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Gentamicin-loaded microspheres for reducing the intracellular *Brucella abortus* load in infected monocytes

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***Objectives*: The intracellular antibiotic efficiency of gentamicin-loaded microspheres in the context of *Brucella*-infected murine monocytes was examined *in vitro* with a view to developing improved therapies for the treatment of brucellosis.**

***Methods*: Biodegradable microspheres made of end-group capped and uncapped poly(lactide-co-glycolide) 50:50 (PLGA 50:50 and PLGA 50:50H) and containing gentamicin sulphate were used to target *Brucella abortus*-infected J774 monocyte-macrophages. The infected cells were treated with 15 μg of free or microencapsulated gentamicin and the efficacy of the treatments was measured after 24 h.**

***Results*: The particle sizes were below 8 μm and *in vitro* release of gentamicin from the microspheres followed a continuous (PLGA 50:50H) or a multiphasic (PLGA 50:50) pattern over 50 days. Treatment with gentamicin microencapsulated into the end-group uncapped PLGA 50:50H microspheres, decreased significantly the number of intracellular bacteria (typically by 2 $\log_{10}$) in comparison with untreated infected cells. Addition of 2% poloxamer 188 to the microsphere dispersion medium further reduced the infection (3.5 $\log_{10}$). Opsonization of the particles with non-immune mouse serum had no effect on the antibacterial efficacy of the microspheres. End-group capped PLGA 50:50 type microspheres containing the antibiotic were less effective at reducing intracellular bacteria (~1 $\log_{10}$ reduction), although addition of poloxamer 188 to the dispersion medium again enhanced their intracellular antibacterial activity. Placebo PLGA 50:50 and PLGA 50:50H microspheres had no bactericidal activity.**

***Conclusions*: The results indicate that PLGA 50:50-microencapsulated gentamicin sulphate may be suitable for efficient drug targeting and delivery to reduce intracellular *Brucella* infections.**

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

Introduction

Brucellosis is an infectious disease caused by *Brucella* spp. Four species, *Brucella abortus*, *Brucella melitensis*, *Brucella suis* and *Brucella canis*, have been recognized as human pathogens each associated with a different natural host animal. These small coccobacilli are mainly localized intracellularly within phagocytic cells making treatment difficult, since most antibiotics, although highly active *in vitro*, do not actively pass through cellular membranes.[1] Treatment for brucellosis remains controversial and requires prolonged therapy with at least two agents. The choice of regimen and duration of therapy is based on the presence of focal disease, underlying patient conditions and age group which may contraindicate certain antibiotics. Attempts at monotherapy with trimethoprim/sulfamethoxazole, macrolides or fluoroquinolones have met with disappointing relapse rates despite excellent *in vitro* activity.[2] A prolonged administration of a tetracycline–aminoglycoside combination (i.e. doxycycline 100 mg twice/day for 45 days, and streptomycin 1 g/day for 14 days or gentamicin 5–6 mg/kg per day for 7 days) has lower relapse rates than the more usual doxycycline–rifampicin combination recommended by the World Health Organization in uncomplicated cases.[2] An extended doxycycline–aminoglycoside regimen is also the preferred therapeutic option in life-threatening forms of the disease, such as endocarditis.[2] However, 3–5% relapse rates, serious side effects (especially in children and pregnant women), and hampered patient compliance, mainly caused

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by the long-term treatment and the inconvenience of parenteral administration of aminoglycosides, still represent major hurdles contributing to low therapeutic efficacy.[2]

Gentamicin sulphate is an aminoglycoside with a wide spectrum of antibacterial activity though important side effects mostly related to nephrotoxicity and ototoxicity restrict its use.[3] It is a highly soluble drug but does not cross cell membranes efficiently, which is an important drawback for the therapy of intracellular infections such as brucellosis, due to the low antibiotic levels achievable inside infected cells. Several reports indicate that gentamicin is more active *in vitro* against clinical isolates of *Brucella* than streptomycin.[4] In combined doxycycline–aminoglycoside regimens, gentamicin appears to be more cost-effective and less toxic given the duration of the administration (14–21 days for streptomycin compared with 7 days for gentamicin), with no increases in clinical relapse or treatment failure.[2] These properties make gentamicin an attractive candidate for the treatment of brucellosis provided that the antibiotic can be delivered intracellularly. Therefore, an optimum strategy to treat brucellosis should target a highly active drug to the intracellular compartment and prolong the release of that antibiotic, thereby reducing the number of doses to be administered and minimizing drug side effects.

