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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.,

Montgomery, TX). In order to determine the concentration of CT-specific IgE, CT-HRP (Bio-rad, Raleigh, NC) diluted to 1:1000 was added and incubated at RT for 60 min. After washing 4 times, 100 μl of Tetramethylbenzidine (TMB) were added and the reaction was stopped with 0.18 M of H_2SO_4. For measuring PC- and KLH-specific IgE levels, PC-BSA (Biosearch, Petaluma, CA) and KLH (Biosearch, Petaluma, CA) were biotinylated with PD-10 desalting column (GE Healthcare, Piscataway, NJ) according to the method which was previously reported to determine tetanus toxoid-specific IgE level [12] and those biotinylated PC and KLH were used as detection solution. After incubating the wells having serum samples with those detection solutions at RT for 60 min, the plates were washed and 100 μl of peroxidase-conjugated anti-biotin (Vector laboratories, Burlingame, CA) diluted to 1:3000 was added as secondly antibodies. After incubation at RT for 60 min, the plates were washed and 100 μl of TMB were added and the reaction was stopped with 0.18 M of H_2SO_4. The OD450 nm absorbance of each well was measured by microplate reader and the levels of CT-, PC-, and KLH-specific IgE were determined by the standard curve prepared from the absorbance of the serially diluted standard IgE.

2.5. Cytokine production from CD4$^+$ T cells

$CD4^+$ T cells were obtained from spleen single cell suspensions by positive sorting using a magnetic bead separation system (Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described [4]. Splenic mononuclear cells treated with mitomycin C (50 μg/ml for 45 min at 37 °C) were used as feeder cells. Purified $CD4^+$ T cells were incubated for 72 h in culture medium containing the feeder cells and PC-bovine serum albumin (10 μg/ml). The supernatant was collected and interferon (IFN)-γ and interleukin (IL)-4 concentrations were measured using ELISA kits (BioSource International Inc., Camarillo, CA) as per the manufacturer instructions. The detection limit of mouse IFN-γ and IL-4 using the kits were 9.4 pg/ml and 7.8 pg/ml, respectively.

2.6. Bacterial cell membrane lysates and cross-reactivity with Spn and NTHi strains

Cell membrane lysates were prepared from 10 strains of Spn and NTHi as previously described [4]. IgA Abs in pooled nasal wash samples collected from immunized mice and PC-specific mouse monoclonal IgA Abs (TEPC 15; Sigma, St. Louis, MO) that were bound to the cell membrane lysates were estimated by ELISA.

2.7. Statistical analysis

Data were compared using the Student's unpaired *t*-test. Cross-reaction data were analyzed using the Pearson correlation coefficient. A p-value of <0.05 was considered as statistically significant.

3. Results

3.1. Mucosal and systemic immune responses induced by SL and IN immunization with PC-KLH plus CT

PC-specific IgA Ab titers in saliva, nasal wash, and vaginal wash were markedly increased with PC-KLH plus CT immunization via the SL and IN routes (Fig. 1). Although

