

A novel adjuvant, a mixture of alum and the general opioid antagonist naloxone, elicits both humoral and cellular immune responses for heat-killed *Salmonella typhimurium* vaccine

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Received 27 July 2010; revised 8 September 2010; accepted 21 September 2010.
Final version published online 5 November 2010.

DOI:10.1111/j.1574-695X.2010.00747.x

Editor: Willem van Eden

Keywords

alum; naloxone; novel adjuvant; *Salmonella typhimurium*.

Abstract

In the current study, we tested the efficacy of the mixture of naloxone, an opioid receptor antagonist, and alum, as a new adjuvant, in the induction of humoral and cellular immunity in response to heat-killed *Salmonella typhimurium* (HKST) as a model vaccine. BALB/c mice were divided into five groups. Mice in the experimental groups received either the HKST vaccine alone or in combination with the adjuvant alum, naloxone or the alum–naloxone mixture. Mice in the negative control group received phosphate-buffered saline. All mice were immunized two times on days 0 and 14. Two weeks after the last immunization, immune responses to *S. typhimurium* were assessed. Our results indicated that the administration of the alum–naloxone mixture as an adjuvant increased the ability of the HKST vaccine to enhance lymphocyte proliferation, shifted the immune response towards a T-helper 1 (Th1) pattern and increased *S. typhimurium*-specific immunoglobulin G (IgG), IgG2a, IgG1 and the ratio of IgG2a to IgG1. This resulted in improved protective immunity against *S. typhimurium*. In conclusion, the administration of the alum–naloxone mixture as an adjuvant, in combination with the HKST vaccine, can enhance both humoral and cellular immunity and shift the immune responses to a Th1 pattern.

Introduction

Over the last century, the use of vaccines has profoundly reduced the morbidity and mortality caused by infectious diseases in both human and animal populations. In fact, vaccination has proven to be the most successful medical intervention ever developed (Schijns, 2003). However, the vaccines that are currently available still fail to protect against certain pathogens. One promising strategy for addressing this challenge is the development of new vaccine adjuvants that enhance the effectiveness of vaccines (Wilson-Welder *et al.*, 2009). These adjuvants should have the ability to elicit a potent immune response. The most effective immune response against multiple pathogens involves a combination of both humoral and cellular components. This is even true for some obligate intracellular pathogens (Casadevall, 2003; Casadevall & Pirofski, 2006). Safety is another important parameter to consider when choosing an appropriate adjuvant, especially for human

vaccination. While many adjuvants have been discovered and used in research, only a few adjuvants have been permitted for use in human vaccination (Gupta & Siber, 1995). The only vaccine adjuvant that is approved by the United States Food and Drug Administration (FDA) is alum (aluminum-based mineral salt) (De Gregorio *et al.*, 2008). In addition to alum, the oil-in-water emulsions MF59 and AS03 and the monophosphoryl lipid A formulated in alum (AS04) have been approved as vaccine adjuvants by the European Medicines Agency (De Gregorio *et al.*, 2008; Perrie *et al.*, 2008; Tagliabue & Rappuoli, 2008). However, alum remains the only adjuvant approved worldwide for human use (Harandi *et al.*, 2010). Alum has been used widely and successfully in many licensed vaccines, and has a good track record of safety. It is considered the adjuvant of choice for vaccines against infectious diseases that can be prevented by the humoral immune response (Lindblad, 2004; Harandi *et al.*, 2010). However, some limitations of alum have been described. Notably, alum is a poor inducer

of cell-mediated immunity and T helper 1 (Th1) responses, which are both required to combat several life-threatening infections (Hem & White, 1995; Harandi *et al.*, 2010). Thus, there is an urgent need to develop new adjuvant formulations for use in the development of effective vaccines against pathogens, which has so far been refractory to current vaccination strategies, and to overcome the limitations of the licensed adjuvants available (Harandi *et al.*, 2010).

Opioid peptides are well-known inhibitors of the immune response and vaccination against microbial agents (Molitor *et al.*, 1992; Carpenter & Carr, 1995; Friedman *et al.*, 2003). Furthermore, exposure to exogenous opioids is known to increase susceptibility to microbial infection (Friedman *et al.*, 2003). It has been suggested that naloxone, an opioid antagonist, can shift the immune response toward a Th1 pattern (Sacerdote *et al.*, 1998, 2000). Naloxone is currently approved by the FDA as a prescription drug (<http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm>) and is routinely used by emergency medical personnel to rapidly and safely reverse opioid-induced respiratory depression (Burriss *et al.*, 2001). We have shown previously that naloxone administration during primary herpes simplex virus type 1 (HSV-1) infection by a nonvirulent strain enhanced protection against a subsequent HSV-1 challenge (Jamali *et al.*, 2007). Additionally, we have shown that naloxone, when administered as an adjuvant in combination with either an HSV-1 DNA vaccine or a heat-killed *Listeria monocytogenes* vaccine, was able to increase the respective vaccines' efficacy and enhance cellular immunity. The addition of naloxone as an adjuvant also skewed the immune response toward a Th1 response and improved protective immunity (Jamali *et al.*, 2009; Jazani *et al.*, 2010).

