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International Journal of Pharmaceutics 271 (2004) 125–135
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Influence of the co-encapsulation of different excipients on the properties of polyester microparticle-based vaccine against brucellosis

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Received 21 May 2003; received in revised form 31 October 2003; accepted 2 November 2003

Abstract

This work evaluates the influence of different pharmaceutical auxiliaries (Pluronic® F68, polyvinylpyrrolidone [PVP] or Tween 20), when mixed with an antigenic extract from *Brucella ovis* (hot saline; HS), on the characteristics of the resulting poly(ε-caprolactone) (PEC) and poly(lactide-co-glycolide) (PLGA) microparticles. In all cases, PEC microparticles were smaller than PLGA ones. Concerning the HS loading, PLGA microparticles were highly dependent on the type of the excipient used, whereas all the PEC formulations displayed similar encapsulation efficiencies. For both types of microparticles, the presence of PVP induced a burst release effect. On the contrary, the use of Tween 20 or Pluronic® F68 dramatically modified this profile. For PLGA–Tween 20 and PEC–Pluronic® F68 microparticles, the HS was released in a pulsatil way during the first 7 days followed by a continuous release for at least 3 weeks. The antigenicity of the HS components was kept in all cases. Phagocytosis by murine monocytes showed a clear difference based just on the hydrophobicity of the polymer, being PEC microparticles better engulfed. Cell activation quantified by the release of H_2O_2 did not showed major differences between batches, however, microparticles of PEC and Pluronic® F68 induced the highest nitric oxide production. Together, these results confirm the advantageous qualities of the "HS–PEC–Pluronic® F68 microparticles" as favorable candidate for vaccine purposes against brucellosis.
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Keywords: Brucellosis; Microparticles; Phagocytosis; Poly(ε-caprolactone); Poly(lactide-co-glycolide); Vaccine

1. Introduction

Brucellosis is a zoonosis caused by bacteria of the genus *Brucella*. Human brucellosis is a serious debilitating disease that also can produce long-lasting or chronic symptoms. Several species cause brucellosis in cattle, sheep, swine, dogs, and other wildlife animals, which is a disease of the reproductive tract which may cause abortion in females, infection of the sexual organs in males, and infertility in both sexes. For last decades, vaccination against *Brucella* infections in animals is usually performed by administration of live attenuated smooth *Brucella* strains such as *B. melitensis* Rev.1 (efficacious against *B. melitensis* and *Brucella ovis* in sheep and goats) and *B. abortus* S19 (against *B. abortus* in cattle). However, both vaccines may

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doi:10.1016/j.ijpharm.2003.11.008

cause abortion in pregnant animals, are pathogenic for humans, and induce antibodies that interfere with serological tests which employ smooth LPS as antigen (Corner and Alton, 1981; Blasco and Diaz, 1993). The rough strain *B. abortus* RB51 has recently been introduced in some countries, but present similar drawbacks and is not so effective (Jiménez de Bagüés et al., 1995; Kreeger et al., 2002). Therefore, development of fully avirulent vaccines appears to be justified.

B. ovis is a stable rough form which lacks of the *O*-polysaccharide side chains characteristic of the smooth strains of *Brucella*, but contains an outer membrane protein (OMP) profile similar to other members of the genus (Gamazo et al., 1989), recently confirmed by genomics and proteomics tools (Vizcaino et al., 1996; Tibor et al., 1999). A hot saline (HS) extract of *B. ovis* is being used as an antigenic complex for diagnosis in lambs infected with *B. ovis* (Marín et al., 1989). The HS extract of *B. ovis* consist in vesicles of lipopolysaccharide, phospholipids and OMPs as constituents (Gamazo et al., 1989; Riezu-Boj et al., 1986). These proteins are exposed on the surface of the bacteria (Riezu-Boj et al., 1990); do not show antigenic diversity (Cloeckaert et al., 1996); there is an homogenicity across strains of *B. ovis* and *B. mellitensis* of different geografic origin (Gamazo et al., 1989); and, they are immunogenic in animals infected with *B. ovis* and *B. melitensis* (Riezu-Boj et al., 1990; Zygmunt et al., 1994; Kittelberger et al., 1995). Moreover, monoclonal antibodies to OMPs have been shown to be highly protective in mice against *B. ovis* (Bowden et al., 2000). Assuming that the smooth-type *B. melitensis* Rev.1 vaccine protects sheep against rough-type *B. ovis*, we hypothesized that a subcellular vaccine containing outer membrane complex of *B. ovis* might be effective in protecting against infections by both rough *B. ovis* and smooth *Brucella*. Previous results demonstrated that protection conferred by HS was greatly enhanced by the incorporation of Pluronic® L121-MDP, QS-21 and other adjuvants (Blasco et al., 1993; Jiménez de Bagués et al., 1994). However, the need for revaccination did not fulfil our criteria for an appropriate vaccine for a field application.

