocytic lymphoma cell line ($U_{937}$) and the murine mastocytoma cell line (P815) were maintained in RPMI supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 1 mM Na pyruvate, 0.075% (wt/vol) Na bicarbonate, and 10% heat-inactivated FCS. Murine T cell hybridomas TH4.4, 24/G1, and 1.19 and the murine fibrosarcomas L929, TU5, and 1023 were maintained in DME supplemented with 2 mM L-glutamine 50 U/ml penicillin, 50 μg/ml streptomycin, 1 mM Na pyruvate, and 10% FCS. Two murine macrophage cell lines were also used: $P388D_1$ was maintained in RPMI and J774 in MEM supplemented with 2 mM L-glutamine, 1 mM pyruvate, 10 mM nonessential amino acids, 0.075% (wt/vol) Na bicarbonate, 50 μg/ml of gentamicin, and 10% FCS.

*Measurement of MAF Activity.* MAF was quantitated by measurement of its ability to induce nonspecific tumorilytic activity in peptone-elicited C3HeB/FeJ macrophages toward P815 mastocytoma cells, as detailed elsewhere (5) with some modifications. Briefly,

reactions were performed in duplicate or triplicate in 96-well flat-bottom A/2 tissue culture plates in a total volume of 100 μl (Costar, No. 3696). Each well contained $1 \times 10^5$ adherent macrophages, serial dilutions of MAF, an excess of a second signal ($1 \times 10^6$ heat-killed *Listeria monocytogenes* (HKLM)), $10^{-6}$ M indomethacin and $1 \times 10^4$ $^{111}$In-labeled P815. $^{111}$In-oxine chelate was purchased from Mediphysics, Inc. (Bloomfield, NJ) and labeling was performed as described by Wiltrout et al. (25). Following incubation for 18 h at 37°C in a humidified 5% $CO_2$ atmosphere, 50 μl of each culture supernatant was removed and analyzed for $^{111}$In content. 1 U of MAF is defined as that amount which produces 50% maximal specific $^{111}$In release in this assay.

*Absorption Studies.* Six-well tissue culture plates (Costar, No. 3506) were used for absorption of MAF. In all the cases, plates were preincubated overnight with medium containing 20% FCS before use. Six million adherent cells were incubated with 1 ml of MAF containing supernatant at 37°C in a humidified 5% $CO_2$ atmosphere. Supernatants were collected and filtered through a 0.2-μm filter (Millipore Corporation, Bedford, MA) to remove cellular debris and the remaining activity quantitated using the tumorilytic assay. As the control for these experiments, 1 ml of MAF-containing supernatant was incubated without macrophages in the culture plates for the same period of time.

*Measurement of Interleukin 2 (IL-2) Activity.* IL-2 activity was quantitated using the method of Smith (26). Serial dilutions of the sample were cultured with 4,000 HT-2 cells, the IL-2–dependent murine T cell line for 20 h in a total volume of 200 μl. The cells were then pulsed for 4 h with [$^3$H]thymidine (New England Nuclear, Boston, MA) and thymidine incorporation determined. The IL-2 standard used in these studies was a gift from Dr. Jacques Chiller, Lilly Research Laboratories, La Jolla, CA and contained 250 U/ml. The amount of IL-2 present in an unknown sample was determined by titration against the IL-2 standard.

*Preparation and Binding of IFN-γ-coated Covaspheres to Macrophages.* 100 μl of a 1.35% suspension of coumarin (green) fluorescent microspheres (Covaspheres, Covalent Technology Corp., Redwood City, CA) in PBS were mixed with 25 μl of purified recombinant IFN-γ (2.6 mg/ml) and incubated overnight at 4°C. The microspheres were pelleted by centrifugation for 10 min at 8,000 *g* in a Beckman microfuge 12 (Beckman Instruments, Inc., Palo Alto, CA) and washed one time with PBS containing 1% BSA. Microspheres were then incubated with 1 ml of 1 M ethanolamine in PBS, pH 7.0 to block any remaining unreacted sites on the beads. After three washes, microspheres were sonicated for 1 min and resuspended in 1 ml of RPMI medium and stored at 4°C until use. Microspheres treated with ethanolamine but not IFN-γ were used as controls. For binding studies, $2 \times 10^5$ EPM were adhered for 2 h at 37°C to coverslips (Coverslip No. 1-THK, Bellco Glass, Inc., Vineland, NJ) in 24-well (16-mm diameter) tissue culture plates (Costar, No. 3424). Coverslips were washed and 20 μl of microspheres was added in 1 ml of medium. After incubation for 2 h at 4°C, coverslips were washed and examined under a microscope (Zeiss, Photomicroscope Type II, Munich, West Germany) with ultraviolet and visible light.

*Binding Studies with $^{125}$I-recombinant IFN-γ.* Five million bone marrow–derived macrophages or the macrophage cell line P388D$_1$ were incubated with different concentrations of $^{125}$I-IFN-γ in a total volume of 150 μl with RPMI-supplemented medium. After 2 h at 4°C, 120 μl was applied to 250 μl of an oil mixture consisting of six parts dioctylpthalate and four parts dibutylpthalate (Aldrich Chemical Company, Inc., Milwaukee, WI) in 400 μl polyethylene microfuge tubes (VWR Scientific, Inc.) and microfuged at 4°C for 1 min at 9,000 rpm in a Beckman microfuge 12. Tubes were sectioned and the radioactivity of the pellet and supernatant counted. Specific binding was defined as the difference between total binding and the nonspecific binding occurring in the presence of a 500-fold excess of unlabeled IFN-γ. Similar studies were carried out using membranes from bone marrow–derived macrophages except that membranes were incubated with IFN-γ in the following buffer: 25 mM Hepes, pH 7/0.1 M NaCl/5 mM sodium phosphate/0.34 units per ml soybean trypsin inhibitor/0.5% saponin. Free and membrane-associated IFN were separated by centrifugation through 20% sucrose.

