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Potent Activation of Mouse Macrophages by Recombinant Interferon-γ[1]

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ABSTRACT

The ability of recombinant interferon-γ (IFN-γ) to activate mouse macrophages was investigated. The use of recombinant IFN-γ has the advantage of being devoid of contaminating lymphokines. Two preparations of IFN-γ were utilized, one which was not glycosylated and which was highly purified from *Escherichia coli* and another which was glycosylated and which was from transfected COS-7 monkey cells. Both preparations of recombinant IFN-γ activated murine macrophages to kill lymphoma and melanoma tumor targets, suggesting that glycosylation of the protein or the presence of other mammalian proteins is not essential for activation. Significant levels of cytolytic activity were induced from IFN-γ (1 to 10 units/ml). This activity was undiminished by treatment of the IFN-γ preparations with polymixin B at doses which neutralized endotoxin (50 μg/ml). Similarly, IFN-γ, at low concentrations, induced an inhibition of migration by macrophages. Based on antiviral activity, IFN-γ was shown to be 100 to 1000 times more potent than was IFN-β as a macrophage-activating agent. Taken together, these results demonstrate that murine IFN-γ is a macrophage-activating factor which is effective at physiological concentrations. Of particular interest is the observation that the nonglycosylated *E. coli*-derived IFN-γ is active and therefore may be of value for therapeutic studies, since it can be easily produced in large amounts.

INTRODUCTION

IFN-γ[3] is a secretory product of activated lymphocytes and differs from IFN-α and -β in cell origin, inducing agents, physical and biological properties (2), amino acid sequence (5), and induction of cellular proteins (18). Initial efforts to define the range of activities of IFN-γ were hampered by the lack of partially purified material. Many investigators were forced to use crude preparations which contained numerous other lymphokines and factors that also affected cellular functions. Recombinant DNA technology has made available large amounts of partially purified human IFN-α and -β for evaluation of their therapeutic potential and for investigations of their biological activities (4, 14). IFN-γ has only recently been produced by recombinant DNA techniques (7), and its role as a lymphokine participating in immunological responses is being investigated extensively. Gray and Goeddel (6) were able to obtain the expression of a cloned murine IFN-γ gene in transfected monkey cells. This recombinant IFN-γ provided a new opportunity to characterize the immunomodulatory properties of IFN-γ, because it is free from other murine lymphokines, including macrophage-activating factor. In addition, IFN-γ was also obtained from *Escherichia coli* harboring the murine IFN-γ expression plasmid (6). This latter source of IFN-γ is free of mammalian proteins, and it is suitable for production of amounts of IFN-γ, large enough for a good purification of this molecule. In these studies, we have utilized a preparation with a specific activity of about 3×10^7 units/mg of protein, which represents one of the highest degrees of purification achieved so far.

We have compared the relative macrophage-activating activities of these cloned IFN-γ preparations with those of natural IFN-β, because of the potentially important role played by macrophages in host defense against growth and metastasis of tumors (3). We have observed eukaryotic-derived IFN-γ to be a remarkably potent activator of tumoricidal macrophages which is 100- to 1000-fold more efficient than IFN-β in terms of antiviral units. IFN-γ is also endowed with MIF activity. Interestingly, IFN-γ derived from *E. coli* exhibited similar macrophage-activating capacity, at the same titers of antiviral activity, despite 1000- to 10,000-fold differences in specific activities. IFN-γ activated macrophages at physiological concentrations, suggesting that it may be involved in the activation of macrophages *in vivo* and pointing to the potential use of IFN-γ in therapeutic protocols designed to augment macrophage-mediated host defenses.

MATERIALS AND METHODS

Animals. Specific-pathogen-free C57BL/6N, 8 to 12 weeks of age, were obtained from the Animal Production Area of NCI-Frederick Cancer Research Facility.

Cell Lines. The following cell lines were used: L5178Y, a chemically induced lymphoma in DBA/2 mice ($H\text{-}2^d$); and the B16 melanoma of C57BL/6 origin ($H\text{-}2^b$).

