Brucella outer membrane complex-loaded microparticles as a vaccine against *Brucella ovis* in rams

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Abstract

Due to the important drawbacks of the *Brucella melitensis* Rev1 vaccine, a safer vaccine based on an outer membrane complex from *Brucella ovis* encapsulated in poly(ε-caprolactone) (PEC) microparticles was developed and tested in rams. Homogeneous batches of microparticles were prepared by a new double emulsion solvent evaporation method called TROMS ("Total Recirculation One-Machine System"). Such microparticles presented a mean diameter of 2 μm and displayed an antigen loading of about 13 μg HS per mg of microparticles. Subcutaneous vaccination of rams with 800 μg HS (hot saline antigenic extract of *B. ovis*) in PEC microparticles induced an adequate serological response against *B. ovis* antigens and conferred similar protection against challenge with *B. ovis* to that induced by the living attenuated *B. melitensis* Rev1 reference vaccine. By contrast, lower doses (80 μg) of HS-PEC evoked reduced serological responses against *B. ovis* antigens and did not induce significant protection. The revaccination with 800 μg of HS-PEC increased the intensity and duration of the serological response against *B. ovis* antigens but did not improve the protection conferred by the single vaccination. Sample sera taken from any of the animals immunized with Rev1 were seropositive in both Rose Bengal and the

Complement Fixation tests used for the diagnosis of smooth *Brucella* infections. By contrast, no positive reactors in both tests were recorded in the animals vaccinated with HS-PEC, being this a target objective of this study. HS-PEC microparticles can be used as a safe vaccine against brucellosis in rams, but further studies using higher doses of antigens are necessary to exploit their full potential for the prophylaxis of brucellosis in sheep.

1. Introduction

Brucella ovis causes a clinical or subclinical chronic disease in ovine that is characterized by genital lesions and low fertility in rams and placentitis and abortions in ewes [3]. With few exceptions, the infection occurs in most sheep raising countries and vaccination is recognized as the most suitable tool for *B. ovis* control in endemic situations [3]. The smooth attenuated *B. melitensis* Rev1 living strain, is the best vaccine available against *B. ovis* [2, 4, 8, 16, 26]. However, the subcutaneous vaccination with this strain elicits a strong serological response interfering with the serological diagnosis of *B. melitensis* [8, 16, 21, 26]. Other disadvantages of Rev1 are that its use is not allowed in some countries free from *B. melitensis*, and that this strain can be pathogenic for humans [5]. Another commercial available vaccine against brucellosis is the live attenuated *B. abortus* RB51 vaccine, however, although does not interfere with the serological diagnosis of *B. melitensis*, it has been proven to be ineffective against *B. ovis* in rams [22]. Consequently, new brucellosis vaccines should be developed, and the acellular preparations offer a safer alternative.

The selection of the antigenic components was based on preliminary studies [17, 34] where we shown that sera from naturally *B. ovis* infected rams developed an intense antibody response against the structural components an outer membrane complex (hot saline extract, HS) of *B. ovis*. Furthermore, these HS extracts incorporated in adequate adjuvants induced significant levels of protection against *B. ovis* in mice, without interfering in serological tests for smooth (S) *Brucellae* [6], suggesting that the HS might be a good subcellular vaccine candidate against *B. ovis*. In fact, a vaccine containing the the HS extracts from *B. ovis*, and incorporated in a multiple emulsion adjuvant based on oil squalene, muramyl dipeptide and block co-polymer Pluronic-L121, was as effective as the Rev1 vaccine against experimental *B. ovis* infection in rams [6], with the additional advantage that the immunization with HS did not induce interferences in the Rose Bengal and Complement Fixation tests used for diagnosing infections due to smooth *Brucellae*. However, the need of a booster dose and the excessive cost of the adjuvant components prevented the practical

application of this vaccine in the field. Therefore, we searched on the development of particulate control release adjuvants. Polymer microparticles prepared from both synthetic and natural biodegradable macromolecules are being of potential interest as adjuvants [24, 32, 38]. They are able to deliver antigens to antigen presenting cells either via phagolysosomes or cytosol, which are processed and presented to specific T cells after homing to secondary lymphatic tissues, thus. The synthetic polyester poly(ε-caprolactone) (PEC) was finally selected among other candidates because of adequate tissue compatibility, biodegradability, low cost and regulatory approval [13]. Preliminary experiments demonstrated that the HS complex incorporated in PEC-microparticles (HS-PEC) induced adequate immune response and protection against experimental brucellosis in mice [31]. In consequence, the objective of this work was to evaluate the efficacy of HS-PEC in rams, the natural host, and, then compare this new vaccinal formulation with the Rev1 commercial reference vaccine. For this purpose, microparticles were prepared after the formation of a multiple emulsion by TROMS (Total Recirculation One-Machine System) [15] and subsequent solvent evaporation. This new method relies on the turbulent injection of the phases, thus avoiding the use of aggressive homogenisation techniques, yielding homogeneous batches of microparticles.

