The aim of this study was to evaluate the activity of gamma interferon (IFN-γ) when it was either adsorbed onto or loaded into albumin nanoparticles. Brucella abortus-infected macrophages and infected BALB/c mice were selected as the models for testing of the therapeutic potentials of these cytokine delivery systems, in view of the well-established role of IFN-γ-activated macrophages for the control of Brucella spp. infections. Whereas the encapsulation of IFN-γ inside the matrix of nanoparticles completely abrogated its activity, adsorbed IFN-γ increased by 0.75 log unit the bactericidal effect induced by RAW macrophages activated with free IFN-γ, along with a higher level of production of nitric oxide. In infected BALB/c-mice, IFN-γ adsorbed onto nanoparticles was also more active than free cytokine in reducing the number of bacteria in the spleens, and the effect was mediated by an increased ratio of IFN-γ-secreting (Th1) to interleukin-4-secreting (Th2) cells. Overall, albumin nanoparticles would be suitable as carriers that target IFN-γ to macrophages and, thus, potentiate their therapeutic activity.

Malfunction 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 macromolecules, 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 U/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.

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-NP Bs). 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-NP Bs).

Briefly, IFN-γ-NP Bs were obtained by the addition of 1.5 ml of ethanol dropwise (ethanol-to-water ratio, 1:5; 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-NPAs were separated from the free cytokine by centrifugation (17,000 rpm/30 min) at 4°C.

For the preparation of IFN-γ-NPAs, 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 coacervate so formed were then hardened with glutaraldehyde that had previously been dissolved in ethanol (1.56 g/mL) 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 process of preparation, either by the encapsulation process or centrifugation, were assayed for the amount of cytokine in the NPs (expressed in mg).

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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.

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 macrophage cultures (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 or centrifugation.

**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-NPAs) did not show antibrucelae activity.

**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 treatment of macrophages with IFN-γ enhanced 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-NPAs, 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 Brucella. 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 Brucella abortus did not significantly alter the number of Brucella inoculated into the spleen or the number of Brucella growing in the spleen, even when IFN-γ-NPAs were used. However, macrophages treated with IFN-γ-NPAs 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-NPBs 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 NPAs. Brucella infection induced an inflammatory response that prevented the analysis of the proliferation induced by the formulations with infected mice.

**TABLE 2. Nitric oxid accumulation in supernatants of RAW 264.7 macrophages treated in vitro with IFN-γ formulations**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>NO production (μM)</th>
<th>I</th>
<th>II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>2.4 ± 1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Empty NPs</td>
<td>5.0 ± 1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>16.4 ± 0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ-NPA</td>
<td>22.4 ± 2.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ-NPB</td>
<td>8.7 ± 0.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The cells were incubated for 24 h with 100 IU/ml of IFN-γ formulations before infection and were treated again at 24 h postinfection with the same dose. The level of NO production was measured after the pretreatment but before the infection (I) and after the treatment and postinfection (II). Data are expressed as the means ± standard deviations of three measurements in an experiment representative of three repetitions (n = 3).

b P < 0.05 relative to the results for IFN-γ.

**TABLE 3. Effects of IFN-γ and IFN-γ formulations on resistance to Brucella abortus in mice**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>CFU log_{10}/spleen at the following time after infection:</th>
<th>Spleen wt (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 wk</td>
<td>2 wk</td>
</tr>
<tr>
<td>Control</td>
<td>5.2 ± 0.1</td>
<td>5.0 ± 0.2</td>
</tr>
<tr>
<td>NP</td>
<td>5.1 ± 0.1</td>
<td>5.0 ± 0.1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5.0 ± 0.1</td>
<td>4.9 ± 0.0</td>
</tr>
<tr>
<td>IFN-γ-NPA</td>
<td>4.4 ± 0.1^c</td>
<td>4.3 ± 0.1^c</td>
</tr>
<tr>
<td>IFN-γ-NPB</td>
<td>5.1 ± 0.1</td>
<td>5.1 ± 0.1</td>
</tr>
</tbody>
</table>

* Mice received free IFN-γ or IFN-γ formulations at 10^4 IU/ml 24 h before infection as well as 2 and 4 days after infection. Numbers of B. abortus CFU per spleen were measured 1 and 2 weeks after infection. The spleen weights refer to the effects in control (noninfected) mice after 1 week following treatment with the IFN-γ formulations. Data are expressed as the means ± standard deviations of three experiments with three mice per group (1 week after infection; n = 9) or two mice per group (2 weeks after infection; n = 6).

b P < 0.05 relative to the results for the control.

c P < 0.05 relative to the results for mice treated with IFN-γ.
spleenocytes of mice treated with IFN-γ or IFN-γ–NPs compared with that detected from the splenocytes of the control mice \( (P < 0.05) \). IFN-γ–NPs 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–NAs, the cytokine was adsorbed onto preformed NPs. In IFN-γ–NPs, 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–NAs and the IFN-γ–NPs 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–NAs released 80% of the loaded cytokine after 20 h; no release of cytokine was detected from IFN-γ–NPs 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–NAs 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–NPs) did not show any effect. The absence of release of the cytokine from IFN-γ–NPs (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–NPs) 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 splenomegaly 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–NAs, 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 also be 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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REFERENCES