Media and Reagents. The basic medium used in these studies was RPMI 1640 (M. A. Bioproducts, Walkersville, MD) supplemented with penicillin (100 units/ml), 2 mM glutamine, streptomycin (100 μg/ml), and 10% fetal bovine serum collected aseptically (Sterile Systems, Logan, UT). The medium was tested routinely for endotoxin contamination, and only batches containing endotoxins at less than 0.05 ng/ml (as assessed by the *Limulus* amebocyte lysate assay) were used. For harvesting of peritoneal exudate cells, the basic medium was supplemented with sodium heparin (100 units/ml) (Grand Island Biological Co., Grand Island, NY). For the macrophage cytotoxicity assay, the basic medium was supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer. In some experiments, macrophages were activated in serum-free medium (Neumann-Tytell; Grand Island Biological Co.). LPS from *E. coli* 0111:B4 (Difco Laboratories, Detroit, MI) and polymixin B (Sigma Chemical Co., St. Louis, MO) were dissolved in phosphate-buffered saline, pH 7.2, aliquoted, and stored at −70°.

IFN-γ was generously provided by Dr. Patrick Gray, Genentech Laboratories Co. (San Francisco, CA) and consisted of supernatant from COS-7 cells transfected with IFN-γ gene or IFN-γ produced in *E. coli* containing the IFN-γ gene. Control supernatants were obtained from cultures of nontransfected COS-7 cells and were used at the same

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[3] The abbreviations used are: IFN, interferon; MIF, migration-inhibitory factor; LPS, lipopolysaccharide; ^{111}In, ^{111}In-oxine.

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dilutions as the IFN-γ-containing supernatants. IFN-β was partially purified by affinity chromatography in Dr. K. Paucker's laboratory and was devoid of IFN-γ activity. The unbound protein fraction from the affinity column (mock IFN-β) was used as a control for the effects of IFN-β and was used at the same dilution as the IFN-β preparation. Antibodies against murine IFN-γ, kindly provided by Dr. Howard Johnson, have been described previously (10) and have been shown not to cross-react with IFN-α or IFN-β. Anti-mock IFN-γ antibodies were used as a control.

Macrophages. Macrophages were collected by harvesting peritoneal exudate cells from mice given injections of 1 ml of 3% proteose-peptone (Difco) 2 days earlier. Cells were washed once and resuspended in complete medium. Differential counts were made on Wright-Giemsa-stained smears prepared by cytocentrifugation. The cell suspension was then adjusted to the desired macrophage concentration.

Macrophage Activation. The ability of IFN to induce cytotoxic macrophages *in vitro* was tested as described previously (19). Briefly, 1.8×10^5 proteose peptone-induced macrophages were purified by adherence in 96-well microtiter plates (Costar, Cambridge, MA) and then treated overnight with dilutions of IFN or control supernatants or with medium alone. At the end of the incubation, the monolayers were washed and assayed for cytotoxicity after 24 hr of incubation with ^{111}In-labeled L5718Y lymphoma cells (19). The percentage of ^{111}In release was calculated from the average cpm of triplicate samples as

$$\frac{\text{cpm with treated proteose peptone-induced macrophages} - \text{cpm with untreated proteose peptone-induced macrophages}}{\text{Total cpm incorporated in target cells}} \times 100$$

The spontaneous release of ^{111}In from target cells cultured alone was between 10 and 15% of the total radioactivity incorporated. ^{111}In release from target cells cultured with untreated proteose peptone-induced macrophages did not differ by more than 5% from the spontaneous release. Alternatively, thioglycollate-stimulated macrophages were collected and activated as described elsewhere (10). Briefly, cell suspensions were centrifuged, and 10^5 cells were plated into the wells of a 96-well plate (Falcon Plastics, Oxnard, CA). Nonadherent cells (less than 10%) were removed by washing with medium 60 min after initial plating. The resultant monolayers of macrophages were >98% pure by morphological and phagocytic criteria. The monolayers were incubated with control medium or with an activating agent for 24 hr, after which the medium was removed and replaced with medium containing 10^4 radiolabeled target cells. Under these conditions and at this population density, normal (untreated) macrophages are not cytotoxic to neoplastic cells. B16 target cells in exponential growth phase were incubated for 24 hr in complete minimal essential medium supplemented with [^{125}I]iododeoxyuridine (0.3 μCi/ml; specific activity, 200 mCi/μmol; New England Nuclear, Boston, MA). The cells were then washed 3 times to remove unbound radiolabel, harvested by a short trypsinization (0.25% Difco trypsin and 0.02% EDTA), and plates onto macrophage monolayers. No significant differences were detected in the plating efficiency of the target cells, either when cells were added to control wells or when cells were added to monolayers of control or activated macrophages. The macrophage-target cell cultures were refed 24 hr after the addition of target cells to remove all nonattached target cells and were incubated for 2 additional days. To terminate the cytotoxicity assay, the wells were washed twice, and the viable adherent cells were lysed with 0.1 ml of 0.1 N NaOH. The lysate was absorbed with a cotton swab, and the radioactivity was monitored using a gamma counter. The cytotoxic activity of the macrophages was calculated as

$$\% \text{ of cytotoxicity} = 100 \times \frac{\text{cpm in target cells cultured with normal macrophages} - \text{cpm in target cells cultured with test macrophages}}{\text{cpm in target cells cultured with normal macrophages}}$$

