Pharmaceutical Research, Vol. 10, No. 6, 1993

Research Article

Interferon-γ in Starch Microparticles: Nitric Oxide-Generating Activity *in Vitro* and Antileishmanial Effect in Mice[1]

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Received May 27, 1992; accepted December 17, 1992

Recombinant mouse interferon-γ (mu IFN-γ) was covalently coupled to polyacryl starch microparticles, a lysosomotropic drug carrier. The microparticle-bound mu IFN-γ was found to activate cultured macrophages for nitrite production and had an anti-leishmanial effect in mice. Low doses of mu IFN-γ, which had no effect in the free form, when bound to microparticles significantly reduced the load of *Leishmania donovani* in infected mice. Further, inducement of nitrite production in cultured macrophages by microparticle-bound mu IFN-γ required intact cell membrane receptors.

KEY WORDS: interferon-γ; polyacryl starch microparticles; *Leishmania donovani;* drug targeting; nitric oxide.

INTRODUCTION

The *Leishmania* are obligate intracellular protozoan parasites causing different forms of leishmaniasis in man and other vertebrates. After entry into the host, the parasites are taken up by macrophages of the reticuloendothelial system (RES)[6] where they differentiate and multiply inside the phagolysosomes (1). The most severe form, visceral leishmaniasis, caused by *L. donovani,* is almost invariably fatal if not treated (2). The treatment of choice is the pentavalent antimonials, which have a high potential toxicity (3) and are not always effective (4). No vaccine is currently available.

Using an experimental model with *L. donovani*-infected mice, Murray *et al.* (5) showed that an effective host defense is primarily T cell dependent and is mediated by activated macrophages. Also, there is a correlation between host control over parasite replication and the capacity of T cells to produce interferon gamma (IFN-γ) and interleukin 2 (IL-2) in response to leishmanial antigen. Acutely infected mice, not yet controlling the infection, are impaired in their antigen-stimulated IFN-γ secretion, and treatment with IFN-γ halts parasite replication (5). The same type of antigen-specific immunosuppression has been shown in patients with active visceral leishmaniasis (6), and clinical trials with recombinant IFN-γ in the treatment of visceral leishmaniasis have started (7).

Unfortunately, IFN-γ has side effects including fever and granulocytopenia as well as a short half-life in blood (8). However, in a study by Hockertz *et al.*, liposome incorporation of IFN-γ decreased its side effects and enhanced its antileishmanial activity in mice (9). These findings are probably due to the fact that parenterally administered liposomes, as well as other particulate matter, will concentrate in the macrophages of the RES.

Polyacryl starch microparticles (10) are a drug carrier with a typical RES distribution. The targeting potential has been shown in experimental visceral leishmaniasis, where the microparticles greatly increase the therapeutic activity of associated antileishmanial drugs (11,12). In the present study, recombinant IFN-γ was associated with microparticles by covalent coupling or physical entrapment, and the antileishmanial effect was studied in mice. Also, the capability of microparticle bound IFN-γ to induce nitrite production in cultured mouse peritoneal macrophages was studied.

MATERIALS AND METHODS

Chemicals. N,N,N′,N′-Tetramethylethylenediamine (TEMED) and pronase E (from *Streptomyces griseus*) were from Merck (Darmstadt, Germany). RPMI 1640 HEPES medium, myoclone plus fetal calf serum, L-glutamine, and penicillin–streptomycin solution were from GIBCO (Gaithersburg, MD). Lactoperoxidase and lipopolysaccharide (LPS) (from *Escherichia coli* 0111-B4) were from Sigma Chemical Co (St. Louis, MO). $Na^{125}I$ (IMS.30) was from Amersham International plc (Amersham, UK). α-Amylase (from *Bacillus subtilis*) was from Boehringer Mannheim GmbH (Mann-

[1] This is paper No. XV in the series: "Biodegradable Microspheres"

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[6] Abbreviations used: hu IFN-γ, recombinant human interferon gamma; mu IFN-γ, recombinant murine interferon gamma; RES, reticuloendothelial system; TEMED, tetramethylethylenediamine; HSA, human serum albumin; CDI, carbonyldiimidazole; DMF, dimethylformamide; PBS, phosphate-buffered saline; LDU, Leishman–Donovan units; LPS, lipopolysaccharide; BSA, bovine serum albumin; TNF-α, tumor necrosis factor alpha; IL 2, interleukin 2; FCS, fetal calf serum.

0724-8741/93/0600-0783$07.00/0

heim, Germany). The human serum albumin (HSA) used in cell culture and in microparticle preparation was kindly provided by Mr. Thomas Österberg, Kabi Pharmacia (Solna, Sweden). The starch used in the microparticle preparation (MD 6) was a gift from Dr. Lars Svensson, Stadex AB (Malmö, Sweden).

