ISSN 1735-1383

Iran. J. Immunol. March 2009, 6 (1), 12-21

Iraj Pakzad, Abbas Rezaee, Mohammad Javad Rasaee, Bahman Tab-baraee3, Ali Delpisheh

Immunogencity of HSA-L7/L12 (Brucella abortus Ribosomal Protein) in an Animal Model

Article Type: RECEARCH ARTICLE

The *Iranian Journal of Immunology* is a quarterly Peer-Reviewed Journal Published by Shiraz Institute for Cancer Research and the Iranian Society of Immunology & Allergy, Indexed by Several World Indexing Systems Including:
ISI, Index Medicus, Medline and Pubmed

For information on author guidelines and submission visit:

www.iji.ir

For assistance or queries, email:

iji@sums.ac.ir

Immunogencity of HSA-L7/L12 (*Brucella abortus* Ribosomal Protein) in an Animal Model

Iraj Pakzad[1]*, Abbas Rezaee[2], Mohammad Javad Rasaee[2], Bahman Tabbaraee[3], Ali Delpisheh[4]

[1]Department of Microbiology, Ilam University of Medical Sciences, Ilam, [2]Faculty of Medical Sciences, Tarbiat Modaress University, Tehran, [3]Department of Bacterial Vaccines, Pasteur Institute of Iran, Tehran, [4]Department of Community Medicine, Ilam University of Medical Sciences, Ilam, Iran

ABSTRACT

Background: The immunogenic B*rucella abortus* ribosomal protein L7/L12 is a promising candidate antigen for the development of subunit vaccines against brucellosis. **Objective:** This study was aimed to evaluate the protection of recombinant Human Serum Albumin (HAS)-L7/L12 fusion protein in Balb/c mice. **Methods:** The amplified L7/L12 gene was cloned in pYHSA5 vector, pYHSA5-L7/L12 construct was transformed in *Saccharomyces cerevisiae* and the expressed protein from supernatant was purified by affinity chromatography. Balb/c mice were immunized in five groups by tHSA-L7/L12 fusion protein (group 1), *Brucella abortus* S19 (group 2), HSA (group 3), recombinant L7/L12 (group 4), PBS (group 5). ELISA to detect antibody production, LTT test to assess antigen specific lymphocyte response were conducted prior to virulent *B. abortus* strain 544 challenge two weeks after the last injection. Bacterial counts from spleens of immunized mice were done four weeks after challenge. **Results:** In ELISA tests, the specific antibodies exhibited a dominance of immunoglobulin IgG1 over IgG2a. In addition, the tHSA-L7/L12 fusion protein and L7/L12 elicited a strong T-cell proliferative response upon restimulation in vitro with recombinant tHSA-L7/L12 and L7/L12, suggesting the induction of a cellular immunity response in vivo. However, there was no significant difference in proliferative response of L7/L12 and tHSA-L7/L12 fusion protein ($p>0.05$). The L7/L12 and tHSA-L7/L12 fusion protein vaccines could also induce significant protection against challenge with the virulent strain *B. abortus* 544 in Balb/c mice ($p\leq0.05$). **Conclusion:** The tHSA-L7/L12 fusion protein, similar to L7/L12 has the ability to induce antigen specific lymphocyte proliferation, stimulate humoral immunity and engender protection.

Keywords: Human Serum Albumin, *Brucella abortus*, Fusion Protein, L7/L12

INTRODUCTION

Brucella abortus is a facultative intracellular pathogen and one of the etiological agents of

*Corresponding author: Dr. Iraj Pakzad, Department of Microbiology, Ilam University of Medical Sciences, Ilam, Iran. Tel: (+) 98 0841 3342272, Fax: (+) 98 0841 2227134, e-mail: pakzad_i2006@yahoo.com

brucellosis infecting humans and domestic animals (1). Currently, live attenuated *B.abortus* strain S19 is used to immunize cattle, despite the disadvantages of abortion and infection (2). Attenuated strains such as *Brucella melitensis* Rev1 and *B. abortus* S19 and RB51 are being used to control brucellosis in domestic animals (3). However, no safe and effective vaccine is available for human use. Several strategies such as development of subunit vaccines (4), utilization of bacterial vectors (5) and over expression of protective homologous antigen (6) have been introduced to develop alternative *Brucella* vaccines. The *B. abortus* L7/L12 ribosomal protein is an immuno-dominant antigen (7). The recombinant L7/L12 protein and plasmid encoding the L7/L12 gene can elicit strong cell mediated immunity and engender protection from *Brucella* infection in mice; however, the protective effect was much lower than what the live attenuated *B. abortus* vaccine S19 provides (4,7,8). The protective role of other types of L7/L12 based-vaccines utilizing different vectors such as vaccinia virus and *Lactococcus lactis* have recently been reported (9-11).