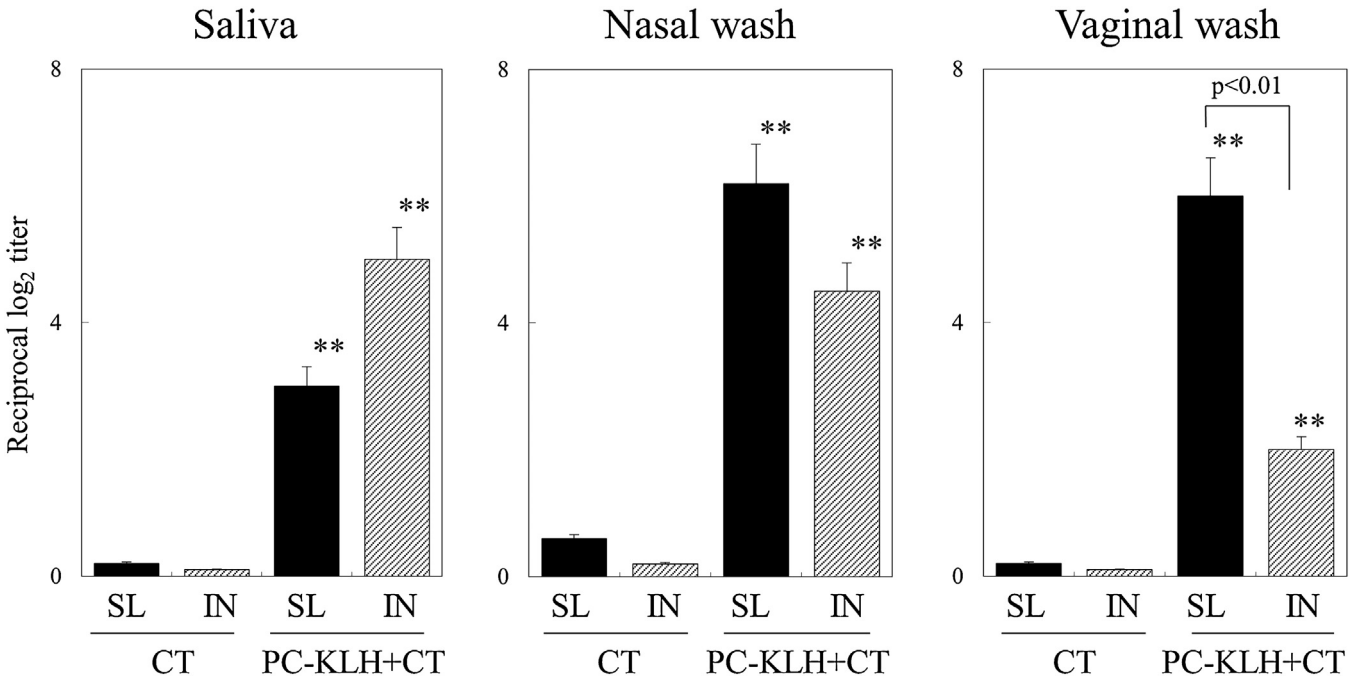

Fig. 1. PC-specific IgA Abs in mucosal samples induced by immunization with PC-KLH plus CT or CT alone via the SL and IN routes.
PC-specific IgA Ab titers in saliva, nasal wash, and vaginal wash were markedly increased with PC-KLH plus CT immunization via both, the SL and IN routes. The levels in vaginal wash were significantly higher with PC-KLH plus CT immunization via the SL compared with the IN route. The results are presented from 3 experiments (5 mice per group) and are expressed as the mean ± standard error (S.E.). **, $p < 0.01$ compared to the titers in mice immunized with CT alone.