*Monoclonal Antibodies.* Monoclonal antibodies to recombinant IFN-γ were produced

by fusion of immune Armenian hamster splenocytes with HAT-sensitive murine myeloma cell lines, as will be described elsewhere. The resulting hybridomas were cloned three times by limiting dilution. Monoclonal antibodies were purified from tissue culture supernatants on a column of staphylococcal protein A Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ).

## Results

*Demonstration of MAF Absorption.* For these studies, MAF has been defined as the lymphokine that primes macrophages for expression of nonspecific tumorilytic activity toward P815 mastocytoma cells. As a source of MAF, we used the supernatant from a Con A–stimulated culture of the murine T cell hybridoma 24/G1. This supernatant contained 55,000 U of MAF activity/ml and 833 IRU IFN-γ/ml. We have previously shown that all the MAF activity in this supernatant was attributable to IFN-γ. By titration against purified recombinant murine IFN-γ, the supernatant was estimated to contain 190 ng IFN-γ/ml.

Fig. 1 demonstrates that MAF activity was removed from the 24/G1 supernatant following exposure to elicited peritoneal exudate macrophages (EPM). In this experiment 1 ml of supernatant was diluted 1/100 (550 U/ml) and was incubated for 4 h at 37°C either in empty wells or in wells containing $6 \times 10^6$ adherent EPM. The supernatant from the control well displayed 460 U MAF/ml as compared with 530 U/ml of unincubated supernatant, indicating that MAF activity was stable to the 37°C incubation. However, after exposure to EPM, the supernatant contained only 92 U/ml of MAF activity. This represents an 80% loss of MAF activity.

*Assessment of Dose, Time, and Temperature Dependency of Absorption.* Removal of MAF activity from 24/G1 supernatants was dependent on both the number of cells and the amount of MAF used in the experiment. Elicited or bone marrow–derived macrophages were equivalent in their ability to bind MAF (Fig. 2). Following 4 h incubation, 50% of the MAF activity was removed by 1.5 ×

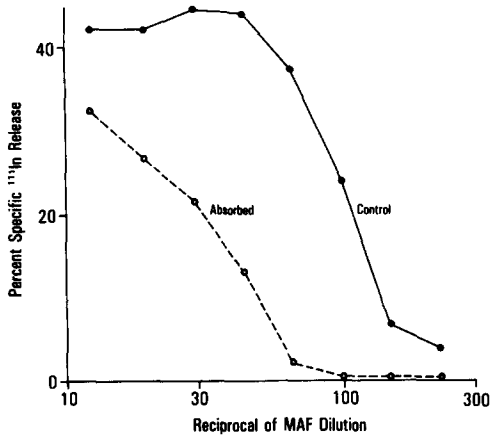


FIGURE 1. Demonstration of MAF absorption by macrophages. $6 \times 10^6$ EPM were incubated for 4 h at 37°C with 1 ml of 24/G1 supernatant diluted 1/100. As control, the diluted supernatant was incubated without macrophages. Following incubation, the supernatants were removed and the remaining MAF activity measured using freshly explanted macrophages.

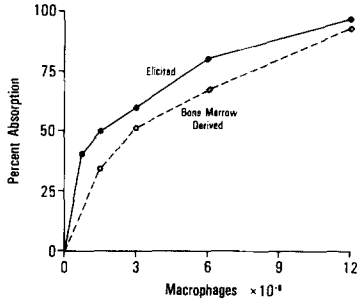


FIGURE 2. Dose-dependent absorption of MAF activity by macrophages. Different numbers of EPM were incubated for 4 h at 37°C with 1 ml of 1/100 diluted 24/G1 supernatant. Controls and quantitation of MAF activity were as in Fig. 1.

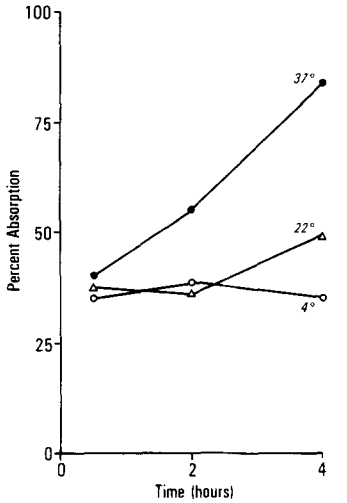


FIGURE 3. Temperature dependence of MAF absorption. Absorption and quantitation were as in Fig. 1.

$10^6$ EPM or $3 \times 10^6$ bone marrow–derived macrophages and $12 \times 10^6$ of either cell population removed 100% of the 24/G1 derived MAF. The reduction of MAF activity was also dependent on the initial input of the lymphokine. At MAF dilutions of 1/25, 1/50, 1/100, 1/150, and 1/200, the amount of activity removed following treatment with $6 \times 10^6$ EPM for 4 h at 37°C was 40%, 57%, 73%, 92%, and 100%, respectively.

Absorption was time dependent at 37°C but not at 4°C or 22°C (Fig. 3). At all three temperatures, exposure of 24/G1 supernatants to $6 \times 10^6$ EPM for 30 min, led to removal of ~40% of the MAF activity. Assuming that the supernatants contained 190 ng IFN-γ/ml, an estimate can be made that EPM bound or consumed ~2,000 molecules of IFN-γ/cell. While longer incubation at 4°C or 22°C did not result in additional removal of MAF activity, incubation at 37°C effected a time-dependent increase in MAF absorption. After 4 h at 37°C, 85% of the MAF activity was lost from 24/G1 supernatants and all the activity was removed when the absorption was carried out for 24 h. Bone marrow–derived

macrophages were equivalent to EPM in these experiments. Similar results were also obtained using other 24/G1 supernatants or supernatants of Con A–stimulated normal splenic cells.

*Controls to Indicate that Removal of MAF Activity was a Result of Absorption.* The ability of macrophages to rapidly (30 min) remove MAF activity from 24/G1 supernatants at 4°C suggested that the loss of activity was the result of IFN-γ absorption. To further substantiate this hypothesis three additional experiments were performed.

