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Poly(D,L-Lactide-Coglycolide) Particles Containing Gentamicin: Pharmacokinetics and Pharmacodynamics in *Brucella melitensis*-Infected Mice[▽]

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Drug delivery systems containing gentamicin were studied as a treatment against experimental brucellosis in mice. Micro- and nanoparticles prepared by using poly(D,L-lactide-coglycolide) (PLGA) 502H and microparticles made of PLGA 75:25H were successfully delivered to the liver and the spleen, the target organs for *Brucella melitensis*. Both polymers have the same molecular weight but have different lactic acid/glycolic acid ratios. Microparticles of PLGA 502H and 75:25H released their contents in a sustained manner, in contrast to PLGA 502H nanoparticles, which were degraded almost completely during the first week postadministration. The values of the pharmacokinetic parameters after administration of a single intravenous dose of 1.5 mg/kg of body weight of loaded gentamicin revealed higher areas under the curve (AUCs) for the liver and the spleen and increased mean retention times (MRTs) compared to those for the free drug, indicating the successful uptake by phagocytic cells in both organs and the controlled release of the antibiotic. Both gentamicin-loaded PLGA 502H and 75:25H microparticles presented similar pharmacokinetic parameter values for the liver, but those made of PLGA 75:25 H were more effective in targeting the antibiotic to the spleen (higher AUCs and MRTs). The administration of three doses of 1.5 mg/kg significantly reduced the load associated with the splenic *B. melitensis* infection. Thus, the formulation made with the 75:25H polymer was more effective than that made with 502H microspheres (1.45-log and 0.45-log reductions, respectively, at 3 weeks posttreatment). Therefore, both, pharmacokinetic and pharmacodynamic parameters showed the suitability of 75:25H microspheres to reduce the infection of experimentally infected mice with *B. melitensis*.

Brucellosis remains a major zoonosis worldwide (17), and it is among the microorganisms most likely to be used as biological weapons (24). Specific therapy reduces morbidity, shortens the duration of illness, and decreases the incidence of complications (31); however, relapse currently presents a significant problem (27). Long-lasting combined drug treatments are difficult to achieve (27); and also the intracellular location of the pathogen in phagocytic cells, mainly in the liver and the spleen, are involved in therapeutic failure (22). Drug delivery systems containing gentamicin (GEN) have been developed as potential alternatives to classical therapy, and the usefulness of these vectors has been shown (5, 7, 10, 23, 30). Although GEN is effective against the pathogen in vitro (11, 18, 25), it shows a low level of intracellular penetration due to its polar nature; hence, its entrapment in particulate carriers might facilitate the entrance of the drug into the cells. Moreover, sustained drug delivery could avoid long-term treatment, eliminating the toxic systemic levels achieved during classical antibiotic therapy with the free drug. Despite the promising results obtained with GEN-containing liposomes (7, 10, 30), important drawbacks are attributed to these vesicles. Their instability in the presence of blood lipoproteins and their osmotic fragility can destabilize them, leading to leakage of the entrapped drug. Additionally, problems with their long-lasting stability upon storage remain (3, 6). On the other hand, microspheres were suitable a priori for this aim because of their higher stability and capture by mononuclear phagocytes, the niche of *Brucella* (23); in fact, microspheres obtained by spray drying produced a significant reduction of splenic infection in mice (21, 22). However, some of the animals died of pulmonary embolism after the administration of multiple doses, with death being attributed to the higher levels of aggregation of the particles (22). Therefore, the aim of the present work was to change the preparation method to obtain more suitable formulations and also, for the first time, to manufacture and study the effects of GEN-containing nanoparticles.

The in vivo fates of these micro- and nanoparticles of different poly(D,L-lactide-coglycolide) (PLGA) copolymers were studied. Finally, the distribution and degradation of particles, as well as pharmacokinetics and pharmacodynamics of encapsulated GEN, were determined. The results demonstrated their therapeutic efficacy against *Brucella melitensis* infections in mice.

