Hydrophobic Gentamicin-Loaded Nanoparticles Are Effective against *Brucella melitensis* Infection in Mice

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The clinical management of human brucellosis is still challenging and demands *in vitro* active antibiotics capable of targeting the pathogen-harboring intracellular compartments. A sustained release of the antibiotic at the site of infection would make it possible to reduce the number of required doses and thus the treatment-associated toxicity. In this study, a hydrophobically modified gentamicin, gentamicin–AOT [AOT is bis(2-ethylhexyl) sulfosuccinate sodium salt], was either microstructured or encapsulated in poly(lactic-co-glycolic acid) (PLGA) nanoparticles. The efficacy of the formulations developed was studied both *in vitro* and *in vivo*. Gentamicin formulations reduced *Brucella* infection in experimentally infected THP-1 monocytes (>2-log10 unit reduction) when using clinically relevant concentrations (18 mg/liter). Moreover, *in vivo* studies demonstrated that gentamicin–AOT-loaded nanoparticles efficiently targeted the drug both to the liver and the spleen and maintained an antibiotic therapeutic concentration for up to 4 days in both organs. This resulted in an improved efficacy of the antibiotic in experimentally infected mice. Thus, while 14 doses of free gentamicin did not alter the course of the infection, only 4 doses of gentamicin–AOT-loaded nanoparticles reduced the splenic infection by 3.23 logs and eliminated it from 50% of the infected mice with no evidence of adverse toxic effects. These results strongly suggest that PLGA nanoparticles containing chemically modified hydrophobic gentamicin may be a promising alternative for the treatment of human brucellosis.

Brucellosis remains one of the world’s most widespread zoonoses (1). It is an infectious disease caused by bacteria of the genus *Brucella*, species of which are facultative intracellular pathogens that localize predominantly in cells and organs of the mononuclear phagocytic system, such as the macrophages of the liver and the spleen. Human brucellosis usually manifests as a febrile illness that may persist and progress to a chronically incapacitating disease with severe complications, and it is an important cause of morbidity worldwide (2). The chronicity of the infection results from the ability of the pathogen to survive within the phagocytic cells, which fail to eliminate the microorganism and act as a reservoir of the bacteria. Although it is a reportable disease, it is estimated that cases of human brucellosis are underdiagnosed and underreported and that the disease continues to be a major human health hazard (3, 4). The disease is also recognized as one of the most common laboratory-acquired infections (5, 6), and owing to its possible airborne transmission and its low infective dose, it is considered a potential bioweapon for bioterrorism (7).

Concomitant with its pathogenicity, the clinical management of human brucellosis is also a difficult task. The intracellular location of the pathogen inside phagocytic cells makes its eradication difficult to achieve, since many *in vitro* active antibiotics fail to reach *Brucella*-infected cells efficiently, lose their antimicrobial activity in the intracellular environment, or do not persist long enough to produce a therapeutic effect (8). Therefore, the current treatment of human brucellosis requires a combination of antibiotics for long periods of time. World Health Organization guidelines recommend doxycycline with rifampin for 6 weeks (9), but more recent recommendations also propose the use of doxycycline for 6 weeks with the aminoglycosides streptomycin for 2 to 3 weeks or gentamicin for 1 week (10). However, despite the reasonable efficacies of current treatment regimens, they often fail to eradicate the infection, with relapse rates of about 5 to 10% (11). On the other hand, the combined therapies are more effective than individual therapies; however, new therapies are necessary due to the difficulties of patient adherence to the treatment itself together with the side effects of combination therapy and the dangers of antibiotic resistance (12).

Nanotechnology has emerged as a promising approach for the treatment of intracellular infections by providing intracellular targeting and sustained release of vehiculized drugs inside the infected cells (13, 14). These drug delivery systems may lead to an improvement in drug cellular accumulation and a reduction of the required dosing frequency which, in turn, will improve patient compliance and the efficacy of the antimicrobial therapy. The aminoglycoside gentamicin is a bactericidal antibiotic with great *in vitro* activity against clinical isolates of *Brucella*, and it has already been encapsulated into particulated systems for the treatment of experimental brucellosis with promising results (15, 16). However, the low encapsulation efficiency obtained limited the dose of particles that could be administered *in vivo*. We recently reported that the hydrophobic ion pairing of gentamicin with the
anionic surfactant AOT successfully improves the antibiotic payload in carriers without affecting its bioactivity (17).