Similar to other facultative intracellular bacteria, protection against *Salmonella typhimurium* requires both humoral and cellular immunity (Mittrucker & Kaufmann, 2000). In the current study, we tested the hypothesis that the adjuvant activity of the mixture of alum and naloxone (a new adjuvant) in the induction of immunity to heat-killed *S. typhimurium* (HKST) is greater than the activity of alum or naloxone alone. We used HKST as a vaccine model against facultative intracellular bacteria that require both humoral and cellular immunity for proper host protection. Because immunogenic doses of HKST without an adjuvant stimulate immune responses efficiently (Jazani *et al.*, 2005), we used a subimmunogenic dose of HKST, as a model vaccine, to evaluate the adjuvant activity of the mixture of alum and naloxone.

Materials and methods

Mice

Six- to eight-week-old male BALB/c mice were obtained from the Razi Institute (Karaj, Iran). Mice were housed for 1

week before the experiment, and were provided with free access to food and water. All experiments were conducted in accordance with the Animal Care and Use Protocol of Urmia University of Medical Sciences (Urmia, Iran).

Preparation of HKST

Salmonella typhimurium Persian Type Culture Collection was grown on blood agar plates (Merck, Germany) overnight at 37 °C. Cultures were then harvested, centrifuged and washed three times in phosphate-buffered saline (PBS). The recovered bacteria were resuspended in PBS and incubated at 80 °C for 2 h to generate a heat-killed preparation. An absence of viable colonies was confirmed by the lack of bacterial growth on blood agar plates. The bacterial concentration was enumerated by comparing the absorbance of a serial dilution of HKST at 590 nm with McFarland Nephelometer Standards. The HKST was then stored at –70 °C. The optimal dose for immunization was determined by preliminary titration (data not shown).

Immunization protocol

The alum–naloxone mixture was prepared by thoroughly mixing 50 µL of PBS containing naloxone (Sigma, Germany) at a concentration of 6 mg kg⁻¹ with 50 µL of alum (aluminum phosphate, Sigma). [Correction added 17 November after online publication: the word 'gel' was removed from the previous sentence]. This mixture was incubated under sterile conditions for 72 h at 4 °C. HKST (10⁶) suspended in 50 µL PBS absorbed in 100 µL of the alum–naloxone mixture was injected subcutaneously into the mice in the Al-NLX-Vac group. The HKST was also suspended in 100 µL of PBS that was absorbed in 50 µL of alum. This preparation was injected subcutaneously into the mice in the Al-Vac group. The mice that were part of the NLX-Vac group received a subcutaneous injection of the HKST suspended in 100 µL of PBS plus naloxone (6 mg kg⁻¹), and then dissolved in 50 µL of PBS. HKST was suspended in 150 µL of PBS and was injected subcutaneously into the mice in the Vac group. The mice that were part of the control group received a subcutaneous injection of 150 µL of PBS. All of the mice were injected with a total volume of 150 µL. All mice were immunized twice: once on day 0 and once on day 14.

Cytokine assays

Two weeks after the last immunization, the spleens of five mice per group were removed aseptically and homogenized in RPMI 1640 medium (Gibco-BRL) supplemented with 10% fetal cow serum (FCS) (Gibco-BRL) and antibiotics. Red blood cells (RBCs) were then osmotically lysed using ammonium chloride buffer (NH₄Cl 0.16 M, Tris 0.17 M). The cells were washed twice with RPMI 1640 and counted,

with viability determined by trypan blue (0.4% w/v) exclusion. A nominal total of 1×10^6 spleen cells were plated in each well of a 24-well plate using RPMI 1640 that was supplemented with 10% FCS, 100 IU mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin and 5×10^{-5} M 2-mercaptoethanol. Two wells were used per mouse. The cells were restimulated *in vitro* with 6×10^4 HKST. The optimal dose of HKST for restimulation had been determined by preliminary titration. Plates were then incubated at 37 °C in 5% carbon dioxide (CO₂). The supernatants were removed 48 h after stimulation and stored at -70 °C. The concentration of secreted interferon- γ (IFN- γ) and interleukin-4 (IL-4) levels in the supernatants were estimated using a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D systems, Minneapolis, MN).