We have previously demonstrated that hydrophobic micelles containing these major membrane antigens from *B. ovis* (HS extract) can be loaded in either poly(ε-caprolactone) (PEC) or poly(lactide-co-glycolide) (PLGA) microparticles when the HS extract was mixed with β-cyclodextrin (Murillo et al., 2001, 2002a,b) in order to prevent the antigen irreversible aggregation in aqueous and organic mediums. However, only HS-loaded in PEC microparticles conferred a protective effect against an experimental challenge with *B. ovis* or *B. abortus* (Murillo et al., 2001). In the present study, in a step forward, the aim was to determine the influence of amphiphilic polymers on the physicochemical characteristics and in vitro properties of the resulting microparticles containing the HS–β-cyclodextrin mixture. Microparticles were prepared from either PLGA or PEC and variables such as size, encapsulation efficiencies and interaction with monocytes were investigated. For this purpose, three pharmaceutical auxiliaries (Pluronic® F68, PVP and Tween 20) were selected by their capacity to contribute, together with β-cyclodextrin, in increasing the aqueous solubility of the HS extract. All of them have been previously used to modify the encapsulation efficiency, release properties and/or to stabilize proteins inside PLGA microparticles.

2. Materials and methods

2.1. Materials

Polyvinylpyrrolidone K-25 (MW: 28,000–34,000) was a gift from ISP Technologies (London, UK). Poly(D,L-lactide-co-glycolide) of 75:25 lactic to glycolic acid copolymer ratio with a MW of 98,000 (PLGA; Resomer RG756) was obtained from Boehringer Ingelheim (Germany). Polyvinylalcohol (PVA; MW: 115,000) and methylenchloride (MC) HPLC grade were purchased from BDH-Supplies (England). Pluronic® F68 and polysorbate 20 were obtained from Sigma Chemical Co. (St. Louis, MO, USA), β-cyclodextrin hydrate and poly(ε-caprolactone) (MW: 42,500) from Aldrich (USA). For cell culture, Dulbelcco's modified eagle medium (DMEM) with glutamax, foetal calf serum (FCS) and antibiotic solution (penicillin/streptomycin) were obtained from Gibco BRL (Paisley, Scotland). Trypan Blue solution, Giemsa stain, $NaNO_2$, sulfanilamide, *N*-(1-naphtyl) ethylendiamine dihydrochloride and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) were from Sigma. Lab-Teck II chamber slides were from Nunc (USA), HCl 1 N analytical grade

from Panreac (Barcelona, Spain), IFN-γ from Peprotech (London, UK) and dihydrorhodamin-123 from Bursttest (ORPEGEN® Pharma, Germany).

2.2. Extraction procedures of HS antigen

Hot saline extract was obtained from whole *B. ovis* cells as described previously (Gamazo et al., 1989). Briefly, 10 g of packed cells per 100 ml saline were heated in flowing steam for 15 min. After centrifugation (12,000 × *g*, 15 min) the supernatant was dialyzed and centrifuged (100,000 × *g*, 5 h) and the pellet (HS) was freeze-dried and stored at room temperature.

2.3. Preparation of antigen-loaded microparticles

Microparticles were prepared by a w/o/w emulsion solvent evaporation method as described previously (Murillo et al., 2001, 2002a,b). Briefly, approximately 4 mg HS were mixed with 4 mg β-cyclodextrin in a mortar and dispersed in 1 ml of either Pluronic® F68 6% (w/v), PVP 5% (w/v) or Tween 20 4% (w/v) in water. The polymer, PLGA or PEC, was dissolved in methylene chloride 4% (w/v) and emulsified with the antigen aqueous phase by probe sonication for 60 s under ice-cooling to form the inner w_1/o emulsion for 1 min at 13 W. Then, this w_1/o emulsion was dispersed into 30 ml of an aqueous phase containing 0.5% PVA by using a rotostator (Ultraturrax® T 20B, Janke & Kunkel & CO IKA Labortechnik, Staufen, Germany) at 1400 rad/s for 2 min. The resulting w_1/o/w_2 emulsion was stirrer for at least 2 h at room temperature conditions to allow the evaporation of the organic solvent. The microparticles were then collected by centrifugation at 11,000 × *g* for 5 min and washed two times with distilled water. Finally the microparticles were freeze-dried and stored at 4 °C.