The aim of this research was to study the efficacy of gentamicin–bis(2-ethylhexyl) sulfo succinate sodium salt (GEN-AOT)–loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) in both human macrophages and mice experimentally infected with \textit{B. melitensis}. Moreover, the \textit{in vivo} pharmacokinetics and toxicities of the formulations were also studied. The results demonstrated that the modification and encapsulation of gentamicin successfully improved its efficacy against brucellosis and reduced the associated toxicological profile.

**MATERIALS AND METHODS**

Materials. Gentamicin sulfate, doxycycline, polyvinyl alcohol (PVA), and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma–Al drich (St. Louis, MO), and AOT was obtained from Sigma (Tres Cantos, Spain). PLGA 502H (Resomer RG 502H, PLGA 50:50, 13.7 kDa) and 752H (Resomer RG 752H, PLGA 75:25, 17 kDa) were supplied by Boehringer Ingelheim (Ingelheim, Germany). For bacterial growth, Tryptic soy broth (TSB) was purchased from bioMérieux (Marcy l’Étoile, France), antibiotic medium 11 was from Difco (Becton, Dickinson, Franklin Lakes, NJ), and American bacteriological agar was from Pronadisa (Madrid, Spain). Reagents for cell culture were obtained from Invitrogen Inc. (Carlsbad, CA), and other reagents were from Sigma–Aldrich (St. Louis, MO) or Merck (Madrid, Spain).

Preparation and characterization of microstructured gentamicin–AOT and gentamicin–AOT. The modified gentamicin, gentamicin–AOT (GEN-AOT), was prepared by the hydrophobic ion pairing of gentamicin with the anionic surfactant AOT, as previously described (18). The obtained GEN-AOT complex was then either microstructured (PCA GEN-AOT) by using a compressed fluid-based technology called precipitation with a compressed antisolvent (PCA) (17) or encapsulated into PLGA nanoparticles by an oil-in-water single-emulsion formation solvent evaporation method, using a nominal drug loading of 20 mg per formulation, as previously reported (17). Briefly, GEN-AOT (20 mg) and the polymer (200 mg of PLGA 502H or PLGA 752H) were dissolved in 1 ml of ethyl acetate and mixed by ultrasonication with 2 ml of a 0.5% (wt/vol) PVA aqueous solution for 1 min (15 W; sonifier 450; Branson Ultrasonics Corp., Danbury, CT). The resulting emulsion was poured into 50 ml of a solution of 0.2% (wt/vol) PVA and stirred for 3 h to allow solvent evaporation and nanoparticle formation. Particles were collected and washed with ultrapure water by three successive centrifugations at 21,000 \( \times g \) for 10 min (3K30; Sigma Laboratory Centrifuges, Osterode am Harz, Germany). Nanoparticles were lyophilized with 5% (wt/vol) mannitol and characterized in terms of size, zeta potential, and encapsulation efficiency (17).

Cell culture and cellular differentiation. The human myelomonocytic cell line THP-1 (ATCC TIB-202; American Type Culture Collection, Manassas, VA), which displays macrophage-like activity (19), was cultured and differentiated into adherent macrophage-like cells as previously described (20).

Bacterial strain and culture conditions. \textit{Brucella melitensis} 16 M (ATCC 23456, biotype 1) smooth virulent strain was used for the different studies. Experiments were performed with fresh bacteria incubated in TSB medium at 37°C under shaking to the exponential growth phase (15).

Macrophage infection with \textit{Brucella melitensis}. THP-1 macrophages (2 \( \times 10^5 \) cells/well in 24-well cell culture plates) were infected with \textit{B. melitensis} at a bacteria-to-macrophage ratio of 100 for 5 h at 37°C with 5% CO\(_2\). Cells were then washed with phosphate-buffered saline (PBS) to remove the extracellular bacteria and incubated in complete RPMI medium. At 48 h postinfection, macrophages were washed with PBS and treated with the different gentamicin formulations at 1 mg/liter of gentamicin (2\( \times \) the MIC of gentamicin against the tested strain [17]) or 18 mg/liter of gentamicin (the peak serum drug concentration in humans during conventional gentamicin treatment [\( C_{max} \) [21]]). Control cells were incubated with 0.25 mg/liter of gentamicin (0.5\( \times \) the MIC) to prevent extracellular bacterial growth.