Lymphocyte proliferation assay

Two weeks after the last immunization, the lymphocyte proliferation rate was measured using an MTT (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl-blue, Sigma) dye assay. The spleens of five mice per group were removed under sterile conditions and single-cell suspensions were prepared in a phenol red-free RPMI 1640 medium. RBCs were lysed using 0.75% ammonium chloride in Tris buffer (0.02%, pH 7.2). The concentration was adjusted to 1×10^6 cells mL⁻¹ in phenol red-free RPMI 1640 that was supplemented with 10% FCS, 2 mM L-glutamine and 25 mM HEPES. One hundred microliters of diluted cell suspensions were dispensed into 96-well flat-bottom culture plates. Mitogen phytohemagglutinin-A (Gibco-BRL) at a final concentration of 5 µg mL⁻¹ (positive control) or 6×10^4 HKST was added to each well and the volume was adjusted to 0.2 mL. After incubating for 48 h at 37 °C in 5% CO₂, cell proliferation was determined using an MTT assay (Mosmann, 1983). Briefly, 20 µL of MTT was added to each well and the plates were further incubated at 37 °C for 4 h. Following incubation, the supernatant was carefully aspirated from each well and formazan crystals were solubilized by adding 100 µL of dimethyl sulfoxide. The absorbance of each well was then determined at a wavelength of 540 nm. Stimulation indices were determined and expressed as differences between the absorbance of treated and untreated wells.

Determination of total immunoglobulin G (IgG) titers and IgG isotyping

Two weeks after the last immunization, the levels of IgG antibodies were measured in the sera of five mice from each group by ELISA using 96-well microtiter plates (Bansal *et al.*, 2010). The optimum dilution of the sera and the optimum dose of HKST to be used in the ELISA were determined using the checkerboard assay. Then, 200 µL of antigen (HKST 6×10^2 per 200 µL) in coating buffer (0.1 M carbonate, pH 9.5) was added

to each well of a 96-well microtiter plate. Coated plates were incubated at 4 °C overnight, washed with PBST (PBS with 0.05% Tween 20) three times and blocked with 5% bovine serum albumin in PBST for 2 h at 37 °C. After washing the plates with PBST, different dilutions (200 µL) of sera were added to the wells. Plates were incubated at 37 °C for 2 h. After washing three times with PBST, the plates were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Sigma), IgG1 or IgG2a (Serotec). After washing with PBST three times, the reaction was developed by adding 200 µL of a TMB/H₂O₂ substrate. The reaction was terminated by the addition of 50 µL of 2N H₂SO₄ and the absorbance was read at 450 nm wavelength. The ratio of IgG2a and IgG1 isotypes was calculated by dividing the OD values for IgG2a by the OD values for IgG1.

Determination of bacterial load in spleens and livers

To evaluate vaccine-induced protective immunity against *S. typhimurium*, an additional experiment was performed to determine the bacterial load in the livers and spleens after challenge with the bacterium. Two weeks after the last immunization, five mice from each group were infected via an intraperitoneal injection with 10^3 live *S. typhimurium* that was suspended in 200 µL of PBS. Forty-eight hours later, the mice were sacrificed and the spleen and liver of each mouse were homogenized individually. Ten microliters of undiluted spleen and liver-cell suspensions, which had been prepared in Triton X-100 (0.05%), were then plated separately on trypticase soy plates. One day after culturing at 37 °C, the log of colony-forming units (CFUs) was determined. The optimal dose of *S. typhimurium* for challenge had been determined by preliminary titration (data not shown).

Survival rate

Two weeks after the last immunization, seven mice from each group were challenged with 10^7 live *S. typhimurium*. The survival rate was then monitored for 3 weeks. The lethal dose of *S. typhimurium* had been determined by preliminary titration (data not shown).

Statistical analysis

The MTT assay, cytokine levels and the bacterial loads in the spleen and liver were analyzed using one-way ANOVA, followed by Tukey's post-test. The survival rate was measured using Kaplan–Meier analysis and the log rank test. A *P*-value of < 0.05 was considered significant.

Results

Cytokine pattern

As shown in Fig. 1a, mice immunized with the alum–naloxone mixture with the HKST vaccine produced significantly

more IFN- γ than mice that received either PBS, the HKST vaccine alone, the HKST vaccine with alum or the HKST vaccine with naloxone. Lymphocytes from the mice vaccinated

with HKST with naloxone produced larger amounts of IFN- γ than those of the mice that received the HKST vaccine with alum, the HKST vaccine alone or PBS; however, the differences between each group were not statistically significant. The mice immunized with the HKST vaccine that contained alum produced more IFN- γ than mice that received PBS or the HKST vaccine alone; however, the difference between the groups was not statistically significant. The mice vaccinated with the HKST vaccine alone produced more IFN- γ than mice that received PBS. Again, the difference between the groups was not statistically significant.

The IL-4 levels were similar among the mice that received PBS, the HKST vaccine alone or the HKST vaccine with naloxone (Fig. 1b). However, mice immunized with the alum–naloxone mixture, in combination with the HKST vaccine, produced significantly more IL-4 than mice that received PBS, the HKST vaccine alone or the HKST vaccine with naloxone. Lymphocytes from the mice that were vaccinated with HKST with alum produced significantly larger amounts of IL-4 compared with the IL-4 levels measured in the mice that received PBS, the HKST vaccine with naloxone or the HKST vaccine alone. The IL-4 levels were similar among the mice that received the HKST vaccine with alum or the HKST vaccine with the alum–naloxone mixture.