Liposomes containing gentamicin are quite efficient for targeting *Brucella*-infected monocyte-macrophages.[5] Liposomes have a membrane-like structure that favours a good cell interaction and their versatility in terms of structure and composition grant their main advantages. However, liposomes suffer from low encapsulation efficiency, stability problems, both during storage and upon injection, their therapeutic efficacy is not always optimal and cannot provide a controlled release of the encapsulated drug.[6] Small biodegradable microspheres are attractive alternatives to liposomes for targeting drug in the monocyte-macrophagic system. Upon phagocytosis of antibiotic-loaded microspheres, intracellularly located and highly protected bacteria, such as *Mycobacterium tuberculosis, Salmonella typhimurium, Listeria monocytogenes* and *Brucella* spp., may become accessible to antibiotic treatment. Biodegradable microspheres made of poly(lactide) (PLA) and its copolymers with glycolide (PLGA) can release encapsulated drugs in a sustained manner, depending on the physicochemical properties of the polymer and drug, and the microencapsulation procedure.[7] Microsphere technology has been widely used for a wide variety of therapeutic compounds including antibacterial agents.[8–11] Thus, PLA/PLGA microspheres should be a potentially suitable and stable delivery system for antibiotic treatment of brucellosis that could provide controlled release of the encapsulated drug and minimize the need for multiple injections.

In this study, we examined the intracellular antibiotic efficiency of gentamicin-loaded microspheres in the context of *Brucella*-infected murine monocytes. Two microsphere types, made of end-group capped PLGA 50:50 and uncapped PLGA 50:50H, were used because they are phagocytosed efficiently by monocytes *in vitro*;[12] the end-group uncapped PLGA 50:50H microspheres also promote cell activation, as measured by oxidative burst.[12,13]

Materials and methods

Preparation of microspheres loaded with gentamicin

Gentamicin was microencapsulated by spray-drying into poly(D,L-lactide-co-glycolide) carrying either capped, i.e. esterified end-groups, or uncapped, i.e. free hydroxyl and carboxyl end-groups,[14] hereafter abbreviated as PLGA 50:50 and PLGA 50:50H, respectively. Briefly, 100 mg of gentamicin was dissolved in 1 mL of PBS (67 mM, pH 7.4 or 6.0), which was then dispersed in 20 g of a 5% (w/w) polymer solution in ethyl formate by ultrasonication under cooling on ice. The water-in-oil emulsion formed was spray-dried (Mini Spray Drier B-190; Büchi, Flawil, Switzerland) and the resulting microspheres were collected on a cellulose acetate filter of 0.8 μm pore size. Microspheres were washed with 0.1% (w/w) poloxamer 188 solution, rinsed with distilled water and dried under vacuum (~10 mbar). Residual water was eliminated by dispersing the particles in n-hexane, followed by additional vacuum drying for 24 h. The final product was stored under dry conditions at 4°C. Placebo microspheres were produced accordingly, but without the gentamicin solution.

Determination of microsphere size and morphology

Microsphere size distribution was determined by laser light scattering (Mastersizer X; Malvern Instruments, Malvern, UK). The morphology of the microspheres was examined in a Zeiss DSM 940A scanning electron microscope.

Gentamicin content in the microspheres

Gentamicin content in the microspheres was determined by dissolving 40 mg of particles in 3 mL of dichloromethane and collecting the undissolved gentamicin on regenerated cellulose filters of 0.2 μm pore size. The dried filters were transferred into test tubes and the antibiotic was eluted with 2 mL of water and assayed photometrically (320–350 nm) after derivation with *o*-phthalaldehyde.[14] Gentamicin extraction from the microspheres was carried out in triplicate and the eluted gentamicin was determined from the replicates.

In vitro release of gentamicin from microspheres

Gentamicin release was determined by suspending ~10–30 mg of microspheres, weighed accurately, in 4 mL of PBS (67 mM, pH 7.4) containing 0.05% polysorbate 20 and 0.02% sodium azide, in borosilicate vials (Chromacol, Welwyn Garden City, UK). The vials were briefly sonicated to facilitate wetting of the particles and placed horizontally on a shaking platform at 37°C. At regular intervals, the vials were centrifuged at 2000**g** for 10 min to obtain a particle-free supernatant that was assayed fluorometrically with a Cytofluor 2300/2350 (Millipore)[5] after derivation of the gentamicin with *o*-phthalaldehyde. At every sampling point, the release medium collected was replaced with fresh phosphate buffer. Burst release was described as the amount of drug released after 24 h. Gentamicin release from the microspheres was determined in triplicate for each microsphere preparation.

J774 murine monocyte-macrophage cell line

The J774.2 murine monocyte-macrophage cell line was obtained from the European Collection of Cell Cultures (ECACC No. 85011428). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 10% (v/v) heat-inactivated fetal calf serum and preserved with 0.1% antibiotic-antimycotic solution (complete medium) at 37°C, 5% CO_2 and controlled humidity.