Empty microparticles were prepared in the same way as described above with the only difference of the HS absence.

2.4. Characterization of microparticles

Microparticles were sized by laser diffractometry using a Mastersizer-S® laser size (Malvern Instruments, Malvern, UK). The average particle size was expressed as the volume mean diameter (V_{md}) in micrometers.

To quantify the antigen content, accurately weighed samples of freeze-dried microparticles were incubated with NaOH 0.1 M by shaking overnight. The sample was centrifuged (25,000 × *g*, 15 min) and the antigen concentration in the supernatant was determined by the BCA protein assay. The entrapment efficiency was determined by relating the total weight of antigen entrapped in the batch of microparticles to the initial weight of antigen. The HS loading was calculated as the amount of antigen entrapped per milligram microparticles.

2.5. In vitro release study

Freeze-dried microparticles (30 mg) were dispersed using a vortex in 1 ml PBS (10 mM, pH 7.4). The samples were maintained under horizontal agitation at 37 °C. At different time intervals after dispersion, 1 ml of the suspension was centrifuged (26,000 × *g*, for 5 min) and then again (47,000 × *g*, for 15 min) to remove extra small particles. Antigen concentration was determined in the supernatant by BCA assay. The medium was replaced with 1 ml fresh buffer after each determination.

2.6. Antigenicity studies

To evaluate the effects of manufacturing processes on the antigen structural integrity and antigenicity, HS-loaded microparticles were dissolved in DCM and evaporated under a vacuum stream. The HS released was resuspended in electrophoretic sample buffer. Proteins profile was determined by SDS–PAGE and its antigenicity by immunoblotting.

SDS–PAGE was performed in 13.5% acrylamide slabs with the discontinuous buffer system of Laemmli and gels stained either with Coomassie blue or alkaline-silver for proteins (Gamazo et al., 1989). Immunoblotting was carried out as described previously (Gamazo et al., 1989) with sera from a pool or rams naturally infected with *B. ovis*; and with peroxidase-conjugated goat anti-rabbit IgG (Nordic) and 4-chloro,1-naphtol as chromogen.

2.7. Phagocytosis studies

Phagocytosis of the different formulations tested by J774.2 murine monocyte-macrophages was deter-

mined as described previously (Prior et al., 2002). Briefly, the cells were cultured in complete medium consisting of Dulbecco's modified eagle medium (DMEM–L-glutamine), 10% heat-inactivated fetal calf serum and 0.1% antibiotic–antimycotic solution, and incubated at 37 °C and 5% CO_2. Monocytes were washed, collected by centrifugation and counted by Trypan blue exclusion; then, 2×10^5 cells in 400 μl of complete medium were added per well into Lab-Teck 8® chamber slides. Cells were incubated during 2–3 h and washed with DMEM–L-glutamine to remove non-adherent cells and FCS. Subsequently, 500 μl of DMEM–L-glutamine medium containing 100 μg of microparticles were added to each well and incubated for 1 h to allow phagocytosis of the particles. After incubation, the cells were washed with DMEM, fixed with methanol for 5 min and stained with Giemsa for 20 min. Microparticles uptake was determined by counting the number of phagocytic cells in which internalized particles could be visualized by light microscopy. At least 200 monocytes were scored per well. Results were expressed as percentage of phagocytic cells capable of phagocytosing at least one microparticle. To differentiate between particle uptake and particle deposition, some experiments were conducted at 4 °C.

2.8. In vitro oxidative burst effect of monocytes upon activation by microparticles

Respiratory burst was determined by the oxidation of dihydrorhodamin-123 to rhodamin-123 green fluorescent product and quantified in a flow cytometer (Becton Dickinson). Briefly, 20 μl of 1 mg/ml microparticles in DMEM–L-glutamine were incubated with 100 μl of 2×10^6 cells/ml for cultured J774 monocytes in test tubes (Falcon, Becton Dickinson) at 37 °C and 5% CO_2 for 4 h. Afterwards, dihydrorhodamin-123 was added, cells were fixed and washed, and the oxidative burst was measured from 10,000 cell events. Fluorescence (FL1-H) was measured using a logarithmic scale of 1024 channels. Monocytes incubated together with a suspension of opsonized *Escherichia coli* and monocytes without previous contact with particulate material were used as high and basal oxidative burst controls, respectively. Results were expressed as fluorescence increase relative to basal control.