To rule out the possibilities that (*a*) the MAF/IFN-γ was degraded or altered by macrophage-derived factors or (*b*) treated macrophages produced substances such as prostaglandins that could interfere with the subsequent quantitation of MAF activity, a mixing experiment was performed as outlined in Table I. Supernatants containing untreated MAF or MAF preincubated either in empty wells or with EPM were mixed in various combinations with medium from EPM-containing wells. In all cases, the amount of MAF activity that was measured in the mixtures closely approximated the theoretical values calculated from the mixture composition. No difference in these results were seen when $10^{-5}$ M indomethacin was present during preparation of the various supernatants. Macrophages incubated with MAF for 2 and 4 h absorbed 46% and 75% of the activity, respectively, in the absence of indomethacin and 42% and 74% of the activity was removed in the presence of the cyclooxygenase inhibitor.

In a second series of experiments, paraformaldehyde-fixed or heat-killed (56°C, 30 min) EPM were compared to normal EPM for the ability to absorb MAF activity (Table II). After 2 h of incubation the untreated cells had removed 61% of the MAF activity, while the fixed cells had removed only 38%. At subsequent time points the native cells continued to absorb MAF activity, while the fixed cells did not. Cells heated to 56°C for 30 min were nonviable as

TABLE I

*Effect of Mixing Macrophage Supernatants on Quantitation of MAF Activity*

| Sample | Measured | Theoretical |
|---|---|---|
| | *U/ml* | |
| Untreated MAF (1/100) | 650 | — |
| Absorbed MAF* | 96 | — |
| MAF incubated 4 h (control)‡ | 480 | — |
| Mϕ incubated with medium§ | 0 | — |
| 50% untreated + 50% absorbed | 385 | 373 |
| 50% untreated + 50% MAF control | 550 | 565 |
| 50% untreated + 50% incubated medium | 300 | 325 |
| 50% incubated medium + 50% MAF control | 225 | 240 |
| 50% absorbed MAF + 50% MAF control | 300 | 288 |

Macrophage supernatants were mixed and incubated for 4 h at 37°C and the MAF activity quantitated.

* $6 \times 10^6$ EPM were incubated with 1 ml of 1/100 diluted 24/G1 supernatant 4 h at 37°C.

‡ 1 ml of diluted supernatant was incubated in an empty well for 4 h at 37°C.

§ $6 \times 10^6$ EPM were incubated with 1 ml of medium for 4 h at 37°C.

TABLE II

*Influence of Fixation or Heat Killing on Absorption of MAF Activity by Macrophage*

| Treatment | Percent absorption | | | Cytotoxic activity |
|---|---|---|---|---|
| | Time (h) | | | |
| | 2 | 4 | 24 | |
| | % | | | *U/ml* |
| None | 61 | 80 | 100 | 46,000 |
| 1% Paraformaldehyde | 38 | 42 | 39 | <100 |
| Heated (56°C, 30 min) | 5 | 0 | 6 | <100 |

$6 \times 10^6$ adherent EPM were treated with 1% paraformaldehyde at 4°C for 1 h or heated 30 min at 56°C. Cells were washed and incubated at 37°C with 1 ml of 24/G1 supernatant diluted 1/100.

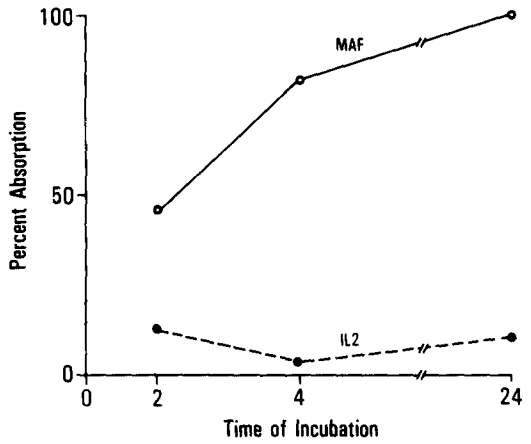


FIGURE 4. Absorption of MAF activity but not IL-2 activity by macrophages. $6 \times 10^6$ EPM were incubated with 1 ml of a supernatant containing 25 U IL-2 activity/ml and 550 U MAF activity/ml. Incubations were performed at 37°C for 2, 4, and 24 h.

evidenced by release of the cytoplasmic marker lactate dehydrogenase and their permeability to trypan blue. These cells did not express tumoricidal activity when treated with 24/G1 supernatants and also absorbed <10% of the MAF activity even when the incubation period was prolonged to 24 h.

A third set of experiments was performed to assess the specificity of the absorption. Macrophages were incubated with a supernatant containing 25 U/ml IL-2 and 550 U/ml MAF for 2, 4, and 24 h at 37°C. After incubation, the remaining MAF and IL-2 activities were quantitated (Fig. 4). When compared to the corresponding control, IL-2 showed 17%, 4%, and 13% absorption after 2, 4, and 24 h, respectively, while for the same periods of incubation, 45%, 83%, and 100% of MAF was absorbed. Similar results were obtained using 50 or 12.5 U/ml of IL-2. The variation between the values obtained for the IL-2 after incubation with macrophages was similar to the variation obtained with the control values, suggesting that IL-2 was not absorbed by macrophages.

*Relationship between Time of Absorption and Development of Cytotoxicity.* To study the relationship between absorption and development of the tumoricidal response, macrophages were incubated at 37°C with the 24/G1 supernatant for

periods of time between 1 and 6 h. After each incubation period, the supernatant was removed and analyzed for MAF activity while the macrophages were washed and then challenged with P815 in the presence of a second signal (HKLM). Fig. 5 shows that although MAF is absorbed progressively over the period of incubation, a minimum exposure of 4 h is required before the macrophage can express tumorilytic activity. During this critical period of incubation, 82% of the MAF has been absorbed. No change was observed in the minimum incubation time needed to elicit a tumoricidal response when higher concentrations of MAF were used.