Brucella strain and preparation of bacteria for infection

B. melitensis is the more frequent cause of *Brucella* human infections. However, as it is a class three pathogen, *B. abortus* was chosen for these studies. *B. abortus* 2308 was isolated from spleens of infected mice by homogenization of the organ in saline solution (Stomacher 80 Lab Blender; Seward Medical, London, UK) and seeding onto trypticase soy agar (TSA) plates (bioMérieux, France). Further, the bacterial colonies were suspended on skimmed milk and maintained in cryovials at –85°C.

For monocyte infection, the brucellae were thawed, seeded onto TSA plates and incubated for 2–3 days at 37°C. Two or three isolated colonies were transferred into 5 mL of trypticase soy broth (TSB) and incubated for 20–24 h at 37°C under shaking, so that the bacteria reached exponential growth phase. The bacterial suspension was first diluted in TSB to an optical density of 0.12 (590 nm) and further diluted in saline solution to a concentration of ~5×10^7 cfu/mL to infect the cultured monocytes.

Infection of monocytes with Brucella abortus 2308

J774 murine monocytes cultured in complete medium were washed, collected by centrifugation and cell viability was determined by Trypan Blue exclusion. Cell numbers were adjusted to 2×10^6 cells/mL and 500 µL of the cell suspension was added to each well of a 24-well culture plate (Nunc, Naperville, IL, USA). The cells were incubated in DMEM supplemented with L-glutamine and 10% (v/v) fetal calf serum for 2–3 h to allow cell adherence. Afterwards, the medium was withdrawn to remove non-adherent cells and replaced. For bacterium-specific opsonization, the *B. abortus* suspension, previously adjusted to an adequate bacterial concentration as described above, was shaken with a sub-agglutinating concentration of antiserum from *B. abortus*-infected mice for 30 min at 37°C. Then, 100 µL of opsonized *B. abortus* was added to the monocytes at a ratio of 5 bacteria per cell and further incubated at 37°C for 1–48 h. After incubation, the culture medium was discarded, the infected cells were carefully washed five times to remove extracellular bacteria and 500 µL of DMEM was added to each well. The number of remaining extracellular bacteria in each well was determined by diluting and seeding the added medium onto TSA plates. The total number of cfu per well was determined after lysing the monocytes with cold 0.2% Triton X-100 for 20 min at 37°C, thorough homogenization and plating of serial dilutions of the lysates onto TSA plates. This Triton X-100 treatment had no effect on viability of the bacteria. Intracellular bacteria were calculated by subtracting the number of extracellular *Brucella* from the total cfu counts in three wells.

Treatment of Brucella-infected monocytes with gentamicin-loaded microspheres

The infected J774 monocytes were treated with 15 µg of free gentamicin sulphate in solution, with placebo microspheres, or with gentamicin-loaded microspheres containing 15 µg of the antibiotic, and the effectiveness of the different treatments in reducing the number of intracellular bacteria was determined. The influences of prior opsonization of the particles in fresh mouse serum and the presence of 2% (w/v) of the non-ionic surfactant poloxamer 188 in the microsphere dispersion medium were also determined. For opsonization, the microspheres were dispersed in DMEM supplemented with L-glutamine and containing 8% (v/v) non-immune fresh mouse serum and incubated at 37°C under shaking for 30 min. In some experiments, the microspheres were simply dispersed in the 8% (v/v) serum solution without incubation.

The J774 monocytes-macrophages were infected by incubating the cells with the opsonized *B. abortus* for 15 h at a bacteria/cell ratio of 5:1 as described above. Afterwards, non-phagocytosed bacteria were removed by washing carefully five times and the effectiveness of the different treatments was studied by incubating the infected monocytes for 24 h at 37°C and 5% CO_2 with 1 mL of DMEM/L-glutamine containing either free gentamicin in solution, gentamicin encapsulated in microspheres or placebo microspheres. DMEM/L-glutamine without fetal calf serum was used to avoid potential effects of the serum proteins on the particle uptake and to moderate the proliferation of monocytes and bacteria. Further, cells were washed twice with DMEM/L-glutamine and extracellular cfu and total cfu after cell lysis with Triton X-100 were determined by plating serial dilutions onto TSA plates as described previously. Intracellular brucellae were calculated by subtracting the number of extracellular bacteria from the total cfu counts in three wells. Results were expressed as $\log_{10}$ of intracellular *Brucella* surviving the treatments and the efficiency of the treatments was tested in one to six independent assays.