MATERIALS AND METHODS

Materials. GEN sulfate, rhodamine B isothiocyanate, and lecithin (L-phosphatidylcholine) were from Sigma-Aldrich Co. (St. Louis, MO). PLGA copolymer with a free carboxyl end group of 13.7-kDa PLGA 50:50 (Resomer RG 502H) was provided by Boehringer-Ingelheim (Ingelheim, Germany), and PLGA 75:25 25 kDa (75/25 ratio of the D and L forms; 2.5 Å; 75:25H) was obtained from Alkermes (Wilmington, DE). Monopotassium phosphate, sodium dibasic phosphate, sodium hydroxide, trichloroacetic acid, and dichloromethane were from

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Panreac (Barcelona, Spain). Polyvinyl alcohol (PVA; molecular weight, 15,000) was purchased from BDH Supplies (Poole, United Kingdom). Pentafluoropropionic acid was from Fluka Chemie (Buchs, Switzerland). Tobramycin (internal standard), procured as Tobra-Gobens (the brand name of the product registered for clinical use in Spain), was purchased from Normon (Madrid, Spain). Methanol (high-pressure liquid chromatography [HPLC] grade) was from Merck (Darmstadt, Germany). For bacterial growth Trypticase soy broth was purchased by bioMérieux (Marcy l'Etoile, France), and American bacteriological agar was from Pronadisa (Madrid, Spain).

Preparation of gentamicin-containing formulations. Poly(lactide-coglycolide) formulations containing GEN were prepared by a water (W)-oil (O)-water solvent evaporation technique (2). Briefly, the antibiotic dissolved in 0.5% PVA in phosphate buffer (pH 6.0) and 200 mg of either 502H or 75:25H copolymers (dissolved either in dichloromethane for microspheres or in ethyl acetate for nanoparticles) were mixed by ultrasonication (sonifier 450; Branson Ultrasonics Corp., Danbury, CT) under cooling for 1 min to form a W_1-O emulsion. For fluorescent formulations, rhodamine B isothiocyanate (0.5 mg/ml) was added to the inner aqueous phase together with GEN. This inner emulsion was added to 2 ml 1% PVA (W_2), and the mixture was homogenized by ultrasonication. The resulting (W_1-O)W_2 emulsion was poured into 50 ml of 0.2% PVA, and the mixture was continuously stirred for 3 h at room temperature to allow solvent evaporation and particle formation. After their preparation, the particulate carriers were isolated by centrifugation (at 7,000 × *g* for 5 min for microspheres and at 12,000 × *g* for 30 min for nanospheres), washed three times with ultrapure water, and freeze-dried.

Particle characterization. The sizes of the microspheres loaded with rhodamine were analyzed by light microscopy (BH-2 microscope; Olympus, Spain) and by laser diffractometry (Mastersizer, Malvern Instruments, United Kingdom) of the microparticles without the fluorescent marker. For the latter, a small amount was dispersed in water and analyzed under continuous stirring, and the average particle size was expressed as the mean volume diameter (in micrometers). The size and polydispersion of the fluorescent nanoparticles were determined by photon correlation spectroscopy with a Zetamaster analyzer system (Malvern Instruments).

Gentamicin loading of PLGA micro- and nanoparticles. Briefly, a known amount of the loaded particles was dispersed in 1 ml of 0.1 N NaOH, the samples were centrifuged (25,000 × *g*, 15 min), and the GEN in the supernatants was quantified by HPLC-mass spectrometry (14).

In vivo studies. Female BALB/c mice (weight, 20 ± 1 g) were supplied by Harlan Iterfauna Ibérica (Barcelona, Spain). The 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).

(i) Distribution and degradation of formulations in vivo. PLGA particle uptake by the liver and the spleen, the two main target organs of *Brucella* organisms, was studied in mice. Two milligrams of fluorescent particulate carriers was intravenously administered in 0.2 ml of freshly reconstituted 1% lecithin in sterile saline (19). At different times postadministration (4 h, 1 week, and 2 weeks), the animals were killed, the liver and the spleen were embedded in a tissue-processing medium (O.C.T., Sakura, The Netherlands), immersed in melted isopentane (Fluka, Buch, Switzerland), and stored at −20°C. The tissue samples were cut into 5-μm sections in a cryostat (2800 Frigocut E; Reichert-Jung, Germany) and were finally visualized by fluorescence microscopy (26).

