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Gentamicin-loaded microspheres for treatment of experimental *Brucella abortus* infection in mice

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***Objectives*: To evaluate the efficacy of gentamicin-loaded poly (lactide-co-glycolide) 50:50H (PLGA 50:50H) microspheres for the treatment of mice experimentally infected with *Brucella abortus* 2308.**

***Methods*: The microspheres were dispersed in either 2% (w/v) poloxamer 188 saline solution, or deionized water with the help of a cell homogenizer to break up particle aggregates, and were administered intravenously or intraperitoneally to *B. abortus*-infected mice 7 days post-infection.**

***Results*: Neither a single intravenous or intraperitoneal dose of 67 µg of gentamicin per mouse, nor three intraperitoneal doses of 100 µg of gentamicin per mouse, reduced the *Brucella* infection in the spleen compared with untreated mice 1 and 3 weeks post-treatment. Histological examination revealed granulation and tissue reaction in the periphery of spleen and liver of animals given three doses of the gentamicin-loaded microspheres.**

***Conclusions*: The lack of therapeutic activity of the gentamicin-loaded microspheres might be related to inappropriate microsphere size and aggregation, resulting also in a poor distribution of the microspheres in the spleen. The results might provide an example of practical problems related to particle size and aggregation for *in vivo* therapy with PLGA microspheres.**

Keywords: biodegradable microspheres, drug delivery systems, *Brucella*-infected mice

Introduction

Brucellosis is a zoonosis of important socio-economic repercussion, since apart from being a major animal health problem, it constitutes a risk to human health. *Brucella* is a facultative intracellular Gram-negative bacterium and its localization within phagocytes makes treatment difficult.[1] *In vivo*, *Brucella* is mainly localized in cells and organs of the mononuclear phagocytic system such as the macrophages of the spleen and the Kupffer cells of the liver. Current therapy is based on a prolonged administration of a tetracycline–aminoglycoside combination that often leads to poor patient compliance, low therapeutic efficacy, frequent relapses and serious side effects.[2] As a consequence, there is a recognized need for improvement of the actual treatment of human brucellosis. Hence, the use of particulate carriers has been proposed to target antibiotics to intracellular sites where the bacteria are located. When targeting is combined with controlled delivery of antibiotic drugs, the number of doses and hence the toxicity of the drug may be decreased.

Gentamicin sulphate is an aminoglycoside with a wide spectrum of antibacterial activity, although nephrotoxicity restricts the use of these compounds. As a cationic and freely water soluble drug, it penetrates poorly into cells, constituting a drawback for treating intracellular bacterial infections. Microspheres are known to accumulate in organs of the monocyte-macrophage system after parenteral administration. In addition, PLGA-microspheres are biodegradable and tissue biocompatible. Microspheres based on poly (lactide-co-glycolide) (PLGA) wherein gentamicin is entrapped, have been prepared to target gentamicin to the cells of the monocyte-macrophage system, reduce drug toxicity and control its release over several days. We have previously shown that PLGA-microspheres loaded with gentamicin

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Table 1. Antibacterial effect of one or three doses of gentamicin (GEN), free or encapsulated into PLGA 50:50H microspheres, on *Brucella abortus* 2308-infected mice (control groups received saline or water, or placebo microspheres)