After 24 h of incubation with the antibiotic treatments, cells were carefully washed with PBS and lysed. The remaining extracellular CFU in the last washing and the total CFU of \textit{Brucella} in the lysates were determined by plating aliquots of 10-fold serial dilutions on TSA plates. The number of intracellular bacteria was calculated after incubating the plates at 37°C and subtracting the extracellular CFU counts from the total CFU counts of each well. Results of CFU counts were log transformed and are expressed as means and standard deviations.

\textbf{In vivo studies. (i) Animals.} Female BALB/c mice (weight, 20 g) were supplied by Harlan Interfana Ibérica (Barcelona, Spain). Following acclimatization, mice were randomized into different groups and kept in cages with a 12-hour light-dark photocycle, and water and food were provided ad libitum. The experimental protocols were revised and approved by the Animal Experimentation Ethics Committee of the University of Navarra (protocol numbers 083-11 and 084-11).

(ii) \textit{Tissue pharmacokinetics study.} Animals (n = 6 mice/group) received a single intraperitoneal dose of one of the different gentamicin formulations, equivalent to 5 mg of gentamicin/kg of body weight, in sterile 0.9% saline. After 4 h, 8, 24 h, 48 h, 4 days, or 7 days, animals were sacrificed by cervical dislocation and their liver, spleen, and kidneys were collected. Organs were then weighed, homogenized in 1 ml of sterile water (Mini-headbeater; BioSpec Products, Inc., Bartlesville, OK) and centrifuged (10,000 \( \times g \), 10 min, 4°C). Tissue supernatants were collected and stored at \(-80°C\) until analysis. Prior to analysis, tissue samples were further processed for protein precipitation by the addition of a 10% trichloroacetic acid solution (1:10, vol/vol). Samples were then vortexed for 1 min, and after centrifugation (10,000 \( \times g \), 10 min, 4°C) protein-free supernatant was collected.

The gentamicin content of the samples was analyzed in a microbiological assay using \textit{Bacillus subtilis} ATCC 6633 as test organism. Fifty-microliter aliquots of the tissue samples were added into 6-mm–diameter wells made in the inoculated antibiotic medium 11 agar, and zones of inhibition were measured after incubation for 12 h at 4°C and 24 h at 37°C. In parallel, standard curves of gentamicin and GEN-AOT in control plasma and tissue homogenates were prepared under the same conditions.

Pharmacokinetic parameters were calculated by noncompartmental methods. All calculations were carried out using WinNonlin Professional version 2.1 (Scientific Consulting, Inc., Mountain View, CA). The area under the concentration-time curve (AUC) from the time of administration to the last measured concentration (AUC\(_{0-\infty}\)) was calculated by trapezoidal integration. The maximum plasma and tissue drug concentration (\( C_{max} \)) and the time to attain the peak drug concentration (\( t_{max} \)) were obtained directly from the raw data.

(iii) \textit{Toxicity studies.} For this study, mice were divided into six groups (n = 6 mice/group) that received for 14 days (i) a 0.9% saline solution, (ii) gentamicin, (iii) a combination of gentamicin and doxycycline, (iv) PCA GEN-AOT, (v) GEN-AOT-loaded PLGA 752H nanoparticles, or (vi) nonloaded PLGA 752H nanoparticles. Saline solution (100 \( \mu \)l/mouse) was administered daily intraperitoneally, doxycycline (200 mg/100 g/kg) was administered daily by oral gavage, gentamicin (100 mg/mouse; 5 mg/kg) and PCA GEN-AOT (300 mg/mouse, equivalent to 100 \( \mu \)g of gentamicin/mouse) were administered daily intraperitoneally, and nanoparticles (4 mg/mouse, approximately, containing the equivalent of 100 \( \mu \)g of gentamicin/mouse) were administered intraperitoneally every 4 days. At the end of treatment, animals fasted overnight were sacrificed, and blood and tissue samples were collected. Hematological, biochemical, and histological parameters were studied.

