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Monophosphoryl lipid A enhances nontypeable *Haemophilus influenzae*-specific mucosal and systemic immune responses by intranasal immunization

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ABSTRACT

Objective: Acute otitis media (AOM) is one of the most common infectious diseases in children. Nontypeable *Haemophilus influenzae* (NTHi) is Gram-negative bacteria that are considered major pathogens of AOM and respiratory tract infections. In this study, we used monophosphoryl lipid A (MPL), a toll-like receptor (TLR) 4 agonist, as an adjuvant to induce mucosal immune responses against NTHi to enhance bacterial clearance from the nasopharynx.

Methods: Mice were administered 10 μg outer membrane protein (OMP) from NTHi and 0, 10, or 20 μg MPL intranasally once a week for 3 weeks. Control mice were administered phosphate-buffered saline alone. After immunization, these mice were challenged with NTHi. At 6 and 12 h after bacterial challenge, the mice were killed and nasal washes and sera were collected. The numbers of NTHi- and OMP-specific antibodies were quantified by enzyme-linked immunosorbent assay.

Results: The MPL 10 and 20 μg group produced a significant reduction in the number of bacteria recovered from the nasopharynx at 12 h after bacterial challenge compared to the control group. OMP-specific IgA titers were also augmented in the MPL groups compared to the control and OMP groups.

Conclusion: MPL is suitable for eliciting effective mucosal immune responses against NTHi in the nasopharynx. These results demonstrate the possibility of an adjuvant that involves stimulation of the innate immune system by TLR4 agonists such as MPL for mucosal vaccination.

1. Introduction

Acute otitis media (AOM) is one of the most common infectious diseases in children. The peak incidence of this disease occurs in early childhood, and it is associated with hearing loss, delayed speech development, permanent middle ear damage, and mucosal changes. Nontypeable *Haemophilus influenzae* (NTHi) is considered a major pathogen in AOM and respiratory tract infections [1]. Current options for treatment depend mainly on the use of antibiotics and are presently facing a serious challenge from emerging antibiotic-resistant strains [2,3]. Thus, developing vaccines to prevent these infections is an urgent goal for public health.

We previously reported that the outer membrane protein (OMP) or P6 from NTHi is a promising candidate target for vaccination, and that intranasal immunization with OMP or P6 effectively induced mucosal IgA immune responses in the upper respiratory tract of mice, including the nose and middle ear [4–7].

Toll-like receptors (TLRs) have emerged as key regulators of innate immune responses to infection in mammals and have been shown to be critical players in both innate and adaptive immune responses [8]. In the TLR family, TLR4 mediates lipopolysaccharide (LPS) or lipooligosaccharide (LOS) responsiveness and recognizes Gram-negative bacteria via the LPS/LOS moiety on the surface of these microorganisms [8]. TLR4 is one of the key regulators of Th2 immune responses, and the significance of TLRs in activating adaptive immunity is well established through the TLR-mediated activation of dendritic cells (DCs) in animal models [9,10]. Monophosphoryl lipid A (MPL), a TLR4 agonist, has been used clinically as an adjuvant for systemic immunization as it has acceptable safety for human use; however, MPL is not used as an adjuvant for mucosal immunization in humans. The MPL used as an adjuvant for

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systemic immunization is a detoxified form of LPS from *Salmonella minnesota R595*, but its synthetic form has not been used. Recently, the effectiveness of MPL to induce a mucosal immune response via intranasal vaccination against viral antigens has been examined [11–13]. However, little is known as to whether a synthetic TLR4 agonist can enhance the mucosal immune response via intranasal immunization with vaccine candidates against NTHi, such as OMP or P6. Clarifying the immune response induced via a TLR4 agonist in the upper respiratory tract may help establish a strategy for intranasal immunization with vaccines against NTHi. In the present study, we investigated the kinetics of mucosal immune responses when mice were immunized intranasally with OMP from NTHi and synthetic MPL, and we also investigated the mechanisms of protective acquired immunity, especially in relation to DCs, via TLR4.

2. Methods

2.1. Animals

BALB/c mice were purchased from Kyudo Japan (Fukuoka, Japan). All mice were maintained in a pathogen-free facility until they were 5 weeks old, at which time they were used for the experiments. A total of 120 animals were used in this study. All experiments were approved by the Committee on Animal Experiments of Oita University (No. Q028063).

2.2. Immunogen and adjuvant

NTHi (strain 76), which was isolated from the nasopharynx of a patient with otitis media with effusion at Oita University, was stored at −80 °C and used for the preparation of antigen and nasal inoculum. The antigen was prepared as described previously [9]. In brief, NTHi was cultured overnight on chocolate agar at 37 °C in a 5% CO_2 incubator. Bacteria were harvested by scraping them from the plate, suspended in EDTA buffer (pH 7.4), and incubated at 56 °C for 30 min. The bacterial cells were then disrupted by sonication on ice, and the unbroken cells and debris were removed by centrifugation at 10,000 × *g* for 20 min. The supernatants were pooled and centrifuged at 80,000 × *g* for 2 h at 4 °C. The clear, gel-like pellet was suspended in distilled water and lyophilized. The resulting powder, referred to as the OMP preparation from NTHi, was stored at 4 °C until used in the experiments.