As shown in Fig. 1c, the IFN- γ /IL-4 ratio from the mice that were immunized with the alum–naloxone mixture, in combination with HKST, was significantly higher than the IFN- γ /IL-4 ratio from mice that were administered the HKST vaccine alone, the HKST vaccine with alum or the HKST vaccine with naloxone. The mice that were immunized with the HKST vaccine containing naloxone had an increased IFN- γ /IL-4 ratio compared with the mice that received the PBS, the HKST vaccine with alum or the HKST vaccine alone; however, the differences between the groups were not statistically significant.

Lymphocyte proliferation

Because lymphocyte proliferation is generally considered to be a measure of cell-mediated immunity, *S. typhimurium*-specific lymphocyte proliferation was evaluated using an MTT assay. As shown in Fig. 2, lymphocyte proliferation was significantly higher in mice treated with the HKST vaccine

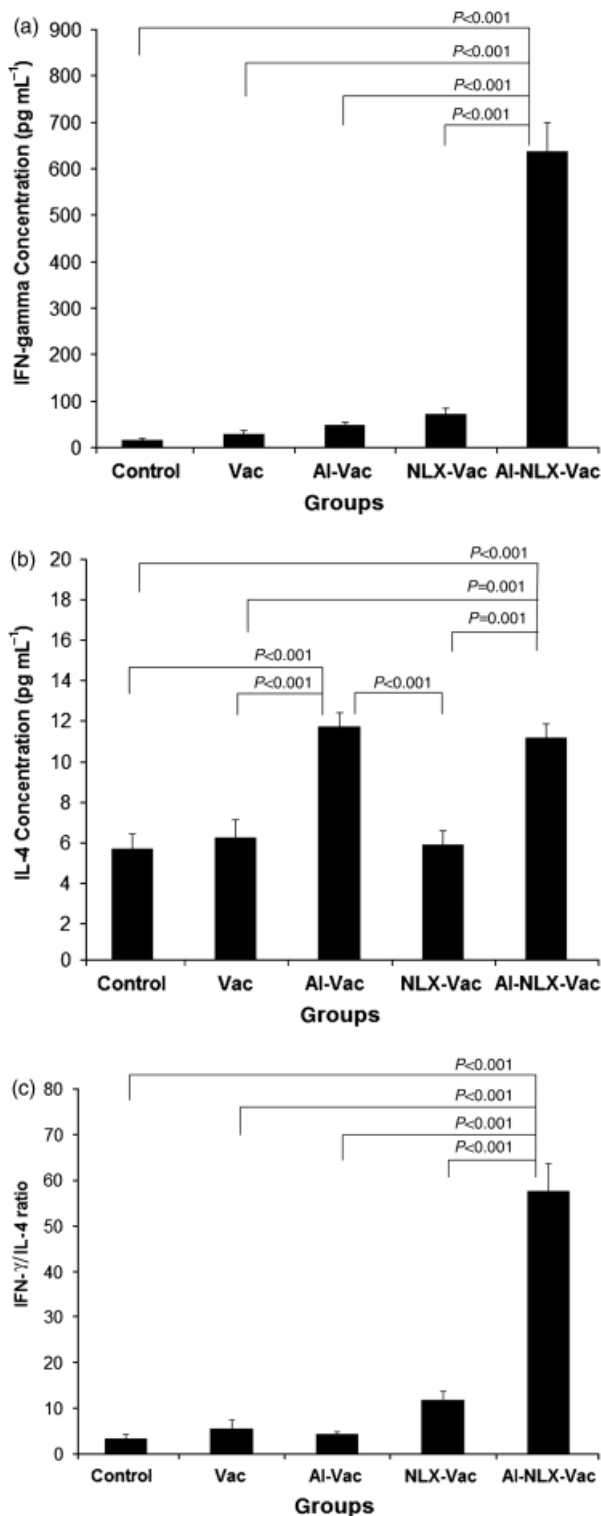


Fig. 1. Effect of administering alum–naloxone mixture on IFN- γ and IL-4 production. Two weeks after the final immunization, the spleens of individual mice (five per group) were removed and IFN- γ and IL-4 production was measured and compared between Al-NLX-Vac (alum–naloxone mixture in combination with the HKST vaccine), control (PBS), Vac (HKST vaccine alone), Al-Vac (alum in combination with the HKST vaccine) and NLX-Vac (naloxone in combination with the HKST vaccine) groups. Values are shown as the mean \pm SE.

