Malfunion of the host defense system renders patients more susceptible to opportunistic infections and can make antibiotic treatment ineffective (15). Activation of cells of the mononuclear phagocyte system, in particular, resident macrophages, by immunomodulating agents has been proved to be beneficial in several infection models (3, 7, 14, 18) and in antitumor therapy (10, 17).

Among them, gamma interferon (IFN-γ) is an important component of the Th1-type cellular immune response and contributes to the control of bacteria through its ability to activate macrophages to increase microbial killing (23, 24). Using an experimental model with Brucella abortus-infected mice, Zhan and Cheers (33) showed that host resistance against these bacteria was primarily mediated by activated macrophages and also by a Th1 profile (4). Also, there is a correlation between host control over parasite replication and the capacity of T cells to produce IFN-γ (11). However, the therapeutic potential of IFN-γ may be limited by its rapid clearance from the circulation and, thus, a brief duration of the activating effect on macrophages. This fact implies that frequent and high doses, which often result in severe systemic side effects, must be used (32).

In order to improve the therapeutic indices of these macro-molecules, one possibility may consist of the use of delivery systems able to both maintain appropriate levels in blood for long periods of time and target the cytokine to macrophages, the reservoir for those microorganisms (1). IFN-γ targeting with colloidal systems, such as liposomes and microparticles, has led to both a reduction in toxicity and an increase in the efficacy of IFN-γ against a great variety of intracellular parasites, such as Leishmania donovani (5) and Klebsiella pneumoniae (28). IFN-γ loaded into liposomes was also effective as an adjuvant with antiviral vaccines (30) and antitumor vaccines (31). Another interesting approach that could increase the therapeutic efficiency of IFN-γ may be to load it into albumin nanoparticles (IFN-γ-NPs). In fact, we have previously reported that these carriers have a high payload of IFN-γ. More importantly, they retain integrity, one of the handicaps currently found during the development of delivery systems for cytokines (26).

On the basis of the deeply understood role of IFN-γ in the fight against infections caused by Brucella spp. (12, 22), we chose experimental infection of RAW macrophages and of BALB/c mice with this pathogen as suitable models for evaluation of the effectiveness of NPs in potentiating the immunomodulatory activities of IFN-γ. Also, the contributions of NO production in vitro and the Th1 response in vivo in the antibrucella effects were examined.

MATERIALS AND METHODS

Materials. Recombinant human and mouse IFN-γ with a specific activity of 1.0 × 10^7 IU/mg and IFN-γ enzyme-linked immunosorbent assay (ELISA) kits were obtained from Biosource International. Bovine serum albumin (BSA; fraction V), concanavalin A (ConA), and glutaraldehyde (25%; grade II) were obtained from Sigma Chemical Co. (St Louis, MO). Absolute ethanol and chlorhydric acid were purchased from Prolabo (Fontenay, France). Sodium nitrite was obtained from Merck (Darmstadt, Germany). Dulbecco modified Eagle medium (DMEM), fetal bovine serum, and L-glutamine were obtained from Gibco-BRL. Bacterial cultures were grown in Luria-Bertani (LB) medium (BBL, USA) at 37°C.

NP preparation and characterization. BSA nanoparticles (NPs) were prepared by coacervation and chemical cross-linkage with glutaraldehyde, as described previously (26). Depending on the step in which the cytokine was added, two different formulations were prepared. In the former, 1 μg IFN-γ/mg BSA was incubated with the albumin NPs (IFN-γ-NPs). In the latter, both the protein and the cytokine (1 μg IFN-γ/mg BSA) were incubated in an aqueous medium prior the formation of the NPs by coacervation (IFN-γ-NPbs).