*Cell and Species Specificity of Absorption.* Elicited and resident peritoneal exudate murine macrophages are known to differ in their responsiveness to IFN-γ. In our experimental assay system, only the freshly explanted elicited cell population developed IFN-dependent nonspecific tumorilytic activity (Table III). This responsiveness was rapidly lost in culture and was also not expressed by freshly explanted or cultured resident peritoneal exudate cells. Table III shows that the defect is not related to IFN-γ binding by the cells, since both the responsive and

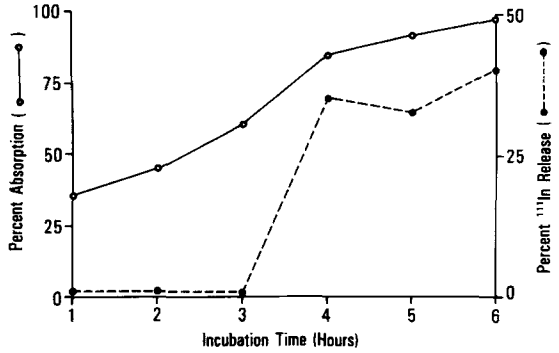


FIGURE 5. Correlation of MAF absorption and development of cytolytic activity. $6 \times 10^6$ EPM were incubated for the time indicated with 1/100 diluted 24/G1 supernatant. The resulting absorbed supernatants were assayed for remaining MAF activity. The macrophages used for absorption were mixed with $6 \times 10^5$ $^{111}$In-labeled P815 tumor cells, $6 \times 10^7$ HKLM (as a second signal) and $10^{-6}$ M indomethacin in a total volume of 1 ml. After 18 h in culture, 0.5 ml of supernatant was removed, the radioactivity counted and the cytolytic activity calculated (●···●).

TABLE III

*Cytotoxic Activity and MAF Absorption by Elicited and Resident Peritoneal Macrophages*

| Macrophages ($6 \times 10^6$) | Condition | Cytotoxic activity (MAF U/ml) | Percent absorption* after 4 h incubation |
|---|---|---|---|
| Elicited | Freshly explanted | 48,000 | 78 |
| | 3-d culture | <200 | 61 |
| Resident | Freshly explanted | <200 | 60 |
| | 3-d culture | <200 | 65 |

* 1 ml of 24/G1 supernatant diluted 1/100 was used for each absorption at 37°C.

unresponsive cells absorbed comparable amounts of MAF activity when incubated for 4 h at 37°C (78% and 60–65%, respectively). In other experiments (data not shown) macrophages from eight different inbred and two outbred strains of mice were tested for their ability to express IFN-γ-dependent tumoricidal activity and to absorb MAF activity from the 24/G1 supernatant. The inbred mouse strains tested included C3HeB/FeJ ($H_2^k$), C3H/HeJ ($H_2^k$), CBA/CaJ ($H_2^k$), A/J ($H_2^a$), C57BL/6J ($H_2^b$), DBA/2J ($H_2^d$), SJL ($H_2^s$), and $C_3D_2$ $F_1$/J ($H_2^k \times H_2^d$). Although the various macrophages expressed different levels of tumorilytic activity, they all absorbed nearly identical amounts of MAF activity during incubation periods of 2, 4, or 24 h. Thus absorption was not H-2 restricted.

Table IV compares a variety of murine cell lines for their ability to absorb 24/G1-derived MAF activity. Murine fibroblasts ($L_{929}$), fibrosarcomas (TU5 and 1023), mastocytomas (P815), and T cell hybridomas (TH4.4, 24/G1 and 1.19) all absorbed MAF activity at 37°C in a time-dependent fashion. Two macrophage cell lines ($P388D_1$ and J774) were identified that were deficient in their ability to bind natural IFN-γ. Even after 24 h incubation, $P388D_1$ absorbed only 37% of the MAF activity and J774 only 60%, while all other cell lines and normal peritoneal exudate macrophages had absorbed 93–100% of the activity. In addition to their reduced ability to bind IFN-γ, $P388D_1$ and J774 also displayed a quantitative defect in their ability to mount an IFN-dependent nonspecific tumorilytic response. When 24/G1 supernatants were titrated on control C3HeB/FeJ macrophages, a value of 43,800 U of MAF activity was obtained. However, values of only 320 U and 4,300 U were obtained when the assay was performed with $P388D_1$ or J774, respectively. When examined after 6 h incubation with the hybridoma supernatant, the two cell lines also showed a defective

TABLE IV
*Absorption of MAF Activity by Murine Cell Lines*

| Cell type | Time of incubation (h) | | |
|---|---|---|---|
| | 2 | 4 | 24 |
| EPM control | 47 | 88 | 100 |
| $L_{929}$* | 22 | 33 | 93 |
| TU5‡ | 33 | 60 | 100 |
| 1023‡ | 35 | 72 | 100 |
| P815§ | 61 | 93 | 100 |
| TH4.4‖ | 37 | 68 | 95 |
| 24/G1‖ | 33 | 97 | 100 |
| 1.19‖ | 52 | 97 | 100 |
| $P388D_1$¶ | 0 | 10 | 37 |
| J774¶ | 12 | 25 | 60 |

In all the cases $6 \times 10^6$ cells were incubated for 2, 4, and 24 h with 1 ml of 24/G1 supernatant diluted 1/100.
* Fibroblast.
‡ Fibrosarcoma.
§ Mastocytoma.
‖ T cell hybridoma.
¶ Macrophage cell line.

spreading reaction. Although 82% of the control cells had spread, only 15% of P388D$_1$ and 22% of J774 had undergone morphological changes.

Table V compares cells from a number of different species for their ability to absorb 24/G1-derived MAF activity. Absorption appears to display a degree of species specificity. When peritoneal exudate macrophages from four rodent species were compared, only murine cells absorbed MAF activity during the first 4 h of incubation. After 24 h of incubation, rat macrophages were also found to absorb or consume a small amount (37%) of MAF activity. Human polymorphonuclear leukocytes, lymphocytes, and erythrocytes did not absorb murine MAF. However, human monocytes absorbed 33% of the activity following in vitro culture for 24 h. This result agrees with the observation that after 1–3 d in culture, human peripheral blood monocytes acquire the ability to respond to murine IFN-γ and can express nonspecific tumoricidal activity toward the human melanoma cell line A357 (Buchmeier, N. A., and R. D. Schreiber, unpublished observation). In contrast to normal human peripheral blood cells, the human histiocytic lymphoma cell line U$_{937}$ was quantitatively equivalent to murine macrophages in effecting absorption of 24/G1-derived MAF activity.

