Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 3688–3692, June 1985
Cell Biology

Biological activity of liposome-encapsulated murine interferon γ is mediated by a cell membrane receptor

(antiviral agent/macrophage tumoricidal agent/antibody neutralization)

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Communicated by I. Robert Lehman, January 28, 1985

ABSTRACT **Recombinant murine γ interferon (rMuIFN-γ) was found to bind reversibly to a specific high-affinity surface receptor on L929 cells; neither murine α or β nor human γ IFN competed for receptor binding. Encapsulation of the rMuIFN-γ in either negatively or positively charged liposomes reduced its immediate ability to bind to this surface receptor. Disruption of liposome integrity with detergent resulted in full ability of the rMuIFN-γ to bind to the membrane receptor. Incubation of the liposomal IFN in serum-containing medium resulted in significant leakage so that the IFN was able to bind to its surface receptor. Assessment of the biological activity of the rMuIFN-γ preparations revealed that full antiviral activity was observed *in vitro* with the liposomal IFN preparations without their prior disruption by detergent. The antiviral activity observed with either free or liposomal IFN was neutralized completely by antibodies against rMuIFN-γ. Both free and liposomal rMuIFN-γ, in conjunction with bacterial lipopolysaccharide, were also able to activate murine peritoneal macrophages to the tumoricidal state. Again, this activity of both free and liposomal IFN could be neutralized completely by antibody. These results indicate that although rMuIFN-γ can be effectively incorporated into liposomes, it must ultimately leak out of the liposome in order to mediate its biological effects; these effects are triggered after the IFN binds to its cell surface receptors.**

Human α, β, and γ and murine α and β interferons (IFNs) have been shown to interact with specific receptors at the surface of target cells (refs. 1–4, reviewed in ref. 5). High-affinity binding of the polypeptides to the cell membrane receptors is the first step leading to their antiviral and antiproliferative effects. IFN-γ has been found to stimulate macrophages with respect to intracellular cytocidal reactions (6, 7) and tumoricidal activity (8–13) in addition to exerting antiviral and antiproliferative properties on mesenchymal cells (reviewed in ref. 14). A cell surface receptor for murine IFN-γ has recently been found on macrophages and is involved in the initiation of the cellular priming effect of macrophage-mediated tumoricidal activity (15).

Liposome encapsulation of polypeptides, including α and β IFNs, can protect against inactivation by proteases such as trypsin (16–18). Liposomes are therefore viewed as potential carrier systems for the *in vivo* administration of IFNs. The use of liposomal–IFN preparations has resulted in altered pharmacokinetics and tissue distribution of IFN *in vivo* when compared to systemic administration of free IFN (17, 19). When these preparations were assayed for antiviral activity *in vitro*, partial to full bioactivity of IFN was obtained surprisingly without the need for prior disruption of the liposome (16–19). These observations prompted us to determine whether or not this bioactivity is caused by IFN that bypasses an initial interaction with the target cell surface. We were particularly interested in studying IFN-γ and its interaction with cells in free or liposomal form in the light of reports of the superior ability of liposome-encapsulated macrophage activating factor (MAF) (20, 21) and, more recently, IFN-γ (22) to prime macrophages for cytolytic effects on target cells. We report that although recombinant murine IFN-γ (rMuIFN-γ) can be efficiently encapsulated in liposomes, it ultimately leaks out of the liposome in order to trigger its biological effects, which in all likelihood are mediated subsequent to binding of the IFN to its cell-surface receptor.

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MATERIALS AND METHODS

Chemicals. Synthetic L-α-lecithins (1,2-diacyl-*sn*-glycero-3-phosphocholine) of compositions dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) were from Avanti Biochemicals; stearylamine (SA) was from Sigma; Enzymobeads were from Bio-Rad; *Limulus* amebocyte lysate was from Mallinckrodt; lipopolysaccharide (LPS; *Escherichia coli* 0111:B4) was from Difco; Wright staining system (Diff-Quick) was from American Scientific Products (McGaw Park, IL); carrier-free $Na^{125}I$ and 5-[^{125}I]iododeoxyuridine (2200 Ci/mmol; 1 Ci = 37 GBq) were from New England Nuclear; rMuIFN-γ [specific activity (s.a.) of different lots ranged from 0.6–1.9 × 10^7 units (U)/mg], recombinant human IFN-γ (rHuIFN-γ; s.a. = 1.2–3.4 × 10^7 U/mg), and rabbit antiserum to rMuIFN-γ were from Genentech (South San Francisco). Natural murine IFN-α (s.a. = 1.7 × 10^6 U/mg) and natural murine IFN-β (s.a. = 2 × 10^8 U/mg) were obtained from Lee Biomolecular Laboratories (San Diego, CA).

