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The Humoral Immun Response of Mice to Liposomes Containing *Brucella melitensis* Outer Membrane Fragments

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Abstract: Brucellosis is an important zoonotic disease and the prevention is made by vaccination of animals. For ewes *Brucella melitensis* REV-1 live attenuated vaccine is widely used but because of some risks such as being infected during the vaccination, re-infection of animals, a new vaccine with less side effect is need to be improved. In this study it was aimed to determine a strong humoral immune response by encapsulating the *Brucella* outer membrane antigens with the optimum liposome formulation and the statistical analyses showed that the *Brucella* outer membrane extract encapsulated with liposome can evoke humoral immune response better than live attenuated *B. melitensis* REV-1 vaccine in mice.

Key words: *Brucella*, liposomes, humoral immunity, vaccine

INTRODUCTION

Brucellosis is an economically important zoonotic disease that is widespread all over the world. It is also an important public health problem because *Brucella* can infect human after direct contact with or drinking and/or eating milk and milk products made from infected animals with brucellosis. Since, *Brucella melitensis* is the most pathogenic strain of the genus *Brucella*, brucellosis that is seen in ewes is very important especially in our country (İyisan *et al*., 2000).

In our country, the prevention has been made by vaccination of animals. For ewes *B. melitensis* REV-1 vaccine has been used. The risk of being infected during the vaccination also makes people and the farmers to be worried (İyisan *et al*., 2000). The need for controlling the *Brucella* infection by vaccination is obvious and in many studies it has been shown that prevention can be obtained by vaccination without any side effects. Recently some new vaccines are prepared by encapsulating the live vaccines by a carrier molecule (Elberg, 1981; Marin *et al*., 1990). Liposomes are attractive candidates as vaccine carriers for several reasons. They can be used as immunological adjuvants to modulate the immune response to the encapsulated antigen because they are made from naturally occurring lipids and they are biodegradable, non-toxic and do not cause granulomas after injection. Phospholipids and cholesterol are themselves non-immunogenic and therefore, animals immunized with liposome do not develop antibodies against to these components. As the antigens are encapsulated in the liposomes, they are delivered in concentrated form directly to macrophages for immunological processing (Alving *et al*., 1986; Richard *et al*., 1998; Wong *et al*., 1992). Also, liposomes protect the encapsulated antigens and the poor immunogenecity of some cellular units are reported to be still effective by utilizing liposomes as vaccine carriers (Wong *et al*., 1992).

In the live attenuated vaccines some problems can be occurred such as having virulence and cause infection and causing false positive results in the diagnostic tests (Limet *et al*., 1993; Monreal *et al*., 2003; Weynants *et al*., 1997).

For these reasons new vaccine studies should be performed to produce an improved effect with lower side effects.

In this study, it was aimed to determine a strong humoral immune response by encapsulating the *Brucella* outer membrane antigens with the optimum liposome formulation.

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MATERIALS AND METHODS

B. melitensis REV1 vaccine strain was obtained from Pendik Veterinary Control and Research Institute. *B. melitensis* vaccine strain was dissolved in sterile sodium chloride (0.9%) and inoculated into *Brucella* agar (Fluka) medium. The media were incubated at 37°C for 3-4 days in aerobic conditions. Pure colonies were inoculated into *Brucella* liquid medium (Fluka) and incubated at the same conditions as described above. After growth, the culture was stored at -20°C. During the study, the stored bacteria were regrown in *Brucella* agar and the colonies were confirmed to be smooth. Rough colonies were not included in the study.

Extraction method of the Hot Saline (HS) *B. melitensis* outer membrane antigenic complex: To obtain the antigenic HS extract, consist of lipopolysaccharide (LPS), phospholipids and Outer Membrane Proteins (OMP) of *B. melitensis*, after incubation the bacteria were washed with sterile saline solution and heated up to 100°C for 15 min. They were then centrifuged at 2000 rpm and the supernatant was removed. The remaining part in the tubes was considered as wet bacterial weight.

