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Intracellular killing of *Brucella melitensis* in human macrophages with microsphere-encapsulated gentamicin

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***Objectives*: Treatment of human brucellosis demands antibiotic targeting into the mononuclear-phagocytic system. The aim of this work was to prepare and characterize particulate carriers containing gentamicin and to study their interactions with phagocytic cells and bactericidal activity against intracellular *Brucella melitensis*.**

***Methods*: Different poly(lactide-co-glycolide) (PLGA) polymers with free carboxylic end-group were used to formulate micro- and nanoparticles containing gentamicin, by a water-oil-water solvent-evaporation technique. PLGA 502H and 75:25H microparticles were selected because they showed the highest gentamicin loadings as well as good physico-chemical properties and sustained release *in vitro*.**

***Results*: Gentamicin-containing microspheres of both polymers were successfully phagocytosed by infected THP-1 human monocytes, and immunocytochemistry studies revealed that the antibiotic reached *Brucella*-specific compartments. A dose of 30 μg of encapsulated gentamicin was able to reduce intracellular *Brucella* infection by 2.2 log.**

***Conclusions*: Altogether, these results suggest that 502H and 75:25H microspheres are suitable carriers for gentamicin targeting inside human macrophages and thus for brucellosis treatment.**

Keywords: microparticles, brucellosis, phagocytosis, drug delivery system

Introduction

Brucellosis is a worldwide zoonosis that affects over half a million individuals every year.[1] Human disease is the accidental expression of the much more widespread disease in animals.[2] Humans may also be infected through exposure to the brucellae in laboratories and, due to their ability to infect humans through aerosol exposure, it is recognized as a biosecurity safety level-3 organism. However, treatment of human brucellosis is not yet solved.

After its entrance in the host, the monocytic-macrophagic system is the target for the pathogen,[3] where it is able not only to survive but also to replicate.[4] The pathogen evades host defences by inhibiting endosome fusion with lysosomes[5,6] and finally may reach the endoplasmic reticulum.[7] In fact, these bacteria may have an extreme preference for the intracellular environment despite their ability to live outside host cells. Thus, brucellae inside the macrophage are protected not only from the immune system (antibodies, complement) but also from *in vitro* active antibiotics that do not reach therapeutic concentrations into those intracellular compartments. As a result, relapse rates of 5–16% are obtained with different protocols in clinical practice.[8,9]

Particulate systems have been proposed for intracellular delivery of antimicrobial agents, therefore targeting the intracellular sites of infection, thereby helping to increase the therapeutic index of antimicrobials in intracellular niches. Gentamicin is an aminoglycoside that is very active *in vitro* against clinical isolates of *Brucella* and has already been trapped in particulate systems for targeting infected monocytes, with promising results.[10–12]

The purpose of the present work was to study the interactions between gentamicin-containing carriers [micro- and nanoparticles of poly(lactide-co-glycolide) (PLGA)] and human macrophages. Among the parameters that control particle uptake by macrophages, size,[13–15] hydrophobicity[13] and zeta potential[15,16] are among the most significant ones. Furthermore, once inside the

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phagocytes, intracellular elimination of the pathogen can be improved not only by the action of released antibiotic from particulate carriers, but also by phagocyte activation triggered by particle uptake. In the case of *Brucella*, reactive oxygen production seems to play an important role in pathogen destruction, while nitric oxide plays a minor role in anti-*Brucella* activity.[17] Therefore, physico-chemical properties regarding particle uptake and macrophage oxidative burst was studied. Finally, intracellular location of particles and delivered gentamicin, and its therapeutic efficacy in the intracellular elimination of *Brucella melitensis* in human monocytes was also determined.

Materials and methods

Cell culture

Two different monocytic-macrophagic cell lines were used. J774 tumorigenic monocytic murine cell line was from the ATCC (ATCC TIB 67) and THP-1 cell line, human myelomonocytic cells displaying macrophage-like activity, was kindly provided by Professor D. Raoult from the Unité des Rickettsies, Université de la Mediterranée, Marseille, France.

J774 monocytes were maintained in DMEM supplemented with 2 mM L-glutamine (Gibco, Invitrogen, Carlsbad, USA) and 10% (v/v) heat-inactivated FCS (Biochrom KG, Berlin, Germany). When adherent cells reached confluent growth (every 3–4 days) the medium was withdrawn, and cells were detached in 5 mL of the medium. After collecting by centrifugation (400 **g**, 4 min), 10^6 viable cells, counted in a haemocytometer (Neubauer Chamber) by the Trypan Blue (Sigma-Aldrich, St Louis, MO, USA) exclusion method, were transferred to 20 mL of fresh medium.