2.9. Determination of nitric oxide production

Monocytes were culture at 2×10^5 cells/ml in a 96-well plate and incubated for 2 h at 37 °C, 5% CO_2. Non-adhered cells were removed by washing, and adherence cells were incubated with 40 μg microparticles either with or without 200 U IFN-γ for 24 h at 37 °C, 5% CO_2. NO was measured by quantifying nitrite (NO_2^-), a stable metabolite of NO, by the Griess reaction using known concentrations of sodium nitrite as standard (Ding et al., 1998).

2.10. Statistical analysis

Data are expressed as the mean ± S.D. of at least three experiments. For the in vitro release study, the means of HS released (ng HS/mg microparticles) were compared using a two criteria ANOVA test (Tukey's DHS test) to assess statistical significance. $P < 0.05$ was considered as a statistically significant difference.

3. Results

3.1. Microparticles characterization

Table 1 summarizes the main physico-chemical properties of HS-loaded microparticles as a function of both the nature of the polymer and the excipient used in the inner aqueous phase. In all cases, PEC microparticles were smaller than the PLGA ones, and Pluronic® F68 resulted the more efficient excipient to reduce the size of microparticles. For HS–PEC microparticles, the use of PVP 5% slightly increased the HS-loading and its encapsulation efficiency. On the other hand, when PLGA was used in the polymeric matrix, the influence of the nature of the excipient used in the inner aqueous phase dramatically influenced the HS-loading (Table 1). In this case, the use of Tween 20 increased two to four times the HS content as compared with microparticles prepared with Pluronic® F68 or PVP, respectively. In addition, when Pluronic F68 was used, the encapsulated efficiency was similar and independent of the type of polymer (around 30%, which represents an HS content of 3.8–5.2 μg/mg). In contrast, PVP affected negatively the HS-loading in PLGA microparticles.

Table 1
Physicochemical characteristics of the different HS-loaded microparticles

	Size (μm)	HS load (μg/mg particles)	Encapsulation efficiency (%)
PLGA (75:25)			
Pluronic® F68 6%	2.8 ± 0.2	3.8 ± 0.7	30.1 ± 0.5
PVP 5%	3.7 ± 0.3	1.9 ± 0.3	14.7 ± 4.0
Tween 20 4%	3.5 ± 0.2	10.8 ± 0.2	63.6 ± 1.5
PEC			
Pluronic® F68 6%	1.3 ± 0.5	5.2 ± 1.5	30.7 ± 5.9
PVP 5%	1.7 ± 0.9	6.4 ± 0.1	34.6 ± 0.7
Tween 20 4%	2.0 ± 0.2	5.4 ± 0.2	32.6 ± 1.3

Data express the mean ± S.D. ($n = 3$).

The stability of HS associated with microparticles was demonstrated by SDS–PAGE and immunoblotting analysis, indicating that the major antigenic proteins of HS were not significantly altered following entrapment in any of the microparticle formulations (Fig. 1).

3.2. In vitro release study

HS release from PLGA and PEC microparticles are shown in Fig. 2. For PLGA microparticles, the presence of either PVP or Pluronic® F68 in the inner aqueous phase displayed a marked burst effect. Effectively, around 70 and 40% of the loaded HS was released during the first hour from PLGA microparticles containing PVP or Pluronic® F68, respectively (Fig. 2). In contrast, PLGA–Tween 20 microparticles showed a burst effect below 20% of the loaded drug followed by a pulsatil and slow pattern release. Actually, after a first pulse during the first hour, a second pulsed appeared on day 2 followed by lag-phase of no antigen release and, then, on day 6 the profile was characterized by a continuous and slow release for 15 days. During this period of time, the amount of HS released was significantly higher than for PLGA–PVP and PLGA–Pluronic® F68 microparticles ($P < 0.05$). At the end of the experiment (day 28), only the 40% of the loaded antigen was released.

For HS-loaded PEC microparticles, the presence of PVP also induced a high burst effect. Around