*Absorption of Recombinant IFN-γ by Macrophages.* We have previously found that the MAF activity displayed by purified recombinant murine IFN-γ was equivalent, on an antiviral activity basis, to the MAF activity expressed by the T cell hybridoma–derived IFN-γ (27). This observation prompted a comparison of the ability of macrophages to absorb natural and recombinant IFN-γ. For these experiments, the recombinant IFN-γ was diluted such that the final solution contained the same amount of MAF activity as a 1/100 dilution of the 24/G1 supernatant (550 U/ml). After 2 and 4 h incubation with normal EPM the amount of absorption of the recombinant IFN-γ (32% and 76%, respectively) was similar to that of the natural IFN (44% and 86%, respectively, Table VI). Moreover, P388D$_1$ that was deficient in absorbing the natural IFN-γ was also deficient in absorbing the recombinant material.

In order to directly visualize the interaction of IFN-γ with the macrophage

TABLE V
*Species Specificity of MAF Absorption*

| | Time of incubation (*h*) | | |
|---|---|---|---|
| | 2 | 4 | 24 |
| Macrophages | | | |
| Mouse | 47 | 88 | 100 |
| Rat | 0 | 0 | 37 |
| Hamster | 0 | 0 | 0 |
| Guinea Pig | ND | 0 | 0 |
| Human cells | | | |
| Monocytes | 0 | 0 | 33 |
| Polymorphonuclear leukocytes | 0 | 0 | 0 |
| Lymphocytes | 0 | 0 | 8 |
| Erythrocytes | 0 | 0 | 0 |
| U$_{937}$ | 47 | 94 | 100 |

Absorption conditions as in Table IV.
*ND*, not done.

TABLE VI
*Absorption of Recombinant IFN-γ by EPM and P388D$_1$*

| | Percent absorption | | | |
|---|---|---|---|---|
| | Recombinant IFN-γ | | 24/G1-IFN-γ | |
| | 2 h | 4 h | 2 h | 4 h |
| | % | | | |
| Control macrophages | 32 | 76 | 44 | 86 |
| P388D$_1$ | 0 | 3 | 0 | 8 |

$6 \times 10^6$ cells were incubated for 2 or 4 h in the presence of 1 ml of 24/G1 supernatant or recombinant IFN-γ diluted such that both preparations displayed 550 U of MAF activity per ml.

surface, recombinant IFN-γ was bound to fluorescent microspheres and incubated at 4°C with macrophage populations. EPM exposed to microspheres bearing IFN-γ bound one to nine beads per cell (Fig. 6*a*). Staining of the cell population was heterogeneous with 35% of the cells binding less than three beads. When microspheres were used that did not carry IFN-γ, but that had been blocked by incubation with ethanolamine, no binding occurred (Fig. 6*b*). Preincubation of EPM with high concentrations of 24/G1 supernatant, abrogated binding of IFN-γ-covaspheres, indicating that binding was IFN mediated (Fig. 6*c*). Fig. 6*d* shows that P388D$_1$ did not bind IFN-γ-coated microspheres. This result thus agrees with the absorption data.

*Demonstration of a Specific IFN-γ Receptor on Macrophages: Quantitation of the Interaction between Recombinant IFN-γ and Macrophages.* The data presented thus far indicate that natural and recombinant IFN-γ bound to macrophages in a similar fashion. Because of its availability, the purified recombinant material was used to quantitate the interaction of IFN-γ with the macrophage surface. Recombinant IFN-γ was first subjected to HPLC gel filtration to remove any degraded material that may have formed during storage. As detected by ultraviolet absorption at 280 nM, two molecular weight species eluted from the column with symmetrical elution profiles (data not shown). The first peak displayed an apparent molecular weight of 32,000, while the second peak corresponded to an $M_r$ of 5,000–7,000. Only the material in the 32,000-dalton peak expressed MAF and antiviral activities and reacted with monoclonal antibodies to IFN-γ.

Fig. 7 shows the results of a binding experiment that used bone marrow–derived macrophages and $^{125}$I-labeled HPLC-purified recombinant IFN-γ. Specific binding has been defined as the binding that is inhibited in the presence of a 500-fold excess of unlabeled IFN-γ. In these experiments ~80% of the total binding was specific. By Scatchard plot analysis, the cells were found to carry, per cell, 15,200 receptors that bound ligand in a homogeneous, noncooperative manner and displayed an affinity ($K_a$) of $1.18 \times 10^8$ M$^{-1}$. The average of three such experiments produced mean values of 12,000 receptors per cell and a $K_a$ of $0.9 \times 10^8$ M$^{-1}$. Analysis of IFN-γ binding to P388D$_1$ indicated that the cell line carried only 760 receptors per cell, which is 6.3% that displayed by bone marrow–derived macrophages. Similar experiments were performed on mem-