Iodination of Human IFN-γ. Recombinant human interferon gamma (hu IFN-γ; a gift from Mme D. Martini, Roussel UCLAF, Romainville, France; 1 mg, 2×10^7 U/mg) in 0.5 mL of 0.2 *M* phosphate buffer (pH = 7.5) was labeled with $Na^{125}I$ (1 mCi) in the presence of 20 μg lactoperoxidase and hydrogen peroxide [0.03% (v/v), 20 μL added three times at 3-min intervals] according to Phillips and Morrison (13). The ^{125}I-hu IFN-γ obtained by this procedure had a specific activity of 1.85 kBq/μg protein after gel filtration.

Preparation of Polyacryl Starch Microparticles. The microparticles were prepared by polymerization of acryloylated starch in an emulsion, as described previously (12,14).

Entrapment of Human IFN-γ in Microparticles. The general method of microparticle preparation was scaled down when entrapping hu IFN-γ. Hu IFN-γ (2 mg) and ^{125}I-hu IFN-γ (0.040 mg) were dissolved in 2 mL of a 0.2 *M* sodium phosphate buffer (pH 7.5, 1 m*M* EDTA) containing 200 mg of acryloylated starch, 200 mg of HSA, and 0.08 *M* ammonium peroxidisulfate. This inner phase was homogenized in 120 mL of toluene:chloroform (4:1), whereupon 40 μL of TEMED was added to initiate the polymerization. HSA was included to minimize the loss of hu IFN-γ due to adsorption onto glassware or plastic tips during preparation and leakage through the pores of the microparticles during storage. The ^{125}I-hu IFN-γ was used as a tracer in order to study the leakage of hu-IFN-γ from the microparticles and to determine the amount of hu IFN-γ incorporated. The yields in two experiments were 10.1 and 8.4%, which correspond to hu IFN-γ contents of 2.82 and 2.15 μg/mg of microparticles, respectively.

The leakage of ^{125}I-hu IFN-γ from the microparticles suspended in PBS stored at 4°C was followed for 10 weeks. The released ^{125}I-hu IFN-γ was determined from the supernatant after centrifugation. The release profile shows that 50% of the entrapped hu IFN-γ had been released after 8 weeks. An earlier study (14) with a low molecular weight protein, lysozyme (MW 14,400), entrapped without HSA showed 50% release of the protein after 3 weeks of storage.

Coupling of IFN-γ to Microparticles. The recombinant murine interferon-gamma (mu IFN-γ; 1×10^7 U/mg), a gift from Dr G. R. Adolf, Bender & Co. (Wien, Austria), was coupled to microparticles using the CDI method by Bethell *et al.* (15). The mu IFN-γ solution (0.05 mL, 1.1 mg/mL) was dialyzed (Pierce Microdialyzer system 500, membrane cutoff MW = 8000) against the coupling buffer 0.250 *M* boric acid with 0.15 *M* NaCl, pH 8.5. Microparticles (5 mg/mL) were activated with CDI (50 mg/mL) in dry DMF for 1 hr at room temperature. After several centrifugal washings with DMF to remove unreacted CDI, the particles (50 mg) were suspended in 10 mL of the coupling buffer containing 0.5 mg of mu IFN-γ. The mixture was rotated end over end at 4–6°C for 48 hr. The particles were then washed to PBS, filtered through a 10-μm filter, and stored at 4–6°C. The average amount of mu IFN-γ coupled was 4.4 μg/mg microparticles ($n = 4$; SE = 0.32) determined by amino acid analysis of the particles. The mu IFN-γ microparticles used in this study had a protein content of 4.16 μg/mg of microparticles. The protein yield in the coupling process was 40.5% ($n = 4$; SE = 2.7).

The hu IFN-γ, provided as a freeze-dried powder, was dissolved in coupling buffer and coupled to the microparticles with the same procedure as described above. The amount hu IFN-γ coupled was 6.7 μg/mg of microparticles ($n = 3$; SE = 0.7) determined by amino acid analysis. The coupling yield of hu IFN-γ was 63.3% ($n = 3$; SE = 3.8). When microparticles were prepared with ^{125}I-hu IFN-γ, no radioactivity could be detected in the supernatant after storage for 5 weeks.

Particle Size Determination. The microparticle size was determined in a Coulter counter Model TAII (Coulter Electronics Ltd., England) equipped with a 50-μm aperture tube, detecting particles from 1 to 20 μm. About 98% of the detected unmodified microparticles had a diameter of <2.5 μm. After coupling with hu IFN-γ and mu IFN-γ, 92 and 94%, respectively, had a diameter <2.5 μm. When hu IFN-γ together with HSA or HSA alone was entrapped, 97% of the microparticles had a diameter <2.5 μm.