Briefly, IFN-γ-NPs were obtained by the addition of 1.5 ml of ethanol dropwise (ethanol-to-water ratio, 1:5.1; by volume) to 1 ml of an aqueous solution of BSA (2% [wt/vol]), adjusted to pH 5.5 with 0.1 N HCl. The coacervates thus obtained were then hardened with glutaraldehyde that had previously been dissolved in ethanol (1.56 μg/mg bulk albumin) for 2 h at room temperature.
After ethanol elimination by evaporation under reduced pressure (water bath B-480; Buchi, Switzerland), the NPs were purified by centrifugation at 17,000 rpm for 30 min (rotor 3336; Biofuge Heraeus, Hanau, Germany) to eliminate the free albumin and the excess of the cross-linking agent. The supernatants were removed and the empty NPs were resuspended in an aqueous solution containing IFN-γ for incubation for 0.5 h. Finally, the IFN-γ–NPs were separated from the free cytokine by centrifugation (17,000 rpm/30 min) at 4°C.

For the preparation of IFN-γ–NPB, IFN-γ was first incubated with the albumin aqueous solution (2% [wt/vol]; pH 5.5) for 0.5 h at room temperature. This aqueous phase was then desolvated with ethanol dropwise (ethanol-to-water ratio, 1.5:1, by volume). The coacervates so formed were then hardened with glutaraldehyde that had previously been dissolved in ethanol (1.56 μg/ml bulk albumin) for 2 h at room temperature, and the resulting NPs were purified by centrifugation as described above. In all cases, the NPs purified by centrifugation were resuspended and dispersed in phosphate-buffered saline (PBS).

The average particle size, polydispersity index, and zeta potential of the NPs were determined with a Nanosizer ZS (Malvern Instruments, United Kingdom), after appropriate dilution in double-distilled water.

The amount of protein transformed into NPs was determined by a standard bicinchoninic acid protein assay. The IFN-γ load was determined by ELISA. For this purpose, aliquots of the clear supernatants obtained during the purification of the NPs were analyzed for IFN-γ content. The amount of the cytokine was estimated from the difference between the amount initially added and the recovered amount in the supernatants. The IFN-γ load was expressed as the ratio between the amount of cytokine in the NPs (expressed in μg) and the NPA yield (expressed in mg).

**Bacteria.** *Brucella abortus* strain 2308, a virulent field strain, was used in all studies. It was grown on tryptic soy agar for infection for 24 h at 37°C.

**Cell culture.** The murine macrophage cell line RAW 264.7 was obtained from ATCC. RAW 264.7 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 50 μg of l-glutamine per ml, 50 IU of penicillin per ml, and 50 μg of streptomycin per ml.

**In vitro infection assay.** RAW 264.7 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 10^5 cells per well. Macrophages seeded at this concentration were incubated with the different IFN-γ formulations at a cytokine concentration of 100 IU/ml for 24 h before they were infected. A control culture, which never received IFN-γ, was also established in parallel. Then, 100 μl of antibody-opsonized bacteria, suspended to the appropriate concentration in DMEM, was added to each well at a bacterium-to-macrophage ratio of 100:1. The microwell plates containing the macrophages and the bacteria were centrifuged at 200 × g for 4°C for 5 min to increase the attachment of *B. abortus* to macrophages and were then incubated at 37°C for 1 h at 50 min, as described previously (12).

To avoid the loss of the macrophages, which became less adherent after infection or cytokine treatment, the macrophage cultures were centrifuged at 200 × g between supernatant treatments.

Following the 2-h infection period, the majority of extracellular bacteria were removed by washing the macrophage cultures three times with PBS, followed by incubation for 1 h in DMEM containing 50 μg gentamicin per ml (time zero of the culture). Following the wash procedure, one set of cultures was treated again with the IFN-γ formulations with a cytokine concentration of 100 IU/ml for a further 24 h.

The infected cells were lysed by pipetting with 200 μl of 0.1% Triton X-100 in PBS. To enumerate the viable intracellular *Brucella*, cell lysates were diluted and plated on *Brucella* agar. The numbers of bacterial CFU were counted after a 3-day incubation at 37°C with 5% CO_2 in air, and the numbers of CFU were transformed to log_{10}.

**NO_2^- measurements.** The culture cell-free supernatants were assayed for the accumulation of NO_2^- by the Griess reaction. The determinations were done after the pretreatment but before the infection and after the treatment and postinfection.