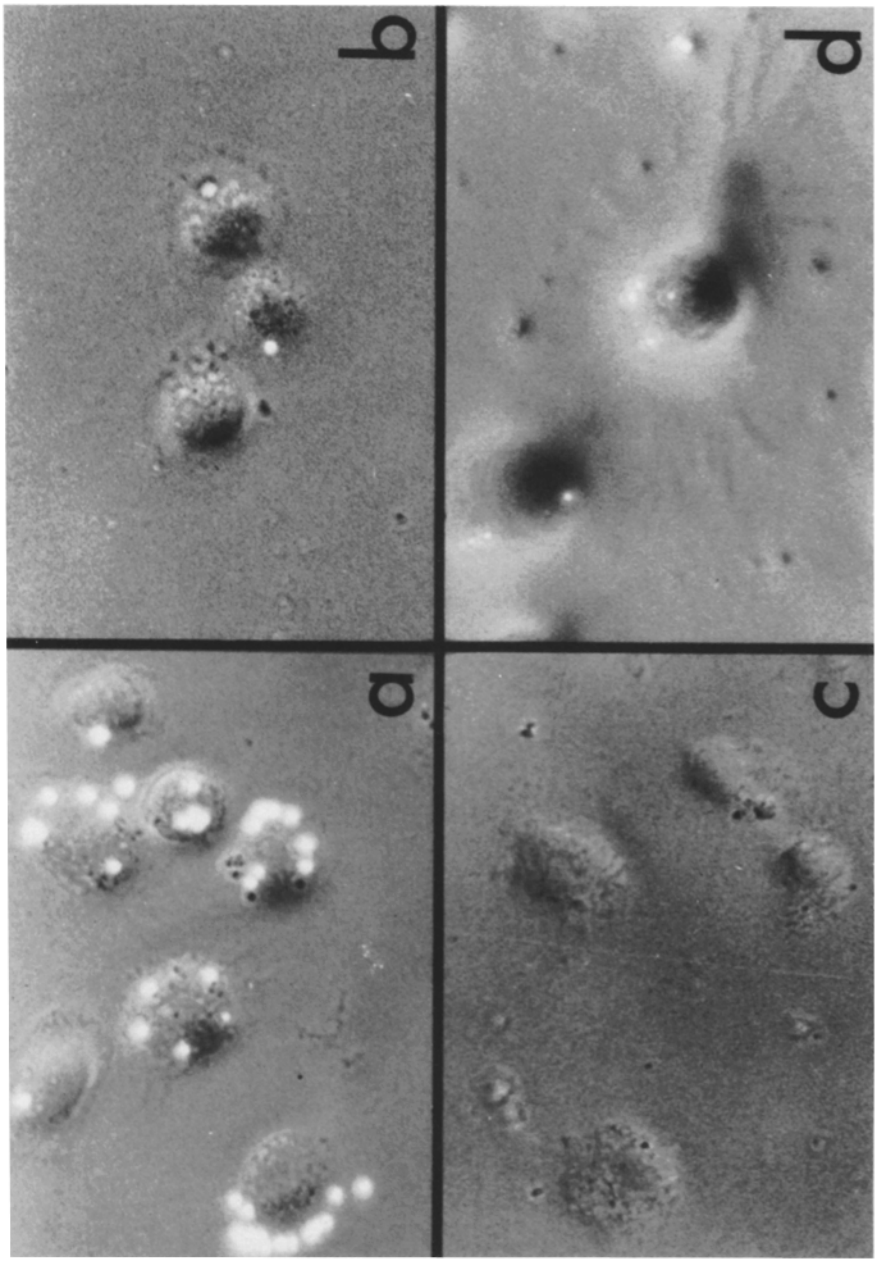


FIGURE 6. Binding of fluorescent microspheres coated with recombinant IFN-γ to macrophages. $2 \times 10^5$ EPM adherent on coverslips were incubated with microspheres for 2 h at 4°C. (*a*) EPM with microspheres bearing IFN-γ. (*b*) Microspheres coated with ethanolamine. (*c*) EPM were first incubated at 4°C with a 1/100 dilution of 24/G1 supernatant and then with IFN-γ bearing microspheres for 2 h at 4°C. (*d*) P388D$_1$ incubated with IFN-γ microspheres.

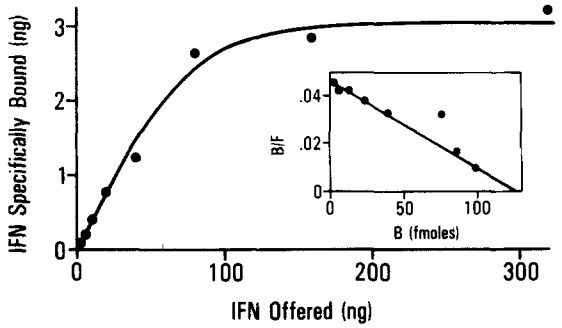


FIGURE 7. Quantitation of IFN-γ binding to murine macrophages. 5 × $10^6$ bone marrow-derived macrophages were incubated at 4°C for 2 h with different amounts of radiolabeled HPLC purified, recombinant IFN-γ. Cell associated and free $^{125}$I-IFN were separated by centrifugation over pthalate oil as outlined in Materials and Methods. Specific binding was defined as the difference between total binding and the nonspecific binding occurring in the presence of a 500-fold excess of unlabeled IFN-γ. The insert represents Scatchard plot analysis of the data. *B*, bound IFN-γ and *F*, free IFN-γ.

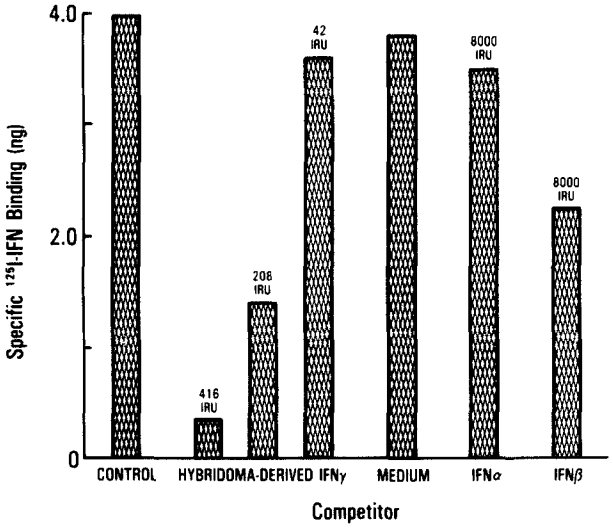


FIGURE 8. Specificity experiments for recombinant $^{125}$I-IFN-γ binding to macrophage. Bone marrow-derived macrophages were preincubated for 30 min at 4°C with designated amounts of competitor in a total reaction volume of 100 μl. 20 μl of $^{125}$I-recombinant IFN-γ (290 IRU) was then added to each tube and incubation continued for 2 h at 4°C. Specific binding and separation of bound and free $^{125}$I-IFN-γ as in Fig. 7. For these studies the T cell hybridoma supernatant or a sham supernatant were concentrated 10-fold before use by ultrafiltration.

brane preparations of bone marrow-derived macrophages. These studies showed that 17 μg of membranes (weight recovered from $10^7$ cells) specifically bound a maximum of 5 ng of IFN-γ.