2. Material and methods

2.1. Bacterial strains

The reference live vaccine *B. melitensis* strain Rev1 and the challenging *B. ovis* strain PA were kindly provided by Dr. J.M. Verger (Laboratoire de Pathologie Infectieuse et Immunologie. INRA, France). For vaccination (Rev1) or challenge (*B. ovis* PA) in rams, freshly bacterial suspensions were prepared as described previously [19]. Briefly, the freeze-dried strains were rehydrated in sterile Buffered Saline Solution (BSS; 0.015 M NaCl, 7mM KH_2PO_4, 10 mM K_2HPO_4; pH 6.85) and grown on Blood Agar Base # 2 (BAB; Biolife) containing 10% sterile bovine serum (Seromed, Biochrom) for 72 h at 37 ºC in 10% CO_2. Cells

were harvested in BSS, spectrophotometrically adjusted in BSS to a A_{600} value of 0.17 (aprox. 10^9 CFU/mL; exact doses were assessed retrospectively by dilution and plating).

The serum and CO_2 independent *B. ovis* REO 198 strain used for antigen extraction was also kindly provided by Dr. J.M. Verger (Laboratoire de Pathologie Infectieuse et Immunologie. INRA, France).

2.2. Extraction and characterization of the HS antigenic complex

HS was obtained from *B. ovis* REO 198 as described previously [17]. To prepare cells for extractions, a thawed vial of stock suspension was streaked onto BAB plates and 24 h cultures inoculated in Trypticase Soy Broth (TSB; Difco, Detroit, Michigan) flasks, which were incubated at 37 ºC for 48 h in air with constant shaking. Live cells were suspended in saline solution (0.85% NaCl) (10 g packed cells per 100 mL), and heated in flowing steam for 15 min. Following centrifugation at 12,000 x *g* for 15 min, the supernatant was dialyzed for five days at 4 ºC against several changes of deionized water (dH_2O). The dialyzed material was ultracentrifuged for 3 h at 60,000 x *g* and the pellet (HS) washed in dH_2O, and freeze-dried. Total protein was determined by the method of BCA [37], with bovine serum albumin as standard. Analysis for 2-keto-3-deoxyoctonate (KDO, exclusive marker of LPS) corrected for 2-deoxyaldoses was performed by the method of Warren modified by Osborn [33]. The batch of antigen used to prepare the vaccine formulation contained 46.2 ± 4.08 % protein and 39.5 ± 3.82% rough lipopolysaccharide (R-LPS).

Immunoblotting. It was carried out as described previously [12]. After SDS-PAGE of 20 µg of HS per well, performed in 15% acrylamide slabs, proteins were transferred to Immovilon membranes (Immovilon P, Millipore Corp., Bedford, Mass.) using a semi-dry transblotter (Bio-Rad Laboratories, Richmond, CA). Blots were placed in a blocking buffer (3% skimmed milk and 0.05% Tween 20 in 10 mM phosphate-buffered saline, pH 7.2) overnight at room temperature. Incubation with sera (1:50 in blocking buffer without milk) was performed

overnight at room temperature. Peroxidase-conjugate rabbit anti sheep IgG, H+L (Nordic Immunological Lab, Tilburg, Netherlands) was used diluted 1:500 in same buffer as sera, and the incubation during 2 h at room temperature. Peroxidase activity was detected by incubation with a solution containing H2O2 and 4-chloro-1-naphtol for 20 min in the dark. Immunoblotting assay was performed with blood samples taken from each animal during the course of the experiment. The apparent molecular masses of the proteins present in the antigenic extracts were determined by comparing their electrophoretic mobility with that of molecular mass markers (Rainbow colored protein molecular weight marker, Amersham Pharmacia Biotech.).

Immunodot blot analysis. Samples of 20 μg HS were spotted onto a nitrocellulose membrane (Schleicher & Schuell). Membrane was blocked with blocking buffer and incubated with monoclonal antibodies (*see* below) for 4 h at 37 ºC. These blots were developed after incubation with peroxidase-conjugated goat anti-mouse immunoglobulins (Nordic) and 4-chloro-1-naphthol-H_2O_2 as the substrate. Monoclonal antibodies used were specific for epitopes of the following strctures: LPS-outer core [11]; LPS-inner core [11]; LPS-lipid A [11]; L-Omp10 [35]; L-Omp16 [35]; L-Omp19 [35]; Omp25 [35]; Omp31 [35]; Omp2b [35].