**Animals.** Female BALB/c mice were purchased at 6 to 8 weeks of age and were used at 8 to 10 weeks of age. The animal experiments were performed in compliance with the regulations of the responsible committee of the University of Navarra, in line with the European legislation on animal experiments (86/609/ EU). All mice were housed in biohazard level 3 facilities in microisolator cages and were handled under sterile conditions, including sterile food, water, cages, breeding conditions, and handling of the animals by laboratory personnel.

**In vivo infection assay.** The mice were infected intraperitoneally with a suspension of 10^7 CFU of *B. abortus* in 0.2 ml of PBS. Treatment of the mice (five mice per group) with single doses of IFN-γ formulations and free IFN-γ at 10,000 IU per day was done intraperitoneally at 1 day before infection and again at 2 and 4 days after infection.

<table>
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<tr>
<th>Table 1. Physicochemical characteristics of albumin nanoparticles containing IFN-γ</th>
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<td>IFN-γ formulation</td>
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“Experimental conditions included an IFN-γ/BSA ratio of 1 μg/mg. Data are expressed as the means ± standard deviations of three experiments. Three measurements were performed in each experiment (n = 3).

At 1 week (three mice per group) and 2 weeks (two mice per group) postinfection, the mice were killed by cervical dislocation, and their spleens were removed and homogenized in plastic bags with 10 ml of sterile PBS by using a stomacher lab blender, and the numbers of CFU were determined.

**In vitro cytokine release.** The spleens from the mice were placed in RPMI 1640 medium and smashed in tissue culture dishes (Iwaki, Japan), and the fat and the capsule were removed. Spleen cells from within experimental groups were mixed in one flask and centrifuged at 400 × g and 4°C for 5 min. The supernatants were discarded, and the pellet was washed twice with PBS and centrifuged at 400 × g and 4°C for 5 min and resuspended in complete medium (RPMI 1640 medium supplemented with 2 μl 2-mercaptoethanol, 500 U penicillin, 500 μg streptomycin, 10 μM HEPES, and 10% heat-inactivated fetal calf serum).

For in vitro cytokine release, isolated splenocytes (8 × 10^6/well) were seeded into a 24-well plate and stimulated with 0 and 5 μg/well of Con A for 48 h at 37°C. The cytokines (interleukin-4 [IL-4] and IFN-γ) in the supernatant were quantified by using ELISA kits from Biosource.

**Statistical analysis of results.** All experiments were repeated at least three times. The results were expressed as means ± standard deviations. Uptake data were analyzed by one-way analysis of variance, with the post-hoc Tukey's test applied for comparisons of several (three or more) group means (SPSS 10; SPSS Inc., Chicago, IL). A P value of <0.05 was considered significant.

**RESULTS**

**Characterization of IFN-γ–NPs.** Table 1 shows the main physicochemical characteristics of the different IFN-γ–NP formulations prepared with an IFN-γ–to-albumin ratio of 1 μg/mg. Both types of NPs displayed a size of about 330 nm. The amount of IFN-γ loaded into the NPs was very high for both procedures of preparation, either by the encapsulation process (IFN-γ–NPB) or by simple adsorption of the cytokine onto preformed NPs (IFN-γ–NPAs).

**Macrophage bactericidal activity against *B. abortus*.** RAW 264.7 macrophages were incubated with free IFN-γ or the IFN-γ formulations before and, eventually, after infection or before infection only (Fig. 1).

When IFN-γ was adsorbed onto the IFN-γ–NPAs, the total reduction of intracellular bacteria was about 2 log CFU relative to the amount in the untreated cells (P < 0.05) and 1 log CFU relative to the amount for macrophages treated with free IFN-γ (P < 0.05) when the cells were treated either only before infection or also after infection. Unloaded NPs and NPs encapsulating the cytokine inside their matrix (IFN-γ–NPBs) did not show antibrucellae activities.