Degradation of Microparticles with α-Amylase. Microparticles with entrapped HSA, or microparticles with ^{125}I-hu IFN-γ together with HSA, and empty microparticles, 7.7 mg from each group, were suspended in 1 mL of α-amylase (1350 U/ml) in RPMI 1640 and incubated for 3 hr at 37°C with reciprocal shaking. In these studies we used microparticles with a hu IFN-γ content of 2.15 μg/mg of microparticles. The clear solutions obtained were then centrifuged (3600*g*) for 15 min. The supernatants were collected and the activity of the released human IFN-γ was measured in an antiproliferative assay. α-Amylase alone was used as control.

Antiproliferative Assay. The human histiocytic lymphoma cell line U 937 (donated by Prof. K. Nilsson, Institute of Pathology, University of Uppsala, Uppsala, Sweden) has been shown to be highly susceptible to the antiproliferative effect of hu IFN-γ (16). U 937 cells were suspended in culture medium (RPMI 1640, supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 292 μg/mL L-glutamine) and were plated to give a concentration of 200,000 cells/well in 24-well culture plates (Flow Laboratories, Irvine, UK), 1.9 mL/well. The cells were treated in triplicate with various concentrations of free hu IFN-γ and supernatants from the degradation with α-amylase and incubated for 72 hr at 37°C in 5% CO_2. To study the antiproliferative effect, 200-μL aliquots were taken out in duplicate from each well and incubated with 0.5 mCi [*methyl*-^{3}H]thymidine (TRK-120, 1 mCi/mL; Amersham International plc, Amersham, England) for 2 hr under the same incubation conditions as above. Cell-bound radioactivity was separated from the medium and the radioactivity was determined. The cell viability estimated after the 72-hr incubation period using trypan blue (0.4% in saline) was not less than 98%.

Parasites. L. donovani MHOM/ET67/HU3 (LV9) was donated by Dr. R. A. Neal, London School of Hygiene and Tropical Medicine, UK. Amastigotes were obtained as described previously (12).

Treatment of Infected Mice. Mice of the BALB/c A Bom strain (Bomholtgård, Ry, Denmark), 8–10 weeks old,

were used. Groups of mice were injected in the lateral tail vein with 0.05, 0.2, or 0.5 mg mu IFN-γ microparticles, empty microparticles in a corresponding amount suspended in 0.2 mL PBS or free mu IFN-γ (10^3, 10^4, or 10^5 U) diluted in 0.2 mL PBS containing 1 mg HSA/mL, per dose and per mouse. Control mice were untreated or treated with 0.2 mL per mouse of 1 mg HSA/mL in PBS before and after infection. The pretreatment was given on days −2 and −1. The mice were infected via the tail vein with 0.2 mL of 1×10^7 viable amastigotes per mouse on day 0 and treatment after infection was given on days 14, 16, and 18. Groups of treated mice were killed and imprints were taken 1 week after final treatment (day 25 after infection). Untreated control groups were killed at day 14 (control 1) and day 25 (control 2) after infection. The degree of visceral infection was determined by examining Giemsa-stained imprints of the cut liver. Liver parasite burdens were expressed as Leishman–Donovan units (LDU) using the formula number of amastigotes per 1000 liver cell nuclei × organ weight (g) (17). The percentage of parasites killed was expressed as $[1 - (b/a)] \times 100$, where a = LDU in control 2 and b = LDU in treatment group. LDU in controls at day 14 in our two experiments shown were 17,806 (Fig. 1) and 8424 (Fig. 2), respectively.

Macrophages. Peritoneal macrophages were harvested in 10 mL of cold PBS from the peritoneal cavity of BALB/c mice, 10–12 weeks old, which had been given an i.p. injection of 1 ml of Brewers thioglycolate medium 3–4 days prior to harvest. The suspension was centrifuged at 150*g* for 5 min, and the cells were resuspended in culture medium (RPMI 1640 supplemented with 5 mg HSA/mL, 100 U/mL penicillin, 100 μg/mL streptomycin, and 292 μg/mL L-glutamine). The number of macrophages was adjusted to 1.2×10^6/mL and added to 24-well culture plates, 0.5 mL/well. After 2 hr of incubation at 37°C in 5% CO_2, the nonadherent cells were removed by washings with prewarmed medium and adherent cells were left with 1 mL culture medium over night. The cell viability after the 72-hr incubation period using trypan blue (0.4% in saline) was not less than 98%.

