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Mucosal and systemic immune response to sublingual or intranasal immunization with phosphorylcholine

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ABSTRACT

Objective: Phosphorylcholine (PC) is a structural component of a wide variety of pathogens including *Streptococcus pneumoniae* and *Haemophilus influenzae*. Here, the immune response in mice to PC immunization via the sublingual (SL) route versus the intranasal (IN) route was investigated in terms of efficacy and safety.

Methods: BALB/c mice were immunized with PC-keyhole limpet hemocyanin (KLH) plus cholera toxin (CT) or CT alone via the IN or SL route. The immune response generated was studied in terms of PC-specific antibody titers, interferon (IFN)-γ and interleukin (IL)-4 production by $CD4^+$ T cells, and cross-reactivity of PC-specific immunoglobulin (Ig)-A antibodies in nasal washes against *S. pneumoniae* and non-typeable *H. influenzae*.

Results: SL and IN immunization with PC-KLH plus CT resulted in a marked increase in the levels of PC-specific, mucosal IgA and serum IgM, IgG, and IgA antibodies. Additionally, SL immunization elicited significantly higher levels of PC-specific IgG2a subclass antibodies and IFN-γ in serum. On the other hand, IN immunization with CT alone remarkably increased the total IgE level in serum compared with SL and IN immunization with PC-KLH plus CT. PC-specific IgA antibodies in nasal wash samples reacted to most strains of *S. pneumoniae* and non-typeable *H. influenzae*.

Conclusion: SL immunization is as effective as IN immunization to induce PC-specific immune responses and more effective than IN immunization to reduce the production of IgE and to prevent the sensitization to allergen causing type I allergy.

1. Introduction

Pneumococcal conjugate vaccines are known to be effective in reducing the incidence of invasive pneumococcal infections. However, the vaccine provides only a moderate amount of protection against acute otitis media due to non-vaccine strains of *Streptococcus pneumoniae* (Spn) and *Haemophilus influenzae* (Hi) [1,2]. Therefore, there is a need to develop a broad-spectrum vaccine that is effective against most strains of Spn and Hi.

Phosphorylcholine (PC) is a structural component of a wide variety of pathogens, including Spn and Hi. The immunomodulatory effects of PC have been demonstrated, wherein intranasal (IN) immunization with protein-linked PC confers protection to mice against a lethal IN challenge with Spn [3]. We previously reported that IN immunization with PC-keyhole limpet hemocyanin (KLH) together with cholera toxin (CT) induced mucosal as well as systemic immune responses in the upper respiratory tract and inhibited the colonization of several strains of Spn and non-typeable Hi (NTHi) in the nasal

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mucosa [4]. Thus, PC is a promising candidate for a broad-spectrum vaccine and the IN route of immunization may be an effective method to prevent upper as well as lower respiratory tract infections.

However, it has been shown that antigens and adjuvants administered via the IN route are potentially redirected to the central nervous system [5,6]. Bell's palsy was frequently reported in a previous clinical trial with inactivated IN influenza vaccine [7]. Such adverse effects with IN vaccines underline the need to establish an alternate vaccination route other than IN. One promising alternative is the sublingual (SL) route of immunization. Song et al. [8] reported that SL vaccination with live or inactivated influenza virus induces both, systemic and mucosal antibody (Ab) responses, and confers protection against a lethal IN challenge with influenza virus. Moreover, live or inactivated influenza virus administered through the SL route does not migrate to or replicate in the central nerve system, as observed with IN immunization. However, differences in the mechanism of immune response induction, when vaccines are administered via the SL and IN routes are not fully understood. Further, the route of administration that is more effective and safe in preventing upper respiratory infection using the same antigen has not yet been investigated.

In the present study, mice were immunized with PC via the SL or IN route. Additionally, the effectiveness of SL immunization was investigated by comparing PC-specific mucosal and systemic immune responses elicited by the SL and IN routes of immunization. Furthermore, immunoglobulin (Ig) E production after SL or IN immunization with PC was examined and the safety of SL immunization was evaluated.