In vitro susceptibility of B. abortus to gentamicin and poloxamer

Gentamicin sulphate and poloxamer 188 as single agents or in combination were tested for their ability to inhibit the bacterial growth of *B. abortus* in broth culture. Gentamicin sulphate was added to the bacterial suspension to obtain final concentrations of 0.007–0.960 µg/mL, alone or in combination with 0.5, 1 and 2% (w/v) poloxamer 188. Bacterial growth in TSB was measured turbidimetrically with an automated Labsystems Bioscreen (Finland) using Bioscreen microtitre plates (100-well honeycomb). Organisms were retrieved from suspensions stored at –85°C in cryoballs (Microbank, Biolab Diagnostics, Canada), plated on TSA and incubated at 37°C overnight. Several colonies were then subcultured in TSB at 37°C for 36 h to reach mid-log phase. The final inoculum suspension was prepared in TSB and adjusted to an optical density of 0.12 (590 nm) (~10^8 cfu/mL). The real inoculum dose was estimated by plating appropriate dilutions of the stock suspension onto TSA plates. Finally, an inoculum of 10^5 cfu per well was added into Bioscreen plates containing serial dilutions of the agents (gentamicin and/or poloxamer 188). Appropriate positive and negative growth controls were also included. The optical density of each well was measured automatically at 30 min intervals using wide band irradiation (420–580 nm) and bacterial growth curves were generated.

Statistical analysis

Comparisons were made between groups by a one-way analysis of variance (ANOVA) and post-hoc Tukey's HSD test. The statistical significance level was defined as $P < 0.05$.

Results

Microsphere size, gentamicin loading and in vitro release

The average diameter of the PLGA 50:50H and PLGA 50:50 microspheres was ~4 µm, except for the placebo PLGA 50:50 microspheres which had an average diameter of 0.5 µm (Table 1). This size appeared adequate for cellular uptake and was mainly a result of the encapsulation method used. Scanning electron micrographs showed spherically shaped and regular particles with smooth surfaces for both the placebo and gentamicin-loaded PLGA 50:50 microspheres. Gentamicin-loaded PLGA 50:50H microspheres were, however, porous and the particles tended to aggregate.

The actual gentamicin loading was 22 and 15 µg/mg microspheres and the *in vitro* gentamicin burst release amounted to 7.8 and 2.0 µg for the PLGA 50:50 and PLGA 50:50H, respectively (Table 1). Additional gentamicin release from PLGA 50:50H microspheres occurred in a continuous fashion and from PLGA 50:50 microspheres in a multiphase-like pattern (Figure 1).

Kinetics of Brucella uptake and replication in cultured J774 monocytes

Opsonized *B. abortus* were incubated from 1 to 48 h with J774 cells in complete medium without antibiotic-antimycotic supplement and afterwards, the number of extracellular and intracellular bacteria was determined. Incubation for 1 h was insufficient for significant phagocytosis of opsonized bacteria at a monocyte/*Brucella* ratio of 1:5. Incubation times between 15 and 48 h allowed an adequate number of

Table 1. Characteristics of the gentamicin (GEN)-loaded microsphere (MS) batches used in this study for the *in vitro* treatment of murine J774 monocytes-macrophages infected with *Brucella abortus* 2308

	Number weighted microsphere diameters (μm)[b]			*In vitro* burst release[d]	
Microsphere formulation[a]	$d_{50\%}$	$d_{90\%}$	Actual loading[c] (μg GEN/mg MS)	(%)[e]	(μg GEN/dose MS[f])
GEN-PLGA 50:50	3.8	7.6	22.4 ± 1.6	50.0 ± 4.1	7.8 ± 0.6
Placebo PLGA 50:50	0.5	0.8	–	–	–
GEN-PLGA 50:50H	3.9	7.7	14.8 ± 0.6	13.5 ± 0.9	2.0 ± 0.1
Placebo PLGA 50:50H	3.5	6.8	–	–	–

[a]GEN, loaded with gentamicin. The commercial products of PLGA 50:50 and PLGA 50:50H were Resomer RG502 and RG502H, respectively.
[b]50% and 90%-undersize diameters.
[c]Nominal loading was 100 μg gentamicin/mg microspheres. Actual loading is expressed as μg of gentamicin per mg of microspheres and represents the mean value ± S.D. of three replicates.
[d]Gentamicin *in vitro* burst release from the microspheres after 24 h incubation in phosphate buffer (67 mM, pH 7.4, 0.05% Tween 20, preserved with 0.02% sodium azide). The values represent the mean value ± S.D. of three replicates.
[e]Percentage of the total gentamicin content in the microspheres.
[f]The gentamicin dose used for *in vitro* treatment of infected monocytes was 15 μg, corresponding to 0.7 mg of PLGA 50:50 and 1 mg of PLGA 50:50H microspheres. The values indicate the absolute amount of gentamicin released *in vitro* within 24 h from the applied quantities of microspheres.