However, there is evidence that polyvalent vaccines, including protein and DNA vaccines, can engender more effective protection than univalent ones (12,13). Thus, polyvalent vaccines combining L7/L12 with other immunogenic antigen(s) such as lipopolysaccharide (LPS) of *Brucella*, are important components in humoral immunity against brucellosis (14). The anionic and amphiphilic nature of the lipid A portion of LPS enables it to bind to numerous substances such as human serum albumin (HSA), providing a positively charged, amphipathic character (15). In order to preparation a natural complex of LPS with L7/L12, a fusion protein tHSA-L7/L12 in *S. cerevisiae* was produced and its protective effect was evaluated compared to recombinant L7/L12 alone in the Balb/c mouse model.

MATERIALS AND METHODS

Strains, Plasmids and Media. *B. abortus* S19, provided by the Pasteur Institute of Iran, was grown on brucella broth (Difco, USA) at 37˚C for 72 h. *S. cerevisiae* strain 2805 (Matα pep4: His3 prb-Δ1.6R can1 his3-20 ura3-52) was used as the host to express the fusion protein (truncated HSA-L7/L12). The yeast was cultured in a rotary incubator at a speed of 200 rpm at 30˚C in SC-Ura, a uracil deficient complete synthetic medium containing 2% glucose (Difco, USA). *Escherichia coli* DH5α (Stratagene, USA) (*f*- gyr A96 Nlar, recA1Thi-1 hsdR17 r- k m+k) was used for cloning, sequencing and maintaining various DNA fragments. E.coli was cultured in Luria Bertani medium (LB) (QUELAB, UK). Shuttle vector pYHSA5 contained the inulinase signal sequence (*Kluyveromyces marxianus*), human serum albumin cDNA, Gal10 promoter and ura3 selective marker (16). Yeast transformation was carried out by the modified lithium acetate method (17).

Amplification of the B. abortus L7/L12 Ribosomal Gene by PCR. According to the L7/L12 nucleotide sequence (Gen Bank accession Number L19101), and nucleotide sequence of pYHSA5 shuttle vector, forward (5'-CCGTCTAGAAAATGGGCTGCTGA TCTCGCAAAG-3') and reverse (5'-CCGTCTAGATTACTTGAGTTCAA CCTTGGG-3') primers were synthesized by the MWG Company (Germany). These primers create two XbaI sites at the 5' and 3' ends of the gene. *B. abortus* S19 DNA was extracted (18) and the *l7/l12* gene was amplified by pfu polymerase (Fermentase, Germany) in 2.5 mM MgSO4 (Fermentase, Germany), 0.5 ρmol primer/1μl reaction mixture at an annealing

temperature of 55˚C in a thermal cycler (FGEN02TD Techneh, UK). The PCR product was purified by gel extraction (Roche, Germany) and confirmed by EcoRI and HindIII digestion. The PCR product was digested with XbaI and ligated to pYHSA5 plasmid. Direction of the *l7/l12* gene in pYtHSA-L7/L12 was determined by enzyme digestion and sequencing. pYtHSA5-L7/L12 plasmid and PCR product was sequenced by MWG company (Germany).

Media and Culture Conditions for Expression. YPD medium (1 % yeast extract, 2 % bactopeptone, and 2 % dextrose) was used for the cultivation of host and yeast transformation. Yeast Nitrogen Base without uracil (Difco, USA) was used for the selection of yeast transformants and for seed culture. For induction of tHSA-L7/L12 expression, the yeast transformants were grown in shaker flasks containing YPDG media (1 % yeast extract, 2 % bactopeptone, and 1 % dextrose at varying concentrations of galactose). The flasks were shaken at 30˚C and 150 rpm for 24 h (19).

Immunoblotting. Western blot analysis of culture supernatant of yeast cells was carried out with the polyclonal antibodies raised against human serum albumin and anti-L7/L12 ribosomal protein antibodies (a donation from Dr. Philippe Langella, France).

Protein Purification. The supernatant of induced cells was collected by centrifugation (1300×g, 3 min), 10mM phenylmethylsulphonyl fluoride for 8 h (PMSF, Merck, Germany) was added and the expressed protein from the supernatant was precipitated by 60 % ammonium sulfate (Merck, Germany), dialyzed against water and purified by affinity chromatography on CN-Br activated sepharose CL-4B (Biogen, Germany) and anti-HSA antibody.