2.3. Preparation of the adjuvant based on microparticles

Microparticles containing HS were prepared by the solvent extraction/evaporation method previously described [30]. However, in this case, the multiple emulsion was prepared by TROMS [15]. This method is easily reproducible and applicable on a semi-industrial scale, minimise human intervention during the production phase and affording homogeneously sized microparticles. All of these properties facilitate the implementation of GMP conditions in the preparation of microparticles. For the preparation, briefly, the first step was to mix the HS with different excipients in order to facilitate its dispersion in the aqueous inner phase (W_1). Thus, the antigenic extract was mixed in a moratr for 30 min with β-cyclodextrin and the mixture was then dispersed in

the aqueous phase containing Pluronic® 6% w/v. On the other hand, the organic phase (O) containing a 4% poly(ε-caprolactone) dissolved in methylenchloride, was injected through a needle (inner diameter of 0.12 mm) into a first vessel containing the aqueous phase by activation of the pumping system (pumping flow of 50 mL/min). Then, the W_1/O emulsion was forced to circulate through the system for 2 min. After this homogenisation step, the first emulsion was injected (maintaining the pumping flow constant) into a second vessel containing the outer water phase (W_2: 0.5% solution of polyvinylalcohol). The turbulent injection through the needle (inner diameter of 0.17 mm) resulted in the formation of a multiple emulsion ($W_1/O/W_2$), which was further homogenised by circulation in the system during 4 min. Then, the multiple emulsion was stirred with a blade stirrer for 2 h to eliminate the organic solvents by evaporation. Finally, the resulting microparticles were washed 3-times with water by consecutive centrifugation at 4 ºC (11,000 x *g*, 5 min.). Finally, the microparticles were suspended in 2 mL ultrapure water, frozen at –80 ºC and lyophilised (Genesis 12EL, Virtis).

2.4. Microparticle Characterization and antigen content

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

Protein content was determined by the BCA assay [37]. Twenty mg of dried MP were treated with 0.1 M NaOH by shaking overnight on a magnetic stirrer. The sample was centrifuged (25,000 x *g*, 15 min) and the BCA assay was used to determined the protein concentration in the supernatant against a series of antigen concentrations in 0.1 M NaOH. Each sample was assayed in triplicate. The entrapment efficiency was determined by relating the total weight of HS entrapped in the batch of MP to the starting weight of HS.

2.5. Animals and vaccination procedure

The experimental test performed in rams was performed according to the method described previously [6] in compliance with the European legislation on animal experiments (86/609/EU). A total of 61 three months old Aragonesa rams belonging to the brucellosis free flock from the unit of animal production (CITA-Zaragoza) were used. Once randomly allotted in separate pens, twelve rams were subcutaneously vaccinated with 1 mL of MP solution in dH_2O containing a total of 0.08 mg of HS antigen. Twenty four rams were subcutaneously vaccinated with 1 mL of MP solution in dH_2O containing a total of 800 μg of the HS complex. Eight weeks after this vaccination, the half of these rams were revaccinated with the same amounts of HS antigen (800 μg) in MP. Twelve rams were subcutaneously vaccinated with 1.6×10^9 UFC (in a volume of 2 mL) of the live *B. melitensis* Rev1 vaccine, and the remaining 13 rams were kept unvaccinated as controls. All animals were vaccinated the same day and in the same anatomic region (left elbow), placed in separated pens and fed *ad libitum* during the whole experiment. During the three days following vaccination all rams were inspected for rectal temperature and local reactions at the inoculation site.

2.6. Serological studies

Each animal was bled before vaccination and then blood samples were taken weekly or fortnightly after vaccination for serological studies. All serum samples were submitted to the modified Rose Bengal test (RBT) and the standard Complement Fixation test (CFT) to evaluate the serological interference induced against smooth *Brucellae*, and to the indirect ELISA with HS antigen to evaluate the serological response against *B. ovis*. The modified RBT using the standard *B. abortus* antigen was performed as described previously [7]. The CFT using the *B. abortus* antigen was performed using the standard warm microtechnique [1]. The indirect ELISA was carried out in standard 96-well polystyrene Maxisorp surface plates (Nunc TM) as described previously [25, 28]. Briefly, the HS antigens were suspended in phosphate buffered saline (PBS; 0.01 M, 0.85% NaCl, pH 7.2),

