Journal of Microbiology, Immunology and Infection (2013) 46, 253–258

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ORIGINAL ARTICLE

Induction of protective immunity against brucellosis in mice by vaccination with a combination of naloxone, alum, and heat-killed *Brucella melitensis* 16 M

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Received 2 February 2012; received in revised form 29 February 2012; accepted 16 March 2012

KEYWORDS
Alum;
Brucella melitensis 16 M;
Naloxone;
Protective immunity

Background/Purpose: A T-helper cell type 1-specific response leads to the elimination of intracellular infection with *Brucella*. Studies have shown that naloxone (NLX) can promote a cellular immune response in this respect. The current study was carried out to evaluate the induction of protective immunity in mice against brucellosis by vaccination with a combination of NLX, alum, and heat-killed *Brucella melitensis* 16 M (HKB).
Methods: Mice were categorized into five groups and received intraperitoneal vaccination on Days 0 and 7. Then serum levels of interferon (IFN)-γ and interleukin (IL)-4, the bacterial load, and the survival rate were measured 2 weeks after the last vaccination.
Results: The serum levels of IFN-γ, IL-4, and immunoglobulin G in the NLX + alum + HKB group were shown to be significantly increased ($p < 0.05$). Furthermore, the lowest bacterial load was observed in this group. The survival rate in groups vaccinated with combinations containing adjuvants was 100%.
Conclusion: The combination of NLX and alum enhanced the immunogenicity of HKB, which can be used in the vaccination of animals and humans at risk of the disease.
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doi:10.1016/j.jmii.2012.03.011

Introduction

Brucella melitensis is the most pathogenic *Brucella* species in human. The bacterium is the cause of abortion in ewes, which leads to a considerable economic loss in most countries.[1] *Brucella melitensis* Rev1 is the vaccine available for the prevention of brucellosis in sheep and goats in endemic areas, producing a higher protective immunity than the killed vaccine. However, in a few cases, the vaccine has led to brucellosis in humans and animals.[2]

In brucellosis infection, macrophages phagocytose the *Brucella* bacteria, which grow and propagate in the macrophages. During the infection, interferon (IFN)-γ is the most important factor for the activation of macrophages to kill the intracellular *Brucella*.[3] A T-helper cell type 1 (Th1)-specific response leads to the elimination of intracellular infections of *Brucella*, while an expansion of the Th2 response is observed in prolonged infections.[4,5] *Brucella* infection can lead to aberrations of the immune system and Th1-specific responses to the infection.[6]

Alum is a commonly used adjuvant.[7] In addition, recent studies have shown that naloxone (NLX) can increase the level of IFN-γ and promote the immune response to produce a Th1 pattern.[8] It has been show that, in infection caused by intracellular bacteria such as *Listeria monocytogenes*, NLX can stimulate IFN-γ production and lead to stronger cell-mediated immunity.[9] Therefore, the aim of the current study was to evaluate the effect of NLX and alum together with whole killed *B. melitensis* 16 M in the development of cellular immunity against the bacterium in mice.

Methods

Bacteria

The strain of *B. melitensis* 16 M used was obtained from the Microbiology Research Center, Islamic Azad University, Zanjan Branch, Iran.

Mice

The experiments were performed according to the Helsinki guidelines. Male BALB/c mice with a mean age of 6—8 weeks were bought from Razi Institute (Karaj, Iran) and received autoclaved water and food.

Preparation of whole killed *Brucella melitensis* 16 M

The bacterium was cultured on *Brucella* agar (Merck, Darmstadt Germany) and inoculated at 37°C for 3—5 days. The colonies were collected and they were washed three times with phosphate-buffered saline (PBS) solution. The precipitated bacteria were again mixed with the PBS solution and then incubated at 65°C for 60 minutes. The solution was evaluated, and it was confirmed that the bacteria were unable to grow. The number of bacteria was measured by comparing serial concentration absorptions at a wavelength of 590 nm.[10]

Immunization of mice

The combination of NLX + alum was prepared by mixing 50 μL of PBS, which contained 6 mg/kg NLX (Sigma, Ansbach, Germany), with 50 μL of aluminum phosphate gel (Sigma). The mice were categorized into five groups and received intraperitoneal (i.p.) vaccine on Days 0 and 7. The first group received a combination of heat-killed *B. melitensis* [HKB; at a dose of 10^8 colony-forming units (CFUs)] + alum + NLX; the second group was vaccinated with a combination of HKB + alum; the third group received HKB + NLX; the fourth group received HKB; and the fifth group, as the control group, received only sterile PBS.[11]

Serum collection

Anesthesia was induced in the mice using i.p. injection of sodium pentobarbital (80 mg/kg). After anesthesia, blood samples were taken, and the sera were kept at −70°C until use.[12]

Evaluation of serum immunoglobulin G

Two weeks after the last vaccination, the immunoglobulin G (IgG) level in serum samples obtained from five mice from each group was measured. Measurement was performed using indirect enzyme-linked immunosorbent assay (ELISA).