The effects of IFN-γ on the migration of murine macrophages *in vitro* (MIF activity) was tested as detailed previously (17). All of the experiments presented were repeated at least 3 times, and representative results are shown in the tables and charts.

RESULTS AND DISCUSSION

Both IFN-γ and IFN-β induced cytotoxic macrophages (Chart 1). However, IFN-γ (10 to 20 units/ml) were sufficient to stimulate maximal levels of cytotoxicity, while IFN-β (5×10^3 units/ml) were needed to obtain comparable levels of cytotoxicity. Supernatants from nontransfected COS-7 cells, mock IFN-β, or human recombinant IFN-γ were inactive (Chart 1). Identical dose-response curves, in terms of antiviral units, were obtained when IFN-γ produced in *E. coli* was used, definitively ruling out the contribution of eukaryotic secretory products in the activation process and indicating that the glycosylation of IFN-γ is not required for the macrophage-activating property of this molecule. The specific activity of IFN-γ from *E. coli* was 3×10^7 units/mg, while that of IFN-γ from Cos-7 cell was $<10^3$ units/mg; therefore, only 1.5 pg/10^5 macrophages of IFN-γ from *E. coli* were needed to reach plateau levels of cytotoxicity. Parallel dose-response curves were obtained when thioglycollate-elicited, IFN-γ-activated macrophages were tested for cytotoxicity against the

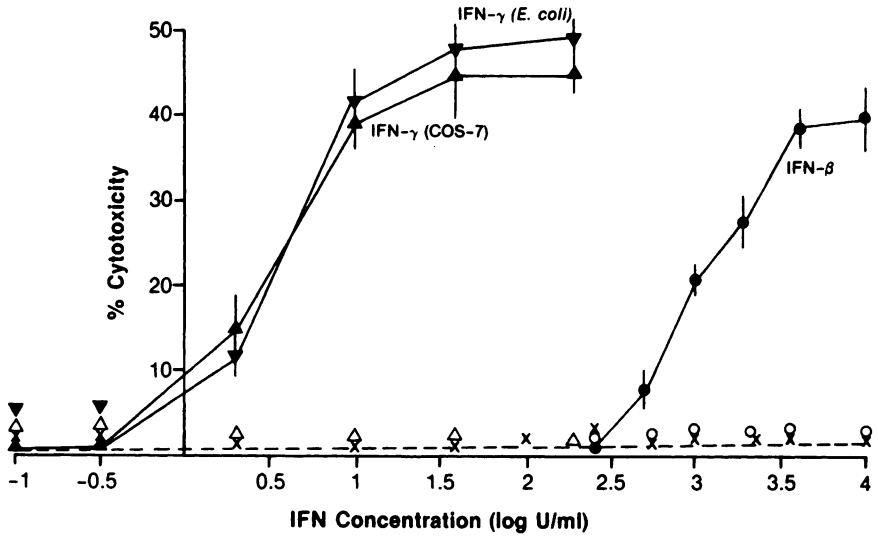

Chart 1. Induction of cytotoxic proteose peptone-induced macrophages by IFN-β and IFN-γ against L5178Y lymphoma. Proteose peptone-induced macrophages were incubated for 18 hr with IFN-γ from COS-7 cells (▲), IFN-γ from *E. coli* (▼), IFN-β (●), control COS-7 supernatant (△), mock IFN-β (○), or human recombinant IFN-γ (×), washed, and assayed for cytotoxicity. The ^{111}In-labeled target cells incorporated 3 cpm/cell, and the spontaneous release of targets cultured in medium alone was 8.5%. The ^{111}In release of target cells in the presence of medium-treated macrophages was 11.3%. The results represent the average cytotoxicity of 4 replicate cultures; *bars*, S.E.; *U*, units.