Total 30 g wet bacterial cells were mixed with 300 mL saline solution and centrifuged at 12000 g for 15 min (Vitas *et al*., 1995). The obtained supernatant was centrifuged at 100.000 g for 5 h and the obtained pellet was lyophilized (Vitas *et al*., 1995). After this procedure, the antigenic extract was obtained that contains the LPS, phospholipids and OMP of the bacterial cell wall and it was kept at + 4°C. To ensure the presence of OMP, SDS-PAGE (Laemmli, 1970) analyses and the presence of lipids, HPTLC (Menlik *et al*., 1989) analyses were performed.

Preparation of the liposome formulation: Total 10 mg cholesterol and 10 mg dipalmitoylphosphatidylholine (DPPC) (Sigma) were dissolved in 10 mL methanol and evaporated in a rotavapor at 45°C. A thin lipid layer was obtained and 10 mL solution of *B. melitensis* outer membrane was added to this layer slowly. They were vortexed for 5 min and multilayered large liposomes were obtained.

Animal experiments: For this experiment, 6 weeks old *Mus musculus* from the Rodentia ordo, Muroidea families were used. They were kept under quarantine for 1 week and the mice, which had not loose weight, were included in the study.

Six experiment groups were designed and in each group there were 8 mice and the experimental groups are shown in Table 1. Before starting the experiment, the mice were weighed and after analyzed with Kruskal-Wallis test no significant difference was found between the groups considering their weights (p = 0.245).

Table 1: Groups of experiment

Group	Injection
1	*B. melitensis* outer membrane
2	Free liposome (control)
3	Live attenuated *B. melitensis* REV-1 vaccine
4	*B. melitensis* outer membrane encapsulated with liposome
5	Live attenuated *B. melitensis* REV-1 vaccine encapsulated with liposome
6	Sterile saline solution

Diet and management: Mice were fed ad libitum consumption with the conventional pellet diet formulated to meet nutrient requirements. During the experiment, temperature and humidity were maintained at 20-24°C and 58%. All mice were exposed to 12: 12 light: Dark cycle. Cages were cleaned twice a week and fresh water was always available via glass bottles with rubber nipples. All the solutions were injected subcutaneous as 200 μg/mouse (Vitas *et al*., 1995).

Sample collection: At the end of the experiment (3 weeks later) blood samples took from the heart under anesthesia using a nonterminal procedure following a 24 h fast. Blood samples were collected into additive-free vacutainers (BD vacutainer SST, BD Vacutainer Systems Preanalytical Solutions, Belliver Industrial Estate, London, UK) for blood chemistry analysis. We obtained serum following centrifugation at 3000 g for 15 min at 20°C, aliquots of which were stored at -20°C until analyses could be done for Wright agglutination method (Alton *et al*., 1988; Gültekin, 2000; Ustaçelebi, 1999) for *B. melitensis* antibodies.

Statistical analyses: Kruskal-Wallis and Mann-Whitney tests were used for statistical analyses.

RESULTS

According to the SDS-PAGE results, in the HS extract 2 major Outer Membrane Proteins (OMPs) of 25-27 kDa weights and 2 minor OMPs of 19 and 10 kDa weights were determined. These OMPs are the proteins that were already shown to be present in *Brucella* cell membrane (Douglas *et al*., 1984; Limet *et al*., 1993). And after the HPTLC analyses, lipids that belong to the *Brucella* cell membrane were determined.