2. Materials and methods

2.1. Mice

Six-week-old, female BALB/c mice (CLEA Japan Inc., Shizuoka, Japan) were housed in the experimental animal facility of Kagoshima University under specific pathogen-free conditions. All mice used in this study were 7–10 weeks of age. The experimental protocol was approved by the Ethics Board of the Institute of Laboratory Animal Sciences of Kagoshima University.

2.2. Immunization and sample collection

The mice were divided into 4 groups: SL immunization with PC-KLH (Biosearch, San Rafael, CA) plus CT (List Biological Laboratories, INC., Campbell CA), SL immunization with CT alone, IN immunization with PC-KLH plus CT, and IN immunization with CT alone. Before immunization, the mice were anesthetized with ketamine/xylazine. The SL vaccine comprised of either 50 μg PC-KLH and 1 μg CT as a mucosal adjuvant, or 1 μg CT alone, diluted in 5 μl phosphate-buffered saline (PBS). During SL immunization, forceps were placed under the tongue of the mouse, its mouth was stretched open, and the vaccine was administered at the SL mucosa using a pipette. Subsequently, the head was maintained in the anteflexion position for 30 min to maintain the antigens on the SL mucosa, according to a previously reported method [9]. For IN immunization, the same antigens were diluted in 10 μl PBS and 5 μl solution was dropped into each nostril using a pipette [4].

SL and IN immunizations were performed once weekly, for 3 consecutive weeks. Saliva, nasal wash, vaginal wash, and serum samples were collected 1 week after the final immunization. Saliva samples were obtained after intraperitoneal injection of pilocarpine (100 μl of 1 mg/ml solution; Sigma, St. Louis, MO; diluted in sterile PBS). Nasal wash specimens were collected by gently flushing the nasal passages with 200 μl PBS [10]. Vaginal wash samples were obtained by gently flushing the vaginal cavity with 200 μl PBS [11]. All mucosal and serum samples were stored at −20 °C until use.

2.3. Isolation of mononuclear cells

Mononuclear cells were isolated from the spleen, collected at the same time as other samples, as previously described [10]. Briefly, spleen cells were isolated by gentle teasing through stainless steel screen and 100 μm nylon mesh. The cells were spin down to make cell pellet and the pellet was re-suspended in Ammonium–Chloride–Potassium (ACK) lysing buffer and incubated 5 min at room temperature to remove red blood cells. Then the cells were washed to remove ACK lysing buffer and re-suspend in complete medium.

2.4. Detection of PC-specific Abs and serum IgE Abs by enzyme-linked immunosorbent assay

PC-specific IgM, IgG, and IgA Ab titers in the saliva, nasal wash, vaginal wash, and serum samples were determined by enzyme-linked immunosorbent assay (ELISA) using horseradish peroxidase (HRP)-conjugated anti-mouse IgM, IgG, and IgA (Southern Biotechnology Associates, Birmingham, AL) and biotin-conjugated anti-mouse IgG1, IgG2a, IgG2b, and IgG3 (BD Biosciences, Franklin Lakes, NJ), as previously described [4,10]. The titers were evaluated by optical density (OD) at 450 nm; $OD_{450\ nm} > 0.2$ was considered as positive. The levels of total IgE Abs in serum were determined by sandwich ELISA using a mouse IgE ELISA Quantitation Kit (Bethyl Laboratories Inc., Montgomery, TX) according to the manufacturer instructions. IgE Ab levels specific to CT, PC, and KLH were measured by sandwich ELISA as followings. Ninety-six wells plate was coated with 100 μl of mouse IgE antibody (Bethyl Laboratories Inc., Montgomery, TX) (1:100) at room temperature (RT) for 60 min. Then the plate was washed with ELISA wash solution (Bethyl Laboratories Inc., Montgomery, TX) 4 times and blocked with 200 μl of blocking solution (Bethyl Laboratories Inc., Montgomery, TX) at RT for 60 min. After incubation, the plate was washed again 4 times. Standard mouse IgE (Bethyl Laboratories Inc., Montgomery, TX: 250 ng/ml) was serially diluted with blocking solution plus Tween 20 to the concentration of 3.9 ng/ml. Each 100 μl of the serially diluted standard IgE and serum sample was added to the well and incubated at RT for 60 min. After washing, the responses of standard mouse IgE were measured according to the manufacturer instructions (Bethyl Laboratories Inc.,

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