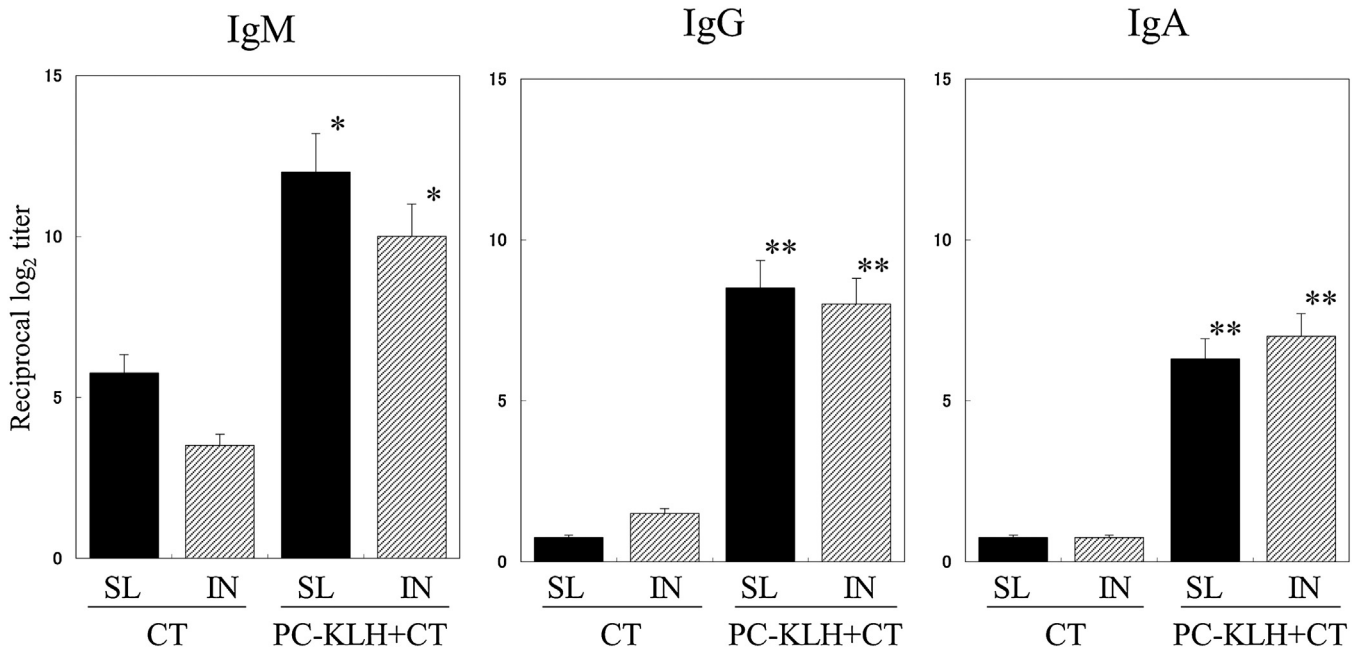

Fig. 2. PC-specific Ab titers in serum after immunization with PC-KLH plus CT or CT alone via the SL and IN routes.
PC-specific IgM, IgG, and IgA Ab titers were significantly higher in mice immunized with PC-KLH plus CT than those immunized with CT alone. There was no significant difference between the SL and IN routes of immunization. The results are presented from 3 experiments (5 mice per group) and are expressed as the mean ± S.E. *, $p < 0.05$ compared to the titers in mice immunized with CT alone. **, $p < 0.01$ compared to the titers in mice immunized with CT alone.

there were no statistically significant differences between SL and IN immunization with regard to PC-specific IgA Ab titers in saliva and nasal wash, the levels in the vaginal wash were significantly increased with PC-KLH plus CT immunization via the SL route. The levels of PC-specific IgM, IgG, and IgA Abs in the serum were significantly higher in mice immunized with PC-KLH plus CT than those immunized with CT alone. There was no significant difference between SL and IN immunization with regard to serum Ab titers (Fig. 2). In addition, abnormal movement and palsy due to the damage of central nerve system and local inflammatory reactions in sublingual mucosa such as edema and swelling were not observed in any mice having IN and SL immunization with PC-KLH and CT.

3.2. PC-specific Th1- and Th2-type immune responses induced by SL and IN immunization with PC-KLH plus CT

Serum IgG subclass analysis showed that PC-specific IgG subclasses were markedly increased with PC-KLH plus CT immunization via both, the SL and IN routes (Fig. 3). Levels of