added to plates (100 µL/well) and incubated at 4 ºC overnight. The optimal antigen concentration (1 µg/well) was determined previously by titration against a panel of sera from culture-positive and *Brucella*-free rams. Non-absorbed antigen was removed by three washings with PBS containing 0.05% Tween 20 (PBS-Tween). Different serum dilutions in PBS-Tween were placed (100 µL/well) on the plates, incubated for 1 h at 37 ºC and washed three times with PBS-Tween. A conjugate protein G-peroxidase was used for the detection of ram IgG (Pierce Chemical Co.). The optimal dilution of conjugate (0.3 µg/mL) was dispensed (100 µL/well) and the plates incubated at 37 ºC for 1 h and washed three times with PBS-Tween. The reaction was developed (100 µL/well) with 0.1% ABTS (Sigma Chemical Co.) in citrate buffer (pH 4) containing 0.004% hydrogen peroxide. The reaction was not stopped and the optical densities at 405 nm (Labsystems Multiskan RC) assessed after 15 and 30 min of incubation. The same reference sera from a culture positive and *Brucella* free rams were tested on each plate as controls. The individual results (duplicate experiments) were expressed as the percentage of the absorbance of the positive serum.

2.7. Challenge and bacteriological studies

Eight months after vaccination, all rams were experimentally infected with 1.16×10^9 CFU of *B. ovis* PA (conjunctivally [25 µL] and preputially [25 µL]). At weekly intervals after challenge all rams were clinically examined for the presence of eventual lesions in testicles and epididymides. Nine weeks after challenge all rams were slaughtered and submitted to individual necropsy for bacteriological examinations. Cultures were performed on portions of spleen and epididymides, and the whole vesicular glands, bulbourethral glands, ampullae, and cranial (submaxillary, parotid and retropharyngeal), iliac, scrotal, prefemoral and prescapular lymph nodes. The samples were homogenized either by using a blender or Stomacher (Seward Medical, London, UK) after adding 20 mL of BSS for spleen and epididymides, or 5 mL for the rest of samples. One mL of each homogenized was smeared onto each of two plates of modified Thayer-Martin's medium containing 100.000 IU Nystatin per liter of culture medium, a

modification that increases sensitivity [27], and incubated for 7-10 days at 37 ºC in a 10% CO_2. *Brucella* colonies were identified by morphology, gram staining, oxidase and urease tests, CO_2 requirement and phage typing [24]. A ram was classified as infected if at least one *B. ovis* CFU was isolated from any of the organs and lymph nodes sampled at necropsy. The *B. melitensis* Rev1 vaccine strain was not isolated from any sample, and all *Brucella* isolates corresponded always to *B. ovis*.

Statistical comparisons were performed using the Chi-Square test (with Yates correction), using the Stat-View Graphics programs for Windows (5.0, SAS Institute Inc. Copyright©).

3. Results

3.1. Physicochemical characterization of the HS-PEC vaccine

The size of microparticles prepared by TROMS was 2.00 ± 0.10 μm. The entrapment efficiency of HS into the HS-PEC vacine was calculated to be 64%, which corresponded with a HS loading of 12.8 ± 0.26 μg/mg. As previously described [9, 17] the HS used was confirmed to contain HS R-LPS and a variety of outer membrane proteins: Omp10, Omp16, Omp19, Omp25 and Omp31, confirmed by Dot-blot analysis with monoclonal antibodies (not shown). The evaluation by SDS-PAGE and immunoblotting desmonstrated that the microencapsulation process was safe and adequate to preserve the antigenicity of the major HS antigens (not shown).

3.2. Animal studies

3.2.1. Immunization

The SC inoculation of the vaccines (HS-PEC and Rev1) produced, in some cases, side effects. The mean rectal temperature was within normal limits in all the rams vaccinated with HS-PEC, but increased slightly during the first week after

vaccination in the animals vaccinated with the live *B. melitensis* Rev1 strain. In order to evaluate the local tissue reactivity, macroscopical and patho-histological examinations of tissues were carried out in the location of adjuvant administration. The check up indicated very low local reactogenity of the experimental HS-PEC vaccine that fully resolved few days after vaccination. In contrast, from moderate to intense inflammatory reaction at the inoculation site was observed in all rams vaccinated with Rev1, although resolved during the first two weeks after inoculation.

3.2.2. Antibody response against S-*Brucella* (CFT/RB tests)

One of the major aims of this work was to develop of new vaccine which, being protective, it should not interfere with the diagnosis of infections due to smooth *Brucellae*. Results indicate that sera taken from any of the animals immunized with Rev1 were seropositive in both RB and CF tests during the first two weeks after vaccination. Moreover, this positive serological response was maintained in over 50% of Rev1 vaccinated animals until the end of the experiment. By contrast, no positive reactors in any of these tests were recorded in the animals vaccinated with HS-PEC at any time post-vaccination.