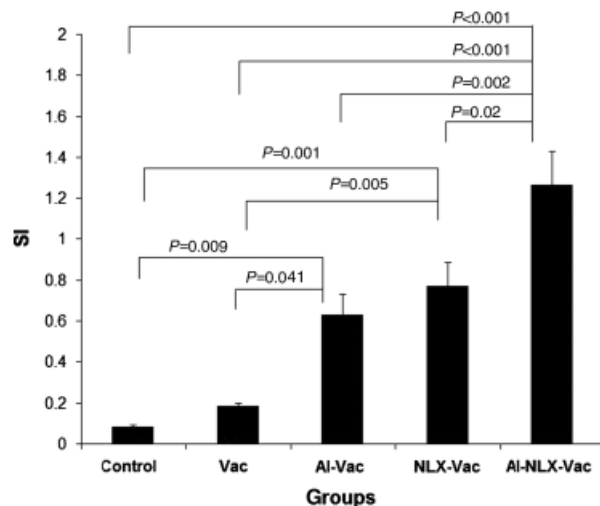


Fig. 2. Effect of administering alum–naloxone mixture on lymphocyte proliferation. Two weeks after the final immunization, spleens from individual mice (five per group) were removed and lymphocyte proliferation was evaluated using the MTT method. The groups are as in Fig. 1. Values represent the mean \pm SE. SI, stimulation index.

in combination with the alum–naloxone mixture compared with the lymphocyte proliferation in mice that were administered the PBS, the HKST vaccine alone, the HKST vaccine with alum and the HKST vaccine with naloxone. Lymphocytes from the mice that were vaccinated with HKST with naloxone showed significantly more proliferation than the lymphocytes harvested from the mice that received the PBS or the HKST vaccine alone. Lymphocyte proliferation was significantly higher in mice treated with the HKST vaccine with alum compared with the lymphocyte proliferation in mice that received the PBS or the HKST vaccine alone. Mice that received the HKST vaccine alone exhibited higher levels of lymphocyte proliferation compared with the levels of lymphocyte proliferation that were observed in the control mice; however, the difference observed between the groups was not statistically significant.

Antibody titer

The sera obtained 2 weeks after the last immunization, from all the groups of mice, were screened for the presence of IgGs against *S. typhimurium*. As shown in Fig. 3, a significant increase in anti-*S. typhimurium* IgG titers was observed in mice vaccinated with the HKST in combination with the alum–naloxone mixture as compared with the mice that were administered PBS, the HKST vaccine alone, the HKST vaccine with alum or the HKST vaccine with naloxone. The mice vaccinated with HKST with naloxone had significantly more anti-*S. typhimurium* IgG titers compared with the IgG titers

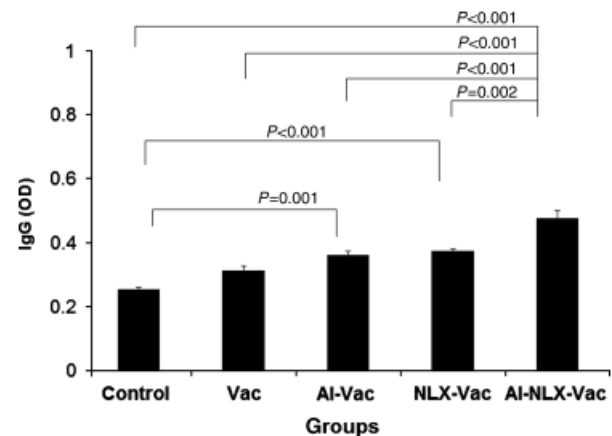


Fig. 3. Effect of administering alum–naloxone mixture on antibody response. The sera obtained 2 weeks after the last immunization, from all of the mouse groups, were screened for the presence of IgGs against *S. typhimurium*. The groups are as in Fig. 1. Values are mean \pm SE of five mice in each group.

observed in the mice that received PBS. The anti-*S. typhimurium* IgG titer was significantly higher in mice that were treated with the HKST vaccine with alum compared with the anti-*S. typhimurium* IgG titer observed in mice that received PBS. Mice that received the HKST vaccine alone had increased anti-*S. typhimurium* IgG titers compared with the IgG titers observed in the control mice; however, the difference between the groups was not statistically significant.

IgG isotyping

The isotype profile of antibody responses is related to the cytokines produced by antigen-specific T cells and is an indirect measure of the Th1/Th2 cytokine profile. We determined the relative levels of anti-*S. typhimurium* IgG2a to IgG1 antibodies in the sera obtained 2 weeks after the last immunization, from all of the immunized groups of mice. As shown in Fig. 4, the IgG2a and the IgG1 levels and the IgG2a/IgG1 ratio were significantly higher in mice that were treated with the HKST vaccine in combination with the alum–naloxone mixture compared with those of mice that received the HKST vaccine alone, the HKST vaccine with alum or the HKST vaccine with naloxone. The mice vaccinated with HKST with naloxone had significantly more IgG2a and more IgG2a/IgG1 ratios compared with the mice that received the HKST vaccine with alum. Furthermore, the IgG2a level was significantly higher in mice treated with the HKST vaccine in combination with naloxone compared with the IgG2a level in mice that were administered PBS. The mice that were vaccinated with HKST with alum had significantly more IgG1 compared with the mice that received HKST vaccine alone or with naloxone. Mice that received the HKST vaccine with alum had lower IgG2a/