For THP-1 cell culture, 2 mM L-glutamine, 10% FCS, 100 U/mL of penicillin base and 100 μg/mL of streptomycin sulphate (Gibco, Invitrogen, Carlsbad, USA) were added to RPMI. Every 4 days, cells were collected by centrifugation (400 **g**, 4 min) and after their resuspension in 2 mL of the medium, 10^6 viable cells, counted by the Trypan Blue method, were transferred to 20 mL of fresh RPMI. For their differentiation into macrophages, THP-1 cells were incubated for 48 h in complete medium with 10 ng/mL of phorbol 12-myristate 13-acetate (PMA).[18]

Bacterial strain and culture medium

B. melitensis 16M (ATCC 23456, biotype 1) smooth virulent strain was used. For bacterial growth, trypticase soy broth (TSB) was purchased from bioMérieux (Marcy l'Étoile, France) and American Bacteriological Agar was obtained from Pronadisa (Madrid, Spain).

Preparation of micro- and nanoparticles

PLGA micro- and nanoparticles containing gentamicin (Sigma-Aldrich) were prepared by a water-oil-water solvent-evaporation technique.[19] Briefly, the antibiotic was dissolved in a phosphate buffer, pH 6.0, solution containing 200 mg of polymer in 0.5% poly-vinylalcohol (PVA, molecular weight 15 000; from BDH Supplies, UK) dissolved in dichloromethane for microspheres or in ethyl acetate for nanoparticle preparations. These two solutions were mixed by ultrasonication for 1 min (Branson sonifier 450, Branson Ultrasonics corp., Danbury, USA) under cooling, to form a W_1/O emulsion. This inner emulsion was added to 2 mL of 1% PVA (W_2) and homogenized by ultrasonication. The resulting $(W_1/O)W_2$ emulsion was poured into 50 mL of 0.2% PVA and continuously stirred for, at least, 3 h at room temperature to allow solvent evaporation and microsphere formation. After preparation, particles were isolated by centrifugation (7000 **g**, 5 min for microparticles and 12 000 **g**, 30 min for nanoparticles), washed three times with ultra pure water and freeze-dried.

The copolymers of PLGA of different molecular weights, with free terminal carboxylic group, were used: 13.7 and 34 kDa PLGA 50:50 (Resomer® RG 502H, named as 502H, and RG 503H, named as 503H) were obtained from Boehringer-Ingelheim (Ingelheim, Germany). Medisorb® of 25 kDa (7525 DL 2.5 A) (named as 75:25H) was provided by Alkermes (Wilmington, USA).

Particle size and zeta potential

Size of microspheres was determined by laser diffractometry (Mastersizer®, Malvern Instruments, UK). A small amount was dispersed in water and analysed under continuous stirring. The average particle size was expressed as the volume mean diameter in micrometres.

Size and polydispersity of nanospheres were analysed by Photon Correlation Spectroscopy (PCS), using a Zetamaster® analyser system (Malvern Instruments, UK).

The zeta potential of all formulations, dispersed in ultra pure water, was performed using the same apparatus.

Both particle sizing and zeta potential measurements were made in triplicate for a single batch of particles and the results were the average of three measurements.

Gentamicin loading

A known amount of accurately weighed loaded particles were dissolved in 1 mL of dichloromethane and the antibiotic was extracted using 2 mL of phosphate buffer, pH 6.0. Drug was assayed in the aqueous phase after its derivatization with *o*-phthalaldehyde (OPA) using a fluorescence assay.[20] Standard concentrations of gentamicin were prepared by serial dilution in boric acid 0.4 M, pH 9.7, and added to a 96-well microplate (TPP, Trasadingen, Switzerland). Samples were also diluted, when needed, in boric acid. Then, OPA solution [0.04% OPA, 0.1% (v/v) diethyl ether, 0.2% (v/v) β-mercaptoethanol in boric acid] was added to the standard solutions. Fluorescence was measured, immediately, in a Tecan GENios fluorimeter (TecanGENios, Tecan Group Ltd, Maennedorf, Switzerland) (excitation: 340 nm, emission 450 nm) and sample concentrations were calculated by means of the standard curve.