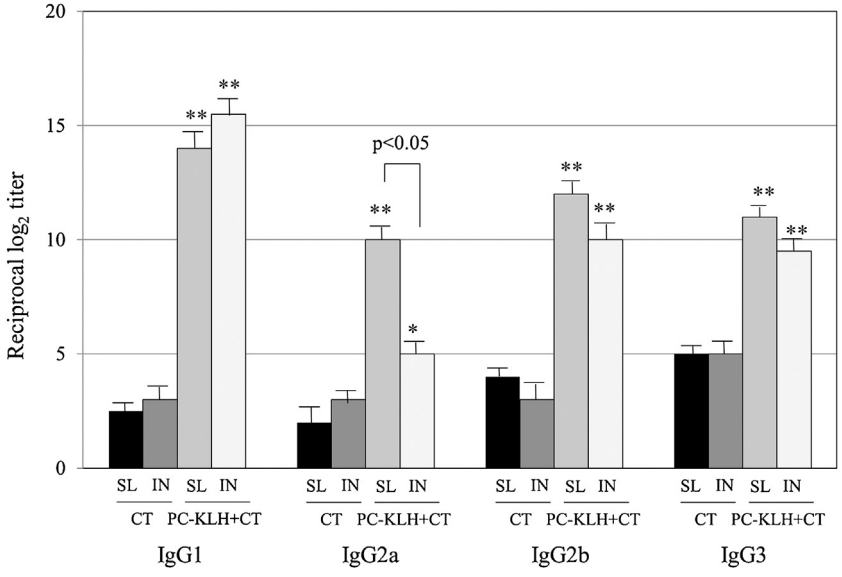

Fig. 3. PC-specific IgG subclass Ab titers in serum.
PC-specific IgG subclass Ab titers were markedly increased with PC-KLH plus CT immunization via both, the SL and IN routes. Immunization via the SL route induced significantly higher levels of IgG2a Abs than that via the IN route. The results are presented from 3 experiments (5 mice per group) and are expressed as the mean ± S.E. *, $p < 0.05$ compared to the titers in mice immunized with CT alone. **, $p < 0.01$ compared to the titers in mice immunized with CT alone.

PC-specific IgG2a induced by PC-KLH plus CT immunization via the SL route were significantly higher than that via the IN route, indicating that immune responses induced by SL immunization tend to shift to Th1 type. To investigate the balance between Th1- and Th2-type immune responses induced by SL and IN immunization with PC-KLH plus CT, the cytokines produced by $CD4^+$ T cells were examined. The results showed that IFN-γ and IL-4 production was remarkably enhanced with PC-KLH plus CT immunization (by the SL and IN routes) than that with CT alone (Fig. 4). Further, IFN-γ levels were significantly higher, while IL-4 levels were significantly lower with SL immunization as compared to IN immunization.

3.3. Serum IgE Abs induced by PC-KLH plus CT immunization via the SL and IN routes

The levels of total IgE Abs in serum were markedly elevated after CT immunization via the IN route but not via the SL route (Fig. 5). On the other hand, PC-KLH administration together with CT reduced the production of IgE Abs in IN immunization group and the levels of serum IgE Abs were similar for both, the IN and SL immunization groups. Additionally, IgE Ab production was not increased with SL immunization of either CT alone or PC-KLH plus CT. When antigen-specific IgE Ab were measured, CT-specific IgE Ab was increased in mice having IN immunization with CT, but not in mice having SL immunization with CT and in that having IN and SL immunization with PC-KLH plus CT. PC- and KLH-specific IgE Abs were not detected in any group of mice having IN as well as SL immunization.

3.4. Cross reactivity of IgA in nasal wash collected after SL and IN immunization, with Spn and NTHi strains

IgA Abs in pooled nasal wash samples from mice immunized with PC-KLH plus CT as well as TEPC 15 Abs were found to be cross-reactive with cell membrane lysates of most strains of Spn and NTHi. The reactivity of nasal wash IgA Abs correlated positively with that of TEPC 15, indicating that IgA Abs in nasal wash were bound to PC of Spn and NTHi (Fig. 6).

4. Discussion

The results of this study demonstrated that PC-KLH plus CT immunization via the SL route significantly increases PC-specific IgA Ab titers in the saliva and nasal wash samples, as well as IgM, IgG, and IgA Ab titers in the serum, to the same levels as those obtained via the IN route. The role of SL mucosa as an inductive site for generating mucosal and systemic immune responses was first reported by Cuburu et al. [9], who investigated the immune responses after SL, IN, and intragastric immunization of mice with ovalbumin (OVA) and CT. They demonstrated that OVA-specific mucosal and systemic immune responses induced by SL immunization are similar to that induced by IN immunization. These findings suggest that the magnitude of mucosal and systemic immune responses induced by SL immunization is comparable to those induced by IN immunization. Although OVA-specific immune responses were observed even in the absence of CT [9], PC-specific responses were not induced without CT in the present study (data not shown). The molecular size and mucosal permeability