Blood samples were analyzed for routine hematological parameters: red blood cells (expressed as 10\(^6\) cells/\( \mu \)l), white blood cells (10\(^6\) cells/\( \mu \)l), hemoglobin (in g/dl), hematocrit (as a percentage), mean corpuscular volume (in fl), mean corpuscular hemoglobin concentration (in pg/cell), and platelets (10\(^9\) cells/\( \mu \)l). The hemogram was investigated by using a
Roche hematolgy analyzer (Sysmex XT1800i). Biochemical parameters were investigated in serum sample: total bilirubin (in mg/dl), creatinine (mg/dl), and urea (mg/dl) levels were studied by using a Roche semi-automated analyzer (Hitachi 911) and Roche analytical kits.

The organs—liver, kidneys, spleen and lungs—were removed, weighed, and preserved in 10% formalin for histological studies.

(iv) Experimental infection of mice with Brucella melitensis. Animals were housed in microisolator cages and handled under sterile conditions in biohazard safety level 3 facilities. Mice were infected with 10⁵ CFU of B. melitensis 16 M in 0.1 ml of 0.9% saline solution. At day 14 after infection, a group of mice was sacrificed and their spleens were aseptically removed for viable bacteria counts. This group of mice was used as a control for baseline infection. Of the other groups of mice, one group was kept untreated and the others received (i) free gentamicin, (ii) a combination of free gentamicin and doxycycline, (iii) PCA GEN-AOT, (iv) GEN-AOT-loaded PLGA 752H nanoparticles, or (v) nonloaded PLGA 752H nanoparticles. The doses were the same as those described in the toxicity studies. At 1 and 3 weeks after the last antibiotic dose (corresponding to days 35 and 49 postinfection), animals were sacrificed and their organs were aseptically collected. Organs were homogenized with 1 ml of 0.9% sterile saline solution and centrifuged for 10 min at 10,000 × g. The efficacy of the treatments was determined by counting the number of CFU per spleen after spreading 0.1 ml of neat supernatant and 10-fold serial saline dilutions on TSA plates.

Statistical analysis. Data analysis and graphical presentations were done using Prism version 5.00 software for Windows (GraphPad Software, San Diego, CA). Comparisons of means between groups were performed by using the Mann-Whitney U test. The statistical significance level was defined as a P value of <0.05.

RESULTS
PCA gentamicin-AOT and gentamicin-AOT nanoparticles. PCA-processed GEN-AOT was obtained as white, powdered solid formed by micron-sized particles with a mean diameter of 1 μm and a zeta potential of around −1 mV. On the other hand, GEN-AOT-loaded PLGA 502H and PLGA 752H nanoparticles presented mean diameters of 289 ± 15 nm and 299 ± 23 nm (means ± standard deviations), with polydispersity indices of 0.10 ± 0.07 and 0.08 ± 0.09 nm and zeta potentials of −3.7 ± 0.4 mV and −3.6 ± 0.7 mV, respectively. Encapsulation efficiencies of 100% were obtained for both types of formulations with drug loadings of 23.8 ± 0.5 and 24.1 ± 0.6 μg of gentamicin/mg of nanoparticles for PLGA 502H and PLGA 752H nanoparticles, respectively (means ± standard deviations).

Efficacy of gentamicin formulations against Brucella melitensis-infected macrophages. Under the experimental conditions, the reduction of intracellular Brucella obtained with GEN-AOT formulations was studied after the treatment of the cells with an equivalent dose of 1 mg/liter or 18 mg/liter of gentamicin for 24 h and compared to that obtained with free gentamicin. As shown in Fig. 1, regardless of the concentration used, free gentamicin did not reduce bacterial growth. The same results were obtained for nonloaded PLGA nanoparticles. In contrast, all GEN-AOT treatments significantly reduced the intracellular infection level (P < 0.01) compared to the control cells (1.23 to 1.41 log₉ unit reductions). On the other hand, although no statistically significant differences were found between the different GEN-AOT formulations at 1 mg/liter, treatment with 18 mg/liter of GEN-AOT 752H nanoparticles was significantly more effective (P < 0.05) than the other gentamicin treatments, achieving a 2.35-log₉ unit reduction of intracellular Brucella. Therefore, and considering previous studies with GEN 502H and 752H microparticles (15), GEN-AOT 752H nanoparticles were selected for in vivo studies. For comparison, the nonencapsulated form of PCA GEN-AOT was also assayed. This particulate formulation was preferred over the as-synthesized GEN-AOT because of its more favorable physico-chemical properties, as PCA GEN-AOT could be prepared as a suspension in aqueous medium after ultrasonication.