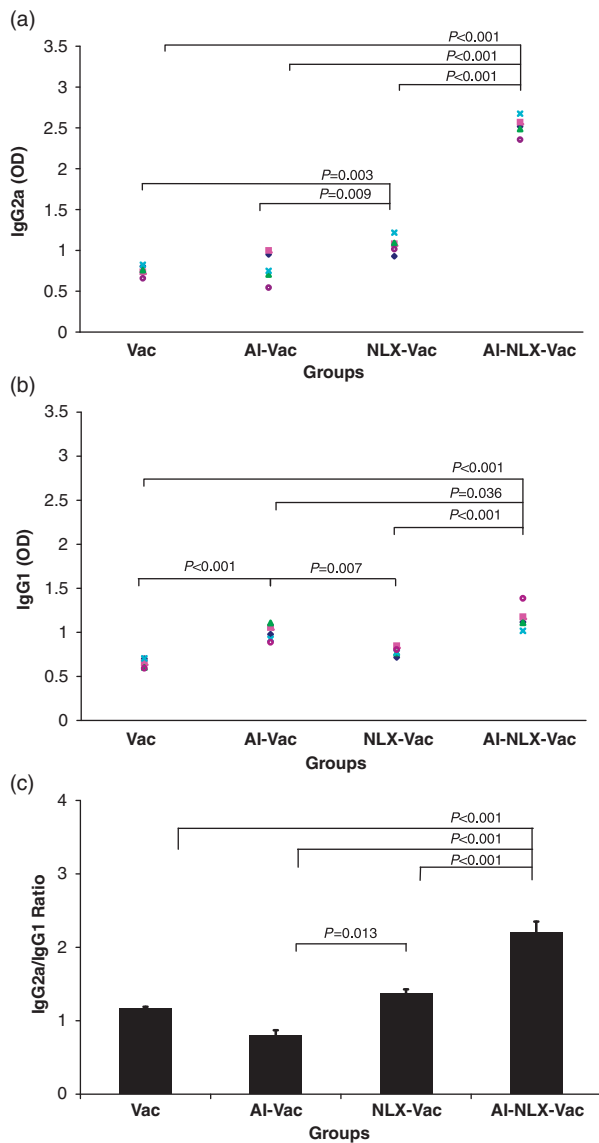


Fig. 4. Effect of administering the alum–naloxone mixture on IgG isotyping. The relative levels of anti-*S. typhimurium* IgG2a to IgG1 antibodies in the sera obtained 2 weeks after the last immunization, from five mice in each group, were determined. The groups are as in Fig. 1. Values in (c) represent the mean \pm SE.

IgG1 ratios compared with the ratios observed in the mice that were administered vaccine alone; however, the difference in the ratios between the groups was not statistically significant.

Bacterial load in the spleen and liver

As shown in Fig. 5a, cultures of homogenized spleens from the mice that received the HKST vaccine in combination with the alum–naloxone mixture had significantly fewer mean bacterial colony counts compared with the

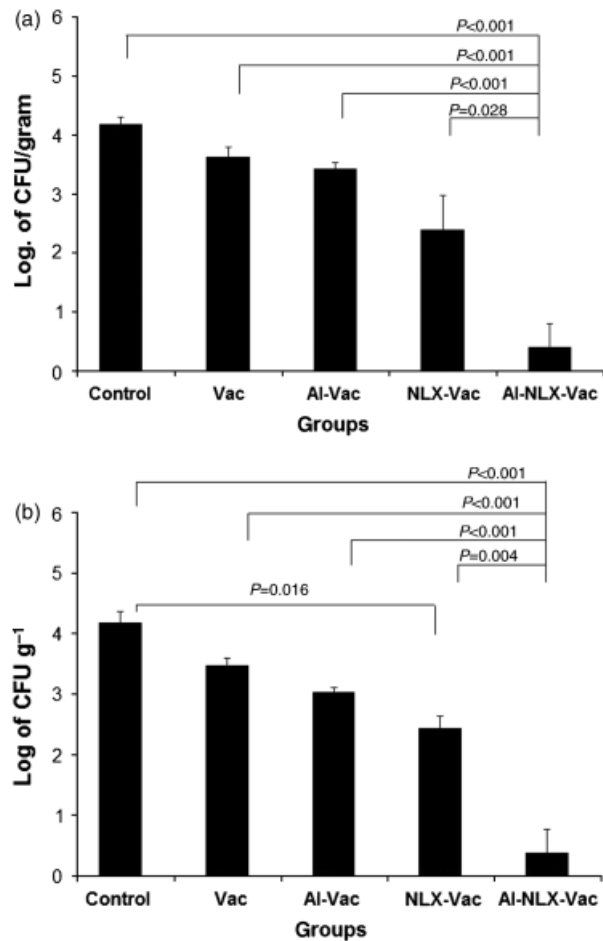


Fig. 5. Bacterial loads in spleens (a) and livers (b) after challenge with live *S. typhimurium*. Two weeks after the last immunization, the mice (five per group) were infected with live *S. typhimurium*. Forty-eight hours post-infection, the spleen and liver from each mouse were homogenized individually and plated on trypticase soy agar plates. One day after culturing at 37 °C, CFUs (log) were determined. The groups are as in Fig. 1. Values represent the mean \pm SE.

mean bacterial colony counts observed in mice that received PBS, the HKST vaccine alone, the HKST vaccine with alum or the HKST vaccine with naloxone. The mean spleen colony counts from the mice immunized with naloxone in combination with the HKST vaccine were less than the spleen colony counts from the mice that received PBS, the HKST vaccine alone or the HKST vaccine with alum; however, the differences were not statistically significant.