Immunization and Challenge of Mice. Five groups of 6-8 weeks old female Balb/c mice (15 mice in each group) were injected intraperitoneally with PBS, HSA (10 µg), tHSA_L7/L12 (10µg), L7/L12(10 µg) (a donation from Dr. Hamid Abtahi, Department of Microbiology, Arak university of medical sciences, Iran) , and *B. abortus S19* (5×10^4 CFU). Injection volumes were 0.2 ml/mouse. A second dose was given 4 weeks after the first one. Blood was collected from five sacrificed mice from each group 2 weeks after the second dose of the vaccine. Sera were collected and stored at -20°C until analyzed for antibody by an enzyme-linked immunosorbent assay (ELISA). Two weeks after the final vaccination, five mice from each group were challenged intraperitoneally (20) with a relatively higher dose of *B. abortus* strain 544 (5×10^5 CFU). Four weeks postchallenge, the mice were killed by cervical dislocation; their spleens were removed aseptically and weighed. Each spleen was homogenized in sterile PBS, serially diluted 10-fold, and plated in triplicate on trypticase-soy agar. *B. abortus* 544 colonies were counted after 3 days of incubation in an atmosphere of 10 % CO_2 at 37°C. The results are presented as the mean log CFU ± SD per group. This experiment was repeated three times. Statistical analyses were performed with a Student's *t* test. Log_{10} of the units of protection were calculated as the mean log_{10} of the numbers of CFU of the negative control group (PBS) minus the mean log_{10} of the numbers of CFU of the experimental group. This study was approved by the Tarbiat Modarres University Ethical Committee.

ELISA. The presence of serum IgG specific to tHSA-L7/L12 and recombinant L7/L12 (a donation from Dr. Hamid Abtahi, Iran) was determined by indirect ELISA, two weeks after the final immunization. The purified tHSA-L7/L12 and L7/L12 were diluted to 5 µg/ml in carbonate buffer (pH 9.6) and used to coat the wells of a polystyrene plate (100 µl/well; Nunc-Immuno plate with MaxiSorp surface). After overnight incubation at 4°C, the plates were washed, blocked, and then incubated with serially diluted sera for 3 h at room temperature. Following another washing, IgG specific rabbit anti-mouse

horseradish peroxidase conjugates were added (100 µl/well) at the appropriate dilutions. After 30 min of incubation at room temperature, the plates were washed, and 100 µl of substrate solution (200 µmol of *o*-phenylenediamine and 0.04 % H_2O_2) was added to each well. After 20 min of incubation at room temperature, the reaction was stopped upon addition of 50 µl of 2M sulfuric acid/well. Absorbance at 450nm was determined with an ELISA reader (Labsystems Multiskan MCC/340; Fisher Scientific, Pittsburgh, PA). The titre, expressed in optical density (OD) was obtained by multiplying the reciprocal dilution of the serum by *A*450nm at that dilution (21). All assays were performed in triplicate.

Splenocyte Culture and Lymphocyte Proliferation. Two weeks after the last immunization, mice were sacrificed, and their spleens were removed under aseptic conditions. Single-cell suspensions were prepared from the spleens, and the red blood cells were lysed with ACK (150 mM NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2 EDTA, pH 7.3) solution. Splenocytes were cultured at 37°C in 5 % CO_2 in a 96-well flat-bottom plate at 4×10^5 cells/well in RPMI 640 medium supplemented with 2 mM L-glutamine and 10 % heat-inactivated fetal calf serum (Sigma, Germany) in the presence of 0.08 µg of purified tHSA-L7/L12 protein and 0.5 µg of L7/L12 protein or no additives (unstimulated control). The cells were cultured for 3 days and pulsed for 8 h with 0.4 µCi of [3H] thymidine (50 Ci/mmol; Amersham, Biosciences, UK) per well. The radioactivity incorporated into the DNA was measured in a liquid scintillation counter. Cell proliferation was expressed as mean counts per min (cpm) from five mice of each group. All assays were performed in triplicate.

Statistical Analysis of the Data. Antibody titers of each group of mice were expressed as means ± standard deviations. The intensities of bacterial infection in spleen were expressed as the mean log CFU ± standard deviation per infected organ. The lower limit for detection of infected spleen was 2 CFU. The differences in ELISA titres and in the log CFU per infected organ were statistically analysed using Student's *t* test.

RESULTS

Construction of pYtHSA-L7/L12 Shuttle Vector. The PCR amplified gene fragment encoding the sequence of L7/L12 contained 400bp when analysed by 1 % agarose gel electrophoresis (Figure1, lane 3). For preliminary confirmation, the fragment was subjected to digestion by EcoRI and HindIII. Digestion with EcoRI yielded 165 and 210 bp fragments, and digestion with HindIII yielded 75 and 300bp fragments at the expected positions (Figure 1, lanes 1, 2). Ligation of the PCR product in pYHSA5 was confirmed by XbaI digestion. The sequencing of the PCR product fragment revealed complete homology at the nucleotide level to the NCBI *l7/l12* gene sequence.