Oxidative burst of monocytes after particle uptake

Monocytes produce reactive oxygen metabolites together with other non-oxygen dependent products, upon stimulation or during phagocytosis, which constitute an essential mechanism of host defence against bacterial and fungal infections.[21] Therefore determination of oxidative burst (intracellular H_2O_2 production) can become a suitable tool to measure monocyte activation after particle uptake. Flow cytometry was used for oxidative burst quantification with a FACSCalibur cytometer and the CellQuest computer program (Becton Dickinson, San Jose, USA), after incubation with particles. Oxidative burst was quantified by oxidation of dihydrorhodamine 123 to rhodamine 123, a green fluorescent product (Bursttest, ORPEGEN® Pharma, Heidelberg, Germany). Briefly, 20 μL of 1 mg/mL of particles in DMEM with L-glutamine (Gibco, Invitrogen, Carlsbad, USA), previously opsonized with 10% fresh serum, was incubated with 100 μL of $1.5–2.5 \times 10^6$ J774 cultured monocytes/mL in test tubes (Falcon, BD, Franklin Lakes, NJ, USA) at 37°C and 5% CO_2 for 4 h. Afterwards, the substrate was added and after vigorous shaking tubes were incubated for 10 min. Cells were washed and the

oxidative burst was measured from 10 000 cell events. Monocytes were discriminated by forward scatter and 90° light scatter signals, further selected by electronic gating in blood samples, and fluorescence (FL1-H) was measured using a logarithmic scale of 1024 channels. PMA incubated with the cells and cultured monocytes or blood without previous contact with particulate material were used as high burst and basal burst controls, respectively. Results are expressed as FL1-H increases or decreases relative to controls.

Cytotoxicity of particles on THP-1 monocytes

Trypan Blue dye exclusion assay. Briefly, THP-1 cells were adjusted to 2×10^6 monocytes/mL and 500 μL per well was added to 24-well plates (Corning Corporation Inc., Acton, USA) and differentiated into macrophages as described above. After removing non-adherent cells they were incubated with 1 mg/mL particle dispersion in RPMI complete medium for 24 h. Incubation medium was withdrawn and cells were carefully detached with trypsin–EDTA (Gibco, Invitrogen, Carlsbad, USA). An aliquot of the cell suspension was diluted 1:1 (v/v) with 0.4% Trypan Blue and viable and dead cells were counted in a Neubauer chamber (Brand, GmbH & Co., Wertheim/Main, Germany). Results were expressed as the percentage of cell viability compared with particle-free medium.

MTT assay. Viable cells are able to reduce MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, St Louis, USA) to coloured formazan serving as an indirect measurement of cell viability.[22,23] Cells were differentiated to macrophages and incubated with particulate carriers as described for Trypan Blue assay. Incubation medium was withdrawn, and cells were washed twice. To each well, 500 μL of the MTT (methylthiazol-tetrazolium) solution was added. Previously, MTT had been dissolved at 5 mg/mL in RPMI complete medium, diluted 10 times and filtered. Cells were incubated with the MTT solution for 1 h, supernatants discarded and formazan crystals dissolved in an extraction solution [10% SDS in dimethyl formamide and water (1:1, v/v), adjusted to pH 4.7 with acetic acid] overnight at 37°C. Formazan quantification was performed by measuring the absorbance at 570 nm after sample dilution in extraction solution and thorough mixing. After centrifugation, particle-free supernatants were obtained. Previous measurements for different initial cell concentrations and sample dilution showed that absorbance was proportional to the number of viable cells. Results of cell viability were calculated as the percentage of formazan: formation of this product by cells co-incubated with particles, over the formation after incubation of cells in a particle-free medium.

Intracellular location of Brucella, microparticles and gentamicin

B. melitensis-infected macrophages which had phagocytosed PLGA microspheres were processed for immunocytochemistry studies. *Brucella* and gentamicin were labelled with immunogold beads as described below and transmission electron microscopy images were obtained.

Several colonies of *B. melitensis* 16M were incubated in TSB for 20 h, to let the bacteria reach exponential growth phase. Absorbance was adjusted to 0.09 (600 nm), further diluted to an adequate bacterial suspension (100 bacteria/cell) and opsonized (30 min, 37°C) with a subagglutinant concentration of serum from a patient infected with *B. melitensis*. Bacterial suspension was added to a well containing 2×10^5 cells/mL of adherent THP-1 macrophages and, after 30 min incubation, five washes were performed to eliminate non-phagocytosed bacteria. At 18 h post-infection, 30 μg/well of either free or particle-loaded gentamicin, resuspended into 1% lecithin in RPMI, was added to infected cells and incubated for further 24 h. Cells were detached with trypsin–EDTA and fixed for 1 h at 4°C in 2 mL of 4% glutaraldehyde (in 0.1 M sodium cacodylate buffer, pH 7.3) After washing with sucrose-cacodylate buffer (0.25 M sucrose in 0.1 M sodium cacodylate), cells were post-fixed with 2 mL of 1% osmium tetroxide (Johnson Matthey Chemicals, London, UK) (in phosphate buffer, pH 7.3) for 1 h at 4°C. One wash was made with veronal buffer (56 mM sodium acetate, 28 mM sodium barbital, 94 mM NaCl, 4 mM KCl, 0.9 mM $CaCl_2$) and cells were embedded in 200 μL of 2% melted agarose and kept at 4°C overnight to harden. Agarose was cut into 1 mm^3 pieces and dehydrated with an ethanol series and two immersions in propylene oxide (15 min each) were performed. Samples were immersed 2 h in prolilene:Epon (1:1) and embedded in pure Epon 812. Afterwards, resin was polymerized by a gradual temperature increase (from 37 to 60°C within 48 h).