Cells and Viruses. Murine L929 cells were obtained from American Type Culture Collection, (ATCC CCL1). Encephalomyocarditis virus (EMCV) was obtained from Interferon Laboratories, Sloan-Kettering Institute. Murine B16-F10 melanoma cells were from Mason Tumor Bank, Worcester, MA.

Liposomes. Lyophilization multilamellar vesicles (MLVs) were prepared by drying lipids (from $CHCl_3$ solution) to a film under N_2, followed by vacuum desiccation for 30 min. Dried lipids (10 μmol) were dissolved in 1 ml of cyclohexane, frozen, and lyophilized. Aqueous phase was then added to the lyophilized lipids, which readily formed liposomes. For receptor-binding and antiviral studies, DOPC/DOPG and POPC/SA (7:3 molar ratio; 10 μmol of lipid/ml of aqueous

Abbreviations: IFN, interferon; rMuIFN-γ, recombinant murine IFN-γ; rHuIFN-γ, recombinant human IFN-γ; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; SA, stearylamine; MLV, multilamellar vesicle; LPS, bacterial lipopolysaccharide; MAF, macrophage-activating factor; EMCV, encephalomyocarditis virus; U, units.

phase) lyophilization MLVs were prepared with rMuIFN-γ at 10^6 or 5×10^4 U/ml, in 0.2 M glycine·HCl (pH 3.5) (DOPC/DOPG preparations) or in phosphate-buffered saline (pH 8.0) in 80% ^2H_2O as indicated (POPC/SA preparations). The pH 3.5 buffer was chosen for the negatively charged liposomes to maximize interactions between positively charged rMuIFN-γ and negatively charged lipids. Conversely, pH 8.0 buffer was employed for preparation of positively charged liposomes to reduce the charge repulsion between lipids and IFN. The pH 3.5 buffer did not inactivate rMuIFN-γ during the assays. SA-containing liposomes caused cellular toxicity at high concentration (≥20 μM), as reported (18, 23, 24). ^{125}I-labeled rMuIFN-γ (40,000 cpm) was added to the aqueous phase before liposome preparation to quantitate liposomal IFN for binding studies. Vesicles were separated from unencapsulated solute by centrifugation, with 2 or 3 buffer washes, at 100,000 × *g* for 30 min in a Beckman Airfuge and were resuspended in buffer. DOPC/DOPG liposomes sedimented, whereas POPC/SA liposomes floated. For POPC/SA preparations used in receptor-binding studies, separation was enhanced by the use of ^2H_2O instead of H_2O.

Liposomal and free IFN aliquots were treated with trypsin, soybean trypsin inhibitor, and 0.1% Triton X-100 as described (18). Samples were assayed directly for antiviral activity, as well as after centrifugation to separate liposomes from free solutes for testing in antiviral and binding studies. Serum-induced liposome leakage was determined by monitoring liposomal vs. free radioactivity after incubation of liposomal IFN, spiked with ^{125}I-labeled rMuIFN-γ, for 24 hr at 37°C in medium containing 10% fetal calf serum; serum-treated liposomes were then tested directly in binding studies.

Antibody Neutralization of Antiviral Activity. Liposomal or free rMuIFN-γ (≈10^4 U/ml) or antibody to rMuIFN-γ (≈10^4 neutralizing U/ml) were titrated by 3-fold serial dilutions against a constant amount of antibody (≈10^3 or ≈10^2 neutralizing U/ml) or rMuIFN-γ (≈10^2 U/ml) in 96-well microtiter dishes: after incubation for 1 hr at 37°, cells were added. Remaining IFN activity was determined after addition of virus.