3.2.3. Antibody response against *Brucella ovis* antigens (HS-ELISAi/Immunoblotting tests)

Figure 1 shows the evolution of the iELISA IgG specific response against the HS antigens elicited after vaccination. At the time of revaccination (week 8), over 70% of the animals vaccinated with the higher dose of HS-PEC (800 μg) resulted positive in this tests, in contrast with rams immunized with the lower dose of HS-PEC (40%), or Rev1 vaccinated ones (50%). At the time of challenge (week 32 post-immunization) all the animals vaccinated with 800 μg HS-PEC or with Rev1 were negative in the HS-iELISA test. On the contrary, 50% of the animals vaccinated with 800 μg of HS-PEC and 90% of revaccinated with the same

preparation remained seropositive, demonstrating the effect of reimmunization on the increase of the specific antibody response. The challenge with *B. ovis* PA induced an anamnestic antibody response to HS antigens in all vaccinated animals, and above 90% of animals in all groups, including the unvaccinated controls, resulted positive in the HS-iELISA until the moment of slaughtering (Figure 1).

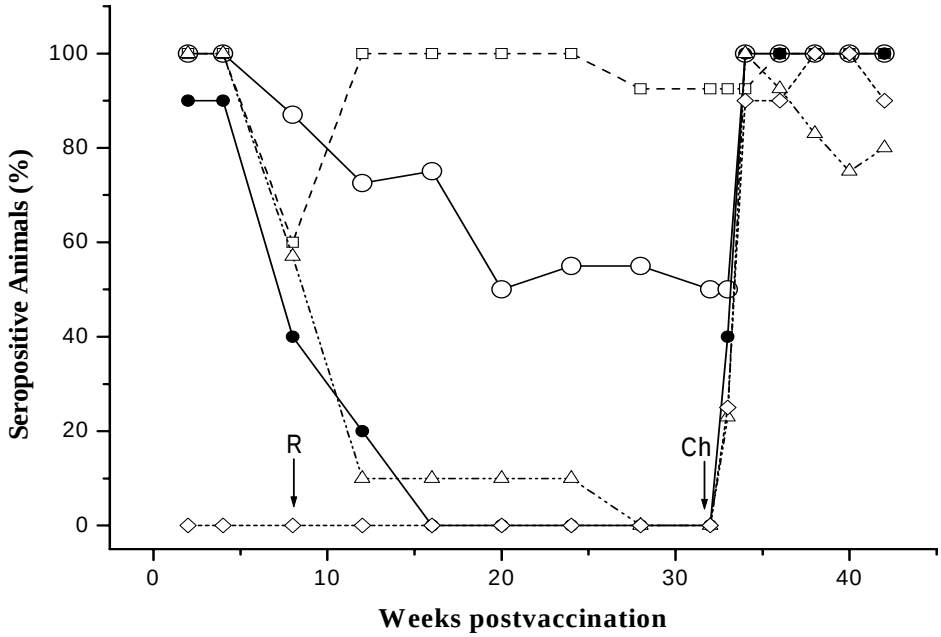

Figure 1. Percentage of reactors in the indirect ELISA with *Brucella ovis* HS antigens after vaccination. Symbols correspond with the experimental groups: (..N..), unvaccinated control group; (..8..), *B. melitensis* Rev1 vaccine; (-Λ-), HS-PEC 80 μg; (-–-), HS-PEC 800 μg; (..∀..), HS-PEC 800 μg revaccinated with the same amounts. Arrows indicate the time of revaccination (R) (performed only in the case of HS-PEC 800 μg), and experimental challenge (Ch) with the virulent *B. ovis* PA strain.

The immunoblotting assays performed (Figure 2) provided information on the development and persistence after vaccination of IgG antibodies directed against the outer membrane proteins contained in the *B. ovis* HS extracts. As it can be seen in Figure 2, the responses induced correlated quite well with HS-iELISA responses. The most immunogenic proteins after immunization with HS-PEC were Omp25 and L-OMP16. The lower dose of HS -PEC microparticles induced an IgG response to L-Omp16 that was only moderate and not persistent on time. In contrast, the higher dose of HS-PEC used elicited a strong response against Omp25, Omp22, Omp19, Omp16 and Omp10, being persistent until the time of challenge (week 32). The revaccination with HS-PEC had a clear effect on the persistence of IgG against Omp25.