Pharmacokinetics study. Pharmacokinetics studies of free gentamicin, PCA GEN-AOT, and GEN-AOT-loaded nanoparticles were performed after a single intraperitoneal administration of a dose equivalent to 5 mg/kg of gentamicin for each formulation. The concentrations of the antibiotic in the liver and spleen (target organs of Brucella) and kidney (because gentamicin is nephrotoxic) were determined. After the administration of free gentamicin, no antibiotic was detected in either the spleen or in the liver (limits of detection, 0.125 μg/g and 0.25 μg/g, respectively) (Fig. 2A and B). In contrast, a high accumulation of gentamicin was detected in the kidneys, with measurable gentamicin concentrations for at least 1 week after the administration of the dose (Fig. 2C).

The AOT coupling and encapsulation markedly altered the distribution of the antibiotic, reducing its accumulation in the kidneys and increasing it in both the liver and the spleen. Cmax and AUC values were decreased in kidneys by 3.5-fold after PCA GEN-AOT administration with respect to the nonmodified free gentamicin (Table 1), whereas relevant concentrations were measured in liver and spleen for up to 7 days. PCA GEN-AOT resulted in similar AUC values in the liver and the spleen, indicating that the drug distributed equally to both tissues. However, the Cmax values achieved in the liver and the spleen with the dose administered were below the MIC against Brucella melitensis (1 mg/liter). As Brucella infection is localized in those tissues, a daily administration schedule was established for PCA GEN-AOT therapy.

FIG 1 Efficacy of 1 mg/liter (horizontal lines) or 18 mg/liter (diagonal lines) of gentamicin in the different formulations against intracellular Brucella melitensis infection in THP-1 human macrophages. Results are expressed as the log₉ intracellular CFU per well and are presented as means value ± standard deviation of at least three independent assays performed in triplicate. The dotted line indicates the intracellular Brucella inoculum at the beginning of the treatment. Statistical analysis: **, P < 0.01 compared to control cells; a, P < 0.05, and b, P < 0.01 compared to gentamicin-treated cells; c, P < 0.05 compared to cells treated with gentamicin-AOT, microstructured gentamicin-AOT, or gen-tamicin-AOT PLGA 502H nanoparticles (Mann-Whitney U test). Abbreviations: GEN, gentamicin; GEN-AOT, gentamicin-AOT; PCA GEN-AOT, PCA microstructured gentamicin-AOT; GEN AOT 502H NP, gentamicin-AOT loaded PLGA 502H nanoparticles; GEN-AOT 752H NP, gentamicin-AOT loaded PLGA 752H nanoparticles; 502H NP, PLGA 502H nanoparticles; 752H NP, PLGA 752H nanoparticles.
Encapsulation of GEN-AOT further enhanced the antibiotic tissue accumulation, especially in the spleen. Nanoparticles increased GEN-AOT’s AUC values by 10.7- and 2-fold in the spleen tissue accumulation, especially in the spleen. Nanoparticles in-loaded PLGA 752H nanoparticles (GEN-AOT 752H NP) equivalent to 5 mg of microstructured gentamicin-AOT (PCA GEN-AOT), or gentamicin-AOT-administration of a single intraperitoneal dose of gentamicin (GEN), PCA particles were studied in mice.

Toxicity of the formulations in healthy mice. In all the treated groups, there was no significant alteration in any of the hematological or biochemical parameters compared to the saline control group (data not shown).

The organ weights were normal, and no significant changes were observed among the different groups. Histological analysis of the liver, the spleen, and the lungs revealed no treatment-related alterations. However, kidney examinations revealed significant differences among the different groups. In the group treated with gentamicin plus doxycycline, all animals presented small foci of tubulonephrosis and one animal showed focal necrosis (Fig. 3B). Moreover, the mice treated with free gentamicin presented a slight tubular lipidosis (Fig. 3A). However, no alterations were found in mice treated with PCA GEN-AOT or GEN-AOT-loaded PLGA 752H nanoparticles (Fig. 3C and D).