**NO production in infected RAW 264.7 cells.** Both NO and reactive oxygen intermediates mediate macrophage anti-*Brucella* effects. To examine the role of NO in IFN-γ-mediated anti-*Brucella* activity, the NO accumulation in infected-cell supernatants was measured.
The infection of macrophages with *B. abortus* increased their levels of NO production. Table 2 summarizes these results. The addition of free IFN-γ to macrophages cultured without *Brucella* enhanced NO production (8-fold), and this production was fairly enhanced (*P < 0.05*) when macrophages were cultured with IFN-γ adsorbed onto NPs (11-fold). When the cells were infected with *Brucella* and treated with free IFN-γ or IFN-γ–NPs, the level of NO production also increased about fourfold. In any case, the IFN-γ–NPA formulation was found to be the best formulation for the induction of NO production by the RAW 264.7 macrophages (*P < 0.05*). In contrast, negligible levels were observed when IFN-γ was incorporated into the NPs. Empty NPs also showed no effect on the activation of macrophages.

**Resistance of mice to infection with *B. abortus*.** Table 3 shows that the treatment of mice with IFN-γ at 1 day before infection and again at 2 and 4 days after infection with *B. abortus* did not significantly alter the number or the growth of intracellular brucellae either 1 or 2 weeks after infection. However, macrophages treated with IFN-γ–NPs reduced by about 1 log the number of intracellular bacteria relative to the amount in untreated mice (*P < 0.05*) both 1 and 2 weeks after infection. Finally, neither unloaded NPs nor IFN-γ–NPs had any effect on the infection rate.

Additionally, the weights of the spleens of noninfected mice treated with IFN-γ and IFN-γ-containing NPs (Table 3) indicated an increase in the proliferative response induced by the cytokine when it was administered in association with NPs. *Brucella* infection induced an inflammatory response that prevented the analysis of the proliferation induced by the formulations with infected mice.

**IFN-γ and IL-4 production in infected mice.** Figure 2 shows the data for the amount of IFN-γ released from isolated splenocytes after in vitro stimulation with ConA. The treatment of splenocytes from mice with free IFN-γ led to significantly enhanced IFN-γ release in response to ConA compared to that for the control cells (*P < 0.05*). Again, the splenocytes isolated from mice treated with IFN-γ–NPA released the largest amount of IFN-γ after stimulation (*P < 0.05*). In contrast, IFN-γ–NPs and empty NPs produced relatively low levels of IFN-γ.

On the other hand, the amount of IL-4 released from splenocytes after stimulation with ConA was also measured. As we can see in Fig. 2, high levels of IL-4 were detected from the infection.
spleenocytes of mice treated with IFN-γ or IFN-γ–NPAs compared with that detected from the splenocytes of the control mice (P < 0.05). IFN-γ–NPBs and empty NPs produced levels similar to those produced by the control.

DISCUSSION

The rationale for the present study is the direct targeting of IFN-γ to its main site of action, the macrophages, by means of the association of the cytokine with drug delivery systems, such as NPs. The strategy could protect the cytokine from rapid enzymatic degradation and clearance and then improve the activities of the cytokines at lower doses, which would allow their toxicity to be avoided (32). A gross body of evidence from previous reports has evidenced the benefits of targeting antimicrobial agents directly to macrophages through their association with lipidic or polymeric particles (2, 16). Also, among the different kinds of delivery systems, albumin NPs are especially suitable for the encapsulation of biomolecules with high sensitivities to media and structural requirements, such as cytokines. The experimental conditions for the optimal association of IFN-γ with NPs have been reported previously (26). We prepared two types of IFN delivery systems. In one of them, IFN-γ–NPs, the cytokine was adsorbed onto preformed NPs. In IFN-γ–NPBs, IFN-γ readily encapsulated into the matrix of the particles by binding to the albumin prior to the NP formation process. Both the IFN-γ–NPAs and the IFN-γ–NPBs had a mean size distribution (about 300 nm) suitable for their optimal uptake by phagocytic cells, such as macrophages. Major differences were related to the cytokine release profile. As determined by a bioassay, meanwhile, IFN-γ–NPAs released 80% of the loaded cytokine after 20 h; no release of cytokine was detected from IFN-γ–NPBs after this incubation time (26). The aim of the present work was to evaluate the activities of both IFN-γ delivery systems against Brucella abortus in infected macrophages and mice, which were used as models of infective disease.