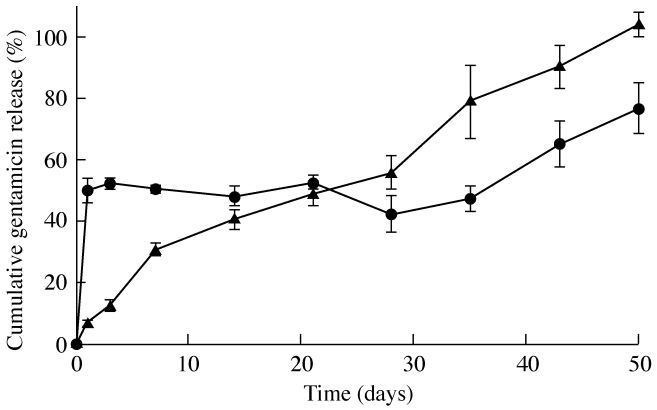

Figure 1. *In vitro* gentamicin release from PLGA 50:50 (filled circles) and PLGA 50:50H (filled triangles) microspheres. Results are expressed as percentage of cumulative gentamicin release and represent the mean value ± S.D. of three replicates.

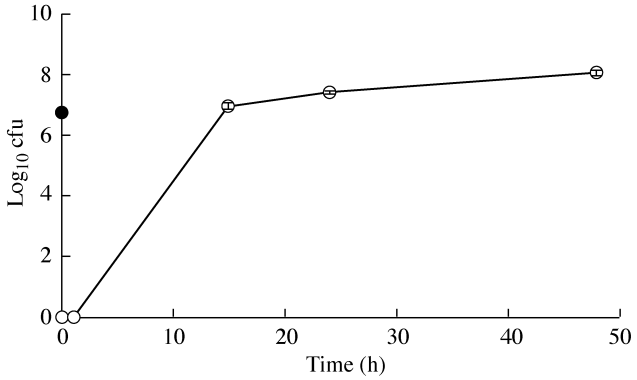

Figure 2. *Brucella abortus* phagocytosis and intracellular growth kinetics in murine J774 monocyte-macrophages. Cells were infected at a cell to bacteria ratio of 1:5 (filled symbols) and incubated for 1–48 h. The number of intracellular brucellae at different time points is shown (open symbols). Results are expressed as $\log_{10}$ of cfu and represent the mean value ± S.D. of three wells.

intracellular brucellae to carry out our study (Figure 2). In this time, the bacteria reached the intracellular compartment and multiplied within the monocytes increasing by approx 1.3 $\log_{10}$ after 48 h. Incubation times above 48 h resulted in excessive cell and bacteria proliferation, as reflected by turbidity and diminished cell viability. Therefore, for all antibiotic activity assays, an infection time of 15 h was chosen. Thus, the cells were incubated with the bacteria for 15 h, non-phagocytosed bacteria were washed off and the different treatments were subsequently applied. Under these conditions, the percentage of intra- and extracellular *Brucella* at the time of the antibiotic treatments was 93–95% and 5–7%, respectively.

Efficacy of gentamicin-loaded microspheres in reducing intracellular Brucella from infected monocytes: effect of polymer type

Monocytes infected with *Brucella* were treated for 24 h with (i) free gentamicin in solution, (ii) gentamicin encapsulated in PLGA 50:50 or PLGA 50:50H microspheres, and (iii) placebo PLGA 50:50 or PLGA 50:50H microspheres. The amounts of microspheres used per well were 0.7 mg for PLGA 50:50 and 1.0 mg for PLGA 50:50H, which corresponded to a gentamicin dose of 15 μg/well for both