A sample of 25 μg sonicated whole-cell antigen of *B. melitensis* 16 M together with carbonate buffer (pH 9.6) was added to each well of a 96-well plate (SPL, Pocheon, Korea). The plate was stored overnight at under 4°C. The wells were then washed three times with PBS-Tween. A blocking step was carried out using PBS containing bovine serum albumin (BSA) 1%, and afterwards the final solution was incubated at 37°C for 2 hours.

Afterwards, the wells were washed five times, and 50 μL of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Millipore, Billerica, Massachusetts, USA), at a concentration of 1:10,000, was added to each well. Before adding Tetramethylbenzidine (TMB) substrate (U-CyTech, Utrecht, The Netherlands), the wells were washed four times, the plate then being incubated at 37°C for 20 minutes. A total of 50 μL of H_2SO_4 (1 M) was added to terminate the reaction. Finally, the optical density (OD) value was read at a wavelength of 450 nm using an ELISA reader (Awareness, Palm city, USA).[11]

Measurement of cytokine levels

Five mice were selected from each group and intraperitoneally challenged with 2×10^4 CFU living *B. melitensis* 16 M 2 weeks after the last vaccination. Serum samples were obtained 48 hours after the challenge, and levels of IFN-γ and interleukin (IL)-4 were measured using the sandwich ELISA method according to the protocol defined for the commercial kit (Mabtech, Stockholm, Sweden).[12]

Determination of bacterial load in spleen using real-time polymerase chain reaction

Two weeks after the last vaccination, five mice from each group were challenged with 2×10^4 CFU of the bacteria;

48 hours after the challenge, the animals were killed and their spleens were resected. Using a DNA extraction kit (Gnet Bio, Nonsan, Korea), DNA was extracted from 30 mg of spleen tissue for each mouse. The amount of DNA extracted was determined by spectrophotometry and measuring the OD ratio A_{260}/A_{280}, and its quality was evaluated using 1% agarose gel electrophoresis.

The bacterial load in mouse spleen tissue was determined using primers specifically designed for the genome segment common among different strains of *Brucella* with the following sequences: F, 5'-CCAGCGCACCATCTTTCAG-3'; and R, 5'-TCGTTGCGCGTAAGGATGC-3'. To prepare the standard curve, we used 0.5 McFarland *B. melitensis* 16 M diluted to 1:100 with sterile distilled water. Using a DNA extraction kit (Cinnagen, Tehran, Iran), bacterial DNA was extracted, and the sample was then diluted 10 times to obtain a range of bacterial presence from 1.5×10^6 to 15×10^6 bacteria per mL (Fig. 1).

For the polymerase chain reaction (PCR) process, Master mix (Pars Tous, Mashhad, Iran), 0.5 μL of each primer, 0.5 μL of SYBR Green (Sigma), and 1 μL of DNA sample in a total volume of 20 μL were used. The PCR process included the following steps: primary denaturation at 94°C for 10 minutes, and reaction cycles of 94°C for 30 seconds, 63°C for 30 seconds, 72°C for 45 seconds, and finally 72°C for 5 minutes. The reactions were carried out using a Corbett Rotor-Gene 6000 (Corbett Life Science, Mannheim, Germany). At the end of the reaction, the melting curve program was run at 72—94°C, and the curve was drawn by the instrument.[13—15]

Determination of survival rate

Ten mice received 5×10^9 CFU (i.p.) living *B. melitensis* 16 M. After 72 hours, all 10 mice had died, so this value was determined to be the lethal dose (LD_{100}). Two weeks after the last vaccination, five mice from each group were intraperitoneally challenged with the lethal dose.[16]

Statistical analysis

Statistical analysis was performed using SPSS software, version 12.0. (SPSS Inc. Chicago, Ilinois, USA) Group comparisons were carried out using Mann—Whitney *U* test. A p value ≤ 0.05 was considered statistically significant.