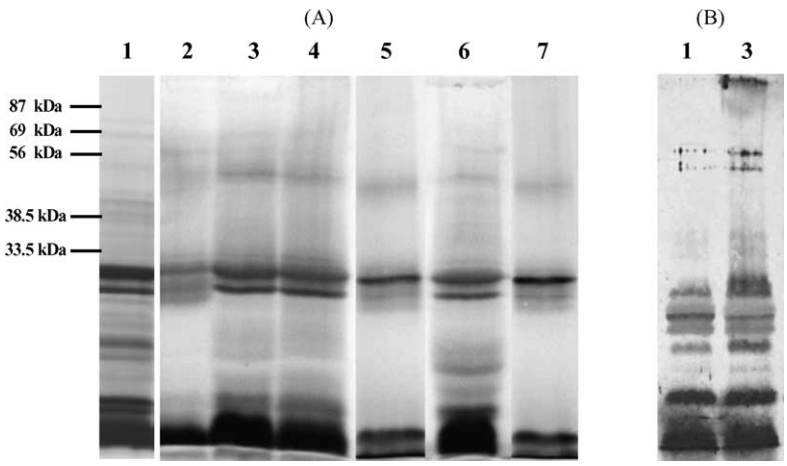

Fig. 1. Panel A shows SDS–PAGE of HS free (1) and HS released from the different HS-loaded microparticles; (2) PEC–PVP; (3) PEC–Pluronic® F68; (4) PEC–Tween 20; (5) PLGA–PVP; (6) PLGA–Pluronic® F68; (7) PLGA–Tween 20). Load was the equivalent to 12 μg HS/well. Silver stain was employed. Panel B shows Western blot analysis with a pool of sera from *B. ovis* infected rams of: (1) free HS, and (3) HS released from PEC–F68 loaded microparticles.

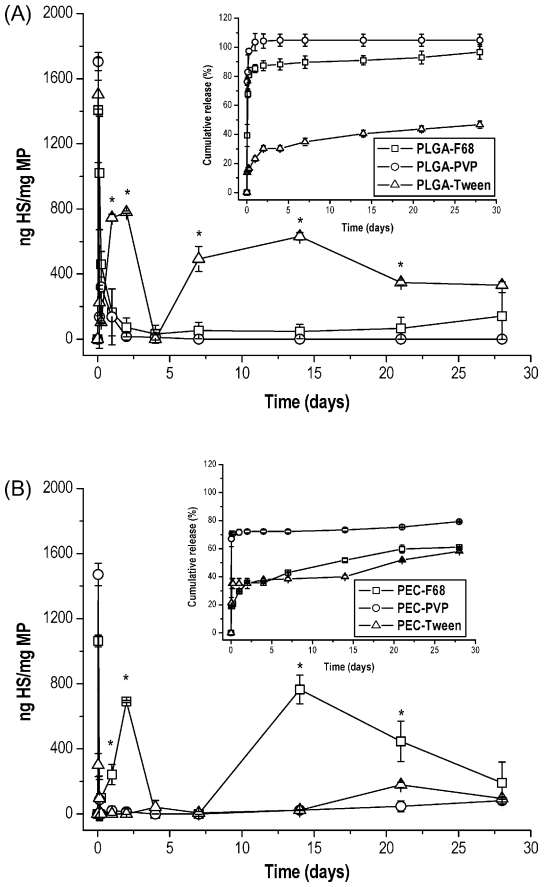

Fig. 2. HS released from PLGA microparticles (A) and PEC microparticles (B). Pluronic® F68 (□), PVP (○), Tween 20 (△). Data express the mean of the amount of HS released per milligram microparticles and day (mean ± S.D., $n = 3$). The means of HS released (ng HS/mg microparticles) were compared using a two criteria ANOVA test (Tukey's DHS test). $P < 0.05$ was considered as a statistically significant difference.

70% of the loaded HS was released in the first hour, and no significant release of the loaded antigen was observed during the remaining14 days of follow-up (Fig. 2). On the other hand, PEC–Tween 20 microparticles displayed a low burst effect (around 25% of the loaded HS) but no further significant release of the loaded antigen was observed till the third week. Finally, HS-loaded PEC microparticles containing Pluronic® F68 showed a first pulse during the first hours of incubation followed by a second pulse on the day 2 and a third pulse on day 14.

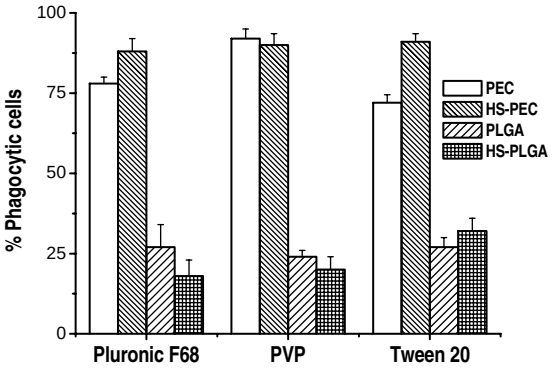

Fig. 3. Influence of excipient used and HS-loaded on the uptake of PEC (left) and PLGA (right) by J774 cells. Phagocytosis was calculated as the percentage of cells capable of uptaking one of more microparticles and expressed as the mean ± S.D. ($n = 3$). Light bars for empty particles and dark bars for HS loaded particles.