Binding Assays. rMuIFN-γ was iodinated by using Enzymobeads (immobilized glucose oxidase/lactoperoxidase system) and purified by sequential gel filtration over Sephadex G-25 and G-75 columns presaturated with gelatin. Bovine serum albumin (1 mg/ml) was added to the radioactive peak fractions. The specific activity was 1–1.5 × 10^5 cpm/ng of protein, with full retention of antiviral activity. Confluent monolayers of L929 cells in 24-well Costar dishes were washed twice with medium containing bovine serum albumin (1 mg/ml) and 50 mM Hepes (pH 7.4). Cells were incubated with ^{125}I-labeled rMuIFN-γ at either 4°C or 37°C for 1 hr in a final volume of 200 μl, washed three times with cold medium, and lysed with 0.1 M NaOH, and the cell-associated radioactivity was determined. Protein concentration was determined with the Bio-Rad protein assay to standardize results. Nonspecific binding was assessed in the presence of a 200-fold excess of unlabeled rMuIFN-γ and was ≤20% of the total binding. In displacement experiments, radiolabeled MuIFN-γ and inhibitor were incubated together with the cells.

Macrophage-Activation Studies. Peritoneal exudate cells were harvested from 6- to 8-week-old C57BL/6 mice basically as described by Fidler *et al.* (25), except that resident rather than elicited cells were used. Viability was ≥95% as determined by trypan blue exclusion; ≈70% of the cells were macrophages (Wright staining system). Cells were seeded at 10^5 macrophages per well in Costar 96-well plates, allowed to adhere for 3 hr at 37°C before washing to remove nonadherent cells, and then incubated with samples for 24 hr. Liposomal samples contained 250 nmol of phospholipid/ml (final concentration). Antibody (when included) was added immediately before addition of IFN. All reagents contained ≤0.025 ng of endotoxin/ml, as determined in a *Limulus* amebocyte lysate test. Tumoricidal activity of the macrophages was determined against syngeneic B16-F10 melanoma cells (26) that had been labeled by 24-hr incubation in exponential growth phase with [^{125}I]iododeoxyuridine (0.2 μCi/ml). Macrophage cultures were washed three times, target cells were added at a 1:15 target/effector ratio, and the cocultures were incubated for 72 hr (refed after 24 hr). Radioactivity of cell supernatants was determined after harvesting onto Skatron filters. Spontaneous release, determined from the radioactivity released into the medium from B16-F10 cells seeded with nonactivated macrophages, was ≈6% of total. The remaining adherent B16-F10 cells were lysed with 0.1 M NaOH to determine total target cell counts. Percent specific release was calculated as follows:

$$\frac{\text{experimental cpm} - \text{spontaneous-release cpm}}{\text{total cpm} - \text{spontaneous-release cpm}} \times 100.$$

Interferon Assay. Antiviral activity was determined in duplicate with a microtiter inhibition-of-cytopathic-effect assay against EMCV on monolayers of L929 cells (27). Titers are reported as antiviral units (U), based on National Institutes of Health murine IFN (α, β) reference reagent G002-904-511.

RESULTS

Antiviral Activity of rMuIFN-γ Liposome Preparations. The antiviral activity of rMuIFN-γ associated with liposomes is shown in Table 1. The negatively charged DOPC/DOPG preparations resulted in association of about 50% of the IFN activity with the liposomal pellet (also quantitated by spiking with ^{125}I-labeled rMuIFN-γ), yet this activity was destroyed to the same extent (≈95%) as that of free IFN by trypsin treatment. Addition of trypsin inhibitor before trypsin completely prevented inactivation of the free IFN (data not shown). Treatment of liposomes with a detergent (Triton X-100) to disrupt liposome integrity did not result in any detectable increase in antiviral activity. However, binding studies (Fig. 3, described below) indicated that most of the IFN associated with this MLV pellet was encapsulated in that it was unavailable for binding to cell-surface receptors unless the liposome integrity was disrupted.

Different results were obtained with positively charged vesicles. With unfractionated POPC/SA preparations, 50%

Table 1. Antiviral activity of rMuIFN-γ–liposome preparations treated with trypsin and/or Triton X-100

Sample	Antiviral activity, $\log_{10}$ U/ml			
	Untreated	Trypsin	Triton	Triton, then trypsin*
DOPC/DOPG–IFN				
Total	4.1 ± 0.2	2.7 ± 0.2	4.2 ± 0.1	≤2.8 ± 0.2
MLV Fraction	3.7 ± 0.2	2.5 ± 0.3	3.4 ± 0.5	≤2.8 ± 0.2
POPC/SA–IFN				
Total	4.6 ± 0.1	4.3 ± 0.2	4.6 ± 0.1	≤2.7 ± 0.0
MLV Fraction	3.6 ± 0.1	3.0 ± 0.3	3.6 ± 0.1	≤2.7 ± 0.0
Free IFN	4.3 ± 0.3	2.6 ± 0.5	4.4 ± 0.4	≤2.6 ± 0.4