Results

Cytokine level

The highest level of IFN-γ was seen in the NLX + alum + HKB group (Fig. 1). The IFN-γ level in this group was significantly higher than in other groups ($p = 0.009$). In addition, the IFN-γ level in the alum + HKB group was lower than that in the NLX + HKB group, although the difference was not significant ($p = 0.34$). Moreover, the difference between the alum + HKB and HKB groups was not significant ($p = 0.75$). Similarly, the difference observed between the NLX + HKB and HKB groups in this respect was not significant ($p = 0.17$). The IFN-γ levels in all the vaccinated groups were higher than the level in the control group ($p < 0.01$) (Fig. 1).

The results also showed that the IL-4 level in the NLX + alum + HKB group was significantly higher than the levels in the alum + HKB, NLX + HKB, and control groups ($p < 0.01$), as well as the HKB group ($p = 0.04$). The IL-4 level in the alum + HKB group was less than that in the NLX + HKB group, but the difference was not significant ($p = 0.10$). The IL-4 level in the alum + HKB group was less than that in the HKB group ($p < 0.01$). A comparison of NLX + HKB with HKB demonstrated that the HKB group had a higher level of IL-4, but the difference was not significant ($p = 0.2$). Finally, the serum level of IL-4 in the control group was significantly lower than the values obtained for other groups ($p < 0.05$) (Fig. 2).

Serum IgG titer

The antibody titer in the NLX + alum + HKB group was higher than the values obtained for the other groups. Compared with the titer in the alum + HKB group, the difference was significant ($p = 0.012$). A significant difference was observed between the IgG level in the NLX + alum + HKB group and the NLX + HKB, HKB, and

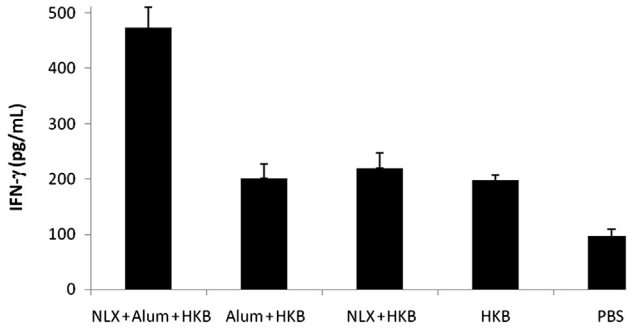

Figure 1. Mean serum level (pg/mL) of interferon (IFN)-γ in the groups under evaluation 2 weeks after their last vaccination. Alum = aluminum phosphate gel; HKB = heat-killed *Brucella melitensis* 16 M; NLX = naloxone; PBS = phosphate buffered saline.

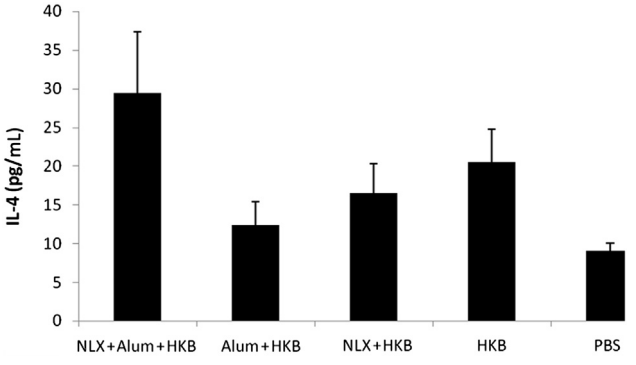

Figure 2. Mean serum level (pg/mL) of interleukin (IL)-4 in the groups under evaluation 2 weeks after last vaccination. Alum = aluminum phosphate gel; HKB = heat-killed Brucella melitensis 16 M; NLX = naloxone; PBS = phosphate buffered saline.

control groups ($p < 0.05$) (Fig. 3). Comparison of the antibody level in the other groups demonstrated that other groups differed in this respect. However, the difference was not significant, although the difference between the vaccinated group and control group was significant ($p < 0.01$). Nevertheless, other vaccinated groups were not significantly different from each other in this respect (Fig. 3).

Bacterial load in spleen

The bacterial load in the NLX + alum + HKB group was lower than the load in the HKB and control groups ($p < 0.01$). Moreover, the bacterial load in the NLX + alum + HKB group was lower than those in the alum + HKB and NLX + HKB groups, although the difference was not significant ($p > 0.05$). The bacterial loads in the alum + HKB and NLX + HKB groups were significantly lower than those in the HKB and control groups ($p < 0.01$). The bacterial load in the HKB group was less than that in the control group ($p < 0.01$) (Fig. 4).