Efficacies of the formulations in Brucella melitensis-infected mice. The efficacies of PCA GEN-AOT and GEN-AOT nanoparticles were studied in B. melitensis-infected mice and compared to that of free gentamicin or the combination of gentamicin plus doxycycline. The administered Brucella inoculum produced a baseline splenic infection of 4.91 log_{10} CFU/spleen, and after the 2-week treatment regimen the course of the splenic infection was monitored. The results are summarized in Table 2.

In comparison with the nontreated group, no significant (P > 0.05) reduction of the infection was obtained in the groups of mice that received free gentamicin or nonloaded nanoparticles. On the other hand, treatment with PCA GEN-AOT, GEN-AOT nanoparticles, or gentamicin plus doxycycline resulted in a significant reduction in the bacterial load in the spleen. Daily treatment with PCA GEN-AOT significantly reduced the splenic infection at both 1 and 3 weeks after the end of the treatment. However, this treatment was not able to sterilize the infected spleens. Regarding the combination of gentamicin and doxycycline, 1 week after the end of the treatment, there was a 5.2-log_{10} reduction and 80% of the infected spleens were sterilized (less than 10 CFU/spleen). However, thereafter, the infection reemerged and a therapeutic failure of 83% was observed at the third week after treatment. Conversely, GEN-AOT nanoparticles exerted their action more progressively, showing improved therapeutic activity over the time of the study. Thus, at 3 weeks posttreatment with the nanoparticles, a 3.23-log_{10} reduction was obtained, and 50% of the animals presented no bacteria in the spleen, a percentage much higher than the 17% observed with the combined therapy.

DISCUSSION

Standard therapy for human brucellosis is based on a 6-week administration of a combination of doxycycline with rifampin or an aminoglycoside, such as streptomycin or gentamicin, which often leads to poor patient compliance, frequent relapses, and serious side effects (22). The doxycycline-plus-rifampin regimen has the advantage of being a completely oral regimen, unlike aminoglycoside-containing regimens, which require intramuscular administration. However, treatments including rifampin have been shown not to be as effective as the regimens that include an aminoglycoside, and they result in higher relapse rates (23–26). On the other hand, although monotherapy could prevent at least some of the side effects and improve compliance with treatment, it has been associated with therapeutic failure and high relapse rates (27–29). As a consequence, there is a recognized need to improve the current treatment for human brucellosis. Appropriate antibiotics should achieve therapeutic concentrations at the sites of infection while maintaining their activity. We have previously reported that hydrophobic modification and nanoencapsulation of gentamicin enhance its cellular accumulation and improve its efficacy against intracellular bacteria, such as Staphylococcus aureus and Listeria monocytogenes (20). Therefore, encouraged by previ-
Intracellular activity studies were carried out in human macrophages, which are the target cells of human brucellosis, using two different concentrations, 1 and 18 mg/liter, corresponding to \( \frac{2}{H} \) the MIC of gentamicin against the tested \( B. \) melitensis strain and the \( C_{\text{max}} \) of gentamicin in human serum, respectively. Regardless of the concentration used, free gentamicin was not able to reduce the intracellular \( Brucella \) infection. This lack of efficacy was attributed to the low gentamicin accumulation inside the cells and the major distribution of the drug into the lysosomes, which impedes the final encounter between bacterium and drug (20). Studies examining the intracellular fate of \( Brucella \) in phagocytic cells have indicated that the \( Brucella \) organisms reside in acidified phagosomes that fuse with components of the early endosomal pathway but not with the lysosomes (30). It has also been suggested that these replicative phagosomes arise through continual interactions with the endoplasmic reticulum (31). Hydrophobic