3.3. Phagocytosis of microparticles

Phagocytosis was determined by optical microscopy, counting the number of phagocytic cells capable of taking up one or more microparticles. The results obtained indicated that neither the antigen loading nor the pharmaceutical auxiliary used in the internal aqueous phase affects the internalization (Fig. 3). In contrast, and confirming the results described previously (Murillo et al., 2002b), significant differences were found depending on the polymer used, with a higher uptake of PEC-microparticles (72.3–92.3%) with respect to PLGA (17.5–32.9%). No microparticles were observed onto the surface of macrophages when the assay was performed at 4 °C, validating a real phagocytosis and not just an adsorption.

3.4. In vitro J774 cell activation

H_2O_2 and NO production were determined in order to compare the degree of activation of J774 cells upon in vitro incubation with microparticulated HS. With respect to H_2O_2 production, no differences were detected among PLGA or among PEC batches; hence, neither the excipients nor the polymers used have any influence on that activation parameter (not shown). Concerning NO, we studied the effect of microparticles on J774 cells in medium alone and in medium supplemented with IFN-γ. The results indicate that PEC–Pluronic® F68 microparticles were the only ones

Table 2
Determination of NO_2^- (μmol) released by 2×10^5 cells after 24 h of incubation with microparticles

	Medium	Medium + IFN-γ
Negative control	0.07 ± 0.02	0.00 ± 0.09
PEC–Pluronic® F68	7.86 ± 2.84	11.19 ± 2.53
PLGA–Pluronic® F68	0.29 ± 0.50	1.22 ± 0.77

Extrapolated from a standard curve for NO_2^-. Data express the mean ± S.D. ($n = 3$).

that induced NO release after incubation with cells (Table 2).

4. Discussion

The use of microparticles for the deliver of subcellular antigens may be a good strategy to prepare new, sure and effective vaccines. This approach would permit to overcome the main drawbacks related with the use of live vaccines and to potentiate the immune response to subcellular antigens, avoiding booster doses. In this context, we have developed some microparticulate formulations containing and antigenic extract from *B. ovis* (HS). Previous results have demonstrated that, in contrast to HS encapsulated in PLGA, the encapsulation of HS in PEC microparticles protected mice against experimental brucellosis induced by *B. ovis*, *B. abortus* (Murillo et al., 2001) or *B. melitensis* (data not published). All of these results were obtained with microparticles prepared by mixing the antigenic extract (HS) with β-cyclodextrin and subsequent dispersion in an aqueous solution of Pluronic® F68. The HS extract shows a very low water solubility and tends to generate irreversible aggregates in a number of organic and aqueous mediums. These problems were solved with the use of β-cyclodextrin (1:1 w/w mixture) (Murillo et al., 2002b).

In the present study, the aim was to determine the influence of amphiphilic polymers and different pharmaceutical auxiliaries on the physicochemical characteristics and in vitro properties of the resulting microparticles containing the HS–β-cyclodextrin mixture. First of all, in all cases, PEC microparticles were smaller than PLGA ones, reflecting the polymeric characteristics used. On the other hand, the size of microparticles prepared in the presence of Pluronic F68 was always smaller than those prepared with either PVP or Tween 20. These results are in agreement with Lin and Huang (2001) who described a similar effect for the encapsulation of BSA as model protein in PEC microparticles. These authors suggest the influence of a high HLB value of the surfactant on the capacity of the emulsifier to stabilize the inner aqueous phase and, thus, to reduce the size of the resulting microparticles. Therefore, it is possible that the lower values obtained with HS–PEC–Pluronic F68 would be due to the high HLB value of this surfactant (29.0 versus 16.7 for Tween 20; Hilbert et al., 1999). In addition, the fact of both dividing and preventing aggregation of the emulsion droplets would also decrease the mean particle size (Benoit et al., 1999). Overall, PEC microparticles obtained were smaller than PLGA ones, within the critical value of 1–2 μm optimal size to be captured either by monocyte-macrophage, dendritic cells, and Peyer's patches cells (Moore et al., 1995; Conway et al., 2001), claiming its potential use through oral administration.