Survival rate of mice after challenge

All mice in the control group had died by 72 hours. Moreover, one mouse from the HKB group died 96 hours after challenge. All the mice from the other groups survived until 2 weeks after the challenge (Fig. 5).

Discussion

The findings showed that a combination of NLX, alum, and whole HKB increased levels of IFN-γ and IL-4 and the specific IgG against *B. melitensis* 16 M. Jazani et al[11] reported that a group of mice vaccinated with a combination of alum, NLX, and Lipopolysaccharide (LPS)-removed lysate of *Salmonella typhimurium* showed a significant increase in IFN-γ level. Furthermore, they found that the highest level of IL-4 was observed in the group vaccinated with alum and lysate of *S. typhimurium*. In the current study, a significant

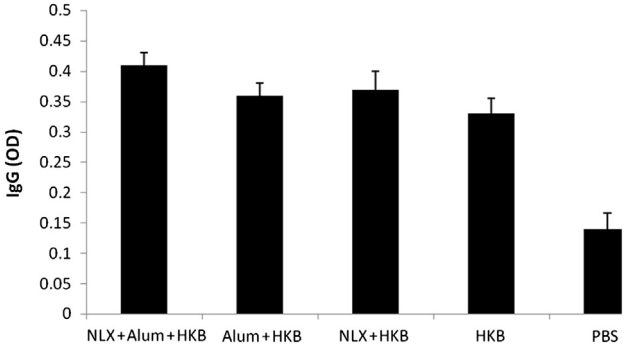

Figure 3. Mean serum level of immunoglobulin G (IgG) against *Brucella melitensis* in different groups 2 weeks after the last vaccination, shown in terms of optical density (OD). Alum = aluminum phosphate gel; HKB = heat-killed Brucella melitensis 16 M; NLX = naloxone; PBS = phosphate buffered saline.

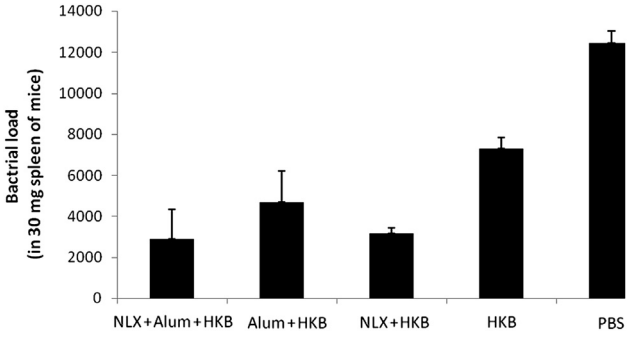

Figure 4. Bacterial load of *Brucella melitensis* (number of bacteria in 30 mg of mouse spleen) in the spleens of mice from different groups 48 hours after challenge with living bacteria (2×10^4 colony-forming units), determined using the real-time polymerase chain reaction. The bacterial load in the NLX + alum + HKB group was lower than that in the HKB and control groups ($p < 0.01$), and in the alum + HKB and NLX + HKB groups. Alum = aluminum phosphate gel; HKB = heat-killed Brucella melitensis 16 M; NLX = naloxone; PBS = phosphate buffered saline.

increase in IFN-γ level was observed in the NLX + alum + HKB group; however, all vaccinated groups showed a significant difference compared with the control group. The highest level of IL-4 production was observed in the NLX + alum + HKB group, and the lowest level of IL-4 was observed in the control group, with the second lowest level in the alum + HKB group. It should be noted that IFN-γ and IL-4 levels were measured using the serum level.

In a study carried out by Wang et al,[17] the immunogenicity of a mutant strain of *B. melitensis* 16 M was compared with that of the vaccinal strain Rev1. These authors showed a significantly increased IFN-γ level in the groups vaccinated with a mutant strain of *B. melitensis* 16 M and the vaccinal strain Rev1; however, the values in the two vaccinated groups were not significantly different. These results indicate that using different mutants of *B. melitensis* did not have a significant effect on the stimulation of IFN-γ production.

In the study published by Al-Mariri,[18] the immunogenicity of P39 protein of *Brucella* expressed in *Escherichia coli* was evaluated. The results of cytokine evaluation demonstrated that the IFN-γ level in the group vaccinated with living *E. coli* expressing P39 protein was higher than that in the control group. Moreover, stimulation of lymphocytes of the control group with *B. melitensis* 16 M showed an increase in the level of IFN-γ, which was lower than the level in the P39 protein vaccinated group. In the current study, the levels of IFN-γ in the vaccinated groups, particularly the group vaccinated with NLX + alum + HKB, were higher than that in the control group.