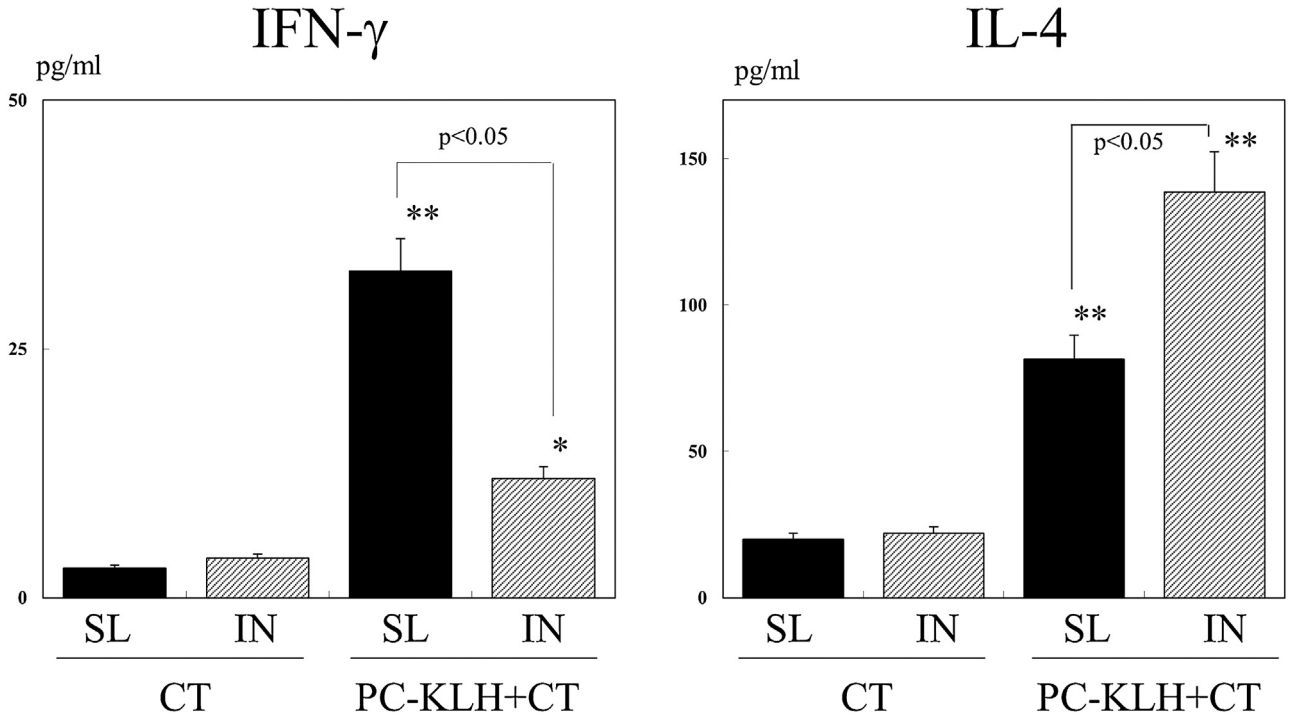

Fig. 4. Cytokines production by $CD4^+$ T cells.
The production of IFN-γ and IL-4 was remarkably enhanced by PC-KLH plus CT immunization via both the SL and IN routes. The level of IFN-γ was significantly higher, while IL-4 levels were significantly lower with the SL route. The results are presented from 2 experiments (5 mice per group) and are expressed as the mean ± S.E. *, $p < 0.05$ compared to the mice immunized with CT alone. **, $p < 0.01$ compared to the mice immunized with CT alone.