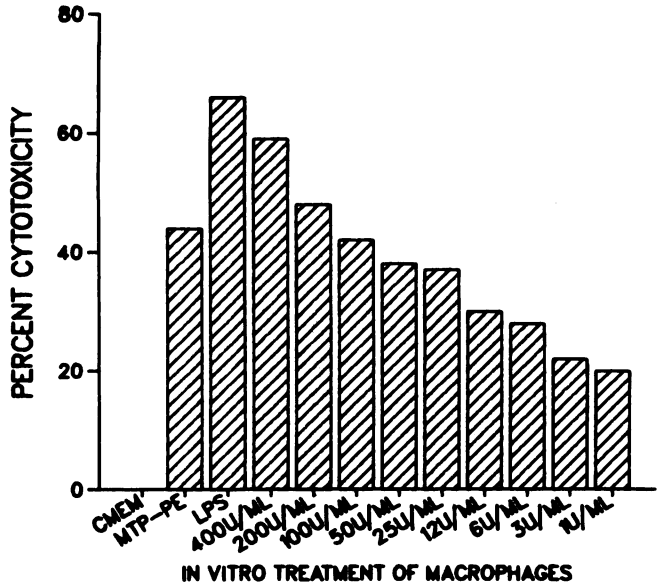

Chart 2. Activation of macrophages by IFN-γ for cytotoxicity against B16 melanoma. Thioglycollate-induced macrophages were activated by different doses of IFN-γ (from Cos-7 cells). All of the dilutions of IFN-γ were mixed with polymixin B (20 μg/ml) for 30 min prior to their addition to the macrophage monolayers. As a positive control, macrophages were activated with multilamillar vesicles (total phospholipid, 100 mmol) incorporating 0.4 μg of muramyl tripeptide-phosphatidylethanolamine or LPS (6.3 μg/ml). The standard errors of the averages of triplicate cultures were less than 10% of the mean. *U*, units; CMEM, complete minimal essential medium.

lymphoma L5178Y in a 24-hr ^{111}In release assay or against the adherent target cell B16 melanoma in a 72-hr [^{125}I]iododeoxyuridine release assay (Chart 2). From the consistent results of 5 replicate experiments, we conclude that IFN-γ is 100 to 1000 times more potent, relative to antiviral activity, than is IFN-β in inducing tumoricidal macrophages. Similarly, at 24 hr after i.p. injection of IFN-γ or IFN-β into C57BL/6 mice, IFN-γ was about 100-fold more efficient than was IFN-β in generating cytotoxic macrophages *in vivo* (data not shown). All of these comparisons were based on units of antiviral activity rather than molarity, since the IFN preparations have not yet been purified to homogeneity.

LPS is known to be an activator and costimulator of macrophage-mediated cytotoxicity (15, 16). The levels of endotoxins in the stock solutions of IFN-γ (600 to 1000 units/ml) derived from COS-7 cells varied, depending on the batches, from ≤0.01 to 1 ng/ml, whereas the IFN-γ preparations used from *E. coli* (≥10^5 units/ml) did not contain any detectable endotoxin (<0.01 ng/ml as measured by the limulus lysate and chromogenic assay). The results presented have been obtained with IFN-γ preparation negative for endotoxins in their concentrated form (*E. coli*-derived IFN-γ) and/or in their working dilutions (COS-7-derived IFN-γ). Even though the levels of endotoxins were as low as was technically possible and were below any detectable level, we examined whether inhibitors of endotoxins could affect the activation of macrophages by IFN-γ. Polymyxin B, an antibiotic which irreversibly binds and neutralizes lipid A, the active moiety of bacterial LPS (8), did not alter the activation of macrophages by IFN-γ at concentrations which completely inhibited the cytotoxicity induced by exogenously added endotoxin (50 μg/ml) (Table 1). Thus, polymyxin B-sensitive endotoxins were neither responsible nor required for the induction of cytotoxic macrophages by IFN-γ. Proteinase K abrogated the macrophage-activating effects of IFN-γ, whereas pretreatment of the IFN-γ preparation with various nucleases (Table 1) failed to affect the induction of cytotoxic macrophages, ruling out the contribution of nucleic acids possibly released by the producing cells. Addition of LPS (10 ng/ml) to the IFN-γ preparation increased about 10-fold the sensitivity of macrophages to IFN-γ stimulation, as shown by the results presented in Chart 3. The augmentation of cytotoxic activity induced by LPS was totally neutralized by polymyxin B (data not shown). These results demonstrated that endotoxins can synergize with IFN-γ in inducing cytotoxic macrophages, although they do not appear to be absolutely required for macrophage activation.