MPL was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL), and the MPL used in this study was a synthetic form of lipid A derived from LPS of *S. minnesota* R595.

2.3. Immunization

The mice were immunized nasally with 10 μL phosphate-buffered saline (PBS) containing a mixture of 10 μg OMP and 0, 10, or 20 μg MPL as an adjuvant (for the OMP, MPL10, and MPL20 groups, respectively). Control mice received intranasal 10 μL PBS alone (control). Each group consisted of 5–10 mice. The mice were immunized on days 0, 7, and 14 for a total of 3 immunizations. On day 21, 2 sets of mice were used for bacterial challenge, while another set was used only for flow cytometric analysis, as described below.

2.4. Bacterial challenge and sample collection

Strain 76 of NTHi was used for the bacterial challenge. NTHi was grown on chocolate agar at 37 °C under 5% CO_2 for 16 h. Bacterial concentration was determined by optical density at a wavelength of 600 nm, and then a bacterial suspension was prepared to a concentration of 1.0×10^9 colony forming units (cfu)/mL in PBS and stored on ice. The mice were inoculated intranasally with a suspension of NTHi (10 μL; 10^7 cfu/mouse). At 6 and 12 h after bacterial challenge, 10 mice from each group were anesthetized by intraperitoneal injection with pentobarbital, and blood samples were collected from the axillary artery. After decapitation and removal of the mandible, the nasal cavity was flushed gently with 200 μL PBS from the posterior opening of the nose and nasal washes were collected from the anterior openings of the nose. The number of NTHi in nasal washes was quantified by standard bacteriologic techniques.

2.5. Detection of OMP-specific antibodies by enzyme-linked immunosorbent assay (ELISA)

Specific anti-OMP antibody levels in nasal washes and serum samples were determined by ELISA with OMP as a coating antigen (5 μg/mL). Samples of fluids from naïve mice (day 0) served as negative controls and gave optical density (A_{405}) readings of less than 0.1 for IgA, IgG, and IgM in serum, and 0.01 in body fluids. The endpoint antibody titer was defined as the highest dilution of samples giving an optical density that was 2-fold greater than that of the negative controls. The levels of IgG subclasses (IgG1, IgG2a, IgG2b, and IgG3) were measured by ELISA using goat anti-mouse IgG1-, IgG2a-, IgG2b-, and IgG3-enzyme conjugates as the second antibody (Southern Biotechnology Assoc., Birmingham, AL), respectively, and evaluated using optical density (A_{405}).

2.6. Flow cytometric analysis of DCs

To analyze the dynamics of DCs in lymphoid tissue, mononuclear cells (MNCs) from nasal-associated lymphoid tissue (NALT), cervical lymph node (CLN), and spleen (SPL) were isolated. Flow cytometric analysis of cellular immunofluorescence was performed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). MNCs from each tissue were harvested from groups of 5 mice on day 21. Fluorescein isothiocyanate-conjugated anti-IA/IE monoclonal antibody (mAb: 2G9; BD Biosciences, San Jose, CA), fluorescein isothiocyanate-conjugated anti-CD80 and anti-CD86 mAbs (BD Biosciences), phycoerythrin-conjugated anti-CD11b mAb (M1/70; BD Biosciences), and PerCP-conjugated anti-CD11c mAb (HL3; BD Biosciences) were used for analyzing DC subsets and activation. This experiment was performed twice independently, each from a pool of 5 mice.

2.7. In vitro MPL stimulation of DCs and cytokine production

To investigate the effect of MPL on cytokine production from DCs, DCs (CD11c-positive cells) isolated from the spleen were purified with an anti-CD11c MicroBead kit and MACS cell sorter (Miltenyi Biotec, Bergisch Gladbach, Germany). A suspension of approximately $2.0–6.0 \times 10^8$ single cells from the spleen was incubated with anti-CD11c microbeads (5 μL per 10^7 cells), and CD11c-positive cells were purified to 1.0×10^6 single cells by positive selection. After isolation, CD11c cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum (Sigma-Aldrich, St. Louis, MO), 2 mM L-glutamine (Invitrogen Life Technologies, Carlsbad, CA), and 1% penicillin/streptomycin (Sigma-Aldrich) in 24-well culture plates. These cells (1.0×10^5 single cells/well) were incubated with 20 μg or 10 μg MPL for 6, 12, and 24 h. Untreated cells served as a control. After incubation, the supernatants were collected for Bio-Plex Pro™ Mouse Cytokine Assays (Th1/Th2 panel). A panel of 13 cytokines were analyzed in culture supernatants using the Bio-Plex Pro™ Mouse Cytokine assay (Bio-Rad Laboratories), according to the manufacturer's protocol, which included interleukin (IL)-2, IL-4, IL-5, IL-10, IL-12p70, interferon-