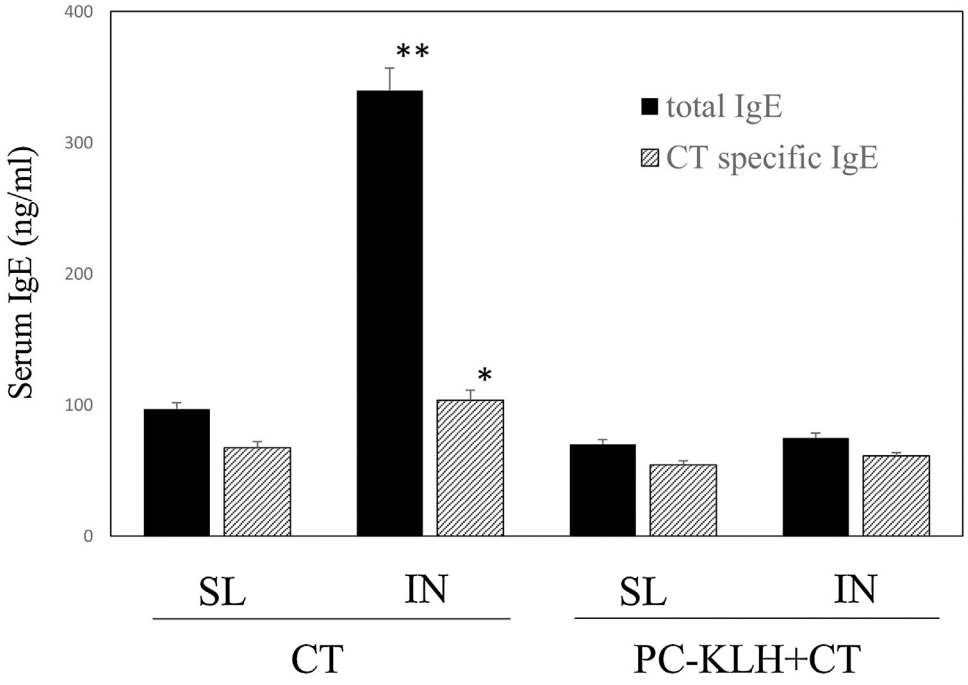

Fig. 5. Serum IgE Abs induced by CT alone and PC-KLH plus CT immunization.
The levels of total IgE Abs in serum were markedly elevated after CT immunization via the IN route. SL immunization with CT and IN as well as SL immunization with PC-KLH plus CT did not increase the levels of total IgE Abs. CT-specific IgE Ab was also significantly increased by IN immunization with CT compared to SL immunization. **, $p < 0.01$ and *, $p < 0.05$ compared to the mice sublingually immunized with CT alone and that immunized with PC-KLH plus CT.