Semi-thin sections of 1 μm were stained with Toluidine Blue [absolute alcohol in deionized water (1:1), 1% Toluidine Blue, 50 mM $Na_2B_4O_7$] to select suitable fields for electron microscopy. Afterwards, by means of a Leica Ultracut R ultramicrotome (Leica, Wetzlar, Germany) ultra-thin sections were prepared. For immunocytochemistry, as primary antibodies, rabbit anti-gentamicin and mouse anti-*Brucella* serum, both diluted 1:10, were incubated overnight at 4°C. After several washings, the secondary antibodies conjugated with different size of colloidal gold particles were used: 20 nm goat-anti rabbit and 10 nm for goat anti-mouse IgG (BBI International, Sigma-Aldrich, St Louis, USA) both diluted 1:20 were incubated for 60 min at room temperature. Samples were washed again and further contrasted with uranyl acetate (4 min, room temperature) and lead citrate (2 min, room temperature). For microscopy studies a Zeiss EM10CR transmission electron microscope (TEM) was used.

Brucella infection in human monocytes and treatment with gentamicin-loaded particles

THP-1 macrophages were infected with *B. melitensis* and further incubated with PLGA microspheres as described above. After incubation of formulations for 24 h, several washes were performed to remove non-phagocytosed particles or bacteria and extracellular fractions were collected. Cells were detached with trypsin–EDTA and after three freeze–thawing cycles macrophages were lysed. The number of intracellular bacteria was calculated by subtracting the number of extracellular bacteria from the total bacterial counts in three wells.

Statistical analysis

For comparisons among more than two groups one-way analysis of variance (ANOVA) was performed and as post-hoc tests Dunnett's test and Tukey B[24] were used. When two groups were compared, the Student's *t*-test was performed. Statistical significance levels were defined as follows: $*P < 0.05$; $**P < 0.01$.

Results

Characterization of particulate carriers

Different PLGA polymers with free carboxylic end-group were used to formulate micro- and nanoparticles, differing in their lactic:glycolic ratio and in their molecular weight.

Table 1. Characteristics of gentamicin (GEN)-containing microparticles (MP) and nanoparticles (NP)

Polymer and particle type		Size (nm)	Zeta potential (mV)	GEN loading (μg GEN/mg particle)[b]
502H NP	GEN-loaded	310 ± 2.0 (0.22)[a]	–15.5 ± 0.2	6.1 ± 0.4**[d]
	unloaded	300 ± 2.1 (0.15)[a]	–19.4 ± 1.1**[c]	–
502H MP	GEN-loaded	3.9 ± 0.2	–21.5 ± 0.5	8.2 ± 0.4
	unloaded	2.2 ± 0.9	–24.3 ± 0.7*[c]	–
503H MP	GEN-loaded	3.4 ± 0.2	–18.2 ± 1.5	9.3 ± 0.6
	unloaded	2.9 ± 0.2	–19.1 ± 0.9	–
75:25H MP	GEN-loaded	3.0 ± 0.2	–17.1 ± 0.4	8.1 ± 0.4
	unloaded	1.4 ± 0.1	–20.2 ± 1.2*[c]	–

Data are expressed as means ± SD of three measurements.
[a]Polydispersion of nanoparticles size.
[b]Nominal loading: 10 mg gentamicin.
[c]Student's *t*-test was performed between zeta potentials of loaded and unloaded particles: *$P < 0.05$; **$P < 0.01$.
[d]Tukey B test was used for comparison among formulations: *$P < 0.05$; **$P < 0.01$.