Extracellular Production of Fusion Protein tHSA-L7/L12 in S.cerevisiae. The pYtHSA-L7/L12 shuttle vector has a signal sequence, and the product was expected to be secreted. Supernatant from induced and non-induced yeast cultures was examined by SDS-PAGE electrophoresis followed by silver nitrate staining. *S. cerevisiae* strain 2805 was used as a negative control. In induced cultures, a band of ~ 52 KD molecular mass was present in the supernatant (Figure 2A). Considering the length of the two fused genes, this weight was expected.

Extracellular Protein Purification. The affinity chromatography purified protein was precipitated by 60 % ammonium sulfate, dialyzed against water overnight followed by Comassie blue-stained (Figure 2, B). The amount of purified fusion protein was 0.5 mg/litre.

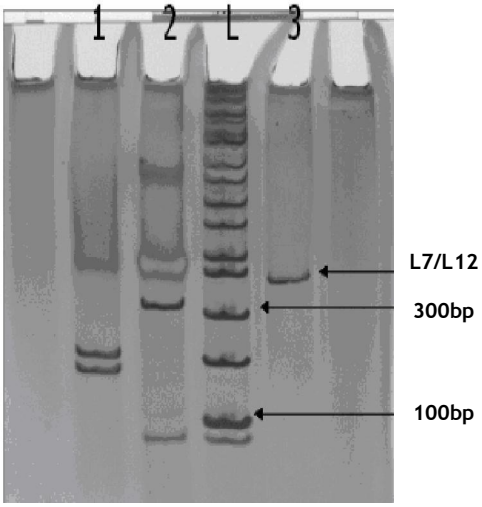

Figure1. Electrophoresis of PCR product on 1% agarose gel (lane 3), PCR product digestion by EcoRI (lane 1) and HindIII (lane 2).

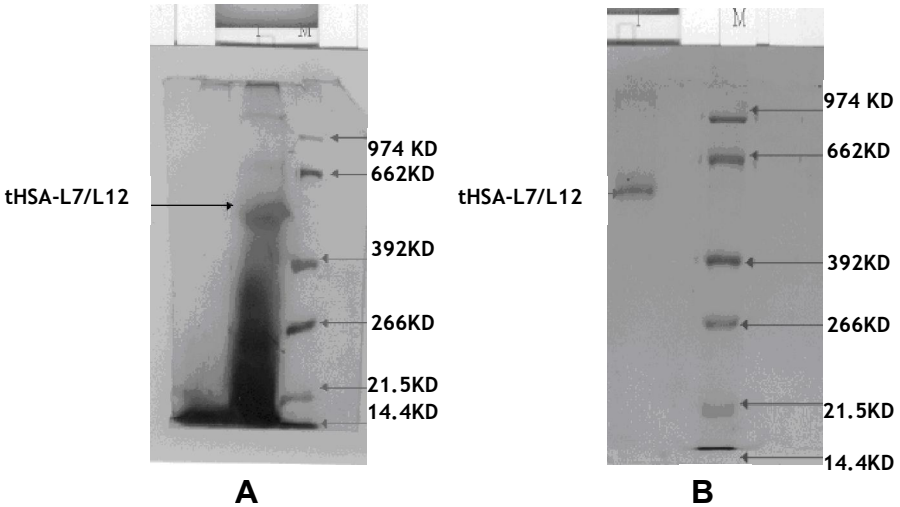

Figure 2. A: silver- stained SDS-PAGE of precipitated supernatant protein by 60% ammonium sulfate, M (marker), lane 1: concentrated fusion protein tHSA-L7/L12. B: Comassie blue-stained SDS-PAGE of purified protein by affinity chromatography, M (marker), lane 1: purified fusion protein tHSA-L7/L12.

Western Blotting of Fusion Protein. Anti-HSA and anti-L7/L12 antibodies from rabbits were used for blotting (Figure 3, A and B). Fusion protein was detected by both antibodies indicating that the antigenic structure of fusion protein is so constructed to permit identification of the two protein fragments.

Antibody Response Elicited by L7/L12 and tHSA-L7/L12 Immunization. Sera collected 2 weeks after the last immunization were assayed for the presence of tHSA-L7/L12 -specific antibodies by ELISA. The total IgG titre of the hyper-immune sera from mice immunized with tHSA-L7/L12, L7/L12, HSA and the live S19 strain reached 1:21800, 1:14800, 1:15100 and 1:54200, respectively. The results show that immunization with tHSA-L7/L12 and the live S19 strain elicits much higher antibody responses

in mice. The amount of L7/L12 present in the S19 vaccination was unknown but probably greater than immunization with L7/L12 alone. Therefore, the finding of a greater titre to S19 might have been anticipated. The ability of S19 to replicate producing even more antigenic material might also be considered. Further, immunization with either L7/L12, or HSA-L7/L12 was without adjuvant, while the adjuvant properties of strain S19 are unknown.