Antiviral activity of rMuIFN-γ preparations was determined on L929 cells challenged with EMCV. Liposomes were prepared with 5 × 10^4 U of rMuIFN-γ per ml per 10 mM lipid. Results are the mean ± SD of four (liposome preparations) or eight (free IFN) experiments.

*Due to cellular toxicity of the Triton, titers in the presence of Triton could only be determined to ≤ the value indicated.

of the IFN antiviral activity remained after trypsin treatment that destroyed >95% of the activity of free IFN. The amount of IFN initially associated with these positively charged MLVs was difficult to quantitate due to their flotation properties, which prevented complete recovery of the liposomal fraction if contamination by the undernatant solution was to be avoided. However, as 50% of the IFN activity of the unfractionated liposome preparation was protected from trypsin digestion, ≥50% of the original IFN apparently had been encapsulated. Receptor binding studies confirmed that the availability of rMuIFN-γ in SA-containing liposomes to bind to cell-surface receptors was only fully achieved after disruption of the liposomes (see below). Thus trypsinization appears to be a valid method of determination of encapsulation of rMuIFN-γ in the positively charged liposomes but not in the negatively charged ones.

As with the negatively charged liposomes, treatment of the positively charged vesicles with Triton did not result in significant increase in IFN antiviral activity. However, after treatment with Triton, the IFN of the SA preparations became fully susceptible to trypsin digestion (Table 1). These results suggested that IFN was leaking out of the liposome preparation before establishment of the antiviral state. To test this hypothesis, antibody neutralization studies were conducted. As shown in Fig. 1, the antiviral activity of free rMuIFN-γ was neutralized by the specific antibodies in a dose-dependent fashion. The antiviral activity of the two liposomal IFN preparations was also neutralized by antibodies to approximately the same extent as the free IFN activity.

Macrophage Activation. The results of a representative assay (of four independent experiments) of macrophage tumoricidal activity are shown in Table 2. rMuIFN-γ by itself or in liposomal formulation showed little ability to activate macrophages, although some activation was obtained at the higher (100–1000 U/ml) concentrations of IFN. Similarly, LPS alone did not result in macrophage activation. In conjunction with LPS, however, preparations either of free or of liposome-encapsulated rMuIFN-γ resulted in a concentration-dependent activation of macrophages to the tumoricidal state; less activation of macrophages was obtained with liposomal than with free rMuIFN-γ. When antibody to rMuIFN-γ was included, the macrophages were not activated to the tumoricidal state either by free or liposomal IFN, whether alone or in combination with LPS. Excess antibody (defined by antiviral neutralization units) was needed for full neutralization of macrophage-priming activity, as evidenced by residual activity with free IFN at 1000 U/ml plus antibody at 1000 U/ml. The fact that full neutralization of the liposomal IFN at 1000 U/ml was obtained with antibody at 1000 U/ml suggested that not all the liposomal IFN was available to antibody and that the IFN fraction mediating the macrophage-priming effect was fully available to antibody, probably corresponding to that fraction which had leaked into the medium.

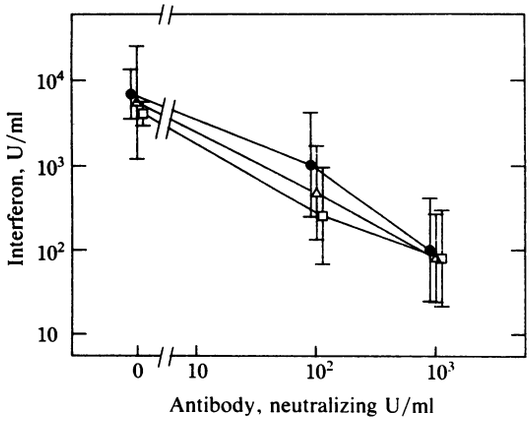

FIG. 1. Neutralization of antiviral activity of rMuIFN-γ preparations by specific antibodies. The titer of rMuIFN-γ preparations was determined, in the presence of a constant amount of antibodies to rMuIFN-γ (10^2 or 10^3 neutralizing U/ml), on L929 cells challenged with EMCV. ●, Free IFN; △, DOPC/DOPG–IFN; □, POPC/SA–IFN. Results shown are the mean ± SD of at least two independent experiments performed in triplicate.