The weight and antibody titers of the mice that belong to the groups were shown in Table 2. After investigating the groups one by one with Mann-Whitney test, statistically significant difference was determined

Table 2: Antibody titers of the experimental groups

Replication	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
1	1/640	(-)	(-)	1/2560	1/640	(-)
2	1/80	(-)	(-)	1/5120	1/640	(-)
3	1/1280	(-)	(-)	1/2560	1/1280	(-)
4	1/1280	(-)	(-)	1/5120	1/1280	(-)
5	1/640	(-)	(-)	1/1280	1/640	(-)
6	1/640	(-)	(-)	1/5120	1/2560	(-)
7	1/640	(-)	(-)	1/5120	1/1280	(-)
8	1/1280	(-)	(-)	1/5120	1/1280	(-)
Average	1/810±433	(-)	(-)	1/4000±1595	1/1200±634	(-)
1	1/640	(-)	(-)	1/2560	1/640	(-)
2	1/80	(-)	(-)	1/5120	1/640	(-)
3	1/1280	(-)	(-)	1/2560	1/1280	(-)
4	1/1280	(-)	(-)	1/5120	1/1280	(-)
5	1/640	(-)	(-)	1/1280	1/640	(-)
6	1/640	(-)	(-)	1/5120	1/2560	(-)
7	1/640	(-)	(-)	1/5120	1/1280	(-)
8	1/1280	(-)	(-)	1/5120	1/1280	(-)
Average	1/810±433	(-)	(-)	1/4000±1595	1/1200±634	(-)

between Group 1 and Group 4 ($p = 0.001$) and between Group 4 and Group 5 ($p = 0.004$) in case of antibody titers.

DISCUSSION

Brucellosis is a zoonotic disease that causes heavy economic losses and human suffering (Monreal *et al*., 2003). Vaccination, serological identification and isolation of the ill animals are seemed to be the best ways for eradication (Monreal *et al*., 2003). However, *Brucella abortus* S19 and *B. melitensis* REV-1 vaccines that have been used today can cause abortions in animals and they are also virulent for human (Monreal *et al*., 2003; Moriyon *et al*., 2004). For this reason, in case of using these live attenuated vaccines, new vaccines that are prepared from bacterial components should be better for animal and public health.

The smooth LPS is also the source of antigenic cross-reactions between smooth *Brucella* sp. and other bacteria which posses an O chain similar to that of *Brucella*. Because of this reason serological tests that determine the S-LPS-Ab cannot differentiate these bacteria from *Brucella* (Limet *et al*., 1993; Monreal *et al*., 2003; Weynants *et al*., 1997). Effective classical vaccines however, induce antibodies to the O chain of the LPS, which may be difficult to distinguish from those resulting from infection and may thus complicate diagnosis (Moriyon *et al*., 2004). Because of these reasons the RB51 vaccine has been evaluated. Strain RB51 is a rough mutant with minimal expression of the O-chain on the cell surface. So serologically positive animal that was vaccinated with this strain are thought to be ill (Ashford *et al*., 2004; Campos *et al*., 2002; Leal-Hernandez *et al*., 2005; Stevens *et al*., 1996).

B. abortus 45/20 R strain was also used as a vaccine but it failed because of its low stability (Monreal *et al*., 2003). Since it is an R colony strain it also has the risk to be virulent again (Arellano-Reynoso *et al*., 2004). It has been known that among the bacterial components OMPs are particularly attractive used for development of vaccine candidates (Ding *et al*., 2005). Therefore, in our research the *Brucella* outer membrane that has the OMPs in its composition has been chosen. Jimenes de Bagües *et al*. (1994) have reported that they have prepared a HS extract obtained from *B.ovis* REO198 contained R-LPS and several proteins including abundant group-3 OMPs. They have used adjuvant rather than liposome to induce the immun response (Jimenes de Bagües *et al*., 1994). They have investigated the Ab titers in the sera of the animals and they have determined a protective immunity in mice (Jimenes de Bagües *et al*., 1994).