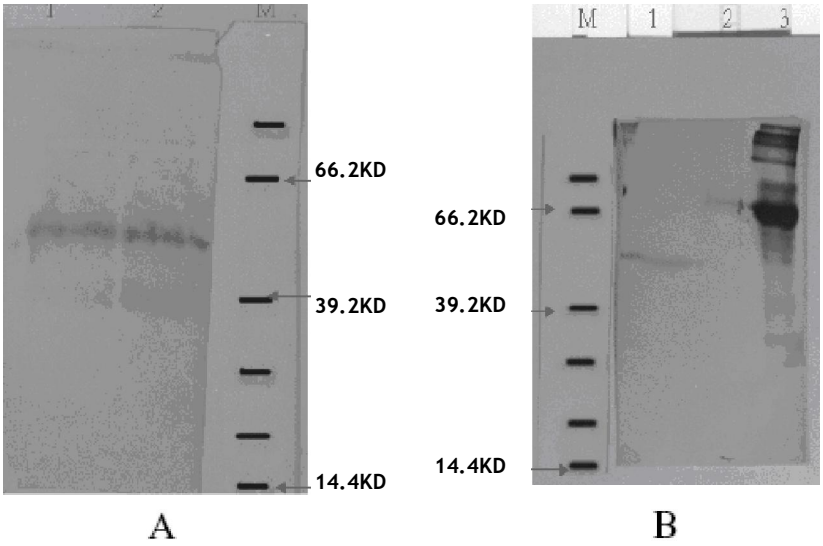

Figure3. A: Western blot analysis of tHSA-L7/L12 by rabbit anti-L7/L12 antibody. B: immuoblotting with rabbit anti-HSA antibody.

To determine the function of IgG subclasses in the mechanism of preventing *Brucella* infection, the proportion of Th1-associated IgG2 and Th2-associated IgG1 in the total serum IgG of each group was examined by ELISA upon diluting the sera 1:100. The analysis of IgG subclasses showed a significant increase in IgG1 and IgG2a subclasses in L7/L12 group, tHSA-L7/L12 group, and the live S19 group compared with HSA ($p<0.01$), (Table 1). Since, the ratio of the Th1-associated versus Th2-associated IgG subclass may reflect a substantial increase in certain IgG subclasses; the IgG2a/IgG1 ratio was calculated for every group. The ratios were 0.63, 0.65 and 0.78 after L7/L12, tHSA-L7/L12 and S19 immunization, respectively. The three different antigens had higher IgG1 and/or IgG2a levels than the HSA, suggesting that the live S19 strain had a more dominant Th1 response than the other groups ($p<0.05$). L7/L12 and tHSA-L7/L12 induced a predominant Th2 response compared to a Th1 response.

Table 1. Anti-HSA-L7/L12 IgG ELISA titers and IgG subclass profiles of mice two weeks after immunization[a]

Group	Vaccine	Anti-tHSA-L7/L12 IgG ELISA titer (mean ± SD)	Anti-tHSA-L7/L12 IgG subclass ELISA titer (mean ± SD)	
			IgG1	IgG2
1	L7/L12	14245 ± 238	8875 ± 238	5667 ± 214
2	THSAL7/L12	21800 ± 715	12800 ± 715	8345 ± 567
3	S19	54257 ± 3867	51257 ± 3867	40256 ± 3126
4	HSA	15178 ± 274	9878 ± 274	4034 ± 245
5	PBS	<2	<2	<2

[a] Mice were immunized intraperitoneally. Two doses of vaccine were given 4 weeks apart.
Sera were collected from five of each group at 2 weeks after immunization.
The data are expressed in OD units.

Lymphocyte Proliferation Induced by tHSA-L7/L12. To further investigate the cell mediated immune response induced by tHSA-L7/L12 fusion protein, L7/L12 alone, and the live S19 strain, we analyzed the proliferative T-cell response. All three vaccins (tHSA-L7/L12, L7/L12 and live S19 strain) induced significant T-cell proliferation in immunized Balb/c mice in response to recombinant tHSA-L7/L12 and L7/L12 compared with PBS or HSA immunization ($p=0.01$), (Figure 4). Though lymphocytes from the tHSA-L7/L12 and L7/L12 groups could significantly prime an antigen specific T-cell proliferative response, this effect was lower than that observed for the live S19 strain ($p=0.05$). The T-cell proliferation response to tHSA-L7/L12 compared to L7/L12 alone was not significantly different ($p>0.05$). Among the three vaccins, the live S19 strain showed the strongest stimulant effect ($p<0.05$).