(ii) Pharmacokinetic studies. In order to perform comparisons of the pharmacokinetics of free and loaded GEN, mice ($n = 3$) received one dose of 30 μg (1.5 mg/kg of body weight) of the antibiotic intravenously either free or loaded into PLGA particles. For calculation of the values of the pharmacokinetic parameters for free antibiotic, higher concentrations had to be administered (three doses of 40 mg/kg of GEN every 2.5 h), and the data were further normalized to the administered doses. At different times (5, 6, 7, 9, 10, 24, and 96 h for free gentamicin; 1, 2, 4, 7, 14, and 28 days for the PLGA formulations), the animals were anesthetized, bled by retroorbital puncture, and killed by cervical dislocation; and the organs (spleen, liver, and kidneys) were removed. Once the organs were diluted in saline solution, they were homogenized and centrifuged (10,000 × *g*, 15 min), and the supernatants were collected. Proteins were precipitated by 1% trichloroacetic acid, and the samples were analyzed by HPLC, as described previously (14).

The values of the pharmacokinetic parameters for GEN were determined by using compartmental and noncompartmental analyses. The concentration-versus-time profiles for each tissue were described by compartmental models, assuming first-order distribution rate constants between the plasma and the liver, spleen, or kidney. GEN showed a two-exponential distribution for the spleen, as can be seen by examination of the raw tissue concentration-versus-time data. In order to describe this phenomenon properly, a model describing the distribution of GEN from plasma to the tissues by the use of two first-order distribution rate constants, one of which was slower, was fitted to the data. With respect to the liver and kidneys, a simpler model was fitted to the data.

The values of the various pharmacokinetic parameters, such as clearance, disposition half-life, and the accumulation rate (maximum concentration in serum [C_{max}]/area under the concentration-time curve [AUC]), were calculated from the estimated parameters by standard procedures. All the analyses were performed with the WinNonlin, version 1.5, computer program (Scientific Consulting, Inc.).

To select the best pharmacokinetic model, a number of criteria, including the Akaike information criterion, the Schwartz criterion, and the *F* test, were used. In addition, the visual inspection of the residual plots and the calculated coefficients of variation of the pharmacokinetic parameter estimates were also used as a guide in selecting the best model. Coefficients of variation lower than 50% were considered acceptable.

The area under the plasma concentration-time curve and the mean residence time (MRT) were determined by use of the linear trapezoidal rule. The times during which the antibiotic concentrations exceeded the minimal bactericidal concentration (MBC) for *Brucella* were also determined for the liver and the spleen.

Experimental *Brucella* infection in mice and treatment with encapsulated gentamicin. BALB/c mice and the reference strain *B. melitenis* 16 M (biotype 1) were used for the experimental infection study. For animal infection, isolated colonies of the bacteria were inoculated in Trypticase soy broth medium and incubated at 37°C under shaking to the exponential growth phase. A washed suspension of the bacterial pellet was diluted in sterile saline solution to obtain the corresponding infective dose.

Mice were infected intraperitoneally with *B. melitensis* 16 M (1×10^5 CFU/mouse) to induce a chronic infection (8). After 2 weeks, the animals ($n = 6$) received different treatments: (i) GEN-loaded microspheres, (ii) empty formulations, (iii) free GEN, or (iv) no treatment (control group). Three doses of free or encapsulated GEN of 1.5 mg/kg were administered at days 14, 17, and 21 postinfection. At 8 days and 3 weeks after administration of the last dose, the animals were killed, and the spleens (the target organs in murine brucellosis) were extracted and homogenized. Appropriate dilutions were plated on Trypticase soy agar medium, and the number of colonies was determined after incubation for 3 to 4 days at 37°C.

Statistical analysis. Student's *t* test was performed for parametric studies and a comparison of the results between two samples. For a comparison of the results among three or more groups, one-way analysis of variance and the post-hoc Tukey B test was performed for homoscedastic groups. The Tamhane test was carried out for heteroscedastic groups. For nonparametric studies, the Mann-Whitney U test was performed. Statistical significance levels were defined as *P* values of <0.05 and <0.01.

RESULTS

Particle characterization. Microparticles formulated with 502H presented the highest loadings (11.9 μg GEN/mg polymer), followed by those formulated with 75:25H (8.3 μg GEN/μg polymer), with mean diameters of 1.84 ± 0.70 μm and 2.5 ± 0.17 μm, respectively. GEN-loaded nanoparticles showed an average size of 310 ± 2.00 nm, with a loading of 6.2 μg GEN/mg of polymer.