Table 2. Macrophage priming by free or liposomal rMuIFN-γ

		% specific lysis (± SD, n = 6)*		
	U/ml	Alone	+ LPS (1 μg/ml)	+ LPS (1 μg/ml) + antibody†
Free rMuIFN-γ	0	0	0	0
	10	2 ± 1	24 ± 4	0
	100	12 ± 2	39 ± 5	0
	1000	17 ± 3	39 ± 5	32 ± 3
DOPC/DOPG–rMuIFN-γ	0	0	0	0
	10	0	0	0
	100	0	7 ± 1	0
	1000	2 ± 1	22 ± 5	0

Resident mouse peritoneal macrophages were incubated for 24 hr with the samples indicated. Tumoricidal activity against [^{125}I]iododeoxyuridine-labeled B16-F10 melanoma cells was determined after a 72-hr cocultivation, as described in *Materials and Methods*.
*Total cpm of B16-F10 targets was 3470 ± 320 cpm. Specific lysis values shown as 0% ranged from −1 to 0 ± 1%.
†Antiviral neutralizing U/ml used: 100 for 0–10 IFN U/ml and 1000 for 100–1000 IFN U/ml.

Receptor Binding Studies. Results of cell surface receptor binding studies with free ^{125}I-labeled rMuIFN-γ and L929 cells are shown in Fig. 2. The binding was fully reversible at 4°C by addition of a 1000-fold excess of unlabeled rMuIFN-γ. At 37°C, after equilibrirm binding, some of the radiolabeled bound IFNs could not be displaced by unlabeled IFN, presumably reflecting receptor-mediated internalization (data not shown). Scatchard analysis (28) of the data [Fig. 2 *Upper* (*Inset*)] indicated approximately 4200 and 4800 binding sites per cell at 4°C and 37°C, respectively, with an apparent K_d of about 1 nM. Neither natural murine α or β IFN nor rHuIFN-γ significantly displaced ^{125}I-labeled rMuIFN-γ, whereas unlabeled rMuIFN-γ or anti-rMuIFN-γ antibodies inhibited binding in a concentration-dependent fashion (Fig. 2 *Lower*).

The binding studies with DOPC/DOPG–rMuIFN-γ are shown in Fig. 3; comparable results were obtained with POPC/SA liposomes (not shown). With both these preparations, full binding displacement by IFN was not obtained unless the liposomes were disrupted with Triton; they then gave results identical to those obtained with free rMuIFN-γ plus buffer MLVs ("empty" liposomes). Preincubation of the intact liposomes with 10% serum at 37°C for 24 hr resulted in more binding displacement than was obtained with the native liposome preparations. Concomitant analysis with ^{125}I-labeled rMuIFN-γ showed that 30–40% of the IFN leaked out of the MLV during this 24-hr incubation with serum (data not shown). Treatment of the liposomal IFN preparations with trypsin reduced the ability to compete with free ^{125}I-labeled rMuIFN-γ for receptor binding, in agreement with results of the antiviral assays.

DISCUSSION

Human IFN-γ has been shown to have a specific high-affinity cell surface receptor (2) that does not crossreact with human α or β IFNs (3), both of which share a common receptor (1). Other workers have reported that murine IFN-γ does not