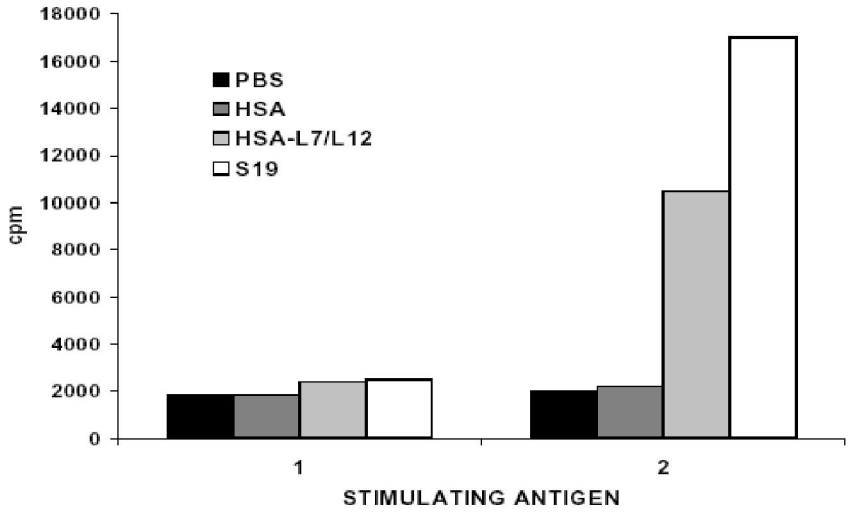

Figure 4. Lymphocyte proliferation assay. Balb/c mice were immunized with tHSA-L7/L12 fusion protein, rL7/L12 strain S19, using PBS and HSA as negative immunization controls. The T-cell proliferation response was measured 2 weeks after the last immunization. Splenocytes from each group were prepared from 4 $\times 10^5$ cells per well in a 96-well flat-bottom plate and stimulated in vitro with purified tHSA-L7/L12 (2µg/ml) or the RPMI 1640 medium (control) as antigens. Each sample was assayed in quadruplicate wells. Data represent the mean cpm ± SD from five mice from each group ($p<0.01$).

Efficacy of tHSA-L7/L12 Immunization in Generating Protective Immunity against B. abortus 544. Two weeks after the last immunization, we challenged the vaccinated mice intraperitoneally with an injection of virulent strain 544. Four weeks after the challenge, the infection level in each mouse was estimated by determining CFU in the spleen. Immunization with tHSA-L7/L12, or L7/L12 resulted in significant protection (1.4 and 1.3-log of increase in protection, respectively) compared to the controls that received PBS and HSA ($p<0.05$), (Table 2). Our results suggest that protection from tHSA-L7/L12 immunization is not significantly different from the protection by L7/L12 alone ($p>0.05$). To compare the extent to which mice could be protected, we included a group immunized with live *B. abortus* strain S19 which induced 2.82-log protection compared with the PBS group ($p=0.001$). No reduction in the frequency of CFU was observed in animals injected with HSA compared to the PBS group (Table 2).

Table 2. Protection of mice against challenge with B. abortus 544 after immunization with various vaccines

Vaccine	Mean ± SD	P-Value	
		Log CFU/spleen	Log protection
PBS	4.96 ± 0.23	0.00	>0.05
HSA	4.86 ± 0.26	0.1	>0.05
L7/L12	3.65± 0.27	1.3	<0.05
HSA-L7/L12	3.56 ± 0.16	1.4	<0.05
S19	2.14 ± 0.18	2.82	<0.01

DISCUSSION

Immunity against brucellosis may require induction of both cellular and humoral immunity. Most individual *Brucella* antigens do not possess the ability to induce a protective response by themselves (22). Therefore, an effective subunit vaccine will likely require a combination of several antigens (23,24).

Outer membrane antigens, specifically LPS, are in direct contact with the host humoral immune system. For this reason, LPS may be one of the main vaccins of a potential subunit vaccine (22,25). Previously, L7/L12 has been reported as a most important protein of this bacterium to stimulate cellular immunity. Immunity induced by L7/L12 recombinant protein has been previously demonstrated (2,7,26). Recently, it has been recommended that a suitable subunit vaccine must contain a combination of LPS with L7/L12. Supporting this concept are reports that polyvalent vaccines can induce a more intensive immune response than a univalent vaccine (12,13).