Distribution of particles and degradation studies in vivo. The optimal formulation should target to a high extent the antibiotic in the organs harboring the pathogen, and afterwards, a sustained release would be desired. Therefore, biodistribution studies were carried out to compare the particle uptakes by phagocytic cells of the liver and the spleen and their degradation rates. The particles were then labeled with rhodamine so that they could be fluorescently quantified in both organs. All particulate carriers were already taken up by both organs 4 h after administration (Fig. 1). PLGA 502H formulated in micro- or nanoparticles was taken up to a higher extent by the liver ($P < 0.01$), while the uptakes of the particles formulated with 75:25H were similar in both organs. One week

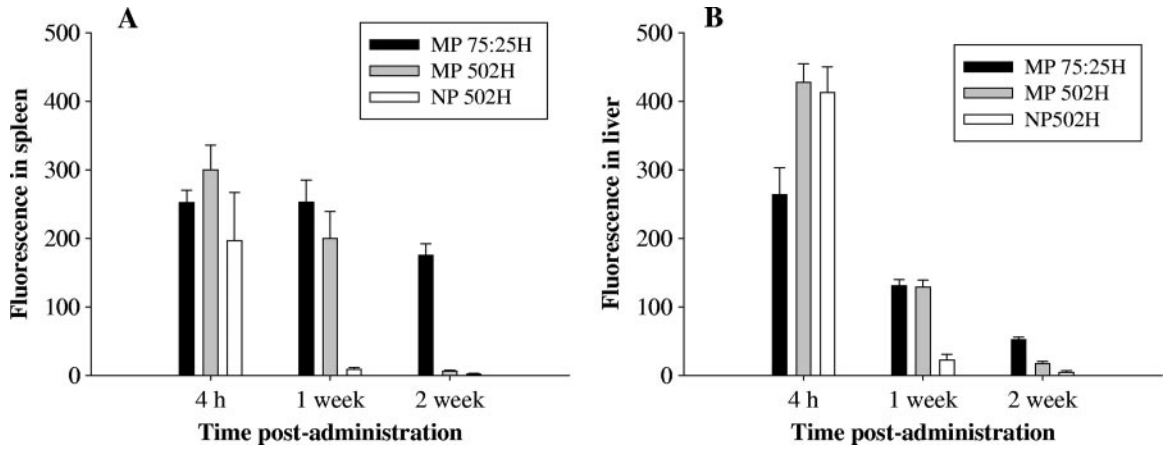

FIG. 1. Biodistribution of particles containing gentamicin in livers and spleens of treated mice at 4 h, 1 week, and 2 weeks postadministration (2 mg of fluorescent particulate carriers was administered intravenously).

after particle administration, most of the nanoparticles had already been degraded in both organs (more than 95%); this resulted in statistical differences with respect to the degradation results for the microparticles ($P < 0.01$). Finally, after 2 weeks, microparticles of the 502H formulation disappeared almost completely (>96%) from both organs, while the 75:25H microparticles had been degraded by only 30% in the spleen. The practical disappearance of nanoparticles within 1 week from both organs made this formulation unsuitable for treatment for brucellosis, since a sustained release is required; therefore, it will be not be used in further studies.

Pharmacokinetic studies. Mice ($n = 3$) were inoculated with GEN (one dose of 30 μg, 1.5 mg/kg) intravenously either in free solution or encapsulated in 75:25H or 502H microparticles; and the concentrations of the antibiotic in the liver and spleen (the target organs of *Brucella* infection) and kidney (as a nephrotoxicity marker) were determined. In order to determine the pharmacokinetic parameters, it was necessary to administer three doses of free GEN (40 mg/kg every 2.5 h), whereas just a single dose of GEN encapsulated in microparticles (1.5 mg/kg) had to be administered. The results indicated that no antibiotic was detected in any of the organs studied 4 days after the administration of free GEN. The effects of the encapsulation of GEN on the distribution of the drug are shown in Fig. 2 and are summarized in Table 1, which show the raw concentration-versus-time profiles (depicted with symbols) for each tissue from the prediction model. The predicted values were adjusted to the real ones, indicating the adequacy of the model in describing the kinetics of GEN in each tissue after 502H and 75:25H microsphere administration. In addition, the pharmacokinetic parameters were accurately estimated (coefficient of variation, <50%) (Table 1) and indicated that when GEN was administered in free solution, GEN levels decreased rapidly within the first 2 days postadministration. In contrast, when GEN was administered in the encapsulated form, the antibiotic concentrations in the three organs were high (above the MBC), even after 28 days. Furthermore, encapsulation in 502H or 75:25H microparticles redirected the antibiotic to both the liver and the spleen and substantially reduced the level of drug accumulation in the kidneys. The increase in the level of antibiotic accumulation was most pronounced in the spleens, in which 502H and 75:25H microparticles increased the AUC by 447- and 800-fold, respectively. In the liver, the factors were 406-fold for 502H microspheres and 370-fold for the 75:25H formulation. On the contrary, the GEN concentration in the kidneys after microparticle administration was significantly lower than that observed in the other organs.