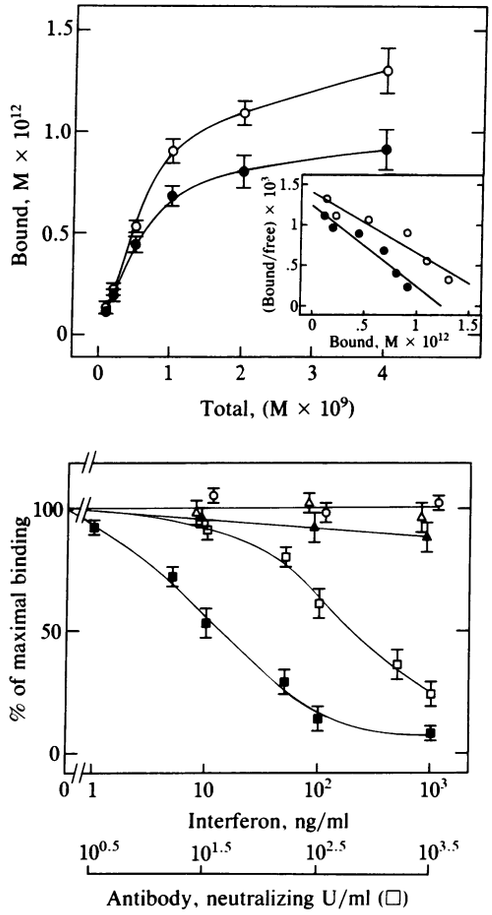

FIG. 2. (*Upper*) Binding of radiolabeled rMuIFN-γ to L929 fibroblasts. Cells were incubated with various concentrations of ^{125}I-labeled rMuIFN-γ for 1 hr at 4°C (●) or 37°C (○) prior to determination of cell-associated radioactivity. Shown are the mean ± SD of three independent experiments performed in triplicate. *Inset*: Scatchard analysis of corresponding binding data. (*Lower*) Specificity of rMuIFN-γ binding to L929 cells. Cells were incubated with ^{125}I-labeled rMuIFN-γ (10 ng/ml) at 4°C for 1 hr in the absence or presence of unlabeled rMuIFN-γ (■), murine IFN-α (△), murine IFN-β (▲), rHuIFN-γ (○), or anti-rMuIFN-γ antibodies (□) prior to determination of cell-associated radioactivity.

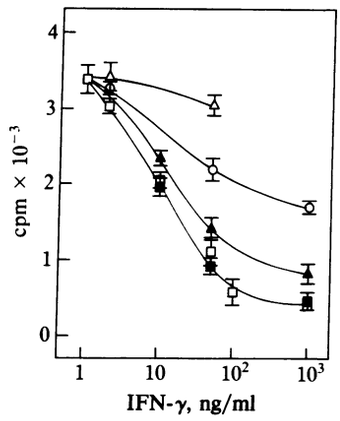

FIG. 3. Displacement of binding of radiolabeled rMuIFN-γ to L929 cells by DOPC/DOPG liposomal preparations. Cells were incubated at 4°C for 1 hr with ^{125}I-labeled rMuIFN-γ (10 ng/ml) in the absence or presence of free rMuIFN-γ (□), free rMuIFN-γ mixed with empty liposomes (■) (which gave the same results as Triton X-100-treated liposomal IFN preparations), liposome-encapsulated rMuIFN-γ (○), or liposome-encapsulated rMuIFN-γ preparations pretreated with either serum (▲) or trypsin (△). Liposome preparations contained 0.43 nmol of lipid per ng of rMuIFN-γ.

compete with murine α or β IFNs for binding to the murine α/β receptor (4). We now have demonstrated that rMuIFN-γ too has a high-affinity cell surface receptor on fibroblasts and that neither murine α or β IFN nor human IFN-γ interact with this receptor. Others have shown recently that a receptor for MuIFN-γ exists on murine macrophages and is involved in the priming stage for macrophage activation (15). Our results indicate that both the antiviral activity and the priming for macrophage activation observed *in vitro* with liposome-encapsulated rMuIFN-γ are mediated after liberation of the IFN from the liposome and most likely subsequent to interaction of the IFN with this cell surface receptor.