Conclusive identification of IFN-γ as the macrophage-activating stimulus was obtained by demonstrating that specific anti-IFN-γ antibodies prepared against natural mouse IFN-γ (12)

Table 1

Characteristics of macrophage activation by IFN-γ and IFN-β

IFN-γ and IFN-β were treated for 45 min at 37° with the various agents and then used to activate proteose peptone-elicited macrophages. Control supernatants, treated in the same way, did not induce any cytotoxic activity (data not shown). Serum-free medium (Neuman-Tytell; Grand Island Biological Co., Grand Island, NY) was used to dilute the IFN and during the activation.

Treatment	Av. % of cytotoxicity[a]	
	IFN-γ (10 units/ml)	IFN-β (10^4 units/ml)
None	35 ± 1.6[b]	28 ± 0.5
Polymyxin B (25 μg/ml)[c]	34 ± 1.5	31 ± 1.6
Proteinase K (50 μg/ml)	−2	1
RNase III (20 units/ml)	36 ± 2.4	30 ± 0.6
RNase H (20 units/ml)	32 ± 1.2	25 ± 0.8
RNase A (50 μg/ml)	38 ± 2.1	27 ± 1
DNase (1000 units/ml)	33 ± 1.1	30 ± 1.4

[a] Average percentage of cytotoxicity above base-line control of 3 replicate samples against L5178Y target cells in a 24-hr ^{111}In-release assay.
[b] Mean ± S.E.
[c] Under the conditions used, Polymyxin B (25 μg/ml) reduced the cytotoxicity induced by LPS (50 μg/ml) from 32 ± 1.3% (S.E.) to 1%.

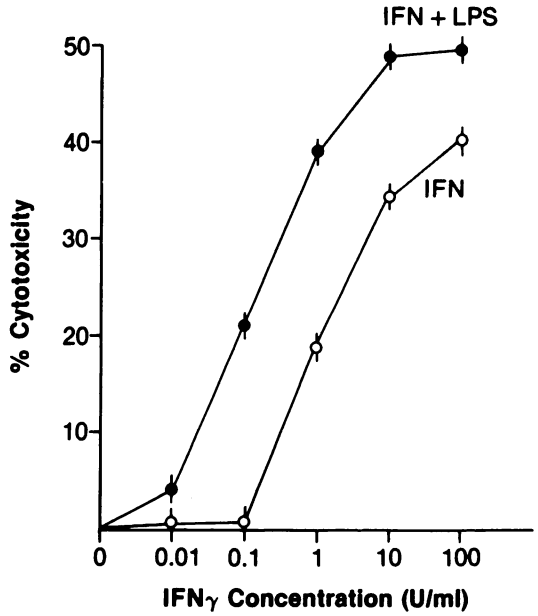

Chart 3. Synergism between IFN-γ and LPS. Proteose peptone-induced macrophages were activated for 18 hr with different concentrations of IFN-γ with the presence (●) or absence (○) of exogenously added LPS (final concentration, 10 ng/ml) and tested for cytotoxicity. Macrophages incubated with medium or human IFN-γ containing LPS (10 ng/ml) were not cytotoxic (≤5 ± 2% cytotoxicity). *U*, units.

prevented the generation of cytotoxic macrophages induced by IFN-γ but not by IFN-β (Chart 4).

Pace *et al.* (13) reported that peritoneal macrophages from C3H/HeN mice required LPS to be activated by recombinant IFN-γ from COS-7 cells. The lack of any detectable LPS requirement in our system might be due to a difference in the susceptibility to activation by IFN-γ between macrophages from C57Bl/6 mice, used in our studies, and macrophages from C3H/HeN. In addition, the LPS requirement might be a function of the dose of IFN-γ used to activate the macrophages. Whereas Pace *et al.* tested IFN-γ to a maximum of 10 units/ml, we extended our titration to 200 units/ml. By comparison of dose-response curves in the short-term cytotoxicity assays, it is evident that the only discrepancy is the LPS-independent activation that they failed to see with 10 units/ml. A difference in the sensitivity of the assays or of the target cells to killing could account for this discrepancy. The present study extends beyond the work of Pace *et al.* (13) by demonstrating that highly purified IFN-γ from *E. coli* activates cytotoxic macrophages. This observation definitely rules out the contribution to activation of enhancing or inhibiting factors possibly present in the COS-7 supernatants.