Microparticles

As shown in Table 1, gentamicin-containing microspheres showed mean diameters in the range of 3.0–3.9 μm. Regarding polymer type, all formulations showed a negative zeta potential, between –17 and –24 mV. Loaded and unloaded microparticles displayed different surface charges ($P < 0.05$), except for 503H microparticles. This fact showed the different distribution of the antibiotic among formulations. For 503H microspheres, gentamicin would be entrapped inside the microspheres, while for the other formulations part of the drug would be also exposed to particle surface.

No statistical differences were observed in drug loading among microspheres. Those made of PLGA 503H presented the highest loading (9.3 μg/mg), while 502H and 75:25H microparticles entrapped 8 μg/mg (Table 1).

Nanoparticles

Size distribution was narrow and monodispersed with an average size of 300 nm (Table 1). No differences were observed between loaded and empty formulations.

Similarly to microspheres, nanospheres showed negative zeta potentials (–15 to –19 mV). Differences ($P < 0.01$) observed between loaded and unloaded particles indicated the presence of antibiotic on particle surface. Finally, compared with microspheres, these formulations showed a lower gentamicin loading (6.1 μg/mg) ($P < 0.05$).

Monocyte activation upon particle uptake: determination of oxidative burst

Particle phagocytosis by monocytes promotes its activation and production of reactive oxygen products[25] among others. J774 murine macrophage activation upon particle uptake was evaluated by measuring oxygen products (H_2O_2) by flow cytometry (Figure 1). Results were expressed as the percentage of cells emitting fluorescence (Figure 1a) and mean fluorescence (Figure 1b). Nanospheres made of PLGA 502H were the most activating particles, followed by microparticles made of the same polymer. Microspheres of 75:25H polymer activated monocytes to a lesser extent, but still differed significantly from basal oxidative burst ($P < 0.05$), while 503H microspheres hardly promoted H_2O_2 production.

Cytotoxicity of micro- and nanoparticles

Since clinical use is the final aim of these particulate carriers, toxicity studies were performed in human monocytic cell line, THP-1. Viable and dead cells can be differentiated by Trypan Blue exclusion assay, since the dye can only enter dead cells. After 24 h incubation of macrophages with particles, cell viability was not compromised. Microspheres showed cell viabilities above 97%, and nanoparticles above 90% (data not shown).

Absence of cytotoxicity was also observed by MTT assay. This technique correlates formazan production with cell viability, since a lower formation of this compound compared with particle-free media would be indicative of cell toxicity. For all formulations, formazan levels were always lower than negative control, indicating absence of toxicity. Even higher amounts ($P < 0.01$) were detected for cells incubated with 502H microspheres. They produced more than twice as much formazan as basal cells. This overproduction may be related to an activation of the metabolic status of the cells[22] by enhanced activity of mitochondrial dehydrogenase enzyme[26] (Figure 2).

Therefore, 502H and 75:25H microspheres, which showed the highest gentamicin loadings and were able to activate monocytes, were selected to perform further studies.

Intracellular location of Brucella, microparticles and gentamicin

In an attempt to determine whether microspheres and/or gentamicin reach pathogen's compartment, gold-labelled transmission electronic microscopy studies were carried out. In these studies microspheres of 502H and 75:25H polymers were selected for additional studies due to their lower cytotoxicity and higher drug loading compared with nanospheres of PLGA 502H as well as their ability for oxidative burst triggering.

Microparticles of both polymers were successfully phagocytosed and no degradation was observed 24 h post-incubation