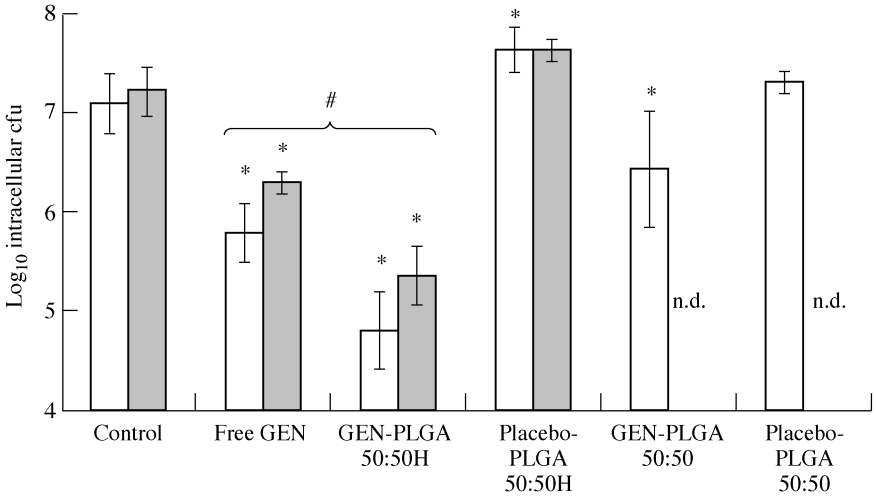

Figure 3. Efficacy of free gentamicin, gentamicin-loaded and unloaded PLGA 50:50 and PLGA 50:50H microspheres in reducing intracellular *Brucella* infection in J774 monocytes *in vitro*. The effect of opsonization with 8% (v/v) fresh mouse serum is also shown (shaded bars). Bars represent the average ± standard error of the means of up to six independent assays; * and # symbols indicate significant differences ($P < 0.05$) in the Tukey test.

microsphere types; corresponding amounts of placebo microspheres were used. A dose of 15 μg of gentamicin was used to ensure the intracellular delivery of the antibiotic at levels well above the MBC (0.06 mg/L, see below). Placebo microspheres had no bactericidal effect on intracellular *Brucella* counts. In contrast, treatment with gentamicin-loaded microspheres reduced intracellular bacteria, compared with untreated controls, by 1.0 and 2.0 $\log_{10}$ for PLGA 50:50 and PLGA 50:50H, respectively. Free gentamicin in solution exerted variable intracellular bactericidal activity in different experiments, varying from a 1.1 to 1.8 $\log_{10}$ reduction. However, treatment with gentamicin-loaded PLGA 50:50H microspheres was significantly more effective at reducing the intracellular infection than free gentamicin treatment (Figure 3).

Efficacy of gentamicin-loaded microspheres in reducing intracellular Brucella from infected monocytes: effect of particle opsonization and addition of poloxamer 188 to the culture medium

Previous results in our laboratory showed that opsonization of gentamicin-loaded microspheres increased significantly the capacity of the particles to induce oxidative burst of monocytes,[12] which may potentially increase the bactericidal activity of antibiotic treatments. With that hypothesis, the effect of opsonization on the antibacterial efficacy of the microsphere treatment was examined in this study. Our hypothesis was, however, not supported by our findings. The antibiotic activity of the gentamicin-loaded microspheres did not increase upon particle opsonization (Figure 3). In contrast, addition of 2% (w/v) of poloxamer 188 to the medium to improve the dispersion characteristics of the microspheres, enhanced significantly the intracellular antibiotic activity of the gentamicin-loaded PLGA 50:50H microspheres (Figure 4). In the presence of the surfactant, the total reduction of intracellular bacteria reached 3.5 $\log_{10}$ cfu, relative to the untreated monocytes, which represented a 1.2 $\log_{10}$ further reduction in intracellular cfu relative to the same treatment without poloxamer 188. A similar improvement, i.e. ~1 $\log_{10}$ further reduction, was also observed with the gentamicin loaded end-group

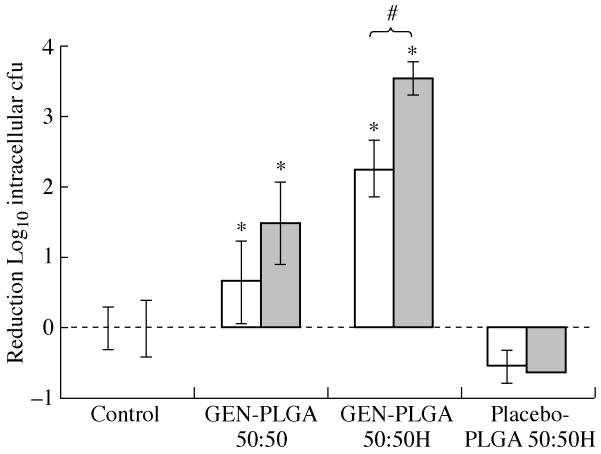

Figure 4. Effect of adding 2% (w/v) poloxamer 188 to the incubation medium (filled bars) on the intracellular bactericidal activity of gentamicin-loaded PLGA 50:50 and PLGA 50:50H microspheres. The effect in medium alone is shown with open bars. Bars represent the average ± standard error of the means of up to six independent experiments; * and # symbols indicate significant differences ($P < 0.05$) in the Tukey test.

capped PLGA 50:50 microspheres when the poloxamer was added to the medium; however, in this case the increase was not statistically significant. Control wells demonstrated the lack of activity of both a poloxamer 188 solution and a suspension of placebo microspheres in poloxamer on the viability of the intracellular *Brucella*.

