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Conjunctival vaccination against *Brucella ovis* in mice with mannosylated nanoparticles

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ABSTRACT

The use of sub-unit vaccines can solve some drawbacks associated with traditional attenuated or inactivated ones. However, in order to improve their immunogenicity, these vaccines needs to be associated to an appropriate adjuvant which, adequately selected, may also offer an alternative pathway for administration. The aim of this work was to evaluate the protection offered by the hot saline complex extracted from *Brucella ovis* (HS) encapsulated in mannosylated nanoparticles (MAN-NP-HS) when instilled conjunctivally in mice. Nanoparticles displayed a size of 300 nm and the antigen loading was close to 30 μg per mg nanoparticle. Importantly, encapsulated HS maintained its protein profile, structural integrity and antigenicity during and after the preparative process of nanoparticles. The ocular immunization was performed on BALB/c mice. Eight weeks after vaccination animals were challenged with *B. ovis*, and 3 weeks later, were slaughtered for bacteriological examinations. Animals immunized with MAN-NP-HS displayed a 3-log reduction in spleen CFU compared with unvaccinated animals. This degree of protection was significantly higher than that observed for the commercial vaccine (Rev1) subcutaneously administered. Interestingly, the mucosal IgA response induced by MAN-NP-HS was found to be much more intense than that offered by Rev1 and prolonged in time. Furthermore, the elicited IL-2, IL-4 and γ-IFN levels showed good correlation with the degree of protection. On the other hand, biodistribution studies in animals were performed with nanoparticles labelled with either ^{99m}technetium or rhodamine B isothiocyanate. The biodistribution revealed that, after instillation, MAN-NP-HS moved from the palpebral area to the nasal region and, the gastrointestinal tract. This profile of distribution was different to that observed for free $^{99m}TcO_4^-$ colloids, which remained for at least 24 h in the site of administration. In summary, mannosylated nanoparticles appear to be a safe and suitable adjuvant for conjunctival vaccination.

1. Introduction

Brucellosis is a zoonotic infection transmitted from animals to humans by direct- or indirect contact with infected animals or their products such as the ingestion of raw milk and other unpasteurized dairy products (i.e. soft cheeses) [1,2]. Other common routes of infection in humans include infection through cuts and abrasions, the conjunctival sac of the eyes or the inhalation of aerosols [3,4]. In livestock, the main route of infection is via the sucking or licking of aborted foetuses and their placentas, as well as vaginal discharges. Infection due to the ingestion of infected milk or feedstuffs may also occur [1]. Human brucellosis remains the commonest zoonotic disease worldwide with more than 500,000 new cases annually [5]. Furthermore this infection is associated with substantial residual disability, and is an important cause of travel-associated morbidity [6].

Mass vaccination of animal populations accompanied by a strict surveillance scheme is a first step to reduce the number of infected animals and hence the infection pressure in regions where the incidence rate of animal brucellosis is high. The most commonly used vaccines are *Brucella melitensis* Rev1 and *Brucella abortus* S19 vaccines [7]. *B. abortus* RB51 vaccine is also used in some countries [8].

Abbreviations: ^{99m}Tc, ^{99m}technetium; $^{99m}TcO_4^-$, ^{99m}technetium pertechnetate; APCs, antigen presenting cells; BALT, bronchial-associated lymphoid tissue; BSS, buffered saline solution; CALT, conjunctiva-associated lymphoid tissue; CFU, colony forming units; DMF, dimethylformamide; EE, entrapment efficiency; H&E, haematoxylin-eosin stain; HS, hot saline subcellular complex extracted from *Brucella ovis*; GALT, gut-associated lymphoid tissue; MALT, mucosa-associated lymphoid tissue; MAN-NP, mannosylated poly(anhydride) nanoparticles; MAN-NP-HS, HS-loaded mannosylated poly(anhydride) nanoparticles; NALT, nasal-associated lymphoid tissue; PBS-T, solution of Tween 20 (0.05% w/v) in PBS; RBITC, rhodamine B isothiocyanate; SALT, skin-associated lymphoid tissue.