As shown in Fig. 5b, cultures of homogenized livers from the mice that were vaccinated with HKST with the alum–naloxone mixture had significantly fewer mean bacterial colony counts compared with the mean bacterial colony counts observed from the mice that received PBS,

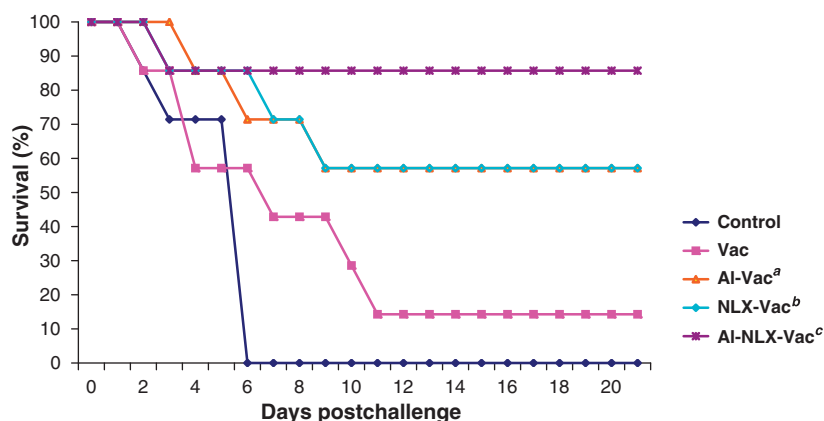


Fig. 6. Survival rates of mice immunized with PBS (control group), HKST vaccine alone (Vac group), alum in combination with the HKST vaccine (Al-Vac group), naloxone in combination with the HKST vaccine (NLX-Vac group) or the alum–naloxone mixture in combination with the HKST vaccine (Al-NLX-Vac group). Two weeks after the final immunization, the mice were infected with live *S. typhimurium*. Their survival rate was recorded daily for 21 days. ^aMice in the Al-Vac group showed a significantly higher survival rate than mice in the control group ($P=0.018$). ^bMice in the NLX-Vac group showed a significantly higher survival rate than mice in the control group ($P=0.006$). ^cMice in the Al-NLX-Vac group showed a significantly higher survival rate than mice in the control and Vac groups ($P=0.006$ and 0.016 , respectively). Values are representative of seven mice per group.

the HKST vaccine alone, the HKST vaccine with alum or the HKST vaccine with naloxone. The mean liver colony counts of the mice that were immunized with naloxone in combination with the HKST vaccine were significantly lesser than those of the mice that received PBS.

Survival after *S. typhimurium* challenge

Two weeks after the second immunization, the survival rates of seven mice per group that were challenged with live *S. typhimurium* were analyzed (Fig. 6). The survival rate of mice that were administered the HKST vaccine in combination with the alum–naloxone mixture was significantly higher than the survival rates observed in mice that received PBS or the HKST vaccine alone ($P=0.006$ and 0.016 , respectively). Furthermore, the mice immunized with the HKST vaccine in combination with alum or naloxone exhibited a significantly higher survival rate compared with the survival rate of mice that received the PBS ($P=0.018$ and 0.006 , respectively). The mean survival rates of the mice that were immunized with HKST in combination with alum or naloxone were more than the survival rates of the mice that received the HKST vaccine alone; however, the differences were not statistically significant.

Discussion

We have demonstrated previously the adjuvant activity of naloxone in combination with vaccine models against a viral infection and an intracellular bacterial infection (Jamali et al., 2009; Jazani et al., 2010). Here, we investigated the

ability of naloxone, and the mixture of alum and naloxone, to enhance the protection of an HKST vaccine, which functions as a vaccine model against a facultative intracellular bacterium.

Our results showed that the administration of the alum–naloxone mixture, when utilized as an adjuvant in combination with the HKST vaccine, significantly increased the vaccine's efficacy. The improved efficacy was associated with the following changes: increased induction of IFN- γ and IL-4 production; a shift towards a Th1 response (by increasing IFN- γ /IL-4 ratio); an increase in lymphocyte proliferation; the production of anti-*S. typhimurium* total IgG, IgG2a and IgG1; a Th1 pattern of humoral immune response (by increasing IgG2a/IgG1 ratio); and improved resistance and survival against an *S. typhimurium* challenge. The adjuvant activity of the alum–naloxone mixture was more than the adjuvant activity of either alum or naloxone alone.