Eistein *et al*. (2003) have reported in their study that recombinant OMP31 protein (rOMP) with adjuvant increases the IgG level in mice (Estein *et al*., 2003). In another study Eistein *et al*. (2004) have shown that OMP31 induces the humoral and cellular immunity in ewes. Estevan *et al*. (2006b) have described different strategies to increase the HS loading and prepare large batches as necessary to test the vaccine in ovine. The mixture of HS with β-cyclodextrin was optimized in order to increase the HS loading in microparticles. On the other hand, TROMS (Total Recirculation One-Machine System) led microparticles with a more homogeneous size than the laboratory or standard procedure. They have reported that β-CD can also be used with large hydrophobic membranous material and TROMS is a valid large production of antigen-loaded microparticles (Estevan *et al*., 2006a). Estevan *et al*. (2006b) have also studied on a sub-unit vaccine encapsulated in microparticles and its efficacy against *B. melitensis* in mice. They have determined that their results confirmed the advantage of F 68-CD-MP to be used as the vector-adjuvant for HS vaccine development. Wong *et al*. (1992) have investigated liposomes effectiveness as vaccine carriers in the potentiation of the BALB/c mouse humoral response to the LPS and OPS antigens of *B.abortus* in 0, 1 and 5th weeks. In this study they have used a liposome formulation of 7: 2: 1 of phosphotidilcholin: cholesterol: Phosphotidilserin. They have determined a fourfold greater IgG levels in LPS encapsulated with liposome injected mouse compared to that of LPS injected mouse. They have also reported that the IgG levels decrease eight times slower in LPS encapsulated with liposome injected mouse than LPS injected mouse. Vitas *et al*. (1995) have prepared a 7: 3: 0.5 M phosphotidilcholin: Cholesterol: Stearylamin liposome

formulation and they have encapsulated the *B.abortus* and *B.melitensis* HS extract. They have searched the immun response before and after infection in mice and they have reported that the HS extracts encapsulated with liposome are protective before 1 day and after 2 days of the injection and they are no longer protective after 10 days.

In our study, liposome formulation containin g 10 mg cholesterol and 10 mg DPPC was used to encapsulate *B. melitensis* outer membrane and a statistically significant differences were found between *B. melitensis* outer membrane injected and *B. melitensis* outer membrane encapsulated with liposome injected groups and between live attenuated *B. melitensis* REV-1 vaccine injected and *B. melitensis* outer membrane encapsulated with liposome injected groups. Thus, it was determined that *B. melitensis* outer membrane encapsulated with liposome induces humoral response stronger than the live attenuated vaccine which has been used to vaccinate the animals. After Wright agglutination test; the antibody titers were found with a mean of 1/800 in the *B. melitensis* outer membrane injected group. In diagnosis 1/160 and upper levels are determined to be significant (Bilgehan, 2000) and these titers are much higher than the significance level. As expected in our control groups. Free liposome and Sterile saline solution, antibody was not determined. The *B. melitensis* REV-1 vaccine encapsulated with liposome group also had no antibody levels. The reason for this was thought to be that the molecule could not be released from the liposome because of that the vaccine is a large molecule. However, this should be investigated in further studies. In *B. melitensis* REV-1 vaccine injected group with a mean of 1/1200 antibody titers were determined as expected. In *B. melitensis* outer membrane encapsulated with liposome injected group antibody titers of 1/2560, 1/5120, 1/2560, 1/5120, 1/1280, 1/5120, 1/5120 and 1/5120 were determined which are higher than the titers of the vaccine applied group. As mentioned in the statistical analyses, results of the titers are significantly different from that of the other groups' antibody titers. Thus, these results show that *B. melitensis* outer membrane encapsulated with liposome was found to be successful in inducing humoral immune response in mice.

In this study we aimed to obtain a strong immune response by encapsulating the HS extract with liposome rather than use an adjuvant. According to the Wright agglutination method results and statistical analyses showed that the humoral immune response induced by *B. melitensis* cell membrane encapsulated by liposome is significant compared to that of the live attenuated *B. melitensis* REV-1 vaccine that is used for vaccination of the animals in our country now.

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