In vitro susceptibility of Brucella abortus to gentamicin and poloxamer

The effect of combining gentamicin sulphate and poloxamer 188 against *B. abortus* was studied in broth culture *in vitro*. When sub-MIC concentrations of gentamicin sulphate and non-inhibitory concentrations of poloxamer 188 were combined, a synergic effect was evidenced between the two agents (not shown). For example, the

MBCs (3 $\log_{10}$ reduction) of gentamicin and poloxamer 188 tested alone were 0.06 mg/L and >2% (w/v), respectively. When gentamicin was combined with the poloxamer at 1% or 2% (w/v), the MBC of gentamicin was reduced to 0.03 mg/L (poloxamer 1%) and 0.015 mg/L (poloxamer 2%).

Discussion

Our previous studies showed that gentamicin-containing PLA and PLGA microspheres are efficiently phagocytosed and that end-group uncapped PLGA 50:50H microspheres stimulated highly the oxidative burst of monocyte-macrophages.[12] In this work, we hypothesized that intracellular oxygen radicals produced upon microsphere internalization might act synergically with the antibiotic in killing intracellular bacteria.

Gentamicin-loaded PLGA 50:50H microspheres decreased significantly the intracellular bacterial levels (typically by 2 $\log_{10}$) compared with untreated monocytes and free gentamicin; the efficiency of PLGA 50:50 microspheres was lower (typically 1 $\log_{10}$ reduction) and normally no different to the free drug; placebo microspheres had no effect. Opsonization of gentamicin-loaded microspheres in fresh mouse serum did not improve the antibiotic efficiency of the treatment although, in previous work, opsonization increased the uptake of placebo microspheres and the cell oxidative metabolism.[12] The release of oxygen metabolites can alter the bacterial membrane permeability and thereby increase drug efficiency.[15] Because *Brucella* spp. alone only slightly increase the oxidative burst in blood leucocytes, we speculated that an enhanced production of oxidative metabolites triggered by the phagocytosis of microspheres should improve treatment efficacy. This hypothesis was supported by the observation that brucellicidal activity of human polymorphonuclear leucocyte granule lysates was enhanced by addition of hydrogen peroxide.[16] In our experiments, no bactericidal effect was observed with placebo PLGA 50:50H microspheres. Nevertheless, the stimulation of oxygen derivatives within the cells may have increased the bacterial membrane permeability and made them more susceptible to the antibiotic.

Free gentamicin sulphate lowered the number of intracellular bacteria by ~1 to 1.8 $\log_{10}$. Because gentamicin cannot diffuse through cellular membranes, its internalization and accumulation inside cells has been ascribed to pinocytosis.[17] The observed variation in the reduction in bacterial counts might result from variations in the pinocytic capacity of the cells. In our investigation, microencapsulated gentamicin achieved bacterial reduction at levels superior or comparable to free drug. Considering that gentamicin release from the microspheres was prolonged over up to 50 days, antibiotic activity may be expected for prolonged periods of time. Incubation times were not prolonged in our studies due to limited cell viability and high bacterial proliferation. We hypothesize that this may provide intracellular bactericidal gentamicin concentrations over prolonged periods of time. Thus, this approach could reduce the multiple injections needed in the aminoglycoside component of current brucellosis therapies, and could ensure efficient clearance of the infection without increasing the possibility of ototoxic and nephrotoxic reactions. It would therefore be interesting to quantify the intracellular concentration of gentamicin after microsphere uptake to improve understanding of the mechanism and kinetics by which the encapsulated drug reduces intracellular *Brucella*. Nonetheless, *in vivo* processing of the microspheres is likely to affect the kinetics of drug release and polymer degradation. Intracellular reduction of *Brucella* was greater for PLGA 50:50H than for PLGA 50:50 microspheres, although *in vitro* burst release within the initial 24 h of incubation amounted to only 14% for the PLGA 50:50H as compared with 50% for the PLGA 50:50 microspheres. Two factors may explain the results. First, the pronounced stimulation of oxidative metabolites by the PLGA 50:50H particles may have enhanced the antibiotic activity of gentamicin. Second, a higher number of PLGA 50:50H particles might have been phagocytosed, because a larger amount of these particles had been added to the infected monocytes to achieve identical gentamicin doses (1 mg of PLGA 50:50H versus 0.7 mg of PLGA 50:50 microspheres, with both types having a similar phagocytosis rate of ~50%).[12] However, the present data are insufficient to conclude that a higher number of phagocytosed particles directly affect antimicrobial efficacy.