Determination of Macrophage Nitrite Production. The day after seeding the macrophages, without changing medium, the macrophages were treated (4 wells/group) with various concentrations of LPS, free IFN-γ (human and murine), IFN-γ (human and murine) microparticles, and empty microparticles with and without 10 ng LPS/mL, in a volume

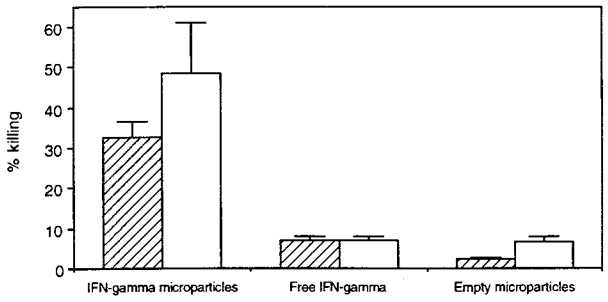

Fig. 1. Reduction of liver parasite burden in mice infected with *L. donovani* after treatment with free mu IFN-γ (2.05×10^4 U/inj), microparticle-bound mu IFN-γ (0.5 mg/inj), or empty microparticles (0.5 mg/inj). Treatment before infection (hatched bars) or treatment both before and after infection (solid bars). Mean ± SE; n = 4–6.

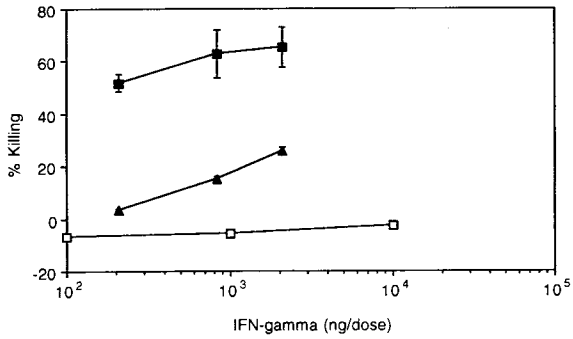

Fig. 2. Reduction of liver parasite burden in *L. donovani*-infected mice. Effect of treatment with various concentrations of free mu IFN-γ (□), microparticle-bound mu IFN-γ (■), or a corresponding amount empty microparticles (▲), given before and after infection. Mean ± SE; n = 6–7.

of 50 μL. Hu IFN-γ, a cytokine similar to mu IFN-γ in molecular weight and composition, was used as a control for the mu IFN-γ specificity in this assay. After 72 hr of incubation the supernatants were collected and the NO_2^- content was analyzed by an colorimetric assay based on the Griess reaction (18). Briefly, 50-μL aliquots of the supernatants were incubated in duplicates with 50 μL of 1% sulfanilamide and 50 μL of 0.1% *N*-1-naphthylethylenediamine dihydrochloride, each in 2.5% H_3PO_4, at room temperature for 5 min. Absorbance at 540 nm was measured and the NO_2^- was quantified by comparison to a $NaNO_2$ standard curve, linear in the range of 1–300 μ*M*.

To study the interaction of the mu IFN-γ with its membrane receptor, macrophages were incubated with the proteolytic enzyme pronase E, 0.02%, for 15 min in serum-free medium. Treatment with pronase E will enzymatically degrade the cell membrane proteins and thereby inhibit the interaction with the receptors and prevent the activation of the macrophages by free mu IFN-γ (19). After pronase pretreatment the macrophages were washed three times with medium and treated with various concentrations of empty microparticles, free mu IFN-γ, and mu IFN-γ coupled to microparticles, in culture medium. LPS (10 ng/mL) was included in all groups. Three treatment schedules were used. In treatment schedule 1, macrophages were treated for 5 hr. After several washings to remove unphagocytosed microparticles, the cells were incubated in culture medium for 72 hr before analysis of nitrite production. Treatment schedule 2 was as schedule 1, but with a 24-hr resting period in culture medium for the cells prior to treatment. Treatment schedule 3 was the same as schedule 2 but the treatment was continued throughout the 72-hr period, before analysis of nitrite production. As a positive control, macrophages in medium without pronase E were treated in the same way.

Determination of Phagocytic Activity. To study whether the phagocytic activity of macrophages was retained after 0.02% pronase E incubation, macrophages were given ^{14}C microparticles (sp act, 2.4×10^5 dpm/mg microparticles) together with 10 ng LPS/mL after the enzymatic treatment, using the same schedules as above. After several washings with prewarmed PBS to remove noningested microparticles, the macrophages were detached from the plastic wells by incubation at 37°C for 2 hr in 0.5 mL of 0.2% pronase E.

Detached macrophages were withdrawn and lysed with 0.5 mL of 2 *M* NaOH overnight, and the radioactivity from ingested microparticles was determined in the liquid scintillation counter. As a positive control, the phagocytic activity of macrophages incubated in medium alone was measured.