High amounts of polymer may induce local inflammatory reactions (Yeh et al., 2002), therefore, high antigen loading per microparticle would be preferred. In this context, amphiphilic polymers have been proposed to modify the loading capacities and release properties of the resulting microparticles. In our work, none of the excipients tested significantly modified the HS loading by PEC microparticles. In all cases, the HS loading was calculated about 5–6 μg/mg, which represented encapsulation efficiencies close to 33%. On the contrary, for PLGA microparticles the type of excipient inside microparticles highly influenced the HS loading. This phenomenon could be associated with the lower hydrophobicity of PLGA, and, thus, with its lower precipitation rate comparing with that of PEC (Cao and Schoichet, 1999).

Therefore, HS–PLGA microparticles containing Tween 20 as the internal stabilizer surfactant achieved the highest loading (between three and five times higher than for Pluronic® F68 and PVP, respectively). These results are in agreement with those obtained from De Rosa et al. (2000) where Tween 20 increased insulin loading with respect to Pluronic® F68. In the same way, Rojas and co-workers also described that the use of Tween 20 in the inner aqueous phase improved the encapsulation of β-lactoglobulin in PLGA microparticles (Rojas et al., 1999a). This fact has been related to the ability of this surfactant to induce the formation of homogeneous aqueous globules in

organic phases containing PLGA than other excipients (Rojas et al., 1999b).

As we mention above, an important issue of delivery systems is the release of the antigen in a way that mimics booster or continuous doses after a single administration. A number of amphiphilic polymers have been proposed to modify the release profile of proteins from microparticles (Lin and Huang, 2001; Rojas et al., 1999a,b; Tobio et al., 1999; Sanchez et al., 1999; Morita et al., 2001). The presence of PVP in both PLGA and PEC microparticles induced a large burst release of HS in 24 h amounting to 100 and 70%, respectively, of the antigen load. A large burst effect may be explained by the presence of a high amount of HS on the microparticle surface and/or its release through pores and channels. In fact, the high hydrophylicity of PVP (as revealed by its low partition coefficient in the methylenechloride/water system) has been related with the capacity of this excipient to migrate to the outer water phase during the solvent evaporation process (Morita et al., 2001), which might accelerate the release of HS by forming aqueous channels.

On the contrary, the use of Tween 20 dramatically reduced the burst effect observed previously. In fact, between 18% (for PLGA microparticles) and 35% (for PEC microparticles) of the loaded HS was released in the first 24 h. The association of Tween 20 to PLGA microparticles permitted to obtain a second release pulse in day 2 and a third pulse in day 7, at which the HS was released in a continuos way at least for 2 weeks more (Fig. 2) and the amount of HS released with these surfactant differs significantly in comparison with both F68 and PVP.

However, for PEC microparticles, after the burst effect a lag time of at least 2 weeks was observed (Fig. 2). All of these findings are in accordance with previous results from Rojas et al. (1999a,b) in which Tween 20 appeared to be able to reduce the number of aqueous channels between the internal aqueous droplets as well as those communicating with the external medium. Therefore, Tween 20 would induce the formation of a more dense structure of microparticles, decreasing the burst release. Concerning the effect of Pluronic® F68 on the release profile of HS from the microparticles, we can observe a different profile depending on the nature of the polymer used. For PLGA microparticles, the burst effect was of about 80% of the loaded antigen whereas PEC microparticles exhibited a burst effect of 20% followed by a second pulse in day 2 and a third pulse in day 7 followed by a continuos release for 2 weeks (see Fig. 2). This profile is quite similar to that observed for PLGA microparticles containing Tween 20. Pluronic® F68 has been widely employed to modify the release pattern of proteins from microparticles (Tobio et al., 1999; Sanchez et al., 1999; Morita et al., 2001; Yeh et al., 1996) and to prevent the possibility of irreversible interactions between proteins and either the PLGA polymers or the acidic degradation products of the polymer (Tobio et al., 1999). Blanco and Alonso (1998) reported that the addition of Pluronic® F68 to PLGA microsperes modified the release of a model protein by inhibiting the interaction between the protein and polymer. On the other hand, different authors have been reported strong evidences for chain entanglement and/or complex formation between polyesters and Pluronics in organic solutions (Yeh et al., 1996; Blanco and Alonso, 1998; Park et al., 1992). This fact would affect the physico-chemical properties of the resulting microparticles (size, HS loading) and the release characteristics. Therefore, the HS release from microparticles containing Pluronic® F68 can be considered to arise from a combination of rapid dissolution of the surfactant from the microparticle and extraction of the antigenic components which basically eliminates the lag phase (Yeh et al., 1996).