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The *B. melitensis* Rev1 strain is currently considered as the best vaccine available for the control of ovine and caprine brucellosis, especially when used at the standard dose by either the subcutaneous or the conjunctival routes [9,10]. However, due to its live attenuated nature, Rev1 displays a large number of drawbacks, including residual virulence and interferences with serodiagnosis [8]. In order to solve some of these drawbacks, the use of sub-unit vaccines has been proposed such as the hot saline subcellular complex extracted from *B. ovis* (HS) [11]. However, due to its non-replicant nature, adequate adjuvants have to be associated. In this context, poly(ε-caprolactone) microparticles containing HS were found to be safe and effective in mice and ram models, when administered by the subcutaneous route [12,13]. However, regarding the behavior of *Brucella* during the infection and colonization processes, the delivery of the antigens (HS) through mucosal surfaces is of remarkable interest in order to both mimic the bacteria pattern and generate immunity at the major portals of entry for these microorganisms.

Mucosal surfaces, mostly the subepithelial regions, are enriched in immunocompetent B and T lymphocytes, as well as antigen presenting cells (APCs). These cells are organized into the mucosa-associated lymphoid tissue (MALT) found in various sites of the body such as the gut (GALT), lung (BALT) or skin (SALT), among others [14]. In the eye, the conjunctiva, the palpebral area and the eye lachrymal drainage system are provided with an associated lymphoid tissue (termed CALT, conjunctiva-associated lymphoid tissue) [15] containing the specialized antigen sampling M-cells [16] present at other mucosal localizations, such as intestinal Peyer's patches. Furthermore, evidences from many studies have confirmed the inter-connected mucosal system, known as common mucosa immune system, allowing that the stimulation at one mucosal site can lead to effector immune cells in local as well as distal mucosal surfaces [14]. In addition to these immunological reasons, mucosa vaccination can also be safer and easier to dispense than traditional (parenteral) vaccines [17,18].

In the last years, nanoparticles made from the copolymer of methyl vinyl ether and maleic anhydride (Gantrez® AN) have demonstrated a remarkable capability to induce immune responses when administered orally [19–21]. This last property can be potentiated and modulated by the "decoration" of the surface of these poly(anhydride) nanoparticles with ligands capable to recognize and bind to specific components of the MALT. Among other ligands, mannose and its derivatives may be of interest due to the capability of these compounds to link with mannose receptors highly expressed in cells of the mucosal immune system (i.e. macrophages and dendritic cells) [22,23].

The aim of this work was to evaluate the protection offered by the HS-loaded mannosylated poly(anhydride) nanoparticles, when administered conjunctivally as eye drops, against experimental *B. ovis* infection in mice. Moreover, the biodistribution of these nanoparticles after their administration was evaluated.

2. Material and methods

2.1. Chemicals

Poly(methyl vinyl ether-co-maleic anhydride) or poly(anhydride) [Gantrez® AN 119; Mw 200 kDa] was gifted by ISP (Spain). Mannosamine hydrochloride, rhodamine B isothiocyanate (RBITC), concanavalin A and Tween 20 were purchased from Sigma-Aldrich (Spain). Antibodies peroxidase/conjugate anti-IgA were supplied from Nordic Immunol. Labs (The Netherlands). BCA™ Protein Assay Reagent Kit was from Pierce (USA). Acrylamide Criterion XT Precast gels (18 Comb, 30 μL, 1 mm) were obtained from Bio-Rad Laboratories (USA). PVDF (pore size of 0.45 μm) sheets were from Schleicher & Schuell (Germany) and 4-chloro 1-naphtol from Merck (Germany). Blood Agar Base plates were from BioMérieux SA (France) and O.C.T.™ was obtained from Sakura (The Netherlands). RPMI 1640 media, β-mercaptoethanol, penicillin, streptomycin, FBS, sterile PBS and sodium pyruvate were purchased from Gibco-BRL (UK). ^{99}Mo-^{99m}Tc generator was from Drytec (GE Healthcare, UK). Acetone, ethanol and dimethyl formamide (DMF) were obtained from BDH-Prolabo/VWR (France). Stannous chloride, methylethylketone and potassium perchlorate, were from Panreac (Spain). T-61 was from Intevet (Spain) and the isoflurane (Isoflo™) from Esteve (UK). All other chemicals used were of analytical grade and obtained from Fluka (Spain).