Previous studies have shown that, because of the low immunogenicity of recombinant proteins from pathogenic bacteria, the appropriate technique for the production of vaccines is to employ complete and intact antigens.[19] This is true for the vaccine against *B. melitensis*. Most vaccination programs for domestic animals are based upon employment of vaccinal strain Rev1.[20] Therefore, studies

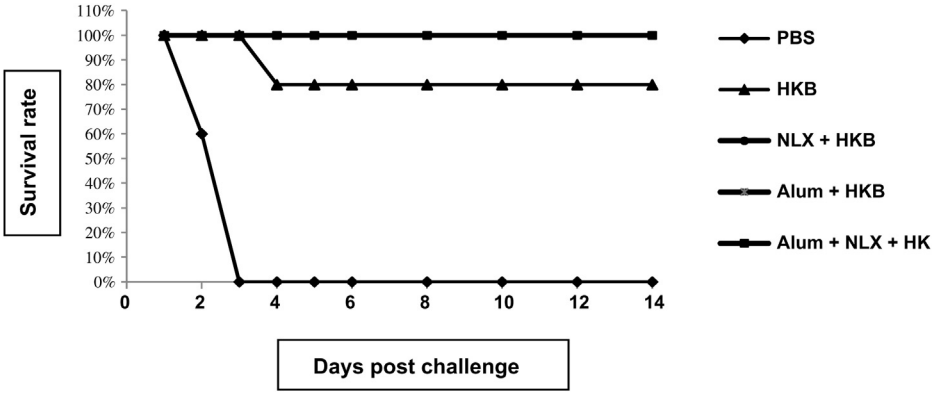

Figure 5. Survival rate of mice after challenging five mice from each group with 5×10^9 colony-forming units (LD_{100}) of living bacteria. Alum = aluminum phosphate gel; HKB = heat-killed Brucella melitensis 16 M; NLX = naloxone; PBS = phosphate buffered saline.

to reduce the problems associated with this type of vaccine and also improve its immunogenicity are of great importance.

Considering the serum-specific IgG level in the study by Jazani et al, the highest level of specific IgG was detected in the alum, NLX, and LPS-removed lysate of *S. typhimurium* group.[11] Wang et al reported that the serum IgG levels increased significantly in groups vaccinated with mutant *B. melitensis* 16 M and Rev1 strain.[17] According to our findings, the serum level of IgG in the NLX + alum + HKB group was significantly higher than that in other groups. Moreover, the IgG levels in all vaccinated groups were significantly higher than in the control group.

We preferred to use the specific and sensitive method of real-time PCR to determine the bacterial load in the spleens of the mice in our study.[20] The lowest bacterial load was identified in the spleens of the groups vaccinated with NLX + alum + HKB, and then NLX + HKB. Jazani et al was reported that the bacterial load measured in spleens from the groups vaccinated with a combination of NLX, alum, and the vaccine, and with NLX plus the vaccine, were significantly lower than in the control group.[11] In this respect, the results of Wang et al's study demonstrated that the bacterial load in the group vaccinated with mutant *B. melitensis* 16 M was reduced, and showed its difference compared with the control group.[17] Furthermore, Al-Mariri showed that the bacterial load decreased, albeit not significantly, in the groups vaccinated with *E. coli* expressing P39. In addition, the bacterial load in these groups was higher than that in spleens of mice vaccinated with vaccinal strain Rev1.[18]

The previously performed studies have shown that secretion of IFN-γ in the early stages of *Brucella* infection plays an important role in limiting and eliminating the bacteria via macrophage activation.[21] According to the results obtained, using a combination of NLX + alum + HKB as a vaccine was strongly effective in reducing the bacterial load in vaccinated mice. It was observed that vaccination with NLX + alum + HKB was able to establish 100% protective immunity against the lethal dose of the bacteria. The current study showed that using NLX and alum, as adjuvants, together with whole killed *B. melitensis* can enhance the cellular immunity response.

NLX and alum have been routinely administered to humans. In addition, the whole killed cells used in the study do not carry a risk of pathogenicity, and the cost of vaccine production in this method is low. Hence, it is hoped that the combination could be used in future vaccines prepared for domestic animals and even humans.

Conflict of interest

No authors in this study have any conflict of interest.

Acknowledgments

The study was financially supported by the Department of Immunology, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran. The authors wish to thank those who kindly supported them.

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