During treatment with 0.2% pronase to detach the macrophages, microparticles unspecifically attached to the bottom of the well could be released. As a control experiment, ^{14}C microparticles (0.029 mg) suspended in culture medium were placed in a cell-free well, incubated with 0.2% pronase E, and washed as above. The release of unspecifically attached microparticles after 0.2% pronase E treatment was found to be at most 300 dpm in this cell-free well and was considered negligible compared to the radioactivity normally found in the wells (1000–3500 dpm).

Protein Determination of Adherent Macrophages. Adherent cell protein was determined by the Lowry assay, with BSA as a standard and performed on aliquots from macrophage monolayers, washed three times with prewarmed PBS, and lysed with 1 mL of 1.0 *M* NaOH overnight (20). Representative adherent cell protein values before treatment were 29.0 ± 2.2 μg/well (n = 4 ± SE) and 27.3 ± 0.3 μg/well (n = 160 ± SE) after 72 hr of culture in medium alone and in medium with different stimulants. The stimulants had no effect on the cell density.

RESULTS

Activity of Free and Microparticle Entrapped Hu IFN-γ in the Antiproliferative Assay. The biological activity of microparticle-entrapped hu IFN-γ was determined in an antiproliferative assay after α-amylase degradation of the microparticle matrix. As shown in Fig. 3, the hu IFN-γ activity was reduced by about 10% units at each dose by α-amylase treatment. This result was confirmed in a separate experiment with a larger number of samples and doses (results not shown). The entrapped hu IFN-γ had almost the activity of native hu IFN-γ, showing that the entrapment process had no major denaturating effect on the protein. The α-amylase itself or control microparticles containing HSA had no antiproliferative effect.

Nitrite Production of Macrophages Treated with Free and Microparticle-Bound IFN-γ. It has been shown previously (21) that peritoneal macrophages treated with mu

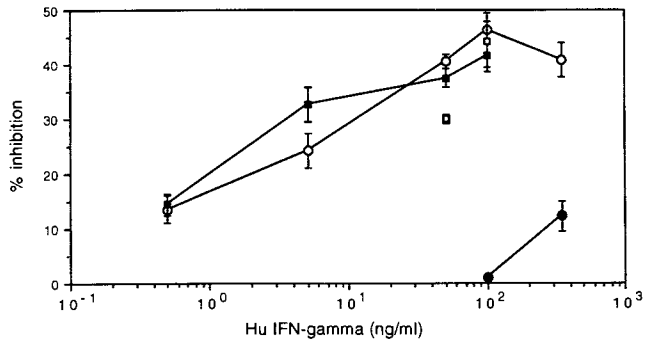

Fig. 3. Antiproliferative effect on U937 cells in the presence of different dilutions of free hu IFN-γ (■), α-amylase-treated free hu IFN-γ (□), α-amylase-degraded hu IFN-γ/HSA microparticles (○), or HSA microparticles (●). Mean ± SE; n = 3.

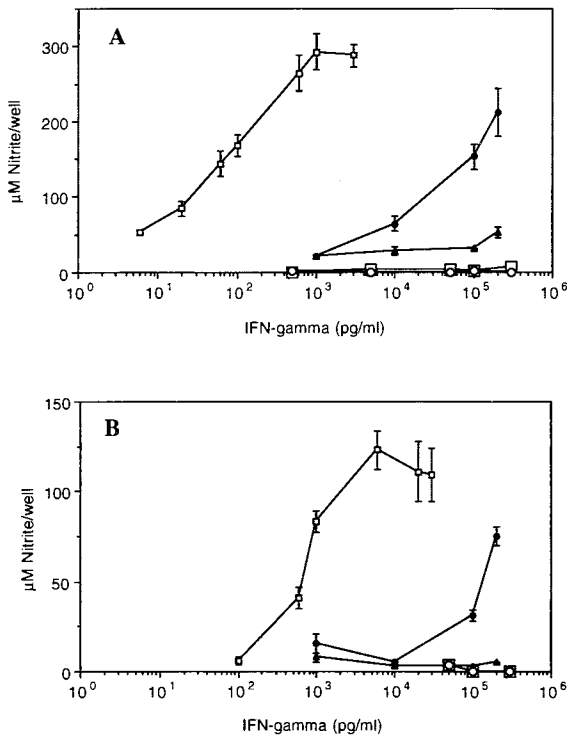

Fig. 4. Nitrite production by macrophages cultured during 72 hr (A) with 10 ng LPS/mL and (B) without LPS. Various concentrations of free mu IFN-γ (□), free hu IFN-γ (○), microparticle-bound mu IFN-γ (●), microparticle-bound hu IFN-γ (□), or a corresponding amount of empty microparticles (▲). Note the different scales on the Y axes. Mean ± SE; n = 4.