These results indicated that the alum–naloxone mixture stimulated both humoral and cellular immune responses. As mentioned above, the most effective immune response against many pathogens, even for some obligate intracellular pathogens, is an immune response that combines both humoral and cellular components (Casadevall, 2003; Casadevall & Pirofski, 2006). The effects of the alum–naloxone mixture on cellular immune and humoral responses are more likely due to naloxone and alum, respectively. However, the IgG response in groups containing vaccine was not high, for example it was only less than twice the control for the highest response, but this was not surprising, because, as mentioned above, we used a subimmunogenic dose of HKST as a model vaccine.

One possible mechanism of naloxone action is to provide a pro-inflammatory milieu by blocking opioid receptors. This inhibition would accelerate local inflammation via a direct effect on innate immune cells, such as monocytes, macrophages and dendritic cells. Another possible mechanism is that naloxone may trigger a low-grade inflammation by increasing the release of local pro-inflammatory neuropeptides, such as substance P (SP), from nerve fibers (Berman *et al.*, 1996; Bileviciute-Ljungar & Spetea, 2001; Mathers *et al.*, 2007). In such a pro-inflammatory milieu, antigen-presenting cells (APCs) could polarize specific immune responses toward cell-mediated immunity and a Th1 pattern. Furthermore, it has been shown that pro-inflammatory neuropeptides such as SP stimulate the maturation and migration of local APCs to the draining lymph nodes and shift immune responses toward a Th1 pattern (for more information, please see Jamali *et al.*, 2009; Jazani *et al.*, 2010). Hence, administration of naloxone as an adjuvant, either alone or in combination with alum, may activate APCs via the above-mentioned mechanisms. This would result in the presentation of HKST antigens by activated APCs. However, because the half-life of naloxone is very brief (Peterson & Poole, 2006), there is probably little or no naloxone in the environment during antigen presentation. Therefore, it is possible that previously activated APCs induce the Th1 immune response.

As mentioned above, naloxone is a Th1-type adjuvant while alum is a Th2-type adjuvant (Hem & White, 1995; Jamali *et al.*, 2009; Harandi *et al.*, 2010; Jazani *et al.*, 2010). However, despite the opposing effects of these adjuvants on shifting the immune system toward a Th1/Th2 pattern, the efficacy of the alum–naloxone mixture in stimulating Th1/cellular immune responses was more than the stimulation ability of naloxone alone. Thus, it seems that alum augmented the adjuvant activity of naloxone, even in stimulating Th1/cellular responses. This finding is in line with the findings of Su *et al.* (2003), which showed that coadministration of alum and IL-12 augmented the ability of IL-12 to induce the production of IFN- γ and IgG2a against a malaria vaccine. Alum-induced augmentation of naloxone-mediated responses could be attributed to a combination of the immunomodulatory effects of naloxone with the depot effect of alum. Another possible mechanism may be associated with the immunomodulatory effects of alum. It has been shown that alum can induce inflammation and activate innate immune cells, including mast cells, macrophages, dendritic cells and eosinophils, to produce cytokines such as IL-1 β , tumor necrosis factor- α , granulocyte–monocyte colony-stimulating factor and IL-4 (Ulanova *et al.*, 2001; Aimanianda *et al.*, 2009; McKee *et al.*, 2009). This, in turn, can increase the expression of neurokinins by innate immune cells. Neurokinins are the receptors of proinflammatory neuropeptides, including SP (Marriott & Bost, 2000;

Guo *et al.*, 2004). Therefore, alum can indirectly increase the expression of neurokinins on local innate immune cells. As mentioned above, one of the possible mechanisms for the adjuvant activity of naloxone is the release of local pro-inflammatory neuropeptides, such as SP, from nerve fibers and the inflammatory effects of these neuropeptides on innate immune cells. The alum-mediated increase in the expression of neurokinins may increase the effects of naloxone-induced proinflammatory neuropeptides on innate immune cells.

In conclusion, the administration of the alum–naloxone mixture as an adjuvant in combination with a HKST vaccine can enhance cellular and humoral immunity and shift the immune response to Th1. As both naloxone and alum are approved for human use (Hem & White, 1995; <http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm>), the combined mixture may provide a new, relatively safe means of eliciting effective vaccine-induced Th1 immune responses to malignancies and microorganisms. Furthermore, our results indicate that administering naloxone, without alum, with the HKST vaccine increased cell-mediated immunity and shifted immune responses to Th1. These findings are consistent with our earlier findings that naloxone coadministration with HSV-1 and *L. monocytogenes* vaccines increased the respective vaccine efficacy (Jamali *et al.*, 2009; Jazani *et al.*, 2010). As mentioned above, the adjuvant activities of naloxone and alum alone were less than that of the alum–naloxone mixture. To our knowledge, this study is the first to evaluate an alum–naloxone mixture for use as an adjuvant for vaccines. Therefore, follow-up studies are needed to confirm these results and test the adjuvant activity of the alum–naloxone mixture when combined with vaccines against other microorganisms that require both humoral and cellular immune responses.

Acknowledgements

This work was supported by a research grant from the Urmia University of Medical Sciences, Urmia, Iran.

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