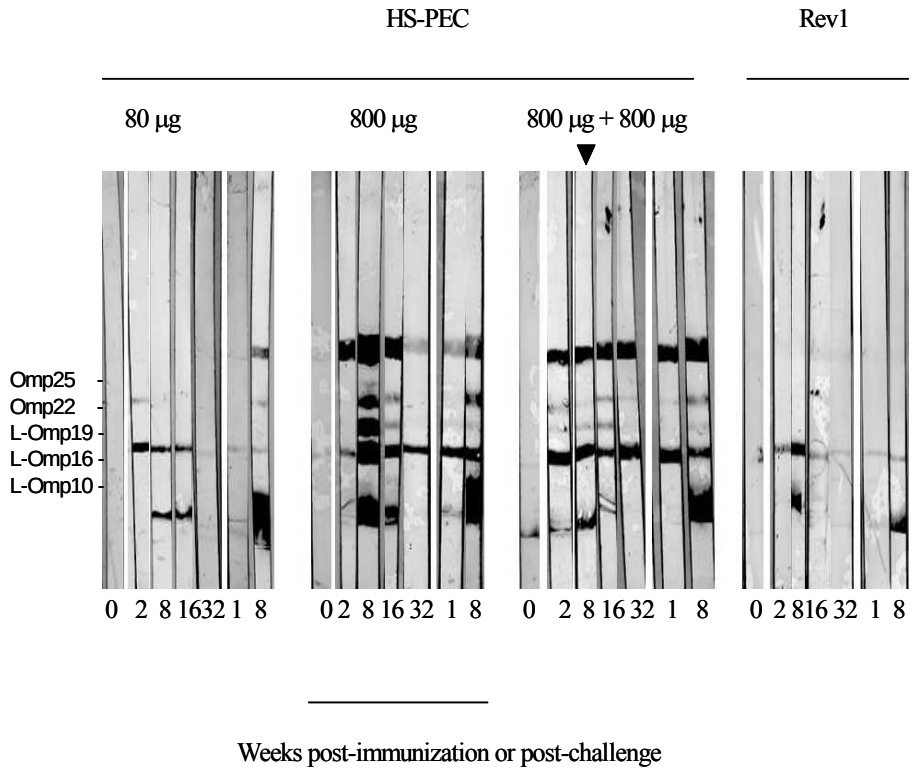

Figure 2. Immunoblotting analysis against outer membrane proteins contained in the *Brucella ovis* HS extracts (20 µg of HS per well) with sera from vaccinated rams (HS-PEC or Rev1). Numbers below blots correspond to the week after

vaccination (0, 2, 8, 6, 16, 32), or challenge (1, 9). The open arrow indicates the time of revaccination (week 8th).

We have not found any relationship between the serological respons either in the HS-i ELISA and immunoblotting and the outcome of protection. In fact, the animals vaccinated with Rev1 (the most effective vaccine) were showing a negative response in the HS-iELISA at the moment of challenge (Figure 1) and presented very low levels of IgG against the OMPs (Figure 2). Moreover, the serological response after challenge with *B. ovis* was not significantly different among all vaccine and control groups.

3.3. Bacteriological results

The bacteriological results obtained after the necropsy of the *B. ovis* challenged animals is summarized in Table 1. The intensity of the challenge procedure was adequate enough for statistical comparisons, even though the relatively low percentage of infection found in the unvaccinated controls (58.8%). The protective effect of microparticles containing the low dose of HS (800 μg) was similar to that found in the unvaccinated controls. In contrast, 9 out of 12 rams vaccinated with the higher dose of HS (800 μg) were protected, at similar statiscally levels than those vaccinated with the live Rev1 reference vaccine. The revaccination with 800 μg of HS-PEC, however, did not improve the efficacy of the single vaccination. None of the immunized and non-immunized animals showed evident testicular alterations after challenge with *B. ovis*, and no lesions were observed in these organs after necropsy.

Table 1. Bacteriological results after challenge with the virulent *B. ovis* PA strain. Rams were vaccinated with HS-PEC microparticles or *B. melitensis* Rev1 strain, and challenged eight months after vaccination.

Vaccine group[a]	Infected rams /total (%)	Positive samples /total (%)	Statistical differences[b] versus			
			Unvaccinated	Rev1	80 μg HS-PEC	800 μg HS-PEC
80 μg HS-PEC	9/12 (75)	31/108 (28.7)	NS	*		*
800 μg HS-PEC	2/11 (18.2)	3/ 99 (3)	*	NS	*	
800 μg HS-PEC + Revaccination	4/12 (33.3)	15/108 (13.8)	NS	*	*	*
***B. melitensis* Rev1**	0/11 (0)	0/99 (0)	*		*	NS
Unvaccinated	7/12 (58.3)	16/108 (14.8)		*	NS	*

a. Each ram was immunized once, except one group that was revaccinated with the same amounts of HS antigen (800 μg) in microparticles. Thirty two weeks after vaccination, all rams were experimentally infected with a virulent strain of *B. ovis* (conjunctivally and preputially). After necropsy (8 weeks after challenge), organs and lymph nodes were cultured in the search for *B. ovis* colonization.

b. Statistical differences either in the number of rams or samples infected between the different vaccine groups (Chi square test results showing at least $P < 0.05$; NS, not significant).