Crude lympokine-containing supernatants have multiple effects on macrophages that may be mediated by distinct molecules or that may be due to different biological responses induced by the same factor. We were interested in determining whether IFN-γ could affect the migration of macrophages *in vitro* and thus contribute to the MIF activity often found in crude lymphokine preparations. The results shown in Table 2 demonstrate that IFN-γ can inhibit the migration of murine macrophages *in vitro* in a dose-dependent fashion. Significant MIF-like activity was still evident at IFN-γ (10 units/ml). These results suggest that IFN-γ may contribute to the biological activities ascribed to MIF (1) such as the homing of macrophages into inflammatory areas.

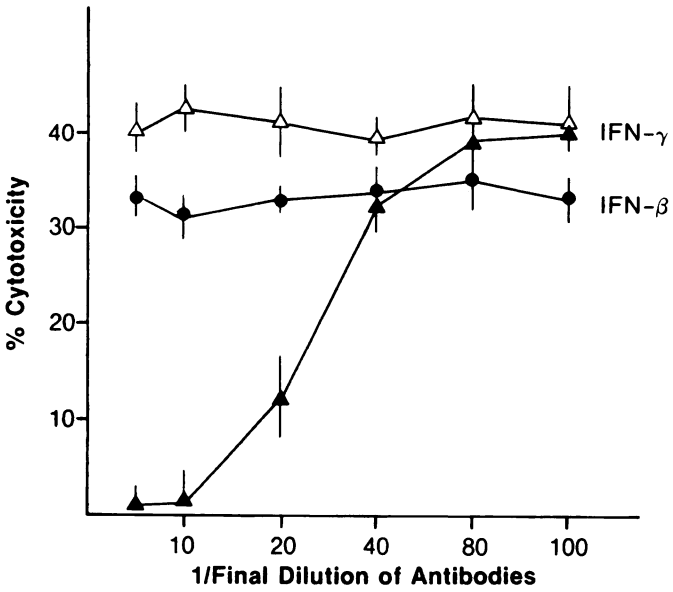

Chart 4. Neutralization of the induction of tumoricidal macrophages by anti-IFN-γ antibodies. IFN-γ (100 units/ml) or IFN-β (10^4 units/ml) were neutralized with different dilutions of antibodies for 30 min at 37° and added to the macrophage monolayers. The cytotoxic activity was tested 18 hr later. ▲, IFN-γ + anti-IFN-γ antibodies; ●, IFN-β + anti-IFN-γ antibodies; △, IFN-γ + anti-mock IFN-γ antibodies. The results represent the average cytotoxicity of 3 replicate cultures; *bars*, S.E.

Table 2

MIF-like activity of recombinant murine IFN-γ measured in the agarose droplet MIF assay with murine peritoneal exudate cells

Concentration of recombinant IFN-γ (from COS-7 cells) (units/ml)	% of macrophage migration inhibition	
	Experiment 1	Experiment 2
1000	NT[a]	77.3
400	52.8	NT
200	47.5	NT
100	35.9	66.4
50	30.6	NT
25	24.7	NT
12	12.8	NT
10	NT	21.6
6	−4.1	NT
1	NT	−4.5

[a] NT, not tested.

The ability of IFN-γ, at very low concentrations, to fully activate macrophages for tumoricidal activity has considerable practical and biological implications. IFN-γ-induced antiviral activity (1 to 10 units/ml) can frequently be detected in serum, biological fluids, or crude lymphokine preparations (9). Moreover, macrophage-activating concentrations of IFN-γ may be present in lymphokine supernatants more often than is generally appreciated, since such levels of antiviral activity are at the borderline of sensitivity for antiviral assays. Therefore, IFN-γ may be responsible for some of the immunomodulatory functions ascribed previously to other lymphokines, such as macrophage-activating factor and MIF. It is conceivable that activated lymphocytes may release more than one factor endowed with macrophage-activating factor and MIF activity. Our results demonstrate definitively that IFN-γ must be considered in this regard.

The ability of nonglycosylated IFN-γ to induce cytotoxic macrophages may be a general property of IFNs, since it has been reported that glycosylation is also not required for boosting of natural killer cell activity (11) by IFN-β.

IFN-α and -β are under evaluation for antitumor activity in clinical trials, and optimal dosages and schedules have not yet been established. The high potency of IFN-γ in activating tumoricidal macrophages suggests that this lymphokine should be tested for therapeutic purposes, at relatively low doses close to physiological concentration, and efficacy at such levels may circumvent toxic side effects.

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