In this work, tHSA-L7/L12 fusion protein was expressed and immune response was investigated. Firstly, this fusion protein was confirmed by HSA and L7/L12 specific antibodies. This finding demonstrated that the designed fusion protein produced by *S. cerevisiae* has retained its antigenic characteristics. In addition, the immunogenic potential of this fusion protein indicates that it is appropriate for immunological evaluation in an animal model.

Immunization with the recombinant fusion protein tHSA-L7/L12 and L7/L12 could induce remarkable titres of total IgG (1:21800, 1:14245, respectively); however, the titres were less than those induced by live S19 strain (1:54257). The IgG subclass assay demonstrated that the ratio of IgG2a/IgG1 in the fusion protein group and L7/L12 group (0.63 and 0.65, respectively) were much lower than that of the live S19 immunization group (0.78), suggesting that fusion protein vaccine similar to L7/L12 elicited a moderate Th1-type cellular immune response.

The lymphocyte proliferation assays demonstrated that tHSA-L7/L12 fusion protein induced a significant T-cell response similar to L7/L12. However, the $CD4^+$ and $CD8^+$ subtypes of T cells that were primed were not identified. However the difference in T-cell proliferation in immunized Balb/c mice with tHSA-L7/L12 and L7/L12 was not statistically significant ($p>0.05$) suggesting that the fusion protein could induce a similar T-cell response as L7/L12.

In this study,The S19 strain conferred the highest protection against *Brucella* infection of all groups. It is possible that S19 can infect the host cells efficiently and produce endogenous antigens in antigen-presenting cells. These results are in agreement with other observations (27). Although the tHSA-L7/L12 fusion protein stimulated a lower immune response than the S19 strain especially in terms of cellular immunity, it still conferred 2.82-log protection conferred by S19. Protection of recombinant L7/L12 was

already confirmed by others (4). Our result showed that the protection of tHSA-L7/L12 was not significantly different from that of L7/L12 ($p>0.05$). This indicates that the fusion of HSA with L7/L12, causes an increase in complexity of this protein without affecting protection. These findings suggest that the tHSA-L7/L12 fusion protein could be a potent target for protein vaccines against *Brucella* infections.

In conclusion, our fusion protein, HSA-L7/L12, could elicit cellular immunity and provide substantial protection for the host against *Brucella* infection. However, the recombinant protein provided less protection than the attenuated *B. abortus* S19 strain. At present, an immunization assay is under investigation with the fusion protein in combination with LPS, and this approach will determine if protection approaching that offered by the current live *Brucella* vaccines could be achieved.

ACKNOWLEDGEMENTS

We are grateful to Mr. Hamid Abtahi (Department of Microbiology, Arak University of medical sciences, Iran) and Mr Philippe Langella (Jouy-en-Josas Cedx, France) for their generous donations. This study was funded by Tarbiat Moddarres University and Ilam University of Medical Sciences.

REFERENCES

REFERENCES

1 Godfroid J, Cloeckaert A, Liautard JP, Kohler S, Fretin D, Walravens K, et al. From the discovery of the Malta fever's agent to the discovery of a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis. Vet Res. 2005; 36: 313-26.

2 Schurig GG, Sriranganathan N, Corbel MJ. Brucellosis vaccines: past, present and future. Vet Microbiol. 2002; 90: 479-96.

3 Moriyon I, Grillo MJ, Monreal D, Gonzalez D, Marin C, Lopez-Goni I, et al. Rough vaccines in animal brucellosis: structural and genetic basis and present status. Vet Res. 2004; 35: 1-38.

4 Oliveira SC, Splitter GA. Immunization of mice with recombinant L7/L12 ribosomal protein confers protection against Brucella abortus infection. Vaccine. 1996; 14:959-62.

5 Onate AA, Vemulapalli R, Andrews E, Schurig GG, Boyle S, Folch H. Vaccination with live Escherichia coli expressing Brucella abortus Cu/Zn superoxide dismutase protects mice against virulent B. abortus. Infect Immun. 1999; 67: 986-8.

6 Vemulapalli R, He Y, Cravero S, Sriranganathan N, Boyle SM, Schurig GG. Overexpression of protective antigen as a novel approach to enhance vaccine efficacy of Brucella abortus strain RB51. Infect Immun. 2000; 68: 3286-9.

7 Oliveira SC, Splitter GA. Subcloning and expression of the Brucella abortus L7/L12 ribosomal gene and T-lymphocyte recognition of the recombinant protein. Infect Immun. 1994; 62: 5201-4.

8 Kurar E, Splitter GA. Nucleic acid vaccination of Brucella abortus ribosomal L7/L12 gene elicits immune response. Vaccine. 1997; 15: 1851-7.