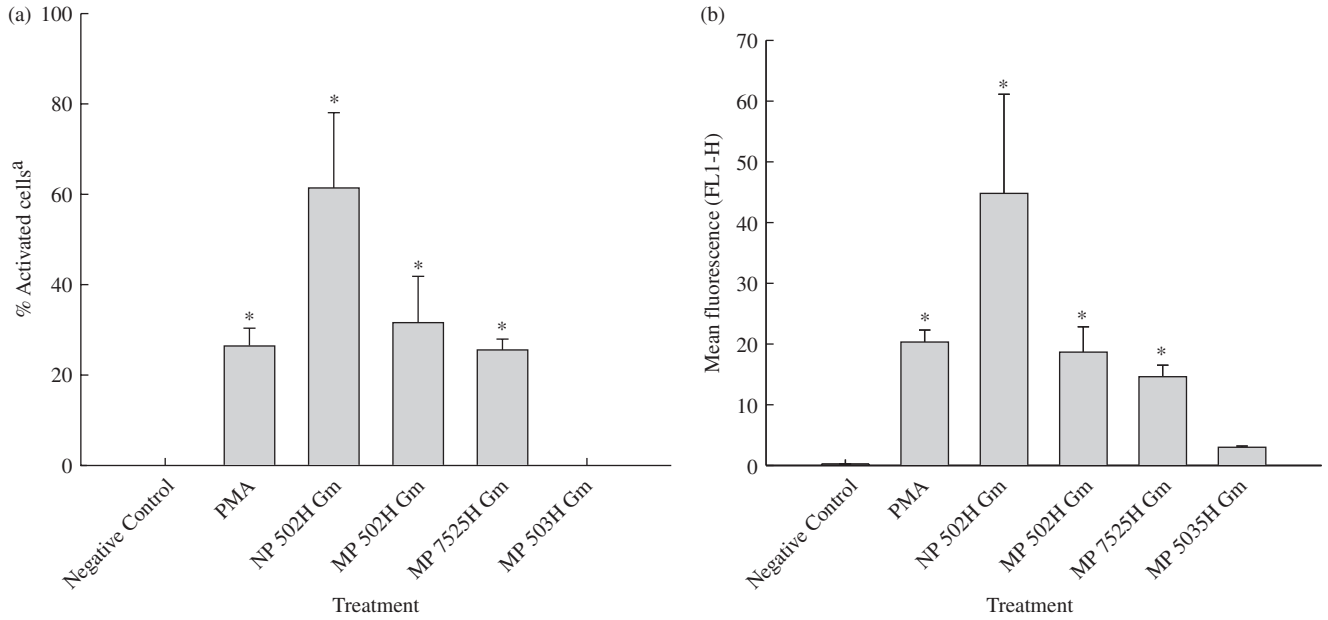

Figure 1. Oxidative burst of J774 macrophages after particle uptake. Results are expressed as (a) the percentage of activated cells ([a]percentage of cells producing oxygen reactives) and (b) mean fluorescence. Bars represent means ± SD of measurements performed on three different days. *$P < 0.05$, Dunnett test, compared with negative control.

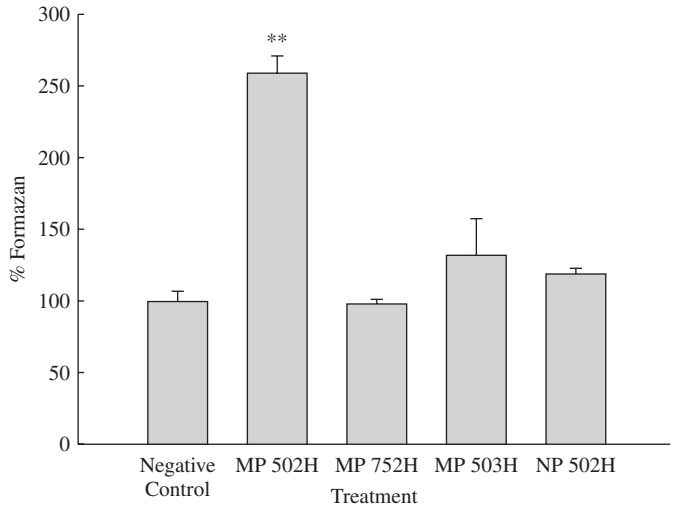

Figure 2. MTT assay: formazan production after incubation of THP-1 macrophages with PLGA formulations. Cells (10^6 cells/well) were incubated with 1 mg/mL of particle suspension for 24 h before the assay was performed. Results were calculated as the percentage of formazan production by cells co-incubated with particles with respect to incubation in particle-free medium. Results are expressed as means ± SD of three measurements. **$P < 0.01$, Dunnett test, compared with negative control.

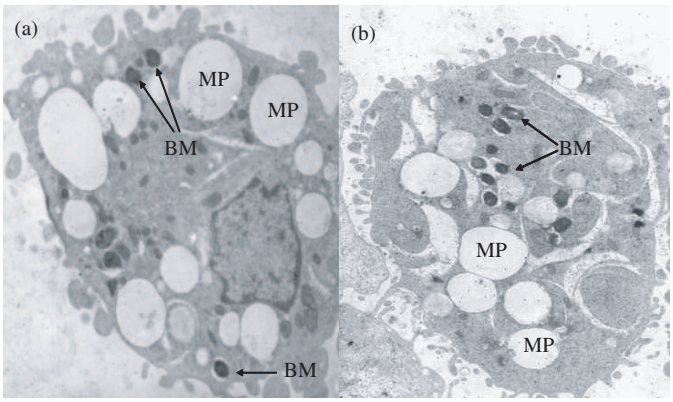

Figure 3. Immunogold labelling of THP-1 macrophages. Cells were infected with *Brucella melitensis* (BM) and further incubated with PLGA microparticles (MP): 75:25H (a) or 502H (b). Many phagocytosed microspheres (MP) can be observed but did not reach pathogen's intracellular compartment.