IFN-γ produce nitrite. Furthermore, the nitrite production is considerably enhanced when LPS is included (21). This was used by us as an assay to quantify the biological activity of mu IFN-γ. A typical dose–response curve for the macrophage nitrite production was seen with increasing amounts of mu IFN-γ, in the presence or absence of LPS (Fig. 4). The addition of 10 ng LPS/mL enhanced the sensitivity in the assay, allowing the detection of 0.06 U/mL (6 pg/mL) of mu IFN-γ. LPS alone gave a low nitrite production response. Treatment with various concentrations of free hu IFN-γ gave no nitrite production response, indicating a species-specific response in our assay (Fig. 4). Medium alone was always included as a control and gave no nitrite production.

To study the biological activity of mu IFN-γ coupled to microparticles, peritoneal macrophages were treated with microparticle-bound and free mu IFN-γ with or without 10 ng LPS/mL. As a control, empty microparticles in corresponding amounts were given with or without 10 ng LPS/mL (Fig. 4). The mu IFN-γ coupled to microparticles induced a dose-related release of nitrite, and as with free mu IFN-γ, the response was higher when LPS was included. In this *in vitro* model, the biological activity of mu IFN-γ coupled to microparticles was about 0.10% compared to that of soluble mu IFN-γ, based on the amount of protein. In a control experiment, hu IFN-γ coupled to microparticles, given with or without LPS, did not induce nitrite production. Empty microparticles gave no response, even when LPS was included. Thus, the microparticle itself could not function as a primer for NO_2^- production.

Table I. Nitrite Production Macrophages Pretreated with Pronase[a]

Macrophage activators with 10 ng LPS/mL	μM nitrite/well with treatment schedule No.					
	1		2		3	
	Medium	Pronase	Medium	Pronase	Medium	Pronase
Free mu IFN-γ (pg/mL)						
1×10^3	7.1 ± 0.6	0.8 ± 0.1	20.8 ± 1.6	0.7 ± 0.4	99.6 ± 7.4	6.5 ± 0.6
3×10^2	2.9 ± 0.7	0.9 ± 0.4	4.8 ± 0.5	1.4 ± 0.4	16.4 ± 1.5	1.7 ± 0.4
1×10^2	6.3 ± 0.8	1.8 ± 0.5	1.4 ± 0.4	1.3 ± 0.6	2.6 ± 0.4	0.8 ± 0.4
Mu IFN-γ microparticles (pg mu IFN-γ/mL)						
2×10^5	21.3 ± 1.0	0.4 ± 0.1	39.8 ± 4.0	3.4 ± 0.8	164 ± 19	29.0 ± 7.1
1×10^5	16.1 ± 1.6	1.0 ± 0.4	25.7 ± 3.3	4.0 ± 0.6	136 ± 17	19.0 ± 1.8
1×10^4	3.6 ± 1.0	1.0 ± 0.4	5.2 ± 1.6	0.5 ± 0.6	—	—

[a] Macrophage monolayers, pretreated with 0.02% pronase E or medium for 15 min, were treated with free and microparticle-bound mu IFN-γ at various concentrations and for different periods of time (see Materials and Methods) before analysis of nitrite production.

Membrane Receptor Dependence of the Nitrite Production. Treatment of macrophages with pronase E, reported earlier (19) to degrade receptors for mu IFN-γ enzymatically, prevented inducement of nitrite production by both free and microparticle-bound mu IFN-γ (Table I). This indicates that macrophage activation by mu IFN-γ microparticles occurs via a membrane receptor, supposedly during attachment and phagocytosis of the microparticles. The results from treatment schedule 3, in Table I, showed that the nitrite production from macrophages pretreated with pronase E was partly restored after the 72-hr incubation period. This indicates a gentle removal of the mu IFN-γ receptor by pronase E, allowing the macrophages to reestablish the cell membrane receptors after the enzyme was removed.

The treatment of macrophages with pronase E did not diminish their phagocytic activity for microparticles. As seen in Fig. 5, the uptakes of microparticles by pronase-treated macrophages and control macrophages were similar.

Can Empty Microparticles Function as a Triggering Signal for Nitrite Production? To find out if the microparticles could replace LPS as a triggering signal for nitrite production, various concentrations of empty microparticles (0.00024–0.048 mg/mL) were given with 10 U mu IFN-γ/mL to peritoneal macrophages and incubated during 72 hr under the same conditions as described above. The control was 10 U mu IFN-γ/mL alone or with 10 ng LPS/mL. Empty microparticles together with IFN-γ gave the same response as IFN-γ alone. Thus, empty microparticles do not synergize with IFN-γ and trigger the nitrite response as LPS did.

Effect of Free and Microparticle-Bound mu IFN-γ on L. donovani *in Mice.* In the treatment of experimental leishmaniasis, liposome-associated mu IFN-γ has a weak antileishmanial effect and free mu IFN-γ in corresponding doses has no effect. Also, liposome-associated mu IFN-γ is more effective when given prophylactically or early after infection (22). In our own preliminary studies, treatment of infected mice with microparticle-bound mu IFN-γ only after infection gave a varying antileishmanial effect (results not shown). Such a treatment schedule was therefore excluded.