The results that led us to these conclusions are as follows: (*i*) Liposomes containing a positively charged lipid (SA) encapsulated rMuIFN-γ so that it was initially resistant to inactivation by trypsin degradation. However, the IFN in these preparations showed full bioavailability for antiviral activity in a 2-day *in vitro* assay (Table 1). (*ii*) No increase in observable antiviral activity was obtained after disruption of the liposome with detergent. However, detergent treatment rendered the liposomal IFN fully susceptible to digestion by trypsin (Table 1). (*iii*) The antiviral activity of liposomal IFN preparations was neutralized by antibody to rMuIFN-γ to the same extent as that of unencapsulated IFN (Fig. 1). The ability of free rMuIFN-γ to activate macrophages to the tumoricidal state in conjunction with LPS (10, 12) also was neutralized by addition of antibody (Table 2). Similarly, the ability of liposome-encapsulated IFN-γ to prime/activate macrophages was fully neutralizable by externally added antibody. (*iv*) Receptor binding studies showed that the liposomal IFN was initially largely unavailable for binding with the IFN-γ receptor. However, incubation at 37°C with 10% serum (i.e., under conditions similar to those of the IFN assay) resulted in release of 30–40% of the IFN from the liposome after 24 hr. This leakage of IFN from the liposome was reflected in a proportional increase in the ability of the liposomal IFN preparation to compete with ^{125}I-labeled rMuIFN-γ for receptor binding. Finally, detergent disruption of the liposomes resulted in full ability of the IFN from the liposomal IFN preparations to compete for receptor binding (Fig. 3). Specific antibody to rMuIFN-γ effectively prevented the rMuIFN-γ from interacting with its receptor, which is consistent with the antibody neutralization of the antiviral activity obtained with liposomal IFN preparations (Fig. 1).

Taken together, these results indicate that the antiviral activity observed with the liposomal IFN preparations was mediated by IFN-γ that had leaked out of the liposomes into the tissue culture medium. This IFN then bound to its specific cell surface receptor to mediate its biological effects. Similarly, the ability of liposome preparations of IFN-γ to prime macrophages for activation to the tumoricidal state was also neutralizable by added antibody, indicating that the IFN productively interacted with the macrophage only after it leaked from the liposome into the medium and not after internalization, in liposomal form, by the macrophage. That less macrophage priming was obtained with liposomal than with free IFN could be the result of incomplete leakage of IFN from the liposome into the medium during the 24-hr incubation with the macrophages and/or phagocytic internalization of some of the liposomal IFN by the macrophage, thereby preventing IFN from interacting with the cell surface receptor. Thus, liposome encapsulation of IFN-γ apparently does not result in its bypassing the need for receptor interaction to mediate the establishment of the antiviral state in the cell or the priming of macrophage activation. These results with IFN-γ are in agreement with previous studies with human and murine α and β IFNs, in which IFN that did not previ-

ously interact with the cell surface was shown to lack antiviral activity. In these studies, either IFN was induced intracellularly by an appropriate stimulus and antibody to IFN was present in the tissue culture medium (29) or IFN was microinjected into the cell (30).

Poste and co-workers (20, 21) reported greater activation of macrophages to the tumoricidal state by liposomal than by free crude MAF preparations. Koff *et al.* (22) reported the increased ability of liposomal rHuIFN-γ preparations to activate macrophages to lyse herpes simplex virus-infected cells. These results are difficult to evaluate both because a mixture of lymphokines was employed in the MAF studies and because no quantitation of the MAF preparation or IFN-γ associated with the liposomal fraction was made. The authors assumed that the amount incorporated was equal to the aqueous internal volume of the MLV ($\approx$5% of the starting volume). As both human (31) and murine (32) γ IFNs are highly positively charged proteins at neutral pH, it is conceivable that considerably more IFN-γ might have been associated with the negatively charged liposomes than would be calculated from the aqueous internal volume. We have shown that $\geq$50% of rMuIFN-γ can be associated with both negatively and positively charged liposomes by adjusting conditions of preparation such as pH, lipid concentration, and initial hydration after lyophilization.

A liposome-delivery system for IFN-γ may be useful in altering pharmacokinetics, tissue distribution, and local concentration of IFN-γ, as was observed for IFN-α (17, 19). However, such a system will not alter the necessity for the interaction of IFN with its cell membrane receptor to mediate direct antiviral and macrophage-priming effects. Along these lines, we have indications that the *in vivo* effect of rMuIFN-γ in inhibiting systemic herpes simplex virus-2 infections in mice, which appears to be mediated largely by immunological mechanisms (33), is unchanged by incorporation of rMuIFN-γ into liposomes (unpublished results). A macrophage activator (such as IFN-γ) that mediates its effects through interation with a cell surface receptor (15) cannot be targeted inside the macrophage to increase its activity.

We thank Drs. J. G. Moffatt and A. C. Allison for critically reviewing the manuscript. C. Kurahara provided excellent cell culture support. We thank Genentech, South San Francisco, for generously supplying rMuIFN-γ, rHuIFN-γ, and antibodies to rMuIFN-γ. This is contribution no. 198 from the Institute of Bio-Organic Chemistry, Syntex Research.

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