(Figure 3). However, after its release from microspheres, gentamicin was observed in cell cytoplasm and even in the nucleus (Figure 4). There was no evidence of fusion of endosomes harbouring *Brucella* and microspheres, but gentamicin was detected inside the pathogen's vesicles. It seemed that microspheres were degraded and the released antibiotic was distributed to cell cytoplasm and from there was able to enter *Brucella*'s niche (Figure 4).

Therapeutic efficacy of gentamicin-containing microspheres in intracellular elimination of B. melitensis in human monocytes

Intracellular *Brucella* reduction after co-incubation with PLGA gentamicin-loaded microspheres in THP-1 human monocytes was studied and compared with free antibiotic. A dose of 30 μg of gentamicin per well, either free or particle-loaded, was added to the cell culture. The same amount of empty particles was used as a control. Free gentamicin was able to eliminate 0.96 log of the intracellular bacteria, which meant that despite its hydrophilic nature it was able to enter monocytes under our experimental conditions, confirming previous results described above. Reduction of infection was higher ($P < 0.01$) when gentamicin was encapsulated into PLGA microspheres, being similar for both

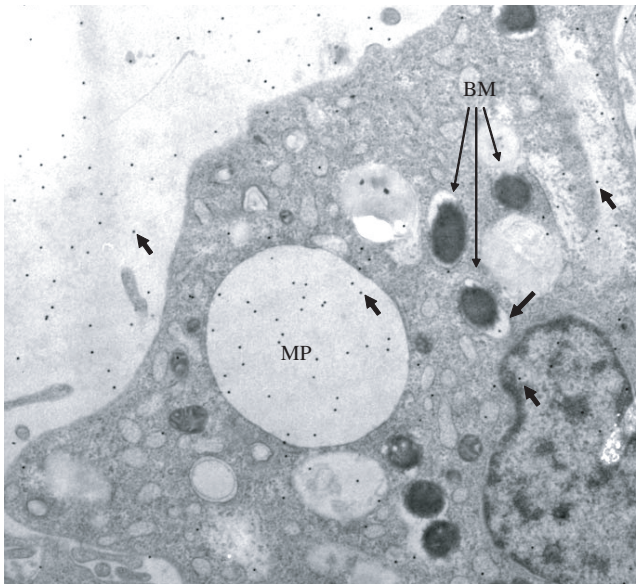

Figure 4. Detailed immunogold labelling of THP-1 macrophages infected with *Brucella melitensis* (BM) and treated with gentamicin-containing PLGA microparticles (MP). Gentamicin was detected (immunogold dots) not only inside microspheres, in cell cytoplasm and in nucleus, but also inside *Brucella*'s compartment (short arrowheads).

polymers: 2.17 logs for 75:25H microparticles and 2.18 for 502H microparticles. Unloaded particles did not show any bactericidal effect (Table 2).

Discussion

Gentamicin is an antibiotic that is rapidly bactericidal against *Brucella in vitro*; however, it crosses cell membranes poorly, and therefore its role in the treatment of human brucellosis is limited, and it must be used in combination with other antimicrobials.[27] Even then, treatment failures and relapses of human brucellosis remain a problem.[8] After its entry into the host, *Brucella* resides mainly in phagocytic cells, being able to survive and even replicate inside them.[4,28] The access of any antibiotic to *Brucella*'s niche is essential and 'drug delivery systems' may help to achieve this goal. Therefore, intracellular antibiotic efficiency of gentamicin-loaded microspheres in the context of *B. melitensis*-infected human monocytes was examined *in vitro* with a view to developing improved therapies for the treatment of brucellosis. We had previously reported that treatment with gentamicin microencapsulated into PLGA 50:50H microspheres decreased the number of intracellular bacteria significantly.[29] However, murine monocytes were used for infection studies with *Brucella abortus*. Now, we have considered the use of the more virulent *B. melitensis* species to *in vitro* infect human macrophages, in order to mimic the natural conditions during human brucellosis. On the other hand, the method of preparation previously used was based on the spray-drying technique that rendered a high degree of aggregation among particles.[30] Therefore, in the current report, solvent-evaporation method was the technique which successfully encapsulated gentamicin in PLGA nano- and microparticles.[31]

Microspheres obtained here, in the range of 2–4 μm, are expected to be successfully taken up by the monocytic-macrophagic system,[15,30] as well as nanoparticles that despite their smaller size should also be cleared by the phagocytic cells.[32] Actually, *in vitro* activation of human monocytes indicated that particles of end-group uncapped polymers triggered reactive oxygen production, with the exception of microspheres made by PLGA 503H, PLGA 502H being the most activating polymer. Nanoparticles made of this polymer promoted the highest burst, which could be related to its higher contact surface with the monocyte. Moreover, in agreement with Prior *et al.*[24] who described that less negatively charged particles were phagocytosed more efficiently, gentamicin-loaded nanospheres with a lower charge than microspheres of 502H polymer led to a higher cell activation upon uptake (Table 1).