In our subsequent studies we used mu IFN-γ microparticles with a protein content of 4.10 μg/mg microparticles and a biological activity of 41.0 U/mg, as determined by the nitrite production assay. Microparticle-bound mu IFN-γ (0.5 mg/injection), empty microparticles (0.5 mg/injection), or free mu IFN-γ (2.05×10^4 U/injection) were given before infection or both before and after infection. The IFN-γ microparticles gave a significant decrease in parasite burdens in infected mice in both treatment schedules, 32.5 and 48.7% killing, respectively (Fig. 1). In contrast, free mu IFN-γ or empty microparticles had a poor antileishmanial activity.

To investigate the dose dependence, different doses of microparticle-bound mu IFN-γ, free mu IFN-γ, or empty microparticles (see methods) were given to mice, before and after infection with *L. donovani* (Fig. 2). Microparticle-bound mu IFN-γ showed no clear dose–response relationship in the concentration range studied. Daily or every-other-day treatment with free mu IFN-γ given for 14 days at doses of 10^5 U/injection has been shown to give an antileishmanial activity *in vivo* (5). With our treatment schedule, the free mu IFN-γ had no effect even at the highest dose (10^5 U/injection), given both before and after infection. This could be due to our shorter and less frequent treatment schedule.

The effect of empty microparticles was somewhat unclear; in one experiment (Fig. 2) the highest doses (0.5 and 0.2 mg) of empty microparticles had a small antileishmanial effect, but in the other experiment (Fig. 1) no effect could be seen. An antileishmanial effect of empty microparticles has

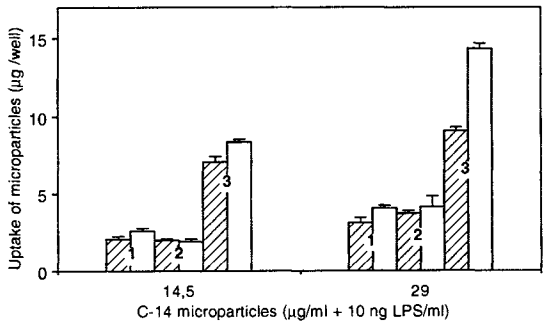

Fig. 5. Uptake of ^{14}C-labeled microparticles during treatment schedules 1–3 (see Materials and Methods) by cultured macrophages. Pretreatment with medium (solid bars) or pronase (hatched bars). Mean ± SE; $n = 4$.

previously been shown *in vitro* (23) but is not in accordance with earlier results *in vivo* (11). The varying effect of empty microparticles in our experiments could be explained by different degrees of infection in the two groups of animals in these experiments. This phenomenon has also been seen by Alving *et al.* (24).

DISCUSSION

The ability of phagocytes to kill intracellular *Leishmania* parasites is dependent on the Th1 subset of CD4+ T cells, secreting IFN-γ in response to antigen (25). IFN-γ activates macrophages for production of reactive oxygen (26) and nitrogen (21) intermediates, toxic to *Leishmania*. However, resident Kupffer cells, the hosts of *Leishmania* parasites, have virtually no capacity for a respiratory burst (27). Nevertheless, in resistant mice, these cells kill *L. donovani* (28). In recent studies of the killing of *L. major* by murine macrophages (as reviewed in Ref. 29), the principal effector mechanism was found to be a nitrogen oxidation of L-arginine where the toxic molecule is NO. It is further postulated that the activation of macrophages to produce NO requires multiple signals delivered in a defined sequence, where IFN-γ can act as a primer, transforming the cells into a receptive state in which small amounts of a second signal, the trigger, e.g., LPS, induce NO release. However, in certain intracellular infections, e.g., tuberculosis, leprosy, and leishmaniasis, these mechanisms are not in function, and no IFN-γ is produced in response to parasite antigen (30). In this work, the possibility of immunoreplacement therapy in visceral leishmaniasis was studied using IFN-γ coupled to starch microparticles.

Two methods for associating proteins with starch microparticles are available: covalent coupling and physical entrapment. Compared to the entrapment technique, where the protein is noncovalently, physically entrapped in the microparticles (14), covalent coupling offers some advantages. Less protein is needed and excess material can easily be regained. Also, as shown here with hu IFN-γ, the binding is stable on storage, in contrast to the leaking of entrapped material. The disadvantage is the obvious risk of losing biological activity of the protein. The binding could block or otherwise sterically hinder sites of the protein necessary for activity, e.g., receptor binding.