4. Discussion

This work is focused on alternative adjuvants, specifically on particulate delivery systems, such as biodegradable microparticles prepared from the polymer PEC, with the objective of making single-dose vaccines. We have previously described the encapsulation in microparticles of a membrane antigenic bacterial complex (HS) from *B. ovis* by using the classical solvent evaporation method [30] and, recently, by the use of TROMS [15], a semiautomatic and reproducible system for the preparation of microparticles. These were the first successful

descriptions in the literature of the encapasulation of strongly hydrophobic antigens. TROMS, as well as the adequate selection of pharmaceutical auxiliaries, such as β-cyclodextrin and Pluronic® F68, enabled us to obtain smooth and spherical microparticles containing bioactive HS antigenic extracts [15, 30]. Overall, PEC microparticles were in the range of 1-3 μm, optimal size to be taken up by the antigen-presenting cells (APC). The effect of microparticle size on the immune response based on their delivery into APCs is widely documented, supported by the observation that large microparticles (>5 μm) failed to elicit adequate immune responses, whereas smaller particles were effective for such purpose [36].

In order to establish the protective value of this acellular vaccine, it was performed a study of immunization and experimental infection with *B. ovis* in rams. In the experimental conditions used, as it will be discussed below, a dose of 800 μg of the antigenic complex HS incorporated in PEC microparticles afforded a level of protection similar to that conferred by the live *B. melitensis* Rev1 reference vaccine. Furthermore, and improving Rev1 properties, the subcutaneous inoculation of HS-PEC into rams did not induce fever or local reactogenicity in the site of vaccine administration. The lack of local reactivity of the HS-PEC vaccine could be due to both the lack of smooth-lipopolysaccharide in the antigenic complex, and the reduced amount of polymer used. It has been reported that high amounts of polymer may induce local inflammatory reactions [39] but the described procedure of encapsulation resulted in high antigenic loading, allowing the reduction of the amount of polymer used. Hence that, apart of reproducibility, the high antigenic loading ability should be considered for the selection of the most adequate vaccine delivery system.

The HS-PEC elicited also a significantly stronger and longer-lasting antibody response as determined by HS-iELISA and immunoblotting. Thus, at the time of challenge (week 32 post-immunization) more than 90% of the animals vaccinated with 800 μg of HS in PEC microparticles were seropositive, in contrast to the animals immunized either with the lower dose of HS (80 μg) or with Rev1, that were seronegative at that same time. However, the elicited antibodies after HS-PEC

immunization did not interfere with the serodiagnosis of ovine brucellosis caused by *B. melitensis* (as proven by the negative results in both RBT and CFT tests). Combined eradication programs based on test and slaughter and vaccination, are the best method of brucellosis control in domestic animals under moderate to high prevalence conditions [29]. Therefore, the assessment that the vaccinal antibodies do not interfere with the conventional serological tests used for diagnosis is considered a gold standard in combined eradication programs. As mentioned above, the HS was extracted from *B. ovis* cells as this species lacks the O-chain polysaccharide, the major antigen involved in *B. melitensis* classical serodiagnostic tests [21]. This has to be considered one of the main benefits of HS-PEC over the classical Rev1 vaccine.

The relevant antigens involved in the protective immunity against *B. ovis* are not completely elucidated yet. Omp31 appear as immunodominant antigen in the course of *B.ovis* infection; however, deletion of *omp31* gene from Rev1 did not affect its protective efficacy against *B. ovis* infection in mice [12]. In spite that Omp31 is a major protein in the HS extract, it was not immunogenic in the immunized rams with HS-PEC, or, at least was not detected by immunoblotting. The absence of antibody reactivity against this protein could be due to the elimination of conformational B-cell epitopes under the denaturing conditions of Western blotting, as it was shown by Cassataro et al. [10]. These authors detected antibodies to Omp31 in both *B. melitensis*- and *B. ovis*-infected animals under the nondenaturing conditions of an ELISA, although false negative results when using Western-blot analysis should not been discarded [10]. Therefore, the negative results obtained in our experimental conditions do not exclude the potential role of the antibodies against Omp31 in protection.