The sustained-release properties of the PLGA microparticles were also reflected in the GEN pharmacokinetic parameters obtained, which showed lower C_{max} values and longer times to C_{max} (T_{max}) and MRTs in all tissues evaluated compared with those obtained after administration of the free antibiotic. Thus, free GEN disappeared very quickly from the liver and the spleen, showing mean residence times of about 0.5 h. Finally, microspheres presented the highest MRT values in the spleen (45 days for the 502H polymer and 60 days for 75:25H).

Similar concentration profiles were observed for both formulations in the liver: on day 4 postadministration, similar plateaus were achieved for both formulations (0.53 μg/g for 75:25H and 0.65 μg/g for 502H) ($P > 0.05$), with AUC values of 14.0 μg · day/g for the 502H microspheres and 12.7 μg · day/g for the 72:25H microspheres ($P > 0.05$) (Fig. 2). In contrast, the parameter estimated for the spleen showed considerable differences between formulations, with the AUC values for the 75:25H microparticles (22.4 μg · day/g) being significantly higher than those for the 502H microparticles (12.5 μg · day/g) ($P > 0.01$). After a peak on day 1 postadministration (1.2 μg/g for 75:25H particles and 0.99 μg/g for 502H particles; $P > 0.05$), the drug concentration decreased to a plateau of 0.45 μg/g for the 75:25H microspheres and 0.35 μg/g ($P > 0.05$) for the 502H formulation.

The MIC and MBC of GEN for *Brucella* were 0.25 and 0.5 μg/ml, respectively (14). The time during which the antibiotic concentrations exceeded the MBC in the spleen was similar over time for the two selected formulations, but GEN remained in the liver longer when it was administered in 502H microspheres (14 day) than in the 75:25H formulation (6.1 days).