The ability of peritoneal macrophages to produce nitric oxides in response to mu IFN-γ (21) was used by us as a sensitive and specific assay for mu IFN-γ. In this assay, coupled mu IFN-γ had about 0.1% of the activity of soluble mu IFN-γ, based on the total protein content (Fig. 4). Empty microparticles given alone or together with LPS had no effect. Nor could empty particles replace LPS as a triggering signal causing enhancement of the mu IFN-γ response. Thus, the possible endotoxin contamination of the particles was too low to have any effect in this system. Also, the phagocytosis as such did not induce NO production. This is in accord with earlier results (29) using latex beads, zymosan, or heat-killed *L. major*.

The activity of entrapped hu IFN-γ activity was measured in an antiproliferative assay after solubilization of the particles. No major loss of activity was seen due to the entrapment (Fig. 3). The ability of the entrapment procedure to retain biological activity has been seen earlier with enzymes (14). Unfortunately, the high amounts of protein needed, even when using albumin as coentrapment agent, prevented us from using this procedure for mu IFN-γ.

The *in vivo* experiments showed a greatly increased efficiency of microparticle-bound IFN-γ compared to soluble. With treatment both before and after infection, a 60–70% killing was reached with the particle-bound lymphokine, but no clear dose–response relationship was seen (Fig. 2). In the dose range of soluble IFN-γ used by us (limited by our supply), no effect was seen. Due to this, the dose reduction factor for the particle bound IFN-γ could not be established but can be estimated to be at least 100. We explain this by the passive targeting of microparticles to liver macrophages (31), earlier demonstrated to increase the efficiency of antileishmanial drugs in experimental visceral leishmaniasis (11,12).

The effect of empty microparticles varied, apparently depending on the degree of infection. In the experiment where empty particles had effect, the LDU was only half of that in the experiment where no effect was seen. Earlier, empty microparticles have shown a small antileishmanial effect in cultured peritoneal macrophages (23) but not in mice (11,12). The mechanism behind this weak activating effect is not known.

Treatment of *L. donovani* with liposomal mu IFN-γ is more efficient if started early after infection or given prophylactically (22,32). In the present study, the importance of prophylactic treatment was also shown for microparticle-bound mu IFN-γ. The antileishmanial capacity of mice treated both before and after infection and mice given only prophylaxis was compared. About 70% of the antileishmanial effect could be attributed to the pretreatment.

The precise mechanisms behind the effect of microparticle-bound mu IFN-γ in leishmaniasis are not known. It has been suggested that IFN-γ primes the macrophages, enabling them to respond to the parasites by releasing TNF-α, functioning as an autocrine trigger signal inducing NO production in the presence of IFN-γ (29). Thus, in activated macrophages, the parasite itself provides the triggering for NO production leading to its destruction.

Macrophages have a specific surface receptor for IFN-γ which also is fairly species specific in its receptor interaction (33). However, after manipulation of hu IFN-γ into murine macrophages via liposomes, thus bypassing the membrane receptor, tumoricidal activity was produced by the macrophages. This indicates a non-species-specific intracellular function of hu IFN-γ, independent of cell membrane receptors (19). In contrast, results of Eppstein *et al.* (34) indicate that the macrophage activating effect of liposomal mu IFN-γ is due to its leakage from the liposomes followed by receptor interaction. Encapsulated mu IFN-γ was largely unavailable for binding.

In this study, microparticle-bound hu IFN-γ did not induce any NO production in cultured mouse macrophages (Fig. 4). Furthermore, the *in vitro* activity of microparticle-bound mu IFN-γ required intact cell membrane receptors. No nitrite production was induced by particles with coupled mu IFN-γ in pronase-treated macrophages. Pronase treatment made the macrophages temporary unresponsive to soluble mu IFN-γ but did not interfere with their capacity to

phagocytose particles. Thus, we found no evidence for an intracellular, receptor-independent function of IFN-γ bound to starch microparticles. Instead, we propose that bound mu IFN-γ interacts with its membrane receptor before and during phagocytosis of the microparticles and that this interaction induces the observed NO production and antileishmanial activity.

In conclusion, we have shown the superior efficiency of microparticle-bound mu IFN-γ compared to soluble in activating macrophages *in vivo* to kill intracellular *L. donovani*. Further, *in vitro* results indicate that the activation is mediated through a cell membrane receptor and not by intracellular delivery of the lymphokine. Obviously, this requires that IFN-γ is localized on the microparticle surface. Thus, coupling to microparticles may offer an advantage compared to liposome encapsulation in targeted delivery of IFN-γ.

ACKNOWLEDGMENTS

The authors thank Ms. Gunnel Arvidsson and Ms. Åsa Bjurling for skillful technical assistance. This work was financially supported by the Swedish Medical Research Council (Project 03X-07318) and the I.F. Foundation for Pharmaceutical Research.

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