			Mean $\log_{10}$ cfu/spleen ± SD[a]			Mean weight (g)/spleen		
Formulation[b,c]	No. of doses	Dose (μg of GEN/mouse)	day 0[d]	1 week[d]	3 weeks[d]	day 0[d]	1 week[d]	3 weeks[d]
Saline (iv)[b]	1	0	6.34 ± 0.60 (6)	5.53 ± 0.22 (6)	6.01 ± 0.23 (6)	0.30 ± 0.09	0.53 ± 0.11	0.37 ± 0.07
GEN (iv)[b]	1	67	–	5.69 ± 0.36 (6)	6.33 ± 0.30 (6)	–	0.41 ± 0.22	0.39 ± 0.09
GEN-loaded microspheres (iv)[b]	1	67	–	5.48 ± 0.69 (6)	4.82 ± 2.01 (6)	–	0.36 ± 0.17	0.27 ± 0.14
Placebo microspheres (iv)[b]	1	0	–	5.86 ± 0.46 (6)	6.08 ± 0.19 (6)	–	0.43 ± 0.09	0.41 ± 0.11
GEN-loaded microspheres (ip)[b]	1	67	–	4.91 ± 1.56 (5)	ND[e]	–	0.43 ± 0.20	ND[e]
Placebo microspheres (ip)[b]	1	0	–	5.64 ± 0.19 (5)	ND[e]	–	0.29 ± 0.12	ND[e]
Water (ip)[c]	3	0	6.73 ± 0.56 (6)	6.08 ± 0.45 (6)	6.39 ± 0.58 (6)	0.37 ± 0.10	0.43 ± 0.07	0.55 ± 0.19
GEN (ip)[c]	3	100	–	6.14 ± 0.28 (6)	6.82 ± 0.23 (6)	–	0.54 ± 0.21	0.70 ± 0.13
GEN-loaded microspheres (ip)[c]	3	100	–	6.20 ± 0.32 (6)	6.22 ± 0.37 (6)	–	0.56 ± 0.21	0.51 ± 0.25
Placebo microspheres (ip)[c]	3	0	–	6.14 ± 0.26 (6)	6.78 ± 0.08 (6)	–	0.51 ± 0.18	0.65 ± 0.17

[a]Five or six mice per group were used.
[b]The different formulations were prepared in a 2% (w/v) poloxamer 188 in saline and administered intravenously (iv) or intraperitoneally (ip) in a single dose (0.2 mL/mouse). Microsphere dispersion was helped by brief sonication. The formulations were injected 1 week after the infection of the mice with *Brucella abortus,* 0.87×10^6 cfu/mouse (in stationary growth phase).
[c]The different formulations were prepared in sterile deionized water helped by 1 min of sonication and 2 min of homogenization by pounding in a glass cell homogenizer and three doses were injected intraperitoneally (ip) during three consecutive days (0.5 mL/dose). The formulations were injected 1 week after the infection of the mice with *Brucella abortus,* 1.8×10^6 cfu/mouse (in exponential growth phase). The inoculum was prepared by first seeding the *Brucella* onto TSB for exponential bacteria growth and diluting the suspension in saline solution to the adequate concentration.
[d]Time post-treatment.
[e]ND, not done.

are readily phagocytosed by monocytes, induce monocyte activation,[3] and transport the drug into cells, thereby decreasing *Brucella abortus* infection in J774 cultured cells.[4]

Here we studied the efficacy of PLGA 50:50H-microspheres containing gentamicin in mice experimentally infected with *Brucella abortus* 2308. Histological studies were also carried out to give insights of microsphere biodistribution and tissue response to these carriers.

Materials and methods

Preparation and characterization of the microspheres

Gentamicin-loaded PLGA 50:50H microspheres were prepared by spray-drying as described elsewhere.[5] Briefly, 100 mg of gentamicin was dissolved in 1 mL of phosphate buffer (67 mM, pH 7.4) and dispersed in a 5% (w/w) polymer solution in ethyl formate by ultrasonication. The water-in-oil emulsion formed was spray-dried (Büchi 190, Flawil, Switzerland) and the resulting microspheres collected, washed and dried under vacuum. The final product was stored at 4°C. Placebo microspheres were produced similarly.

Microsphere size distribution was determined by laser light scattering (Mastersizer X, Malvern, UK) and morphology was examined by scanning electron microscopy (Zeiss DSM 940A).

Measurement of gentamicin content in the microspheres was achieved by dissolving the microspheres in dichloromethane and collecting the undissolved gentamicin on cellulose acetate filters. The gentamicin was eluted from the dried filters with water and assayed photometrically after derivation with *o*-phthalaldehyde.[5]

In vivo experimental Brucella abortus infection and treatment with microencapsulated gentamicin

Animals: Female Swiss-Webster mice (20 ± 1 g) were used under Protocol 039/00 (Ethics Committee for Animal Experimentation, CEEA, University of Navarra, Spain, 2000).

Brucella strain: *Brucella abortus* 2308 isolates from spleens of infected mice and maintained on skimmed milk at −85°C were used. For infection of the mice, the brucellae were thawed, seeded onto trypticase soy agar (TSA) plates and incubated for 2–3 days at 37°C. Isolated colonies were transferred into 5 mL of trypticase soy broth and incubated for 24 h at 37°C under shaking (exponential growth phase). The bacterial suspension was then diluted in saline solution to the adequate bacteria concentration to infect the animals. In some experiments, the bacteria were thawed and, immediately before injection, the suspension was diluted in saline (stationary growth phase).