TABLE 2 Antibacterial efficacies of the different gentamicin formulations in \( Brucella melitensis \) 16 M-infected BALB/c mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1 wk posttreatment</th>
<th>3 wk posttreatment</th>
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<tbody>
<tr>
<td></td>
<td>Log CFU/spleen</td>
<td>Log reduction</td>
</tr>
<tr>
<td>Untreated</td>
<td>5.54 ± 0.50</td>
<td>0/5</td>
</tr>
<tr>
<td>GEN + DOX</td>
<td>0.37 ± 0.83**d</td>
<td>5.17</td>
</tr>
<tr>
<td>GEN</td>
<td>5.10 ± 0.25</td>
<td>0.44</td>
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<tr>
<td>PCA GEN-AOT</td>
<td>4.85 ± 0.10**</td>
<td>0.69</td>
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<tr>
<td>GEN-AOT 752H NP</td>
<td>3.69 ± 1.86**</td>
<td>1.85</td>
</tr>
<tr>
<td>752H NP</td>
<td>5.49 ± 0.30</td>
<td>0.05</td>
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\( \text{a} \) Statistical analysis (Mann-Whitney U test): *, \( P < 0.05 \), and **, \( P < 0.01 \) compared to the untreated group at the same time of sacrifice; lowercase c, \( P < 0.05 \), and d, \( P < 0.01 \) compared to the untreated group 2 weeks postinfection (baseline infection, 4.91 log CFU/spleen).

\( \text{b} \) Limit of detection was <10 CFU/spleen. Abbreviations: GEN, gentamicin; DOX, doxycycline; PCA GEN-AOT, PCA microstructured gentamicin-AOT, GEN-AOT 752H NP, gentamicin-AOT loaded PLGA 752H nanoparticles; 752H NP, PLGA 752H nanoparticles.
modification and encapsulation of gentamicin significantly enhanced the intracellular killing of *Brucella*, reducing the intracellular infection below the baseline control infection. Such a finding has also been previously reported for *Staphylococcus aureus* and *Listeria monocytogenes*-infected cells and was attributed to the higher cellular accumulation achieved by GEN-AOT formulations, particularly by GEN-AOT 752H nanoparticles, and the altered subcellular distribution of the encapsulated antibiotic, which may enable it to reach the pathogen-harboring compartments at therapeutically relevant concentrations (20). In addition, it has been demonstrated with gentamicin PLGA formulations that particle uptake stimulates the oxidative burst of macrophages, which may also account for the better efficacy of the encapsulated GEN-AOT in comparison to the free form (32, 33).

On the other hand, after their entrance into the host, *Brucella* cells are taken up by the local tissue lymphocytes and transferred into the general circulation, whence they are disseminated throughout the body, with special tropism for the cells and organs of the mononuclear phagocytic system, such as the macrophages of the liver and the spleen (34). In sharp contrast, most gentamicin injected into the body is eliminated by renal clearance without being metabolized, and the rest of the dose accumulates mainly in the renal cortex, leading to the well-known nephrotoxicity of aminoglycosides (35). Consistent with these observations, after the administration of 5 mg/kg of free gentamicin, no antibiotic was detected in the livers or the spleens of the mice. In contrast, a high drug accumulation was observed in the kidneys (30 μg/g).

Both conjugation of gentamicin with AOT and its encapsulation managed to increase the gentamicin concentration level in the liver and more pronouncedly in the spleen, while decreasing antibiotic accumulation in kidneys. Aminoglycosides present a concentration-dependent antimicrobial activity and thus require elevated peak levels to reach the pharmacodynamic objectives. The plasma drug $C_{\text{max}}$-to-MIC ratio has been shown to be a good predictor of aminoglycoside therapeutic efficacy, and it is generally accepted that optimum bactericidal activity is achieved when the peak concentration is approximately $10 \times$ the MIC (36, 37). Taking into account the *in vivo* distribution of the bacteria, special attention was paid to the $C_{\text{max}}$/MIC ratios in the liver and the spleen.