2.2. Extraction and characterization of the hot saline antigenic complex (HS)

The hot saline antigenic complex (HS) was obtained from the strain *B. ovis* REO 198 incubated in a bioreactor as described previously [24]. Total protein and lipopolysaccharide content of each batch of the antigenic extract were determined by the BCA™ Protein Assay and the Warren modified method [24], respectively. The HS used to prepare the nanoparticles contained 66.4 ± 10.6% total proteins and 39.5 ± 3.8% rough lipopolysaccharide.

2.3. Preparation and labeling of nanoparticles

2.3.1. Preparation of HS-loaded mannosylated nanoparticles

Poly(anhydride) HS-loaded mannosylated nanoparticles (MAN-NP-HS) were prepared by the solvent displacement method as described previously [20,25]. Briefly, 4 mg of the HS antigenic extract was dispersed in acetone and added to 100 mg of Gantrez® AN 119 dissolved in acetone, previously incubated overnight with 1 mg of mannosamine. After 30 min of incubation, nanoparticles were formed by the addition of an ethanol/water mixture (1:1 v/v). Once the organic solvents were eliminated under reduced pressure (Büchi R-144, Switzerland), the aqueous nanosuspensions were magnetically stirred for 1 h with mannosamine. The resulting nanoparticles were purified twice at 3000×*g* for 20 min by centrifugal filtration in tubes VivaSpin® 20 300,000 MWCO (Vivascience, Germany). Filtrates were collected for the quantification of HS and mannosamine. Finally, formulations were freeze-dried with sucrose at 5% as cryoprotector (Genesis 12EL, Virtis, USA).

Control mannosylated nanoparticles (MAN-NP) were prepared using the same methodology without the use of HS.

2.3.2. HS-loaded mannosylated nanoparticles labeled with RBITC

HS-loaded nanoparticles were fluorescently labeled by incubation with 1.25 mg of rhodamine B isothiocyanate (RBITC) for 5 min at room temperature [20,25]. After adsorption of the marker, the nanoparticles were purified by centrifugation and, finally, freeze-dried as described above.

2.3.3. HS-loaded mannosylated nanoparticles labeled with ^{99m}Tc

Nanoparticles were radiolabeled with ^{99m}Tc by reduction of ^{99m}Tc-Pertecnetate ($^{99m}TcO_4^-$) with stannous chloride as described previously [26]. Briefly, 20 μL of a stannous chloride solution were added to 1 mg of freeze-dried nanoparticles followed by addition of 74 MBq of freshly eluted $^{99m}TcO_4^-$ in 0.5 mL. The mixture was vortexed for 30 s and incubated at room temperature for 10 min. The pH of the final suspension was adjusted to 4.

2.4. Characterization of nanoparticles

2.4.1. Size, zeta potential, morphology and yield

The size and zeta potential of the nanoparticles were determined by photon correlation spectroscopy and electrophoretic laser Doppler anemometry, respectively, using a Zetamaster analyser system (Malvern Instruments, UK). The yield of the nanoparticles preparation process was determined by gravimetry from freeze-dried nanoparticles as described previously [20].

The morphological characteristics of the nanoparticles were observed by electron microscopy in a Zeiss DSM 940A microscope