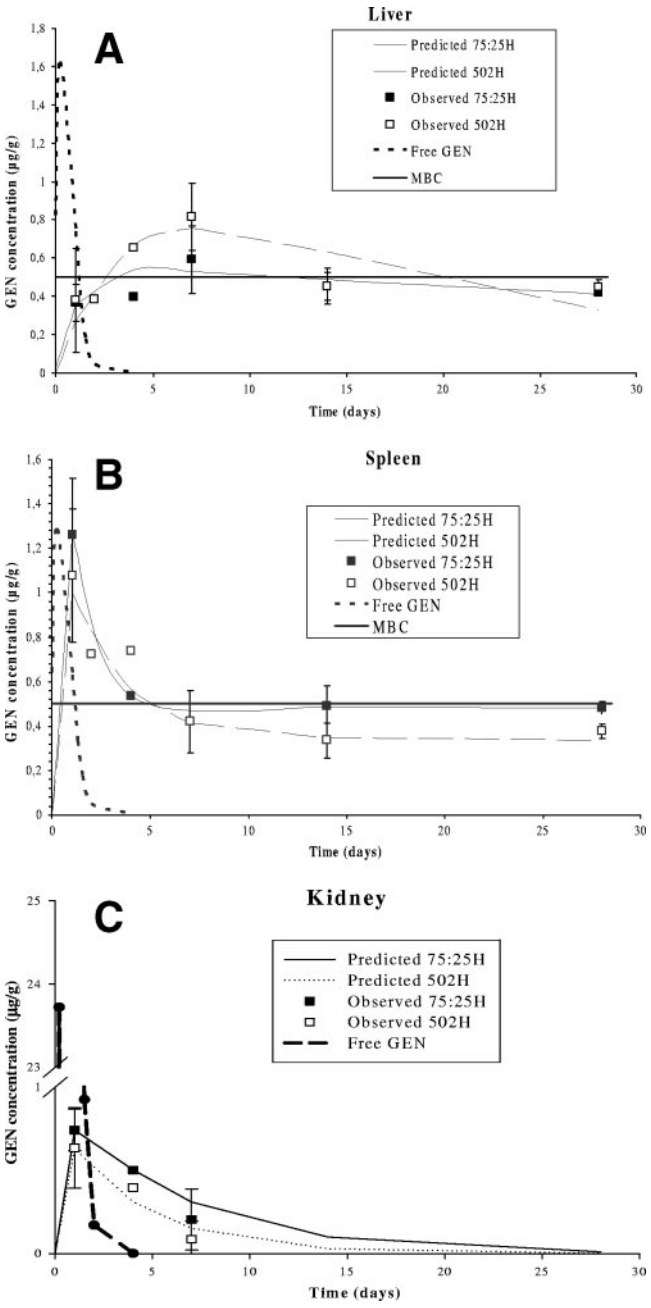

FIG. 2. Predicted and observed GEN concentrations ($n = 3$) (μg/g) in spleen, liver, and kidney after administration of three doses of 40 mg/kg of free GEN or one dose of 1.5 mg/kg of microparticle-loaded antibiotic and mean ± standard deviation ($n = 3$) of the observed concentration of loaded GEN.

Protective effect of microspheres containing gentamicin against *Brucella melitensis* infection in mice. Under these experimental conditions, mice were infected with *B. melitensis* and the treatment was started 2 weeks later. The results indicated that neither the empty formulations nor free GEN produced any bactericidal effect. In contrast, both microsphere formulations showed bactericidal effects during the two studies (Table 2). After 1 week from the time of last administration of the dose, the 75:25H microspheres were more effective than those made of PLGA 502H in reducing the *B. melitensis* load in the spleen. They achieved a 0.72-log reduction, while for the 502H microparticles, the reduction was 0.41 log; both of these values are statistically different from the results achieved for the negative control. At 3 weeks postinfection, the 502H formulation did not improve the therapeutic activity (0.45-log decrease). On the contrary, 75:25H was able to reduce the *Brucella* infection level by 1.45 log units (range, 0.38 to 4.01 log units of protection in individual animals).

DISCUSSION

Intracellular bacterial infections still constitute a challenge for classical antimicrobial therapies due to the limited penetration of active antibiotics. Thus, gentamicin is very active in vitro against intracellular bacteria, such as *Brucella melitensis*, but it is not effective in vivo due to its poor intracellular penetration and inactivation by lysosomal enzymes (12). The development of gentamicin delivery systems capable of intracellular delivery has been described previously (22, 30). Nevertheless, drawbacks like the osmotic fragility of liposomes (20) and aggregation and embolism induction of microspheres (22) suggested the need for the development of new formulations of micro- and nanoparticles prepared with different copolymers of PLGA; however, on the basis of physicochemical properties, adequate drug loading, and the capability to interact with *Brucella* host cells to promote their oxidative burst (15), three formulations were selected: micro and nanoparticles of 502H and microparticles of 75:25H. Here we report the results of the in vivo and in-cell behaviors obtained with these formulations.

On the subject of the particle distribution in vivo, it was interesting to study the fate and permanence of particles after administration by looking for them at a similar location as the *Brucella* organisms. After invasion, the organism multiplies in regional lymph nodes, draining the site of entry, the spleen and the liver. In fact, an enlarged spleen and an enlarged liver are usual features during brucellosis. Some differences in particle distribution were observed after their uptake by the spleen and the liver, depending on the polymer type. Microspheres prepared with PLGA 75:25H showed a similar distribution in both organs, while for the 502H polymer the liver was the preferred organ, regardless of the particle size, since both nano- and microspheres showed the same distribution patterns. These results suggest that hydrophilicity may play a role in the fate of particles and their distribution to the liver, while for the more hydrophobic polymer, no differential distribution to the two organs was observed. The general rule is that hydrophilic surfaces decrease macrophage uptake and enhance the outcome for the liver. In contrast, particle degradation and, therefore, antibiotic release seem to depend on several factors, such as the polymer used and the particle size. Several authors have reported that as the glycolic acid content decreases, the rate of degradation also diminishes (1, 13, 16), which is in agreement with our results. The more hydrophobic 75:25H copolymer presented a more sustained release, with only 30% degradation in splenic tissues at 2 weeks postadministration of the particles, whereas the PLGA 502H particles were degraded more than 96% over the same time. On the other hand, particle size is an important parameter that could affect the deg-