As previously observed in cell culture models (20), nanoparticles yielded higher antibiotic levels in all the tissues studied, which were translated into higher $C_{\text{max}}$ and AUC values compared to those for PCA GEN-AOT. At equivalent drug doses, nanoparticles achieved $C_{\text{max}}$-to-MIC ratios of 9 and 109 in the liver and the spleen, respectively, while these ratios were below 1.5 in both tissues for PCA GEN-AOT. Moreover, as a result of nanoparticle treatment, sustained drug levels above the MIC were achieved for 4 days in the liver and the spleen, which formed the basis for designing a treatment schedule based on a lower dosing frequency. This is an important issue to take into account, as several studies have demonstrated that extended-interval dosing, or administration of aminoglycosides in higher and less frequent doses, allows for increased bacterial killing while minimizing the associated nephrotoxicity (38–40). The toxicological and therapeutic aspects of the PCA GEN-AOT and GEN-AOT-loaded nanoparticles were therefore studied and compared to those observed for gentamicin and the reference treatment of gentamicin plus doxycycline. During the treatment, no hematological or biochemical alterations were observed. However, histological examination revealed characteristic aminoglycoside-induced renal alterations, such as tubular lipidosis and tubulonephrosis foci in those mice that had received a gentamicin solution, alone or in combination with doxycycline, respectively (35, 41). Conversely, no alterations were found in mice that received PCA GEN-AOT or the GEN-AOT-loaded nanoparticles. Aminoglycoside-derived nephrotoxicity has been associated with the accumulation of a small percentage of the administered dose in the proximal renal tubular epithelial cells. Because of its polycationic properties, gentamicin binds to the negatively charged membrane phospholipids and enters tubular cells via endocytosis mediated by the megalin/cubilin complex (42). Therefore, the complexation of gentamicin with the anionic AOT surfactant may decrease the affinity of gentamicin to the membrane phospholipids and endocytic receptors and, thus, the uptake of GEN-AOT by these cells, and may reduce its interaction with the lysosomal or endosomal phospholipids, inhibiting the phospholipidosis, as has also been observed after the coadministration of gentamicin with some polyanions (43). Regarding nanoparticles, both a different uptake mechanism and the extended dosing interval used could protect the tubular cells from continuous exposure to high antibiotic concentrations, minimizing the chances of renal damage.

Finally, in accordance with the undetectable gentamicin levels in the spleens, it was observed that gentamicin monotherapy did not affect the course of the murine infection. On the other hand, despite the initial finding that combined therapy promisingly sterilized 80% of the infected mouse spleens, 83% of the animals became reinfected at the end of the study. Previous studies by Sha-sha, Lang, and Rubinstein showed sterile spleens in 100% of infected animals after a 14-days treatment with doxycycline (6 to 10 mg/kg) combined with either streptomycin (44) or rifampin (6 mg/kg) (45). However, these results were obtained just after the conclusion of the treatment period. Further data at different times of sacrifice will be required in order to compare the efficacies of the different combined therapies.

Interestingly, both GEN-AOT treatments significantly reduced the splenic infection, particularly GEN-AOT-loaded nanoparticles. Remarkably, with 4 nanoparticle doses, the splenic *Brucella* infection was reduced by 3.23 log$_{10}$ units and eliminated in 50% of the mice. Furthermore, the trend toward a greater therapeutic efficacy observed over the time of the study could indicate that the peak efficacy of the nanoparticles may not have been reached. The gentamicin-AOT-loaded nanoparticles had superior efficacy to other drug delivery systems, such as gentamicin PLGA microparticles (15) and polymeric nanoparticles containing streptomycin and doxycycline (46), and some free antibiotics, such as quinolones, co-trimoxazole (47), and a ciprofloxacin plus streptomycin combination (44) against *Brucella melitensis* infection in mice.

**Conclusions.** The present study demonstrates the potential of PLGA nanoparticles for delivering sustained therapeutic GEN-AOT concentrations in the liver and the spleen, the target organs for intracellular infections such as brucellosis, with no associated toxicity. These high and sustained tissue drug concentrations resulted in a reduced dosing frequency and improved therapeutic efficacy compared to the free drug. Therefore, the polymeric nanoparticles developed in this study are promising tools to meet the current challenges in the treatment of human brucellosis. This result, alongside the well-accepted use of PLGA by medical regulatory agencies, opens promising perspectives for this novel nano-
medicine. Presently, research is being carried out to optimize the dose and the duration of the nanoparticle therapy, with the aim of maximizing its therapeutic efficacy. Importantly, these nanoparticles may also be useful platforms for the encapsulation of other antibiotics and the treatment of diseases caused by intracellular bacteria, such as tuberculosis.

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