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Original article

Evaluation of the adjuvant activity of naloxone, an opioid receptor antagonist, in combination with heat-killed *Listeria monocytogenes* vaccine

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Abstract

We have previously demonstrated the adjuvant activity of naloxone (NLX), a general opioid antagonist, using a DNA vaccine for herpes simplex virus type 1. Here, the adjuvant activity of NLX has been evaluated using a heat-killed *Listeria monocytogenes* (HKLM) vaccine as a model for general immunization against intracellular bacteria. BALB/c mice were divided into three groups: the Vac group received the HKLM vaccine alone; the NLX–Vac group received the HKLM vaccine in combination with the adjuvant NLX; and the control group received phosphate buffered saline (PBS). Our results indicate that the administration of NLX as an adjuvant enhances the ability of the HKLM vaccine to increase lymphocyte proliferation, delayed type hypersensitivity, and skewing of the immune response toward a T-helper 1 (Th1) pattern. Additionally, combination of NLX with the HKLM vaccine improves protective immunity against *L. monocytogenes*. In conclusion, administration of NLX as an adjuvant for the HKLM vaccine can enhance cell-mediated immunity and shift the immune response to Th1.

Keywords: Opioid; Naloxone; Adjuvant; Vaccine; Intracellular bacteria

1. Introduction

Protection against intracellular bacteria requires T-helper 1 (Th1)-oriented immune responses [1]. Unfortunately, strong Th1 adjuvants, including those based on oil/BCG (bacille Calmette–Guerin) formulas like Freund's adjuvant, are not tolerated by humans [2]. AS04 (monophosphoryl lipid A plus aluminum salts) and MF59 (an oil-in-water emulsion) [3], which are European Medicines Agency-approved vaccine adjuvants [4,5], can also stimulate Th1 immune responses [6,7]. Meanwhile, aluminum salts, which are approved by Food and Drug Administration (FDA) as a vaccine adjuvant, enhance the Th2-specific response [8].

It has been suggested that opioids have considerable immunomodulating effects that are mediated directly by opioid receptors on immune cells and/or indirectly by the central nervous system and the hypothalamic–pituitary–adrenal (HPA) axis [9]. Opioid receptors are part of an immunomodulatory system that responds to both opioid neuro- and immunopeptides secreted locally by innervating neurons and immune cells, respectively [10]. Opioid peptides are well known inhibitors of the immune response and vaccination against microbial agents [11–13]. Furthermore, exposure to exogenous opioids is known to increase susceptibility to microbial infection [13].

It has been suggested that naloxone (NLX), an opioid antagonist, can shift the immune response toward a Th1 pattern [14–16]. NLX is currently approved by the FDA as

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a prescription drug [17] and is routinely used by emergency medical personnel to rapidly and safely reverse opioid-induced respiratory depression [18]. We have shown previously that NLX administration during primary herpes simplex virus type 1 (HSV-1) infection by a non-virulent strain enhanced protection against HSV-1 challenge [19]. Additionally, we have shown that NLX administered as an adjuvant in combination with an HSV-1 DNA vaccine increased the latter's efficacy in enhancing cellular immunity, skewing the immune response toward Th1 and improving protective immunity [20]. As in the case of other intracellular bacteria, protection against *Listeria monocytogenes* requires cell-mediated immunity and a Th1 response. In the current study, we tested the hypothesis that NLX, when used as a Th1-inducing adjuvant in combination with heat-killed *L. monocytogenes* (HKLM), a model vaccine against intracellular bacteria, increases the vaccine's efficacy. More specifically, we postulated that this new adjuvant would help to enhance non-cytotoxic T lymphocyte (CTL)-mediated cellular immunity, shift the immune response toward Th1, and improve protective immunity. It should be noted that the purpose of this study was not to assess a vaccine formulation against *L. monocytogenes* infection, but rather to evaluate the efficacy of NLX as an adjuvant. Since immunogenic doses of HKLM without adjuvant efficiently stimulate Th1 immune responses [21], we used a sub-immunogenic dose of HKLM as a model vaccine.

2. Materials and methods

2.1. Mice

Six- to eight-week-old male BALB/c mice were obtained from the Razi Institute (Karaj, Iran). Mice were housed for one week before the experiment, given free access to food and water, and maintained in a light/dark cycle, with light exposure from 6:00 A.M. to 6:00 P.M. All experiments were conducted in accordance with the Animal Care and Use Protocol of Urmia University of Medical Sciences (Urmia, Iran).

2.2. Preparation of HKLM

L. monocytogenes Persian Type Culture Collection (PTCC) 1298 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 lack of growth on blood agar plates. Bacterial concentration was enumerated by comparing the absorbance of a serial dilution of HKLM at 590 nm with McFarland Nephelometer Standards. The HKLM was then stored at −70 °C.

2.3. NLX treatment and immunization

BALB/c mice were immunized twice subcutaneously with 3×10^6 HKLM in a total volume of 100 μL on Days 0 and 7 (Vac group). NLX (Sigma, Germany) was dissolved in 100 μL PBS containing 3×10^6 HKLM at a concentration of 6 mg/kg and injected into each mouse in combination with HKLM on Days 0 and 7 (NLX−Vac group). PBS was injected into a third group of mice as a negative control (control group).

2.4. Delayed type hypersensitivity (DTH) assay

Two weeks after the last immunization, 5×10^7 HKLM in 25 μL was injected into the right footpad of each mouse and 25 μL PBS was injected into the left footpad as a negative control. Footpad thickness was measured with a dial caliper after 24 h and the results were expressed as the mean percentage increase in footpad thickness in five mice. The mean percentage increase was calculated according to the following formula: [(Thickness of right footpad challenged with HKLM) − (Thickness of left footpad injected with PBS)] × 100/(Thickness of left footpad injected with PBS).

2.5. Cytokine assays

Two weeks after the last immunization, the spleens of individual mice 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 (NH_4Cl 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 was plated in each well of a 24-well plate using RPMI 1640 supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/mL streptomycin, and 5×10^{-5} M 2-mercaptoethanol. Two wells were used per mouse. The cells were restimulated *in vitro* with 1×10^4 HKLM. Plates were then incubated at 37 °C in 5% carbon dioxide (CO_2). At 72 h after stimulation, supernatants were removed and stored at −70 °C before measurement of secreted interferon (IFN)-γ and interleukin (IL)-4 levels. The concentration of IFN-γ and IL-4 in the supernatants was estimated using a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D systems, Minneapolis, MN).

2.6. Lymphocyte proliferation assay

Two weeks after the last immunization, the lymphocyte proliferation rate was measured using an MTT (3[4,5-dimethylthiazol-2-μl]-2,5-diphenyltetrazolium bromide; thiazolyl-blue, Sigma, Germany) dye assay. The spleens of the mice were removed under sterile conditions and single-cell suspensions were prepared in 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 in phenol red-free RPMI 1640 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