The ability of phagocytes to kill intracellular Brucella is dependent on the Th1 subset of CD4+ T cells, which secrete IFN-γ in response to antigen. In the current study, we found that in vitro, the pretreatment of RAW 264.7 macrophages with 100 IU/ml of IFN-γ for 24 h resulted in an increased ability of the macrophages to kill B. abortus (Fig. 1). The greatest difference in killing potential was observed when IFN-γ was added both before and after infection. In this way, Jiang and Baldwin (12) showed that the pretreatment of J774.2 macrophages with 100 IU/ml of IFN-γ and the addition of IFN-γ again after the infection resulted in more effective treatment.

Whereas IFN-γ–NPAs were more effective than the free cytokine for the prevention and eradication of the infection (about 0.75 log of CFU reduction relative to that for macrophages treated with free IFN-γ), the cytokine encapsulated into the NPs (IFN-γ–NPBs) did not show any effect. The absence of release of the cytokine from IFN-γ–NPBs (26) could be the cause for this lack of effectiveness, in agreement with the findings of similar studies performed with IFN-γ adsorbed onto and/or encapsulated in liposomes (29). So far, the good correlation between the amounts of NO released by macrophages activated with the different IFN-γ formulations and the decrease in the number of colonies corroborate a decisive role of NO from macrophages as the mediator of the killing of intracellular Brucella spp., as described previously (8, 9, 13, 21).

Finally, our formulations were tested in vivo. BALB/c mice were treated with IFN-γ 1 day before and again 2 and 4 days after infection with B. abortus, but the treatment was not effective either 1 or 2 weeks after infection. The higher doses administered by other authors could explain a certain amount of the activity reported 1 week after infection (6). Anyway, this effect was transient and it was not observed for periods of time longer than 1 week (19, 20). The adsorption of IFN-γ onto NPs (IFN-γ–NPAs) not only made the cytokine effective at this low dose but also increased the duration of the effect, that is, for almost 2 weeks. In this way, van Slooten et al. (29) showed that IFN-γ-loaded liposomes were active against influenza virus infection 3 weeks after infection, while IFN-γ showed efficacy only during the first week of treatment. This greater efficiency can be due to the NPs that actually reach the infection site before their degradation, with the IFN-γ then gradually desorbed close to the parasites. In that sense, mice treated with NPs containing IFN-γ showed significantly increased spleen weights, probably indicating a major accumulation of macrophages that engulfed the NPs in this organ and that were armed to fight the parasite (25). The inflammatory response induced by IFN-γ depends on the incubation time (27). At short times, IFN-γ mediates a transiently increased splenomegalic indicative of an immunostimulatory response. One week later, if the treatment is effective, the bacteria will be cleared from spleens; and thus, a significant reduction in the spleen weight will result (27).

The patterns for the splenic cytokines from the mice treated with free or associated IFN-γ also confirmed the increased activity of IFN-γ when it was loaded onto NPs. We found a mixed response in mice treated with free IFN-γ and IFN-γ–NPAs, with cells producing Th1 cytokines as well as cells secreting Th2 cytokines. However, the ratio of IFN-γ to IL-4 was higher in spleen cells from IFN-γ–NPA-treated mice. This switching in the type of response dominance correlated well with the better resistance with this treatment and underscored the protective role exerted by the cytokines IFN-γ and IL-2 in the face of a Brucella infection (11).

In summary, the present study demonstrates the feasibility and efficacy of targeting IFN-γ by means of BSA NPs. NP delivery was found to enhance the nonspecific activation of RAW 264.7 macrophages, resulting in an increase in the level of NO production and the killing of Brucella. The efficacy of IFN-γ in protecting mice against Brucella was also significantly improved by its adsorption on NPs. In this study, brucellosis was selected as a model only to test the potential therapeutic activities of exogenous IFN-γ and to demonstrate that NP-adsorbed IFN-γ was more active than the free cytokine against Brucella in vitro and in vivo. Albumin NPs would be also suitable for the coencapsulation of therapeutic agents when an additional or synergistic effect of chemotherapeutics (i.e., conventional antibiotic therapy) along with immune system components is sought. The use of exogenous IFN-γ may accelerate the response to conventional therapy; hence, the potential applications for IFN-γ NPs may include not only infections which typically respond poorly to available treatment or infec-
tions which require prolonged therapy for cure but also long-term prophylaxis in patients with T-cell-deficient states.

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