Infection of mice with Brucella abortus 2308 and treatment with microencapsulated gentamicin: Mice were infected intraperitoneally with $\sim 1 \times 10^6$ cfu per mouse (300 μL). On day 7 after infection, the mice received free gentamicin, gentamicin-loaded microspheres, placebo microspheres or a blank solution. The gentamicin-treated animals received a single dose of 67 μg of gentamicin per mouse (0.2 mL) administered intravenously or intraperitoneally, or three doses of 100 μg of gentamicin/dose/mouse (0.5 mL) administered intraperitoneally on three consecutive days. Poloxamer 188 (2%, w/v) was used in the 1-dose treatments to facilitate the dispersion of the microspheres and as control in the blank and free gentamicin solutions; water was used in the 3-dose treatments and microspheres were dispersed with the aid of a cell homogenizer. The efficacy of the treatments was determined by counting the number of viable *Brucella* in spleen homogenates 1 and 3 weeks post-treatment after diluting and seeding onto TSA plates.

Histological examination

Healthy mice were injected intraperitoneally with three doses of gentamicin-loaded microspheres containing 100 μg of gentamicin/dose/mouse on three consecutive days. The spleen and liver were removed aseptically 24 h after the treatment and the organs were prepared for histological examination.

Results and discussion

Efficacy of gentamicin treatment on Brucella abortus-infected mice

The intravenous route of administration limited the amount of microspheres injected (4.5 mg of microspheres) and, thus, the gentamicin dose to only 67 μg. The mean diameters of the microspheres (∼ 3 μm) should have been suitable for intravenous administration, however, dispersibility, concentration, homogeneity and stability in the dispersed state also need to be adequate. Typically, PLGA 50:50H particles exhibited a strong tendency to aggregate, especially when loaded with gentamicin.[5] Although rarely reported, dispersibility of PLA/PLGA microspheres is influenced by many factors such as size, shape, drug/polymer charge, pH and viscosity of the vehicle.[6] Poloxamer 188 was used to improve the dispersion characteristics; moreover, a beneficial effect of the poloxamer on the antibiotic activity of the gentamicin-loaded microspheres *in vitro* was previously observed.[4] After 1 or 3 weeks, none of the 1-dose treatments, intravenously or intraperitoneally administered, had a significant effect on the bacterial counts in the spleen compared with the saline control group ($P > 0.05$, Student's *t*-test) (Table 1). However, the mice treated with the encapsulated gentamicin showed high SD values due to a few individual mice exhibiting low bacteria numbers per spleen. This correlated with a slightly lower weight of the spleens of animals treated with gentamicin-loaded microspheres at 3 weeks post-treatment compared with the saline group, but the effect was not statistically significant ($P > 0.05$, Student's *t*-test). Despite the good dispersion state of the microspheres, increasing the number of doses or the microsphere concentration administered intravenously led to respiratory problems for the animals, which became very weak and lethargic or died from pulmonary embolism.

When the antibiotic dose was increased by treating the infected mice with three doses of 100 μg of gentamicin, given intraperitoneally in solution or encapsulated in the PLGA 50:50H microspheres, no effect on the number of viable bacteria in the spleen or on spleen weights was observed (Table 1). Besides a possible sub-therapeutic dose of gentamicin, poor accumulation of the microspheres in the spleen, considered as the target organ, may have been responsible for the results. Accumulation of particles in the spleen is generally lower than in the liver, probably also due to the respective weights of these organs. Hence, we may suggest that the amount of PLGA microspheres reaching the spleen in our model could have been insufficient to produce a significant decrease in the bacterial counts.