Table 2. Efficacy of gentamicin (GEN)[a] free or loaded in PLGA microparticles against intracellular *Brucella melitensis* infection in THP-1 human macrophages

Treatment	Intracellular cfu (log) ± SD[b]	Reduction (log)[c]	Statistical significance[d]	
			e	f
Negative control	6.46 ± 0.03	0.00		
Free GEN	5.50 ± 0.02	0.96	**	
GEN-loaded 502H MP	4.28 ± 0.30	2.18	**	**
GEN-loaded 75:25H MP	4.29 ± 0.11	2.17	**	**
Unloaded 502H MP	6.45 ± 0.07	0.01		
Unloaded 75:25H MP	6.46 ± 0.06	0.00		

[a]Gentamicin concentration: 60 μg/mL.
[b]Data are the average ± SD of three measurements.
[c]Bacterial count reductions were performed compared with negative control.
[d]Tukey B test was performed for statistical analysis.
[e]Comparisons were performed between all treatments and control without treatment: $*P < 0.05$; $**P < 0.01$.
[f]Comparison between free and loaded microparticles with free gentamicin: $*P < 0.05$; $**P < 0.01$.

Molecular weight of PLGA polymers seems to influence reactive oxygen formation, since particles with higher molecular weight (503H microspheres; molecular weight 34 kDa) exerted no oxidative burst and 75:25 H (24 kDa) resulted in less activation than 502H (13.7 kDa).

Macrophage viability was not compromised after incubation with the PLGA formulations. Our results agree with literature data about lack of toxicity of these copolymers.[19,33,34] A slight 10% loss of viability observed with the nanoparticles could be due to extremely high oxidative burst triggered by these particles which would have released a high quantity of toxic free radicals from these cells. In addition, an increased activation status after 502H microsphere uptake was observed by MTT assay. This enhanced metabolic status was related to mitochondrial dehydrogenase activity, and our results suggested that it was independent of oxidative burst, since nanoparticles were unable to promote high formazan production but activated oxidative burst to a high extent.

Oxidative burst upon phagocytosis is known to play an important role in intracellular *Brucella* elimination since reactive oxygen production enhancers increased anti-*Brucella* activity of these cells, while inhibitors of these intermediates reduced their bactericidal activity.[17] Thus, optimal formulations should be able to trigger reactive oxygen production of macrophages.

Therefore, based on these observations, 502H and 75:25H microspheres, which showed the highest gentamicin loadings and were able to activate monocytes, were selected to perform further electron microscopy and immunocytochemistry studies.

By transmission electron microscopy lack of fusion was observed between both microspheres and the pathogen's niche. However, the antibiotic released from the particles was observed in the cytoplasm and nucleus and also entered the vacuoles harbouring *Brucella*. Once inside lysosomes, upon particle degradation,[13] the antibiotic would be released to cytoplasm.[35] Aminoglycosides are known to be lysosomotropic, because of their protonation state at acidic pH, but different amino acid transporters have been described in lysosomal membranes[36,37] which could help antibiotic translocation to the cytoplasm and subsequently enter the pathogen's compartment. These findings agree with Sandoval *et al*.,[38–40] who proposed that the antibiotic would follow a retrograde traffic from Golgi to endoplasmic reticulum and after its release in cytoplasm it would bind to mitochondria and nucleus.

Finally, the *in vitro* experimental infection demonstrated that the released gentamicin from particles was active, being able to exert its bactericidal effect inside the macrophagic cells. No differences were found between both 502H and 75:25H microsphere formulations.

In conclusion, gentamicin-loaded microspheres were effective in killing intracellular bacteria by way of efficiently targeting microencapsulated antibiotic into *B. melitensis*'s niche and by activation of oxidative burst in the macrophagic cells. These results support further studies in animal models with 502H and 75:25H microspheres carrying gentamicin for brucellosis treatment.

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Transparency declarations

None to declare.

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