This hypothesis agrees well with the in vitro release results displayed here. The high burst effect displayed by PLGA microparticles could be correlated with a high degree of chain entanglement between the non-ionic surfactant and the amorphous polymer. However, the interaction between Pluronic® F68 and PEC would be minimized by the low mobility of the crystalline domains of this polymer and, thus, the possibility of a extensive pore structure of the resulting microparticles would be impaired. In addition, since the crystalline phase of PEC is essentially impermeable to water (Yeh et al., 2002), the HS rate release would be lower from these microparticles than from PLGA ones. This explanation is supported from evidences concerning both the lower burst effect and the longer lag-time observed for PEC microparticles containing PVP and Tween 20, respectively, in comparison with PLGA microparticles.

Protein stability is considered to be of crucial importance for biological activity, SDS–PAGE and

immunoblotting analysis indicate that the protein profile of HS and antigenicity were not altered following entrapment in microparticles. The combination of β-cyclodextrin and the other excipients may be responsible for the maintenance of the antigenic properties of the HS extract, like reported by other authors by using different biomolecules (Lin and Huang, 2001; Rojas et al., 1999a,b; Tobio et al., 1999; Sanchez et al., 1999; Morita et al., 2001; Yeh et al., 1996; Blanco and Alonso, 1998). Some quantitative differences were appreciated comparing the respective pattern profiles obtained by the different formulations. It is possible that physico-chemical nature of HS protein components could mediate on those differences, but we do not have any experimental data to support it yet. However, the similar pattern of antigenicity revealed by immunoblotting indicates that those differences probably are not biologically conspicuous.

Particle uptake by phagocytic cells is largely affected by physicochemical properties of the particle surface, particularly by its size (Ahsan et al., 2002; Men et al., 1999), as was mentioned above, and hydrophobicity (Müller et al., 1997). In general, an increase in particle surface hydrophobicity leads to an enhanced uptake (Ahsan et al., 2002; Torché et al., 2000). In our study, neither the antigen loading nor the excipient used in the internal aqueous phase affects the internalization of microparticles by macrophages. In contrast, significant differences were found depending on the polymer used, with a higher uptake of PEC-microparticles with respect to PLGA, probably due to the higher hydrophobicity of PEC.

In addition to drug delivery, the ingestion of microparticles may result in the activation of macrophages and, subsequently, enhance its properties as antigen presenting cells (Artursson et al., 1997). We studied H_2O_2 and NO production in order to compare the degree of activation of J744 cells upon in vitro incubation with microparticulated HS. With respect H_2O_2 production, no differences were detected between PLGA or among PEC batches; hence, the excipients used did not have any influence on that activation parameter. Similar results were obtained by Jackson et al. (2000) who studied the activation of PMN with PLA and PEC microparticles.

On the contrary, PEC–Pluronic® microparticles were the most active NO inducers. It has become increasingly apparent that RNI (reactive nitrogen intermediates) are potentially important regulators of the immune system (Babior, 1983). Activation of macrophages by membrane activation induces the release of oxygen radicals; whereas, activation caused by internalization is when RNI are produced. This means that there may exist a correlation between NO production and real phagocytosis. Our results confirm that hypothesis, as PEC microparticles were more phagocytosed that the PLGA ones. Additionally, these results confirm our previous findings concerning IFN-γ production after HS–PEC immunization in contrast to using HS–PLGA microparticles (Murillo et al., 2002b). Finally, the interaction of macrophages with PEC-microparticles, and further NO production, should be considered also as a direct way to control the infecting organism, as a major role of NO in the intracellular killing of *Brucella* by murine macrophages has been described (Gross et al., 1998; Lopez-Urrutia et al., 2000).

In conclusion, the in vitro release profile, antigenic conservation, and activation of macrophages, it is suggestive that HS–PEC–Pluronic® F68 microparticles may represent a serious alternative to the conventional attenuated vaccines against brucellosis. Taken together with results obtained in the mouse model (Murillo et al., 2001), that this subcellular preparation would comply the requisites for testing it the natural hosts. Our results demonstrate again that HS–PEC–Pluronic® F68 microparticles would comply better for vaccine purposes as exhibited a pulsatil release of the antigen (see Fig. 2).

Acknowledgements

M. Murillo was financially supported by a fellowship grant from “Departamento de Educación y Cultura” (Gobierno de Navarra, Spain). This work was supported by grants from the “Ministerio de Ciencia y Tecnología, CICYT” (Program Project Research Grants BIO097-0777-C03 and AGL 2000-0299-CO3), and from the Spanish “Instituto de Salud Carlos III” (Project Red G03/204).

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