Fig. 8 demonstrates the specificity of the binding reaction. Preincubation of the bone marrow macrophages with different amounts of 24/G1-derived IFN-γ inhibited the uptake of $^{125}$I-recombinant IFN-γ in a dose-dependent fashion. Addition of 208 IRU of natural IFN-γ to the reaction mixture caused a 62% reduction in binding of a comparable amount of the recombinant material (290

IRU), indicating that the two products bound to the cells with comparable affinity. Crude preparations of IFN-$\beta$ displayed a weak ability to inhibit binding of recombinant IFN-$\gamma$ when added in large excess (8,000 IRU), while IFN-$\alpha$ displayed no inhibitory activity at all.

*Relationship between Receptor Binding and Macrophage Activation.* Two monoclonal antibodies to IFN-$\gamma$ (H2 and H21) that differentially modulate IFN-$\gamma$-dependent biological responses in macrophages were used to demonstrate the involvement of the IFN-$\gamma$ receptor in macrophage activation. H21 has been found to inhibit the ability of IFN-$\gamma$ to induce nonspecific tumorilytic activity or Ia expression in macrophages, while H2 was found to enhance these activities.[2] Fig. 9 shows that inhibition or enhancement of IFN-induced macrophage functions correlates with the interaction of IFN-$\gamma$ with the IFN-$\gamma$ receptor. Depending on the input of IFN-$\gamma$, an excess of H21 inhibited 100% of the cellular uptake of IFN-$\gamma$, while H2 enhanced binding as much as 3.3-fold. No alteration of binding was observed when normal hamster IgG was substituted for the monoclonals.

## Discussion

This report documents the existence of a IFN-$\gamma$-receptor on murine macrophages and indicates that receptor engagement is a necessary first step in the induction of nonspecific tumorilytic activity in these cells. Macrophages bound either purified recombinant IFN-$\gamma$ produced in bacteria or natural IFN-$\gamma$ derived from either a murine T cell hybridoma or normal murine splenic cells. Binding was specific, saturable, of high affinity, and resulted in the induction of a biological response in appropriate macrophage populations. These parameters thus indicate that IFN-$\gamma$ was binding to a specific cell surface receptor.

Although substantial quantities of purified natural murine IFN-$\gamma$ were not available for these studies, the interaction could be demonstrated by quantitating the macrophage-dependent absorption of IFN-$\gamma$ from supernatants of stimulated T lymphocytes. We chose to quantitate MAF activity as an indicator of IFN-$\gamma$,

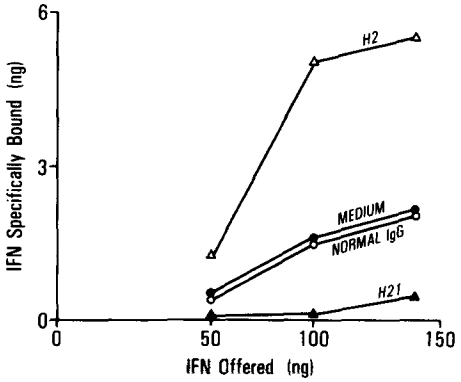


FIGURE 9. Effect of monoclonal antibodies on IFN-$\gamma$ uptake by macrophages. $5 \times 10^6$ bone marrow–derived macrophages were incubated for 2 h at 4°C with the indicated amounts of $^{125}$I-IFN-$\gamma$ in the presence of medium (●), normal hamster IgG (○), monoclonal anti-IFN, H2 (△) or monoclonal anti-IFN, H21 (▲). Specific binding and separation of cell-associated $^{125}$I-IFN-$\gamma$ as in Fig. 7.

since the macrophage was the target cell for this activity and since the MAF assay was more quantitative and 10 times more sensitive than the antiviral activity assay. For most of the studies, a supernatant of the 24/G1 T cell hybridoma was used. Previous studies have shown that the MAF activity produced by 24/G1 was largely due to IFN-γ (6, 8, 27). Recently, using monoclonal antibodies to recombinant IFN-γ we have found that IFN-γ represented the only MAF in these supernatants and in Con A–stimulated supernatants of normal murine splenic cells.[2] The absorption experiments showed that the ability of macrophages to remove MAF activity from culture supernatants was dependent on cell number, temperature, time of incubation, and displayed species specificity. The data also indicated that the activity was removed by cellular absorption and not by extracellular degradation or production of macrophage-derived factors that interfered with MAF quantitation. This conclusion was supported by the observations that (*a*) absorption occurred at 4°C, (*b*) paraformaldehyde-fixed murine macrophages absorbed MAF activity, (*c*) absorption of 24/G1 MAF was effected by murine macrophages but not hamster- or guinea pig-derived cells, (*d*) absorption was specific since treated supernatants showed a selective reduction in MAF activity but not IL-2 activity, and (*e*) mixing 24/G1 supernatants with macrophage culture supernatants did not affect the levels of MAF activity.

While these results strongly suggested the existence of an IFN-γ receptor on murine macrophages, they had to be validated with quantitative uptake experiments using radiolabeled IFN-γ. This was accomplished with $^{125}$I-labeled purified recombinant IFN-γ. Scatchard plot analysis of the binding data showed that the binding of IFN-γ to macrophages at 4°C was noncooperative (linear Scatchard plots), saturable (~12,000 receptors/cell), specific (binding was inhibitable by unlabeled IFN-γ), and of moderately high affinity ($K_a = 0.9 \times 10^8$ $M^{-1}$). Binding could be demonstrated to membrane preparations as well as to intact cells.