TABLE 1. Mean pharmacokinetic parameters of free or microsphere-loaded GEN in mice

Dosage form	Organ	Half-life (days)	T_{max} (days)	C_{max} (μg/g)[a]	AUC (μg · day/g)[a]	C_{max}/AUC (h^{-1})[a]	MRT (days)
Free GEN	Liver	0.2 ± 0.1	0.1 ± 0.1	1.4 ± 0.2	1.2 ± 0.0	1.2 ± 0.2	0.6 ± 0.1
	Spleen	0.37 ± 0.1	0.1 ± 0.1	1.9 ± 0.0	0.9 ± 0.1	2.1 ± 0.3	0.6 ± 0.1
	Kidney	0.2 ± 0.19	0.1 ± 0.0	16.6 ± 7.1	15.0 ± 4.2	1.1 ± 0.2	2.5 ± 1.3
502H MP[b]	Liver	14.5 ± 7.6	7.3 ± 1.6	25.1 ± 2.7	468.3 ± 58.7	0.05 ± 0.01	13.7 ± 0.4
	Spleen	ND[c]	0.9 ± 0.1	33.3 ± 5.7	416.0 ± 168	0.08 ± 0.02	45.0 ± 5.15
	Kidney	2.9 ± 1.3	0.7 ± 0.5	11.7 ± 7.0	109.3 ± 3.3	0.11 ± 0.08	1.7 ± 0.6
75:25H MP	Liver	38.0 ± 10.8	4.5 ± 1.1	18.0 ± 1.7	425.0 ± 55.0	0.04 ± 0.01	13.9 ± 1.0
	Spleen	ND	0.8 ± 0.5	43.0 ± 5.0	745.3 ± 55.7	0.06 ± 0.02	60.4 ± 0.2
	Kidney	4.3 ± 0.4	1.2 ± 0.5	24.3 ± 3.7	221.7 ± 30.7	0.11 ± 0.02	1.6 ± 0.1

[a] C_{max} and AUC values were normalized to the doses of GEN administered (1.5 mg/kg of loaded GEN and three doses of 40 mg/kg every 2.5 h of free GEN).
[b] MP, microsphere.
[c] ND, the half-life could not be determined because over the time interval of the study, the GEN concentration in the spleen was maintained and did not vary.

radation of the PLGA matrix. As the particle size is reduced, the surface area-to-volume ratio increases, resulting in a larger surface area available for the autocatalytic hydrolysis of the ester bonds of the PLGA polymer. Thus, under our experimental conditions, the PLGA 502H nanospheres were degraded within the first week postadministration, while microspheres of the same polymer were still in the liver and the spleen for 2 weeks. The sustained release of the antibiotic is extremely desirable for treatment for brucellosis, since sustained release could reduce the duration of treatment and, hence, facilitate the effectiveness of treatment. Therefore, nanospheres were discarded because of their rapid elimination from the target organs.

Determination of the efficiency of the particles for the targeting of GEN in vivo was completed by the performance of pharmacokinetic studies, and at the same time, the correlation between pharmacokinetic parameters and the effectiveness of the formulation for elimination of *Brucella* from the spleen was assessed. The pharmacokinetic parameters illustrated the markedly altered distribution of GEN-loaded PLGA compared to that of the free drug, with higher concentrations of GEN observed in the spleen and the liver when it was administered loaded in microspheres. At the same time, serum samples had undetectable concentrations. Taken together, these results make evident the microparticle removal from the circulation and accumulation primarily in the liver and the spleen, precluding drug accumulation in the kidneys, where tubular necrosis may occur in a concentration-dependent manner (13).