The relevant role played by T cells and antibodies in the immunity against *B. ovis* has been object of discussion and has not been fully elucidated. It has been demonstrated the participation of both cellular and humoral components in *B. ovis* protection in murine models [14, 23]. Further studies with *B. ovis* monoclonal antibodies provided new insights toward the recognition of antibodies in protection against *B. ovis* infection [9]. Results obtained in mice suggest that

immune T cells elicited by vaccination with the live Rev1 vaccine induced passive protection against *B. ovis*, while antiserum raised against Rev1 did not [23]. Nevertheless, the absence of demonstrable protection in passive antibody experiments does not neccesarily mean that antibodies do not play a significant role in protection, since this conclusion cannot be made from a negative experimental result. In a previous work performed in immunized rams with different *B. ovis* subcellular antigenic extracts incorporated in a Pluronic L-121/muramyl dipeptide adjuvant, the most effective vaccines elicited strong specific antibody responses, but declined with time and were negative or almost negative by the time of challenge. Moreover, there was not correlation between protection and intensity of the antibody response [6]. However, as it was the case here, the animals vaccinated with the most effective vaccine (Rev1), despite inducing intense responses to the *B. ovis* HS antigen during the first two months after vaccination, were seronegative in the HS- iELISA by the time of challenge. Thus, the presence of specific circulating antibodies does not seem the exclusive mechanism of protection against *B. ovis* in rams. Therefore, the participation of both humoral and cell mediated immune mechanisms in *B. ovis* immunity would be the most probable hypothesis. In this context, microparticle delivery systems allow the stimulation of both branchs of the immune system. The high concentration of antigens achieved in local lymph nodes will promote antigen uptake into motile APCs; a strong Th response is then elicited following their differentiation into mature dendritic cells. Our previous results obtained in mice immunized with HS-PEC confirm that potential [31].

The ideal vaccine for active immunization should confer strong and prolonged immunity in vaccinated animals. The replicative nature of the classical live vaccines favour the induction of long-lasting B and T memory cells due to booster priming APCs. Microparticles, due to their ability to degrade slowly and to release entrapped antigens, mimick the effect of replicative organisms inducing long-term immune responses after a single dose of inoculation [20, 38]. Immunoblotting results obtained with serum samples taken at different times after vaccination with HS-PEC confirm that particular behaviour. However, in spite of the higher antibody levels reached at the time of challenge, the revaccination with HS-PEC

did not improve the protective efficacy of a single dose. These results are in compliance with the efficacy of memory T cells in protection against *B. ovis* in ovine.

The assessment of vaccine efficacy should be based on the degree of bacterial colonization but also in histopathological examinations, to determine the degree of tissue damage, such as inflammation. In fact, the inflammatory processes that undergo brucellosis produce the major symptoms in ovine brucellosis: infertility and abortion. The pathologic fate of brucellosis, in particular the transient and recurrent nature of the disease's inflammatory episodes, depends on a delicate balance between pro- and anti-inflammatory responses. It has been described that surface exposed lipoproteins Omp16 and Omp19 and, of less extent, Omp10, play an important role in such processes [18]. Then, it is conceivable the hypothesis that antibodies against these proinflammatory Omp16 and Omp19 would interfere with the inflammatory process. Consistently with this hypothesis are our current data on the lack of reactogenicity at the HS-PEC inoculation site (see above). Moreover, previously published results showed that, after a challenge with *B. ovis*, rams previously vaccinated with HS were showing lower and less intense inflammatory responses than rams previously vaccinated with Rev1, which developed severe lesions in one or more organs after challenge [6].

Finally, a key issue to be considered in the preliminary assessment of candidate vaccine efficacy is the infective dose used in these vaccination-challenge studies. To be considered acceptable, the challenge should result in a percentage of infection in the control unvaccinated animals being significantly higher than that induced in the animals vaccinated with the reference vaccine used. In this context, to be considered effective the experimental vaccines should significantly minimize the percentages of infection with respect to that of unvaccinated controls, and ideally, result in similar levels than those obtained in the animals vaccinated with the reference vaccine. The percentages of infection in the unvaccinated controls and vaccinated with the low dose of HS were in the range of 60 to 75%, similar to those reported in similar experiments [2, 4, 8, 16, 21, 26]. Moreover, the reference Rev1 vaccine was able to reduce the levels of infection

with enough statistical significance with respect to control unvaccinated rams. Altogether, both results confirm the validity of our experiment.

In summary, our results indicate that one single dose of HS-PEC confers similar protection against *B. ovis* than the Rev1 reference vaccine, and that a dose-response effect exists in the rams immunized with HS-PEC microparticles. When using the higher dose of HS-PEC only two animals resulted infected, in contrast with the lower dose of vaccine that resulted in infection levels similar to that found in unvaccinated controls. The lack of interference in *B. melitensis* diagnostic tests and the intrinsic avirulence and innocuousness of HS-PEC, make this formulation an attractive anti-*Brucella* vaccine candidate. Further evaluation in large field trials using higher doses of HS loaded into PEC-microparticles is required to establish the real interest of this preparation for the effective prophylaxis of *B. ovis* infection in sheep.

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