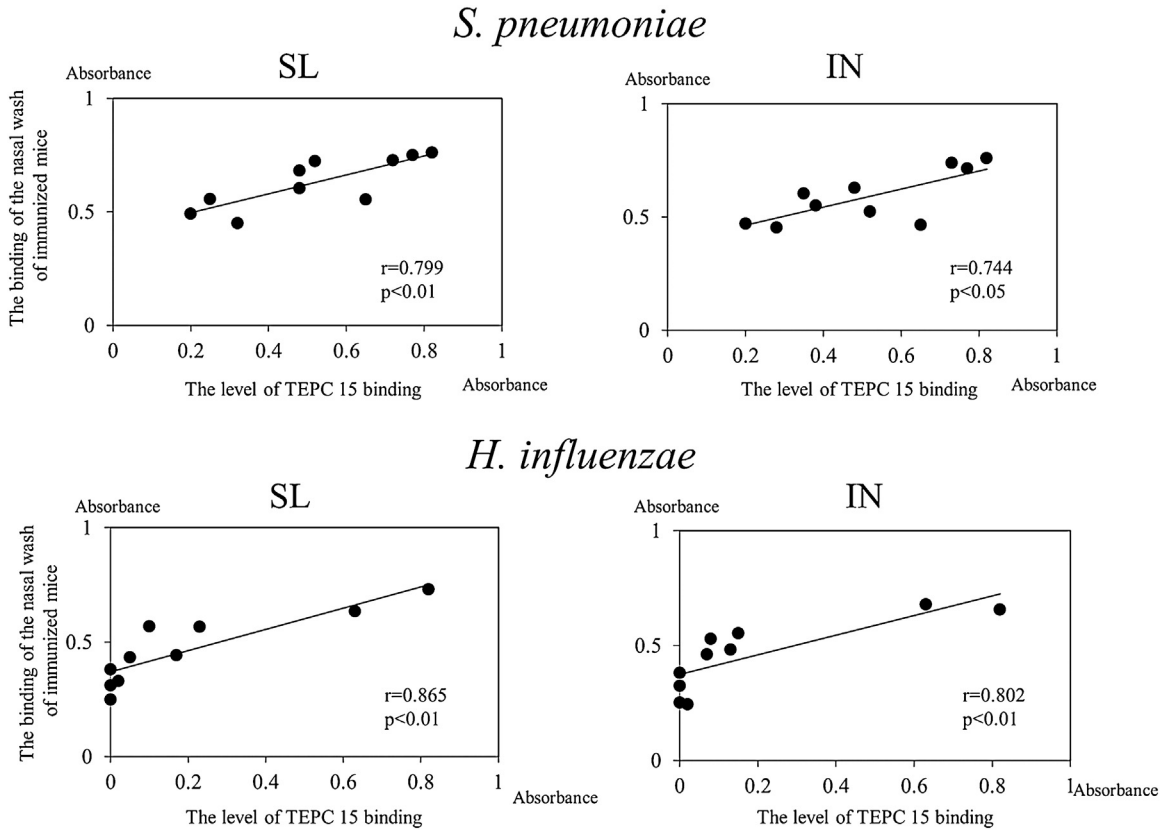

Fig. 6. Cross-reactivity of nasal wash IgA Abs and TEPC 15 against multiple strains of Spn and NTHi.
The IgA Abs in nasal wash samples of mice immunized with PC-KLH plus CT as well as TEPC 15 Abs cross-reacted with 10 strains of Spn and NTHi. The reactivity of IgA Abs in nasal wash correlated positively with that of TEPC 15.

of the antigen may be associated with the difference in Ab responses induced by OVA and PC-KLH.

A significant finding in the present study is the difference in PC-specific IgA Ab levels in vaginal wash samples between SL and IN immunization. The IgA Ab titers were significantly higher with SL compared to that with IN immunization, though the titers were increased in both. In contrast, when OVA plus CT was administered, there was no significant difference in the level of OVA-specific IgA Ab titers in vaginal wash between SL and IN immunization [11]. However, the number of OVA-specific IgA Ab secreting cells in the genital tract has been shown to be remarkably higher with SL than with IN immunization [11], indicating that immunization via the SL route is superior to that via the IN route in terms of inducing a mucosal immune response in the genital tract. Those findings suggest that SL immunization might be more useful than IN immunization to prevent gynecological infectious diseases.

Although serum IgG Ab response to PC-KLH immunization via the SL route was comparable to that via the IN route, some differences were observed in the subclass IgG Ab responses. PC-KLH-specific IgG1, IgG2b, and IgG3 subclass Ab titers with SL immunization were similar to those with IN immunization; however, IgG2a Ab levels were significantly higher with SL immunization, indicating that Th1 immune responses were predominant after SL immunization. In order to investigate the Th1- and Th2-type immune responses obtained with SL and IN immunization, we measured IFN-γ and IL-4 production by splenic $CD4^+$ T cells. We found that SL immunization elicited higher levels of IFN-γ production. Conversely, IN immunization elicited higher production of IL-4, although the levels of serum IgE Abs were almost similar in both immunizations. As the reason for those results, it can be speculated that immunocompetent cells other than $CD4^+$ T cells such as dendritic cells are associated with the production of IgE Abs and the immunological profiles might be different between SL and IN immunization. In a previous study with OVA and CT immunization via the SL and IN routes, strong IgG1 and IgG2a subclass Ab responses were observed and the Ab levels were comparable between the two groups [9]. Although cytokine production was not compared between the groups receiving SL and IN immunization, OVA immunization via the SL route induced an increase in both, IFN-γ and IL-4 production [9]. Since the dose used in the previous study was 2 μg CT, which is twice the dose used in the present study, it is possible that the magnitude of Th2-type immune response was enhanced by CT itself, rather than the vaccine antigens [13]. These observations suggest that SL immunization of PC-KLH plus CT induces a mixed Th1- and Th2-type immune response, which is in favor of Th1 type when compared to that induced by IN immunization.