Histological examinations

Structures resembling abscesses were observed macroscopically in livers and spleens of healthy mice 24 h after they were given three doses of the gentamicin-loaded microspheres intraperitoneally. Histological examination under light microscope revealed

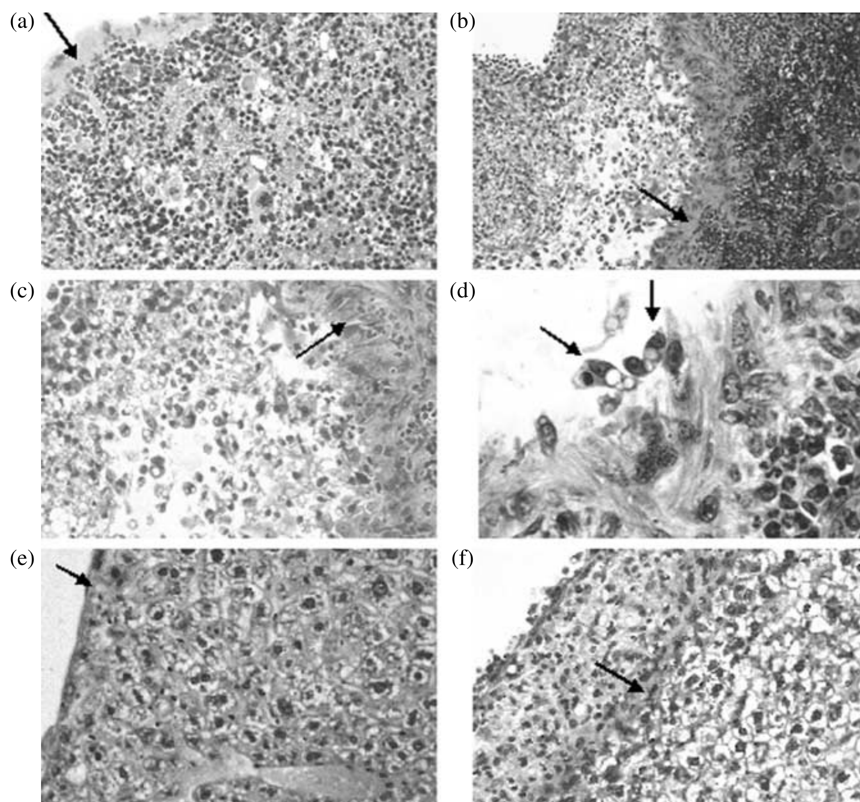

Figure 1. Histological micrographs of splenic (a, b, c and d) and liver (e and f) tissue. Control mice (arrow pointing at the capsule) are shown in (a and e). A healthy mouse given three doses of gentamicin-loaded PLGA 50:50H microspheres intraperitoneally is shown in (b, c, d and f). In (b, c and f) the arrows show the capsule of the organ (×100, ×1000, ×400 magnification, respectively); and in (d) a detail of phagocytic cells containing empty vacuoles localized in the periphery of the spleen is shown.

inflammation of the spleen and liver capsule with localized presence of polymorphonuclear leucocytes (Figure 1). Granulation tissue was found in the periphery of these organs, with typical tissue necrosis, new blood capillary formation, fibroblast proliferation and the accumulation of high numbers of leucocytes. Empty vacuoles were observed in the interior of monocyte-macrophage cells from the periphery granulation tissue of both organs (Figure 1). Similarly, Schmidt *et al.*[7] observed empty vacuoles in macrophages of tissue surrounding PLGA/PLA implants and associated them with reabsorbed detached polymer. These cellular and tissue host reactions may indicate that the administered microspheres reached the spleen and liver. The peripheral location of the response may also indicate that the microspheres reached only the periphery of the organ because they may have been trapped at the capillary level irrigating the spleen. In agreement with this suggestion, Jani *et al.*[8] observed the presence and alignment of large polystyrene particles in the trabeculae of the spleen but not small ones (<1 μm), suggesting the fenestrations between trabeculae and reticular spaces may have trapped particles of micrometre size but not smaller. Furthermore, intraperitoneally-injected PLA microspheres have been shown to spread over the whole peritoneal cavity and to distribute in the serosal membranes of the mesentery, internal organs or the peritoneum.[9] Besides, important particle accumulation in lungs and lymph nodes draining the peritoneal cavity should be expected depending on characteristics of the particles.[10] In view of this, microsphere distribution to various organs (liver, spleen, lung, lymph nodes, peritoneal cavity), along with a poor intrasplenic distribution, possibly due to entrapment in the peripheral capillary network, could explain the lack of efficacy of the systems studied here for reducing *Brucella abortus* counts in the spleen of mice. Nevertheless, evaluation of spleen counts at different or longer times post-treatment may have produced a different result. It may be speculated that particle aggregates could gradually decrease in size upon degradation and deglomeration. Therefore, a redistribution and further cellular uptake of particles of appropriate size might be feasible, which may finally yield antibacterial effects.

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