An interesting result of this study was the increased antibacterial activity (up to 1.2 $\log_{10}$) of microencapsulated gentamicin when the dispersion medium for the microspheres contained 2% (w/v) poloxamer 188. Several mechanisms may be responsible for this phenomenon: (i) adsorption of the surfactant on the microsphere surface thereby altering the surface polarity and the potential for cell adhesion; (ii) enhanced phagocytosis due to a higher degree of particle dispersion or another mechanism; (iii) non-specific cell activation by the surfactant; and (iv) synergic enhancement of antibiotic activity. Particle coating by poloxamer type surfactants is well known to suppress phagocytosis *in vitro* and alter the opsonization by serum proteins *in vivo*, slowing down the elimination of particles from the blood by the mononuclear phagocytic system.[18] The efficiency of poloxamer types to reduce particle phagocytosis depends on the molecular weight and chain length of the propylene oxide (PO) and ethylene oxide (EO) blocks.[18] Thin coatings conferring poor steric stabilization were obtained on highly polar surfaces[19,20] where the PO blocks were adsorbed preferentially onto the particle surface, whereas the EO chains stretched out into the dispersion medium.[18] On larger particles, such a thin layer was probably adsorbed flatly so that the hydrophobic PO units remained exposed to the medium. With the relatively polar and hydrophilic PLGA 50:50 and PLGA 50:50H microspheres studied herein, adsorption of poloxamer probably occurred through interaction with the EO chains, resulting in a flat arrangement of adsorbed poloxamer. As a result, the exposed PO chains possibly increased the hydrophobicity of the particle surface, leading to increased particle uptake by monocytes, as observed by optical microscopy (data not shown). Further, improved particle dispersion in 2% poloxamer 188 should have also contributed to the increased phagocytosis. Finally, mechanisms involving interaction between poloxamer and cell membrane[21,22] or cell activation cannot be excluded. Some poloxamers have indeed increased phagocytic activity, and altered bacterial cell wall integrity and permeability yielding a synergic effect with antibiotic agents.[23–26] Our susceptibility studies in culture broth demonstrated that poloxamer can potentiate the antibacterial activity of gentamicin. Enhancement of antibiotic susceptibility was observed with subinhibitory concentrations of poloxamer. However, no bactericidal effect of 2% soluble poloxamer alone or 2% poloxamer incubated with placebo microspheres was observed on the intracellular bacterial counts. The results indicate that only non-inhibitory amounts of poloxamer may reach the intracellular bacteria. Nevertheless, non-inhibitory concentrations of the surfactant may have synergically enhanced the anti-*Brucella* activity of gentamicin delivered intracellularly from microspheres by as yet unknown mechanisms.

Finally, an important aspect for discussing the effectiveness of encapsulated gentamicin is the location of the *Brucella* inside the cell. Intracellular bacteria can evade the host defences by different

mechanisms and prevention of phagosome–lysosome fusion has been proposed for intracellular survival of virulent *Brucella* spp., e.g. *B. abortus* 2308.[27] *B. abortus* is located in the perinuclear region within compartments resembling autophagosomes and is then delivered to the endoplasmic reticulum where intracellular bacterial replication takes place.[28–30] This special localization of *B. abortus* might prevent contact between bacteria and antibiotic-loaded microspheres. On the other hand, virulent *Brucella* can transit from early endosomes to autophagosomes, which apparently did not fuse with endosomes loaded with exogenous material.[30] Nonetheless, fusion between phagosomes and other endocytic vesicles can generally occur, so that phagosomes loaded with particles or pinocytic endosomes have the potential to eliminate *Brucella* that reside in accessible phagosomes.[17] Two localizations for *B. abortus* have been found at 24 h post-infection: accessible single membrane phagosomes and multimembranous autophagosomes,[28] where the bacteria may escape from the antibiotics delivered to phagosomes.

In conclusion, we suggest that microsphere internalization is responsible for the significant reduction in the intracellular bacteria by gentamicin-loaded PLGA 50:50 and PLGA 50:50H microspheres within 24 h. Microencapsulated gentamicin was efficiently targeted to infected monocytes and reduced intracellular *Brucella* infections. In addition, gentamicin may still be available for further release from the microspheres for prolonged periods of time. Poloxamer 188 added to the dispersion medium for the microspheres significantly enhanced the efficiency of the antibiotic treatment. Further studies should focus on determining the mechanisms by which microencapsulated gentamicin is capable of reducing intracellular viable *Brucella* and investigate the efficacy of gentamicin-loaded microspheres in the treatment of brucellosis *in vivo*.

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