The difference in the balance between Th1- and Th2-type immune responses with SL immunization compared with IN immunization may have affected the increased levels of total and CT-specific IgE Abs in serum after CT immunization via the IN and not the SL route. SL immunization is well known to be effective in suppressing IgE production by inducing regulatory T cells (Treg). Therefore, this route is used as allergen immunotherapy for the treatment of Type-I allergy such as allergic rhinitis [14]. Although the induction of Treg cells and the production of IL-10 and TGF-β were not examined in the present study, it can be speculated that Treg cells induced by SL immunization suppressed IgE production while maintaining the production of IgA Abs even using CT which mediates Th2-type immune responses [15]. Those finding indicate that SL immunization might be more effective than IN immunization to reduce the production of IgE Abs and to prevent sensitization to allergen causing type I allergy. Additionally, when PC-KLH was administered together with CT, the production of IgE Abs induced by IN immunization with CT alone was remarkably reduced, indicating that PC-KLH may suppress IgE Ab production. Since IL-12p40 production by $CD11c^+$ cells isolated from spleen of naïve mice was increased by stimulation with PC (Miyashita et al., in preparation), dendritic cells activated by IN and SL immunization with PC-KLH might have enhanced Th1-type immune responses. This speculation is supported by the increased IFN-γ production with PC-KLH plus CT immunization via both, the SL and IN routes. However, in the future study, it is needed to examine the role of Treg and dendritic cells present in spleen and cervical draining lymph nodes in order to clarify the mechanism in suppressing IgE Ab production by IN and SL immunization with PC-KLH.

IgA Abs in the nasal wash of mice immunized with PC-KLH via the SL and IN routes not only cross-reacted with cell membranes isolated from all 10 stains of Spn and NTHi, the binding ability also correlated positively with that of TEPC 15. These results indicate that the components binding to PC-specific IgA in nasal wash were almost identical to those binding to TEPC 15. However, IgA Abs in nasal wash and TEPC 15 were less reactive against some strains of NTHi as compared to Spn. This might be due to the differences between Spn and NTHi with regard to PC expression on the bacterial cell surface. Since PC is a structural component of the cell membrane lipoteichoic acid and capsular polysaccharide, all Spn strains possess PC [16]. In contrast, although PC is incorporated into lipooligosaccharide and lipopolysaccharide of Hi, NTHi lack capsular lipopolysaccharide. Additionally, the expression of lipooligosaccharide is controlled, in part, by phase variation, and therefore, varies among NTHi strains [17,18]. NTHi strains that reacted weakly with IgA Abs in nasal wash and TEPC 15 might express a small amount of PC in their cell membrane. However, this could be considered as an advantage for PC vaccination via the SL and IN routes. Bacterial invasion into respiratory endothelial cells and nasopharyngeal colonization by Spn and NTHi are associated with the enhanced PC expression [19–21]. Therefore, PC-specific IgA Abs induced by immunization with PC components via the SL or IN routes may prevent invasion by highly virulent Spn and NTHi strains without affecting commensal bacteria in the respiratory tract. In addition, as Treg cells affect both humoral and cell-mediated immune responses and may be associated with bacterial clearance, further studies are required whether Treg cells affect bacterial clearance from nasal cavity as well as the mucosal immune responses against bacterial antigens.

5. Conclusion

The present study demonstrated that PC-KLH plus CT immunization via the SL route induces PC-specific mucosal and systemic immune responses in the same way as the IN route. The levels of IgG2a subclass and IFN-γ production by $CD4^+$ T cells were significantly higher with SL compared with IN immunization, indicating the tendency of immune responses to SL immunization to shift to Th1-type. Further, significant production of serum IgE Abs was not observed with SL immunization even by the administration with CT alone. IgA Abs in nasal wash cross-reacted with all 10 strains of Spn and NTHi analyzed in this study, in case of both, the SL and IN routes of immunization. Those results suggest that SL immunization might be as effective as IN immunization in inducing PC-specific mucosal as well as serum Abs and more effective than IN immunization to reduce the production of IgE and to prevent the sensitization to allergen causing type I allergy.

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