Several lines of evidence indicated that the interaction of the receptor with natural IFN-γ was comparable to that observed with the recombinant material. Both types of IFN-γ expressed equal MAF activities when compared on an antiviral activity basis (8, 27). Hybridoma-derived and recombinant IFN-γ were absorbed in an identical fashion by normal macrophages and were not absorbed by the P388D$_1$ cell line. Subsequent quantitation of $^{125}$I-recombinant IFN-γ binding to this cell line indicated that P388D$_1$ carried only 6.3% as many receptors as were expressed on normal macrophages. The number of receptors on normal macrophages determined by the absorption experiments with natural IFN-γ (2,000–5,000 receptors/cell) was comparable to the number obtained by measurement of $^{125}$I-recombinant IFN-γ binding (12,000 receptors/cell). Finally, unlabeled, natural IFN-γ could quantitatively compete with the labeled recombinant material for receptor binding. This result indicated that the two IFN-γ preparations bound to the receptor with similar affinities.

The monoclonal antibody studies indicate that binding of IFN-γ to its cell surface receptor is a necessary step for the induction of a biological response in macrophages. Modulation of the binding reaction correlated with appropriate increases or decreases in the eventual induction of tumoricidal activity. It is most likely that the inhibition produced by H21 reflected its ability to directly or

sterically block regions of IFN-γ that interact with the receptor. The enhancement observed with H2 probably reflects the formation of soluble immune complexes that were multimeric with respect to IFN-γ and that formed multipoint attachments to the macrophages through IFN-γ receptors. These possibilities are currently under examination.

The results presented in this communication are consistent with observations made in other laboratories. The ability of macrophages to remove MAF activity from lymphokine preparations was previously reported by Yamamoto and Tokunaga (28). However, this earlier study used a MAF of unknown biochemical identity and failed to establish absorption as the mechanism for MAF removal. A number of other studies have indicated that MAF-dependent induction of tumoricidal activity is not H2 restricted (29). Our experiments support this conclusion, since macrophages from mice with different H2 backgrounds absorbed MAF activity to comparable degrees.

The species specificity of the binding agrees with other results obtained independently. Although murine IFN-γ did not bind to hamster or guinea pig macrophages, it did bind to cultured normal human monocytes and to the human histiocytic lymphoma cell line $U_{937}$. Both natural and recombinant murine IFN-γ have been found to activate normal human peripheral blood monocyte-derived macrophages for nonspecific tumoricidal activity (Buchmeier, N. A., and R. D. Schreiber, unpublished data). Moreover, unlabeled recombinant *murine* IFN-γ can inhibit binding of $^{125}$I-*human* recombinant IFNγ to human mononuclear phagocytes (Celada, A., and R. D. Schreiber, manuscript in preparation). These results thus clearly differentiate IFN-γ-dependent MAF activity from IFN-γ-antiviral activity on fibroblasts. The latter displays strict species specificity.

To date only one other series of studies exists that suggests the existence of a cellular IFN-γ receptor. Anderson et al (30, 31) have demonstrated binding of purified natural human IFN-γ to human GM-258 fibroblasts. These fibroblasts expressed 8,000–20,000 receptors/cell and bound ligand with a $K_d$ of 2–6 × $10^{-9}$ M (or a $K_a$ of 1.7–5 × $10^8$ $M^{-1}$). These parameters are similar to the values obtained in our study. We have also documented the presence of an IFN-γ receptor on the murine fibroblast cell line $L_{929}$ as well as several other cell types using the MAF absorption technique. However, it is not yet known whether the receptors on the different cell types are identical or distinct. More structural and immunochemical data about the IFN-γ receptor is needed before this question can be answered. Work is currently underway to isolate and characterize the macrophage IFN-γ receptor.

The identification of a IFN-γ receptor on macrophages that participates in the induction of nonspecific tumoricidal activity is the first step at understanding macrophage activation at the molecular level. Work is currently in progress to determine the fate of the receptor-bound ligand and whether receptor engagement is sufficient to initiate a tumoricidal response. The quantitation of this receptor-ligand interaction should also provide a means of defining the mechanisms of action of other factors that have been reported to have MAF activity but not antiviral activity (32–34).

## Summary

Gamma-interferon (IFN-γ) is the macrophage-activating factor (MAF) produced by normal murine splenic cells and the murine T cell hybridoma 24/G1 that induces nonspecific tumoricidal activity in macrophages. Incubation of 24/G1 supernatants diluted to 8.3 IRU IFN-γ/ml with $6 \times 10^6$ elicited peritoneal macrophages or bone marrow–derived macrophages for 4 h at 37°C, resulted in removal of 80% of the MAF activity from the lymphokine preparation. Loss of activity appeared to result from absorption and not consumption because (*a*) 40% of the activity was removed after exposure to macrophage for 30 min at 4°C, (*b*) no reduction of MAF activity was detected when the 24/G1 supernatant was incubated with macrophage culture supernatants, and (*c*) macrophage-treated supernatants showed a selective loss of MAF activity but not interleukin 2 (IL-2) activity. Absorption was dependent on the input of either IFN-γ or macrophages and was time dependent at 37°C but not at 4°C. With four rodent species tested, absorption of murine IFN-γ displayed species specificity. However, cultured human peripheral blood monocytes and the human histiocytic lymphoma cell line $U_{937}$ were able to absorb the murine lymphokine. Although the majority of murine cell lines tested absorbed 24/G1 MAF activity, two murine macrophage cell lines, $P388D_1$ and J774, were identified which absorbed significantly reduced amounts of natural IFN-γ. Purified murine recombinant IFN-γ was absorbed by elicited macrophages but not by $P388D_1$. Normal macrophages but not $P388D_1$ bound fluoresceinated microspheres coated with recombinant IFN-γ and binding was inhibited by pretreatment of the normal cells with 24/G1 supernatants. Scatchard plot analysis showed that 12,000 molecules of soluble $^{125}$I-recombinant IFN-γ bound per bone marrow macrophage with a $K_a$ of $0.9 \times 10^8$ $M^{-1}$. Binding was quantitatively inhibitable by natural IFN-γ but not by murine IFNα. IFN-β competed only weakly. Monoclonal antibodies against IFN-γ either inhibited or enhanced MAF activity by blocking or increasing IFN-γ binding to macrophages, respectively. These results indicate that IFN-γ reacts with a receptor on macrophage in a specific and saturable manner and this interaction initiates macrophage activation.

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