9 Baloglu S, Boyle SM, Vemulapalli R, Sriranganathan N, Schurig GG, Toth TE. Immune responses of mice to vaccinia virus recombinants expressing either Listeria monocytogenes partial listeriolysin or Brucella abortus ribosomal L7/L12 protein. Vet Microbiol. 2005;109: 11-7.

10 Pontes DS, Dorella FA, Ribeiro LA, Miyoshi A, Le Loir Y, Gruss A, et al. Induction of partial protection in mice after oral administration of Lactococcus lactis producing Brucella abortus L7/L12 antigen. J Drug Target. 2003; 11: 489-93.

11 Ribeiro LA, Azevedo V, Le Loir Y, Oliveira SC, Dieye Y, Piard JC, et al. Production and targeting of the Brucella abortus antigen L7/L12 in Lactococcus lactis: a first step towards food-grade live vaccines against brucellosis. Appl Environ Microbiol. 2002; 68: 910-6.

12 Schirmbeck R, Kwissa M, Fissolo N, Elkholy S, Riedl P, Reimann J. Priming polyvalent immunity by DNA vaccines expressing chimeric antigens with a stress protein-capturing, viral J-domain. FASEB J. 2002; 16: 1108-10.

13 Johnston D, Bystryn JC. Heterogeneous antibody response to polyvalent melanoma vaccines in syngeneic mice. Cancer Immunol Immunother. 2005; 54: 345-50.

14 Huang L, Krieg AM, Eller N, Scott DE. Induction and regulation of Th1-inducing cytokines by bacterial DNA, lipopolysaccharide, and heat-inactivated bacteria. Infect Immun. 1999; 67: 6257-63.

15 David SA. The interaction of lipid A and lipopolysaccharide with human serum albumin. In: H.Brade, S.M.Opal, S.N.Vogel, and D.C.Morrison, (Editors). Endotoxins in health and disease. New York: Marcel Dekker; 1999. p. 413-22.

16 Kang HA, Choi ES, Hong WK, Kim JY, Ko SM, Sohn JH, et al. Proteolytic stability of recombinant human serum albumin secreted in the yeast Saccharomyces cerevisiae. Appl Microbiol Biotechnol. 2000; 53: 575-82.
17 Rezaee MA, Rezaee A, Moazzeni SM, Salmanian AH, Yasuda Y, Tochikubo K, et al. Expression of Escherichia coli heat-labile enterotoxin B subunit (LTB) in Saccharomyces cerevisiae. J Microbiol. 2005; 43: 354-60.
18 Wilson K. Preparation of genomic DNA from bacteria. Curr Protoc Mol Biol. 2001; Chapter 2: Unit 2 4.
19 Choi ES, Sohn JH, Rhee SK. Optimization of the expression system using galactose-inducible promoter for the production of anticoagulant hirudin in Saccharomyces cerevisiae. Appl Microbiol Biotechnol. 1994; 42: 587-94.
20 Zhan Y, Yang J, Cheers C. Cytokine response of T-cell subsets from Brucella abortus-infected mice to soluble Brucella proteins. Infect Immun. 1993; 61: 2841-7.
21 Winter AJ, Schurig GG, Boyle SM, Sriranganathan N, Bevins JS, Enright FM, et al. Protection of BALB/c mice against homologous and heterologous species of Brucella by rough strain vaccines derived from Brucella melitensis and Brucella suis biovar 4. Am J Vet Res. 1996; 57: 677-83.
22 Cloeckaert A, Zygmunt MS, Bezard G, Dubray G. Purification and antigenic analysis of the major 25-kilodalton outer membrane protein of Brucella abortus. Res Microbiol. 1996; 147: 225-35.
23 Cloeckaert A, Vizcaino N, Paquet JY, Bowden RA, Elzer PH. Major outer membrane proteins of Brucella spp.: past, present and future. Vet Microbiol. 2002; 90: 229-47.
24 Ko J, Splitter GA. Molecular host-pathogen interaction in brucellosis: current understanding and future approaches to vaccine development for mice and humans. Clin Microbiol Rev. 2003; 16: 65-78.
25 Jacques M. Role of lipo-oligosaccharides and lipopolysaccharides in bacterial adherence. Trends Microbiol. 1996; 4: 408-9.
26 Oliveira SC, Splitter GA. CD8+ type 1 CD44hi CD45 RBlo T lymphocytes control intracellular Brucella abortus infection as demonstrated in major histocompatibility complex class I- and class II-deficient mice. Eur J Immunol. 1995; 25: 2551-7.
27 Onate AA, Cespedes S, Cabrera A, Rivers R, Gonzalez A, Munoz C, et al. A DNA vaccine encoding Cu,Zn superoxide dismutase of Brucella abortus induces protective immunity in BALB/c mice. Infect Immun. 2003; 71: 4857-61.