Although higher AUC values were obtained for the kidney after GEN-loaded PLGA administration, this could not be attributed to microsphere accumulation in this organ but was the result of the continuous release of antibiotic from the formulations after their accumulation in the liver and the spleen, as assessed in a previous study (22). In fact, the rate of gentamicin accumulation in the kidneys after free gentamicin administration was 10-fold greater than that obtained after administration of GEN-loaded PLGA, with absolute C_{max} values 24-fold greater than the values observed in the kidney after PLGA microparticle administration.

MRT and AUC values were sustained for both the 502H and the 75:25H formulations compared to those for free GEN, confirming the sustained release of the microencapsulated antibiotic. By comparison of the two formulations, the highest MRT and AUC values were observed for the 75:25H particles, consistent with the lower level of degradation of this polymer in the spleen.

As discussed above, distribution studies showed that at 2 weeks postadministration, 75:25H microspheres were observed mainly in the spleen and, in discrete quantities, in the liver. However, GEN was detected up to 4 weeks postadministration in both the liver and the spleen. This long persistence of the drug beyond the half-life of the vector could be expected, since

TABLE 2. Protective effect of three doses of GEN-loaded microparticles against sublethal infection with *Brucellamelitensis* 16M administered intraperitoneally

Treatment	Result for the following time after administration of last dose[a]:					
	1 wk			3 wk		
	Spleen wt (g)	Log CFU/spleen	Reduction (log)	Spleen wt (g)	Log CFU/spleen	Reduction (log)
Untreated	0.87 ± 0.08	6.85 ± 0.17	0.00	0.93 ± 0.11	6.73 ± 0.18	0.00
Free GEN	0.86 ± 0.05	6.77 ± 0.12	0.08	0.83 ± 0.26	6.77 ± 0.12	0.04
502H empty MP[b]	0.80 ± 0.05	6.83 ± 0.16	0.02	1.04 ± 0.14	6.87 ± 0.30	0.13
75:25H empty MP	0.78 ± 0.28	6.80 ± 0.12	0.05	1.16 ± 0.19	6.70 ± 0.18	0.04
502H GEN MP	0.92 ± 0.07	6.44 ± 0.05	0.41**	1.12 ± 0.44	6.28 ± 0.09	0.45
75:25H GEN MP	0.87 ± 0.16	6.13 ± 0.08	0.72**	0.70 ± 0.36	5.29 ± 1.58	1.45**

[a] Groups of six mice each were infected intraperitoneally with *B. melitensis* 16 M (1×10^5 CFU/mouse). After 2 weeks, the animals received three doses of free or encapsulated GEN (1.5 mg/kg). At 1 and 3 weeks after administration of the last dose, the animals were killed. **, $P < 0.01$ (Mann-Whitney U test).
[b] MP, microsphere.

aminoglycosides are highly stable and are not metabolized in the liver. Because of their polar nature, they penetrate cells very poorly, but once they are inside cells, their intracellular retention is very high (28).

From a pharmacodynamic point of view, antibiotics are frequently divided into two major groups: those that exhibit time-dependent (concentration-independent) killing and reduced persistent effects and those that exhibit a concentration-dependent killing and prolonged persistent effects (4, 5, 9). Therefore, for the second group of antibiotics, which includes the aminoglycosides, the goal should be to maximize the drug concentration. Interestingly, the concentrations of GEN in the liver and the spleen were in the range of the in vitro MBC for *B. melitensis* (14).

BALB/c mice were chronically infected with a virulent strain of *B. melitensis* and treated with the selected GEN-containing formulations. The results indicated that the treatment with free GEN was ineffective, in agreement with the undetectable levels of GEN in the liver and the spleen. Empty microspheres did not produce any antibacterial effect. In contrast, both microparticle formulations significantly reduced the level of infection in the spleen from 1 week after administration, and at 3 weeks after administration, 75:25H microspheres managed to reduce the counts in the spleen more efficiently (1.45 logs) than those made of 502H (0.45 log). These data agree with the higher AUC and MRT in spleen shown by the former formulation.

In conclusion, 75:25H microspheres were the most suitable formulation for the treatment of brucellosis among the PLGA micro- and nanoparticles studied. Nevertheless, GEN loading improvement or the administration of more doses would be required before the formulation could be used for the treatment of human brucellosis.

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